KINETICS OF CONSECUTIVE ENZYME CATALYZED REACTIONS WITH SPECIAL REFERENCE TO STUDIES OF ENZYMES OF THE UREA CYCLE

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The research work described in this thesis was undertaken in the Department of Physical Biochemistry in the John Curtin School of Medical Research, Australian National University, between March 1972 and February 1975. All experimental results reported in the text, except the amino acid analyses which were performed by Dr D.C. Shaw, were obtained by the author alone, while theoretical considerations of the kinetics of steady-state coupled enzymic reactions were made in collaboration with Professor L.W. Nichol and Dr P.D. Jeffrey. The work reported in Appendix II, and the computing performed in relation to the urea cycle simulation were done in collaboration with Dr D.V. Roberts.

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CONTENTS

ABSTRACT

The work commences with consideration of a linear sequence of two reactions, each catalyzed by an enzyme of the Michaelis-Menten type. The steady-state differential equations are integrated using an approach employing Maclaurin polynomials to yield a solution, in open form, which expresses the concentrations of each reactant and of final product as explicit functions of time. The formulation suggests that experimentally available results of the increase of final product concentration with time be plotted with t^2 as the abscissa, since the slope of the limiting tangent to this curve, drawn as t approaches zero, is then given by a defined analytical function from which the steady-state kinetic parameters relating to the first enzyme catalyzed reaction may readily be evaluated.

The analysis procedure is extended by consideration of the following: (1) more than two enzymes in a linear sequence; (2) mechanisms involving single substrates other than the simple Michaelis-Menten mechanism (especially those pertaining to control phenomena); (3) reactions involving enzymes acting on two substrates (Appendix I); and (4) sequences of reactions forming a cycle (Appendix I). The general finding emerges that in all cases a plot of final product concentration versus time raised to the power of the number of reactions in the sequence, be constructed to permit evaluation of the slope of the limiting tangent : this slope is given by an analytical function which specifies the contribution to final product formation of the steady-state kinetic parameters of each of the enzymes involved.

detection of such macromolecular interactions by simple

The above concepts embodied in Chapter II were explored in relation to consecutive reactions catalyzed by arginase and urease. As a prelude to this study the physico-chemical properties of beef liver arginase were determined and are reported in Chapter III. In addition to confirming previously established values of basic parameters such as molecular weight (114,000) and of establishing others such as the apparent specific volume (0.734 $g^{-1}ml$), the findings in Chapter III also show that arginase does not dissociate on dilution to concentration levels generally employed in enzymic assays, and that it does not heterogeneously associate with sulphite modified urease in environments used for kinetic analysis. Accordingly, the arginase-urease couple was judged suitable as a system to explore the application of the theory presented in Chapter II. A report on the kinetic studies is given in Chapter IV where it is concluded that the suggested analysis procedure is indeed pertinent to results obtained in a coupled assay. While the results also confirm the suggestion that the urease and arginase do not heterogeneously associate, the opportunity is taken at the end of Chapter IV to formulate theory pertaining to the effect of enzyme-enzyme interaction on results obtained in coupled assays. It is shown, for example, that such an interaction between two enzymes which exhibit Michaelis-Menten kinetics when studied alone, can result in behaviour paralleling that found when an enzyme of the Michaelis-Menten type is coupled with one of the control type. The finding is discussed in relation to possible biological implications and to experimental detection of such macromolecular interactions by simple

kinetic measurements.

Chapter V commences with a characterization of the molecular and kinetic properties of beef liver argininosuccinase. The physico-chemical findings include support for the suggestion that the enzyme, of molecular weight 203,000, comprises four ellipsoidal subunits arranged with tetrahedral D_2 symmetry : dissociation of the 203,000 unit does not occur in the environment and dilution range used for the conduct of subsequent kinetic experiments. The kinetic findings indicate that the Eadie-Hofstee plot obtained with argininosuccinate as initial substrate is not linear, as suggested previously, but is interpretable in terms of models which involve either cooperativity (both positive and negative) between the active sites, or isomerization of the enzyme with preferential binding of substrate to one isomeric state which possesses two non-equivalent sites. The reverse reaction with arginine and fumarate as initial substrates was less extensively investigated but application of numerical integration methods permitted estimation of the relevant steady-state kinetic parameters. Both physico-chemical (sedimentation velocity) and kinetic (coupled assay) findings show that arginase and argininosuccinase do not form a complex in the environments studied.

Chapter V concludes with a description of fluxes associated with the urea cycle and utilizes parameters found for arginase and argininosuccinase together with literature values for ornithine transcarbamylase and argininosuccinate synthetase in a numerical integration of flux equations. The integration is considered in relation

to both steady-state differential rate equations and to a more extensive set which does not assume a steady-state of enzyme-substrate complexes. It is concluded that both approaches lead to the same intermediate metabolite pattern, defined as the concentration of relevant species, found as early as 10 sec from the time of commencement of the operation of the cycle. Specified kinetic parameters are modified in an attempt to simulate certain inborn errors of metabolism, the resulting intermediate metabolite patterns being correlated with observed clinical-biochemical findings. Although it is stressed throughout this Section that several clearly specified assumptions must necessarily be made, it is felt that the results demonstrate the potentiality in both explanatory and predictive areas.

A basic theme of the work is the expression of the concentrations of species in consecutive reactions as a function of time, either by applying numerical integration methods or by utilizing Maclaurin polynomial expansions of the relevant differential equations to obtain open solutions. The theme is continued in Appendix II where it is shown that application of Laplace-Carson transforms may lead in certain circumstances to closed solutions describing both pre-steady- and steady-states.

Considerable interest has been shown in the kinetic behaviour of consecutive reactions catalyzed by enzymes in solution (Garfinkel, Garfinkel, Pring, Green and Chance, 1970; Tschudy and Bonkowsky, 1972; Heinrich and Rapoport, 1974a,b; Rapoport, Heinrich, Jacobasch and Rapoport, 1974). There are two basic reasons for this interest. First, metabolic pathways considered as sequences of reactions in linear or cyclic array may be examined in terms of the concentrations of intermediate compounds as a function of time (e.g. Garfinkel and Hess, 1964) or of initial substrate concentration (London, 1966). Secondly, the use of coupled enzymic assays is of wide occurence in laboratory practice, a situation which has arisen since it is not convenient to monitor the concentrations of either substrates or products of certain enzymes studied alone. This work is concerned with both aspects, but that of *in vitro* coupled enzymic assays is considered first since these form the simplest examples of the operation of linear sequences of reactions.

One established method for the conduct of a coupled enzymic assay, involving two enzymes, proceeds in two stages, a denaturing agent being introduced to terminate the first reaction prior to the adjustment of environmental conditions and introduction of the second enzyme. The procedure is laborious, introduces dilution steps and requires controls to ensure that complete conversion of the first substrate to the final product has been achieved. There are obvious advantages in employing a reaction mixture in which both enzymes operate together. This is done in the second established procedure for conducting a coupled assay, typified by the work of McClure (1969) and Easterby (1973),

which utilizes a relatively large concentration of the second enzyme in an attempt to maintain a low and steady-state concentration of the intermediate compound. With the additional assumption that the amounts of substrates bound to the enzymes are small it may be shown, by differentiating the equation expressing conservation of mass, that the rate of depletion of initial substrate equals the rate of formation of final product. This procedure is limited by the following considerations: (1) since the kinetic parameters of the first enzyme are unknown there is little to guide the experimenter in the choice of the concentration of the second enzyme, to ensure a steady-state of the intermediate compound; (2) the procedure is inapplicable if active sites catalyzing consecutive reactions, reside on the same protein molecule (e.g. Koch, Shaw and Gibson, 1971, 1972); and (3) there is a danger that the two enzymes may heterogeneously associate to form a complex exhibiting different catalytic activity than the unbound forms of the enzymes (Hess and Boiteux, 1972) and in this instance the use of an excess of the second enzyme is clearly undesirable if the kinetic parameters pertaining to the unbound form of the first enzyme are being sought. A third established method attempts to fit the curve of concentration of final product versus time obtained experimentally (with no particular restriction on the enzyme concentrations employed) with theoretical curves simulated by numerically integrating the differential rate equations describing the consecutive reactions (Curtis and Chance, 1972; Bates and Frieden, 1973). It suffers from the disadvantage that sets of kinetic

parameters must be selected for the simulation, and these sets might have to include parameters describing complicating effects, such as product inhibition, if results obtained at relatively long times are to be fitted. One purpose of the present work is to formulate an alternative means for the interpretation of results obtained from a coupled enzymic assay. Thus in Chapter II the possibility is explored that the concentration of all reactants in a sequence may be expressed as explicit analytical functions of time. Such expressions are obtained and are examined in the limit as t approaches zero (when no inhibition effects operate) to define a procedure for interpreting the available experimental results directly without recourse to numerical integration (although this proves to be a useful complementary procedure). In addition it will be shown that the formulation of such expressions permits comment on the effects of coupling enzymes of the control and Michaelis-Menten types and on enzyme-enzyme interactions. The integration procedure is based on the use of Maclaurin polynomials and assumes a steady-state of all enzyme-substrate complexes, but not necessarily of intermediate compounds: it is therefore applicable to coupled assays involving a single protein with two active sites and does not place restriction on the enzyme concentrations when two separate enzymes are involved. For simplicity of presentation, the development in Chapter II is in terms of enzymes acting on single substrates; but the procedure is generalized in Appendix I, to encompass two substrate and cyclic sequences of reactions. Consideration is also given to analytical expressions which describe the concentration

of final product as a function of time in the pre-steady-state period (Appendix II).

It was decided to explore the theoretical concepts presented in Chapter II with an experimental kinetic study of a coupled assay where the final product concentration could be estimated with precision. This and the additional requirement that the individual enzymes could be studied separately, was met by the system in which arginase (L-arginine amidino hydrolase, E.C.3.5.3.1) catalyzed the conversion of arginine to ornithine and urea, and urease (urea amido hydrolase, E.C.3.5.1.5) hydrolyzed urea to form ammonium and bicarbonate ions in solution. It is of historical interest that this particular coupled assay was employed in studies with arginase as early as 1924 (Hunter and Dauphinee, 1924). As a prelude to this kinetic investigation it was necessary to physico-chemically characterize arginase: the behaviour of urease in solution is well documented (Creeth and Nichol, 1960; Nichol and Creeth, 1963; Mamiya and Gorin, 1965; Lynn, 1967; Blakely, Webb and Zerner, 1969; Reithel, 1971). Chapter IIIcommences with a review of the relevant background theory pertaining to the physico-chemical methods employed, and continues with a description of the method used to prepare arginase from beef liver which was based on that described by Harell and Sokolovsky (1972). The results of the characterization which follow are compared with the recent findings of Harell and Sokolovsky (1972) and others (Hirsch-Kolb, Heine, Kolb and Greenberg, 1970; Vielle-Breitburd and Orth, 1972) and in extending these pay particular attention to the aggregation state of arginase at high dilution and

to the possibility that complex formation, between arginase and urease, might complicate the interpretation of the coupled assay kinetic results which are reported in Chapter IV.

It is timely to note that a second theme of this work is the study of the ornithine-urea cycle enzymes, arginase and argininosuccinase (L-argininosuccinate arginine lyase, E.C.4.3.2.1); for the studies begun in Chapters III and IV with arginase are extended in Chapter V to consider the coupled reaction catalyzed by argininosuccinase and arginase. It is relevant therefore to outline briefly the available information on the occurence and postulated biological roles of these enzymes.

It is now well established, by the use of sensitive assay procedures for arginase (Van Slyke and Archibald, 1946; Ceriotti and Spandrio, 1963) that the occurence of the enzyme is wide spread in relation to both different tissues and different species (Reddy and Campbell, 1970; Carlisky, Sadnik and Menendez, 1972). This point was not appreciated in earlier studies which used relatively insensitive assays (Richet, 1894, 1897; Kossel and Dakin, 1904a,b; Baldwin, 1936) where for example it was erroneously thought that the enzyme was absent from the livers of uricotelic animals (Clementi, 1914, 1919). The most abundant source of arginase is mammalian liver (Fuchs, 1921) where its role in the ornithine-urea cycle is now well known (Krebs and Henseleit, 1932). The existence, to a greater or lesser degree, of arginase in virtually every other mammalian tissue (Levin, 1971) where frequently the other enzyme members of the urea cycle are

not present (Mepham and Linzell, 1966, 1967) suggests the possibility of several alternative metabolic roles for arginase. The second most abundant source of arginase is mammary tissue (Folley and Greenbaum, 1946) where its activity is especially high during lactation (Glass and Knox, 1973). Arginase produces ornithine which in turn is converted by the coordinately induced ornithine aminotransferase to yield finally proline. The implication is that proline provided by this source is beneficial to infant growth. Another source of arginase is brain tissue where, unlike its cytoplasmic distribution in the liver, it is found associated with nuclei (Davtyan and Bunyatyan, 1970). It has been suggested that this arginase may interact with arginine containing histones which themselves interact with DNA to perform a role in the control of DNA transcription and cellular differentiation. Another interesting discovery was that arginase levels are greatly enhanced in the skin of rabbits infected with Shope papilloma virus (Rogers, 1959). The enzyme is coded for by the viral genome and has no metal requirement. The virus which is said to be non-pathogenic in humans, has been used (as initially suggested by Rogers in 1964) in attempts to treat patients suffering from Hyperarginaemia, a congenital deficiency of arginase (Colombo, Terheggen, Lowenthal, Van Sande and Rogers, 1973). It is clear that certain of these postulated roles for arginase are somewhat speculative and the significance of the iso-enzyme pattern found with rat (Porembska, Jachimowicz and Gasiorowska, 1971; Farron, 1973) is as yet unclear. In this connection it is noted that the

present studies concentrate solely on an electrophoretically homogeneous single species of arginase found in beef liver and comment will only be made on the role of the enzyme in the ornithine-urea cycle which is now established without doubt.

Argininosuccinase is the most recently discovered enzyme associated with the ornithine-urea cycle (Ratner, Anslow and Petrack, 1953) and its substrate, argininosuccinate was the last component of the cycle to be positively identified (Ratner and Petrack, 1953). It is not surprising therefore that less is known about its distribution although it is known to occur in microorganisms (Walker, 1953; Newmeyer, 1957) as part of an arginine biosynthetic pathway. It is not the aim of this work to search for possible alternative roles for argininosuccinase, but rather to comment on its physico-chemical characteristics and kinetic behaviour. Reports on these aspects are given at the beginning of Chapter V where, as with arginase, information is provided which permits correlation with earlier findings (Ratner, 1973) and conclusions are drawn pertaining to its behaviour at high dilution in frontal gel chromatography, and in sedimentation velocity in the presence of arginase.

The remainder of Chapter V is concerned with the topic mentioned at the beginning of this introduction, the consideration of sequences of enzyme catalyzed reactions comprising a metabolic pathway. Thus the results obtained with arginase and argininosuccinase are combined with information obtained from the literature on ornithine transcarbamylase (carbamylphosphate L-ornithine carbamy-

transferase,E.C.2.1.3.3) and argininosuccinate synthetase (L-citrulline: L-aspartate ligase,(AMP) E.C.6.3.4.5), in an attempt to numerically integrate a set of differential equations describing internal fluxes within the ornithine-urea cycle and external fluxes related to it. The purpose of performing such numerical simulations was to determine the concentrations of certain intermediary metabolites which might pertain to the operation of the cycle in normal circumstances, and in patients suffering from certain inborn errors of metabolism, relating to the kinetic parameters of the enzymes concerned. It must be stated at the outset that, as with similar simulations, of the glycolytic pathway (Garfinkel and Hess, 1964) and glycogen synthesis (London, 1966), several assumptions must be made. At the same time it will become apparent that the simulations do lead to the formulation of intermediate metabolite patterns which correspond to those observed in clinical syndromes and, perhaps of even greater interest, suggest the potentiality of using such simulations in the predictive-diagnostic area. It could also be noted that this Section represents the first attempt to describe, in kinetic terms, this particular metabolic pathway which is a vital one, as a detoxification process, in the body. In general terms the particular calculations to be presented serve to illustrate some of the broad concepts which have been evolved in an attempt to give a descriptive and all-encompassing view of fluxes and their control within pathways (Hearon, 1949a,b, 1950,a,b and 1953; Waley, 1964; Kacser and Burns, 1973; Reich and Sel'kov, 1974; Heinrich and Rapoport, 1974a,b; Rapoport, Heinrich,

Jacobasch and Rapoport, 1974).

In summary, the aims of this thesis are four-fold.

(1) To develop equations which will aid in the interpretation of coupled enzymic assay results and clearly display the combined effects of the enzymes present in terms of kinetic parameters appropriate to the particular mechanisms associated with each enzyme.

(2) To apply these ideas to the coupled systems arginase-urease and argininosuccinase-arginase and to extend them to explore the possibility that enzyme-enzyme interactions (or lack of them) may be detected with kinetic measurements.

(3) To utilize the quantitative information obtained in the characterization of arginase and argininosuccinase to explore the kinetic behaviour of the sequence of reactions which comprise the ornithine-urea cycle.

(4) To comment generally on the relation between the concentrations of the final product of a reaction sequence, and time, in terms of open, closed, and numerically obtained solutions.

CHAPTER II

STEADY-STATE KINETICS OF CONSECUTIVE ENZYME CATALYZED REACTIONS INVOLVING SINGLE SUBSTRATES: THEORY

The purpose of the present Chapter is to present sets of differential rate equations, describing sequential enzyme catalyzed reactions, and to formulate approaches for their integration. The solutions obtained describe the time dependence of the concentrations of initial substrate, intermediate compounds, and final product. It will be seen that these relations are useful for obtaining kinetic parameters from experimental results, in elucidating enzyme-enzyme interactions, and in correlating the new approaches to be presented with established procedures for the conduct and interpretation, of for example, a coupled assay (Easterby, 1973; McClure, 1969; Bergmeyer, 1953). The Chapter commences by considering the simplest possible scheme, where two enzymes of the Michaelis-Menten type, E_1 and E_2 catalyze the consecutive reaction $S_1 \xrightarrow{E_1} S_2 \xrightarrow{E_2} S_p$, in which the product of the first reaction is the substrate for the second reaction which produces the final product S_p. The integration procedures are therefore introduced in the simplest possible context prior to their application to more complicated reaction schemes, which may certainly operate in practice.

1. Coupled systems involving two enzymes of the Michaelis-Menten type.

(a) The differential rate equations

A full description of the coupled system may be written as follows,

$$E_1 + S_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_1S_1 \xrightarrow{k_2} E_1 + S_2 \qquad \text{(II - 1a)}$$

$$E_2 + S_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E_2S_2 \xrightarrow{k_4} E_2 + S_p \qquad \text{(II - 1b)}$$

where the symbol k denotes a rate constant.

A full set of rate equations written in terms of the individual rate constants may readily be written, but the analytical integration of this set is possible only in special cases (Appendix II), or in general by numerical methods. Thus it would be possible to present particular numerical illustrations of the time dependence (including the pre-steady-state) of the concentrations of the compounds S_i (i = 1,2 or p) and of the enzyme-substrate complexes. The use of such numerical examples will be discussed later. It is however possible to integrate in more general terms a set of differential equations which have been written, assuming that each of the enzyme-substrate complexes is in a steady-state. In these terms the rate equations may be written as,

$$\frac{d[S_1]_t}{dt} = - \frac{V_1[S_1]_t}{(K_1+[S_1]_t)} \qquad \text{(II - 2a)}$$

$$\frac{d[S_2]_t}{dt} = + \frac{V_1[S_1]_t}{(K_1+[S_1]_t)} - \frac{V_2[S_2]_t}{(K_2+[S_2]_t)} \qquad \text{(II - 2b)}$$

$$\frac{d[S_p]_t}{dt} = + \frac{V_2[S_2]_t}{(K_2+[S_2]_t)} \qquad \text{(II - 2c)}$$

where V_1 and V_2 are the maximal velocities, $k_2[E_1]_o$ and $k_4[E_2]_o$ respectively, and K_1 and K_2 are Michaelis constants given respectively by $K_1 = (k_{-1} + k_2)/k_1$ and $K_2 = (k_{-3}+k_4)/k_3$.

Equations (II-2a and b) follow from the Briggs-Haldane treatment (Briggs and Haldane, 1925) for which a steady-state of enzyme-substrate complexes is assumed, while equation (II-2c) is obtained directly by differentiation of the conservation of mass condition;

$$[S_1]_o = [S_1]_t + [S_2]_t + [S_p]_t \qquad (II - 2d)$$

It is noted that a steady-state of the intermediate compound S_2 has not been assumed, but that it is implicit in equation (II-2d) that the amounts of substrates bound to the enzymes is negligible. Equation (II-2d) also gives the initial condition that at $t = o$, $[S_2]_t = o$, and $[S_p]_t = o$. These initial conditions will be used in the integration of the set of non-linear differential equations (equation II-2) to give $[S_i]_t$ ($i = 1,2$ or p) as a function of time.

(b) Methods of integration

(i) Expansion of the logarithmic term. Equation (II-2a) may be directly integrated by separating the variables to give (Dixon and Webb, 1958),

$$V_1 t = ([S_1]_o - [S_1]_t) - K_1 \ln\left(\frac{[S_1]_t}{[S_1]_o}\right) \qquad (II - 3)$$

This equation is valid only over the time domain in which the enzyme-substrate complex can be considered to be in an approximate steady-state, a point which has been considered by many workers, (Laidler, 1955; Hommes, 1962; Walter and Morales, 1964; Walter, 1966; Wong, 1965). It emerges that equation (II-3) cannot pertain at very early times (the pre-steady-state period) or at very long

times when the percentage depletion of substrate is very large. Laidler and Bunting (1973) have suggested that in general the steady-state hypothesis is satisfactory provided $[S_1]_t$ / $[E_1]_t$ exceeds 10^3 : this however is only an approximate guide since the value of $d[E_1S_1]_t/dt$ (which equals $(d[E_1S_1]_t/d[S_1]_t)(d[S_1]_t/dt)$ and must be less than $(d[S_1]_t/dt)([E_1]_o/K_1)$) also depends on the value of K_1.

Figure (II-1) explores the applicability of equation (II-3) in relation to a particular system. The solid curve was computed using equation (II-3) and is compared with that obtained by numerically integrating the rate equations pertinent to equation (II-1a) (dots) in which a steady-state of the enzyme-substrate complex E_1S_1 was not assumed. With the parameters chosen the pre-steady-state was of such short duration that it could not be represented graphically in Figure (II-1) with the result that for both treatments the percentage depletion of substrate was described by the solid line until only approximately 0.1% of the initial substrate remained : the slight deviation of the non-steady-state solution at this point, however, is too small to be shown in Figure (II-1). It could be noted in relation to this example, where the parameters were chosen to resemble those pertinent to arginase catalysis, that ninety nine percent of the substrate was depleted in approximately seven seconds. This short time arose because a value of $[E_1]_o$ of 1×10^{-6}M, much larger than utilized in experimental work to be presented, was selected in order to minimize the computing time of the elaborate programme which involved

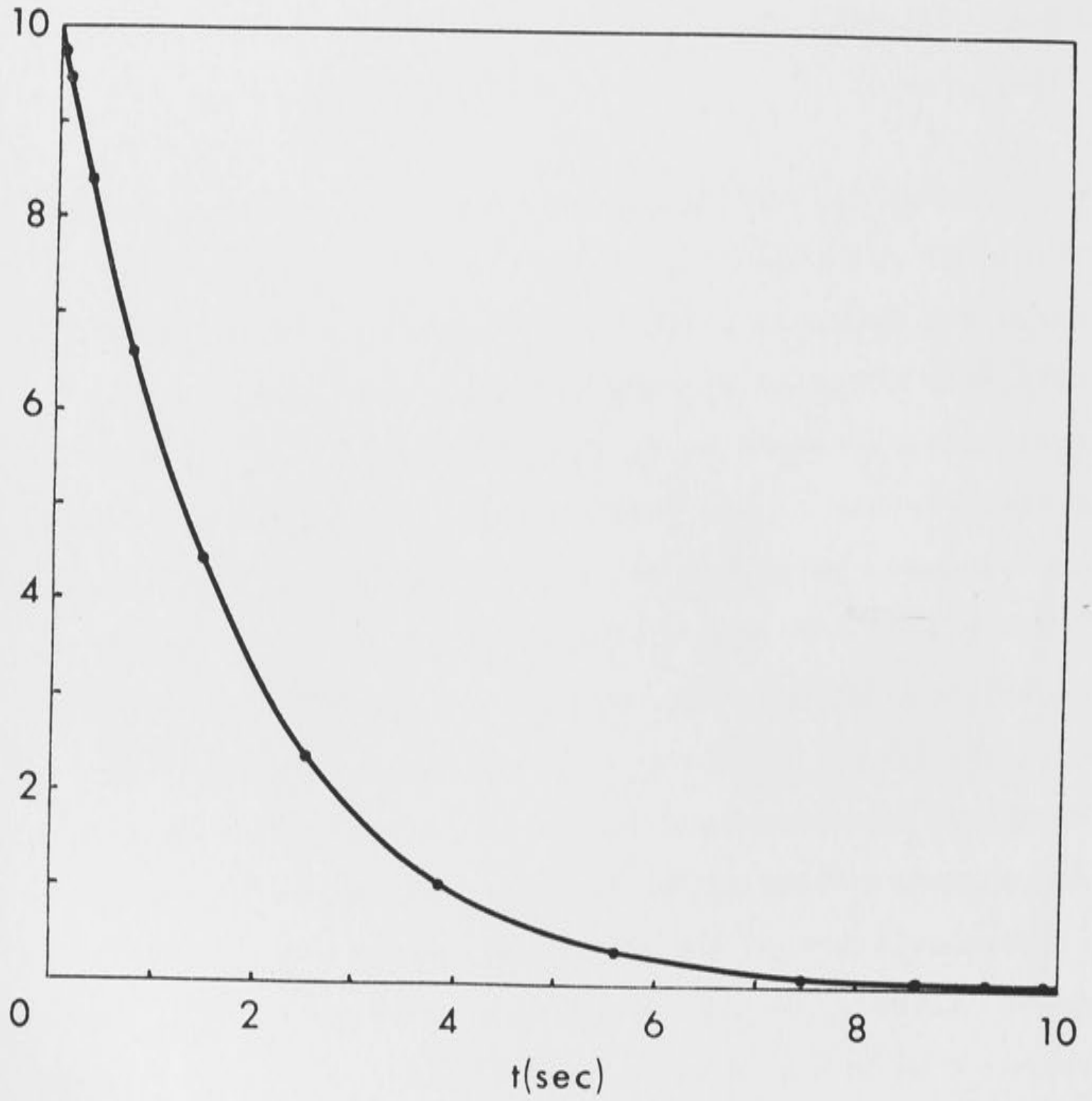

10
8
6
4
2
0
2
4
6
8
10
$(S_1)_t$, M×10⁴
t(sec)

Figure II - 1

A comparison of the depletion of substrate with time predicted by steady-state and pre-steady-state treatments. The solid line refers to a Michaelis-Menten mechanism ($[S_1]_o = 1x10^{-3}M$, $V_1 = 3.3x10^{-3}Msec^{-1}$, and $K_1 = 5.165x10^{-3}M$) and was computed using equation (II-3) which assumes a steady-state of the enzyme-substrate complex. The same result was obtained by numerically integrating equation (II-2a). The solid points were found by numerically integrating the differential rate equations corresponding to equation (II-1a) without assuming a steady-state of the enzyme-substrate complex. The latter calculations were performed with $[S_1]_o = 1x10^{-3}M$; $k_1 = 2.0x10^{7}Msec^{-1}$; $k_{-1} = 1.0x10^{5}sec^{-1}$; $k_2 = 3.3x10^{3}sec^{-1}$; $[E_1]_o = 1x10^{-6}M$, chosen to correspond to the Michaelis-Menten parameters cited above.

the Gear subroutine DIFSUB for handling stiff differential equations (Gear, 1969). The important point emerges, however, that the illustration in Figure (II-1) confirms the previous prediction that equation (II-3) is valid until $[S_1]_t$ / $[E_1]_t$ decreases to a value of 10^3. At the same time it is noted that equation (II-3) expresses $[S_1]_t$ as an *implicit* function of t, (i.e. $t = t([S_1])$), a format which is not useful in relation to further integration of equation (II-2).

In order to obtain $[S_1]_t$ as an *explicit* function of t it is required to expand the logarithmic term to obtain

$$V_1 t = ([S_1]_o - [S_1]_t) + K_1 \left\{ \left(\frac{[S_1]_o - [S_1]_t}{[S_1]_o} \right) - \left(\frac{[S_1]_o - [S_1]_t}{[S_1]_o} \right)^2 \frac{1}{2} + \ldots + (-1)^n \left(\frac{[S_1]_o - [S_1]_t}{[S_1]_o} \right)^n \frac{1}{n} + \ldots \right\} \qquad \text{(II - 4)}$$

Application of the ratio test shows that this series converges absolutely for all $[S_1]_t$ if $[S_1]_t \in (o, 2[S_1]_o)$, which is non-restrictive since $[S_1]_t$ always satisfies these conditions experimentally. The series for ln is rapidly convergent, so it is reasonable to truncate the series after the squared term and still obtain a reasonably accurate expression. The result of such truncation is a quadratic in $[S_1]_t$, which can be solved for $[S_1]_t$, and after expansion of the square root of the discriminant, the following expression obtains,

$$[S_1]_t = [S_1]_o - \frac{V_1 [S_1]_o t}{(K_1 + [S_1]_o)} + \frac{V_1^2 K_1 [S_1]_o t^2}{(K_1 + [S_1]_o)^3 2} \qquad \text{(II - 5)}$$

Retention of higher degree terms in equation (II-4) gives polynomials in $[S_1]_t$ which do not yield an accessible general solution. Moreover this method of integration, which involves a double expansion is inapplicable to equation (II-2b) and thus, while equation (II-5) may reasonably describe the depletion of initial substrate, in order to obtain the time dependence of $[S_2]_t$ and $[S_p]_t$, an alternative method of integration must be found.

(ii) Integration in series. This method assumes that $[S_i]_t$ (i = 1, 2 or p) can be expressed as an infinite power series in the dependent variable t, convergent for values of t in a domain about t = o.

$$[S_i]_t = [S_i]_o + A_1 t + A_2 t^2 + \ldots + A_n t^n + \ldots \quad (II - 6)$$

It suffices to illustrate the application of equation (II-6) in relation to $[S_1]_t$ for which equation (II-2a) may be rewritten as,

$$V_1[S_1]_t + K_1[S_1]_t^{<1>} + [S_1]_t [S_1]_t^{<1>} = 0 \quad (II - 7a)$$

where <1> denotes the first derivative with respect to time. Application of equation (II - 7a) requires evaluation of $[S_1]_t$, $[S_1]_t^{<1>}$ and their product which follow directly from equation (II-6)

$$[S_1]_t = [S_1]_o + A_1 t + A_2 t^2 + \ldots + A_n t^n + \ldots \quad (II - 7b)$$

$$[S_1]_t^{<1>} = A_1 + 2A_2 t + 3A_3 t^2 + \ldots + (n+1)A_{n+1} t^n + \ldots \quad (II - 7c)$$

$$[S_1]_t [S_1]_t^{<1>} = A_1[S_1]_o + (A_1^2 + 2A_2[S_1]_o)t + (3[S_1]_o A_3 + 3A_1A_2)t^2 + \ldots + \left(\sum_{j=o}^{n} (j+1)A_{j+1}A_{n-j} \right) t^n + \ldots \quad (II - 7d)$$

where $A_o = [S_1]_o$. It is possible to substitute the latter three equations, into equation (II-7a) and to expand each term to form a new power series in t : each coefficient of this series is now equated with zero to yield explicit expressions for A_1, A_2, to A_n. Application of this method leads to equation (II-8)

$$[S_1]_t = [S_1]_o - \frac{V_1[S_1]_o t}{(K_1+[S_1]_o)} + \frac{V_1^2 K_1[S_1]_o t^2}{(K_1+[S_1]_o)^3\ 2}$$

$$+ \frac{V_1^3 K_1[S_1]_o\ (2[S_1]_o - K_1)\ t^3}{(K_1+[S_1]_o)^5\ 3!}$$

$$+ \frac{V_1^4 K_1[S_1]_o(K_1^2 - 8K_1[S_1]_o + 6[S_1]_o^2)t^4}{(K_1+[S_1]_o)^7\ 4!} + \ldots \quad (II - 8)$$

It is immediately noted that the first three terms of this equation are those of equation (II-5) and that the present method has generated even higher terms. Nevertheless it is a cumbersome procedure and one extremely difficult, but not impossible, to apply in the evaluation of $[S_2]_t$ and $[S_p]_t$ from equations (II-2b and c). When it is recalled that the latter equations pertain to only one particular model, the need for a more easily applied method becomes even more apparent.

(iii) Maclaurin polynomials. Since the concentration of each species S_i (i = 1,2 or p) in a reaction scheme can be described by a continuous and differentiable function in t, it may be expressed as a Taylor series whose coefficients are evaluated at the origin (i.e. a Maclaurin polynomial (Maclaurin, 1742),)

$$[S_i]_t = [S_i]_o + [S_i]_o^{<1>} t + [S_i]_o^{<2>} \frac{t^2}{2} + \ldots$$

$$+ [S_i]_o^{<n>} \frac{t^n}{n!} + \ldots \qquad \text{(II - 9)}$$

where again < > denotes the order of the derivative with respect to time. The value of $[S_1]_o$ used in evaluating $[S_1]_o^{<1>}$ directly from equation (II-2) will be visualized as the concentration of the first species prior to the commencement of the reaction, implying as is frequently and reasonably done, that the amount of S_1 bound to E_1 is small, and that a negligible amount of S_2 is formed during the first, pre-steady-state period of extremely short duration. Expressions for other first derivatives are directly obtainable from equation (II-2) simply by placing $[S_i]_t = [S_i]_o$. Expressions for higher order derivatives are more difficult to obtain since they require successive differentiation of equation (II-2) *prior* to the substitution $[S_i]_t = [S_i]_o$. This procedure is however facilitated by noting that equation (II-2) involves only terms of the form $V_i[S_i]_t/(K_i + [S_i]_t)$ whereupon expressions for $[S_i]_t^{<2>}$ and $[S_i]_t^{<3>}$ for example, involve only the respective terms,

$$\frac{V_i K_i [S_i]_t^{<1>}}{(K_i+[S_i]_t)^2} \quad \text{and} \quad \frac{V_i K_i \left\{ [S_i]_t^{<2>} (K_i+[S_i]_t) - 2([S_i]_t^{<1>})^2 \right\}}{(K_i+[S_i]_t)^3}$$

(II - 10)

TABLE II - 1

Expressions in terms of $[S_1]_o$ and $\alpha = (K_1 + [S_1]_o)$ for successive derivatives of equation (II - 9) for the consecutive enzyme system $S_1 \longrightarrow S_2 \longrightarrow S_p$ involving two enzymes of the Michaelis-Menten type.

i	$[S_i]_o$	$[S_i]_o^{<1>}$	$[S_i]_o^{<2>}$	$[S_i]_o^{<3>}$
1	$[S_1]_o$	$-\frac{V_1[S_1]_o}{\alpha}$	$\frac{V_1^2K_1[S_1]_o}{\alpha^3}$	$-\frac{V_1^3K_1[S_1]_o(K_1 - 2[S_1]_o)}{\alpha^5}$
2	0	$\frac{V_1[S_1]_o}{\alpha}$	$-\frac{V_1(V_1K_1K_2 + V_2\alpha^2)}{K_2\ \alpha^3}$	$\frac{V_1^3K_1[S_1]_o(K_1-2[S_1]_o)}{\alpha^5} + \frac{V_2V_1[S_1]_o}{K_2}\left\{\frac{V_2}{K_2\alpha} + \frac{2V_1[S_1]_o}{K_2\ \alpha^2} + \frac{V_1K_1}{\alpha^3}\right\}$
p	0	0	$\frac{V_2V_1[S_1]_o}{K_2\ \alpha}$	$-\frac{V_2V_1[S_1]_o}{K_2}\left\{\frac{V_2}{K_2\alpha} + \frac{2V_1[S_1]_o}{K_2\ \alpha^2} + \frac{V_1K_1}{\alpha^3}\right\}$

The problem of obtaining expressions for the coefficient in equation (II-9) therefore becomes one of successive substitution.

Table (II-1) summarizes expressions for $[S_i]_o$, $[S_i]_o^{<1>}$, $[S_i]_o^{<2>}$ and $[S_i]_o^{<3>}$ so obtained for the coupled assay system $(S_1 \xrightarrow{E_1} S_2 \xrightarrow{E_2} S_p)$ under discussion.

It follows from the first row of Table (II-1) and equation (II-9) that,

$$[S_1]_t = [S_1]_o - \frac{V_1[S_1]_o t}{(K_1+[S_1]_o)} + \frac{V_1^2 K_1 [S_1]_o t^2}{(K_1+[S_1]_o)^3 2} ; \qquad (II - 11)$$

$t \in (o, \delta t)$

where the alternating series has been truncated after the third term. Equation (II-12) on page 18a shows that higher degree terms, in agreement with equation (II-9) may readily be obtained. In an entirely analogous manner the time dependence of $[S_2]_t$ may be obtained, and this is given on page 18b (equation II-13). The corresponding expression for $[S_p]_t$, given on page 18c(equation II-14) is obtained by difference using the conservation of mass condition $[S_p]_t = [S_1]_o - [S_1]_t - [S_2]_t$.

The coefficients of t in equation (II-12) evidently become very complicated as the degree of t increases, and thus no general algebraic recursion formula could be found, which implies that it is unlikely that an analytical test can be made for convergence. Accordingly recourse must be made to a numerical example to illustrate likely convergence. The solid line in Figure (II-2) was

$$[S_1]_t = [S_1]_o + \left\{ - \frac{V_1[S_1]_o}{(K_1+[S_1]_o)} \right\} t + \left\{ \frac{V_1{}^2 K_1[S_1]_o}{(K_1+[S_1]_o)^3} \right\} \frac{t^2}{2}$$

$$+ \left\{ - \frac{V_1{}^3 K_1[S_1]_o(K_1 - 2[S_1]_o)}{(K_1 + [S_1]_o)^5} \right\} \frac{t^3}{6}$$

$$+ \left\{ \frac{V_1{}^4 K_1[S_1]_o(K_1{}^2 - 8K_1[S_1]_o + 6[S_1]_o{}^2}{(K_1 + [S_1]_o)^7} \right\} \frac{t^4}{24}$$

$$+ \left\{ - \frac{V_1{}^5 K_1[S_1]_o(K_1{}^3 - 22K_1{}^2[S_1]_o + 58K_1[S_1]_o{}^2 - 24[S_1]_o{}^3}{(K_1 + [S_1]_o)^9} \right\}$$

$$\frac{t^5}{120} \qquad \text{(II - 12)}$$

$$[S_2]_t = 0 + \left\{\frac{V_1[S_1]_o}{(K_1+[S_1]_o)}\right\} t + \left\{-\frac{V_1[S_1]_o}{K_2}\,\frac{(V_1K_2+V_2(K_1+[S_1]_o)^2)}{(K_1+[S_1]_o)^3}\right\}\frac{t^2}{2}$$

$$+\left\{\frac{V_1{}^3K_1[S_1]_o(K_1-2[S_1]_o)}{(K_1+[S_1]_o)^5} + \frac{V_2V_1[S_1]_o}{K_2}\left[\frac{V_2}{K_2(K_1+[S_1]_o)}\right.\right.$$

$$\left.\left. + \frac{2V_1[S_1]_o}{K_2(K_1+[S_1]_o)^2} + \frac{V_1K_1}{(K_1+[S_1]_o)^3}\right]\right\}\frac{t^3}{6}$$

$$+\left\{-\frac{V_1{}^4K_1[S_1]_o(K_1{}^2 - 8K_1[S_1[S_1]_o + 6[S_1]_o{}^2}{(K_1 + [S_1]_o)^7}\right.$$

$$-\frac{V_2V_1[S_1]_o}{K_2}\left[\frac{V_2{}^2}{K_2{}^2(K_1+[S_1]_o)} + \frac{8V_2V_1[S_1]_o}{K_2{}^2(K_1+[S_1]_o)} + \frac{V_1(V_2K_1K_2+6V_1[S_1]_o{}^2}{K_2{}^2(K_1+[S_1]_o)^3}\right.$$

$$\left.\left.\left. + \frac{6V_1{}^2K_1[S_1]_o}{K_2(K_1+[S_1]_o)^4} + \frac{V_1{}^2K_1(K_1-2[S_1]_o)}{(K_1+[S_1]_o)^5}\right]\right\}\frac{t^4}{24}$$

(II - 13)

$$[S_p]_t = 0 + 0 + \left\{ \frac{V_2 V_1 [S_1]_o}{K_2 (K_1 + [S_1]_o)} \right\} \frac{t^2}{2}$$

$$+ \left\{ - \frac{V_2 V_1 [S_1]_o}{K_2} \left[\frac{V_2}{K_2 (K_1 + [S_1]_o)} + \frac{2 V_1 [S_1]_o}{K_2 (K_1 + [S_1]_o)^2} + \frac{V_1 K_1}{(K_1 + [S_1]_o)^3} \right] \right\} \frac{t^3}{6}$$

$$+ \left\{ \frac{V_2 V_1 [S_1]_o}{K_2} \left[\frac{V_2^2}{K_2^2 (K_1 + [S_1]_o)} + \frac{8 V_2 V_1 [S_1]_o}{K_2^2 (K_1 + [S_1]_o)} + \frac{V_1 (V_2 K_1 K_2 + 6 V_1 [S_1]_o^2)}{K_2^2 (K_1 + [S_1]_o)^3} \right.\right.$$

$$\left.\left. + \frac{6 V_1^2 K_1 [S_1]_o}{K_2 (K_1 + [S_1]_o)^4} + \frac{V_1^2 K_1 (K_1 - 2 [S_1]_o)}{(K_1 + [S_1]_o)^5} \right] \right\} \frac{t^4}{24}$$

(II - 14)

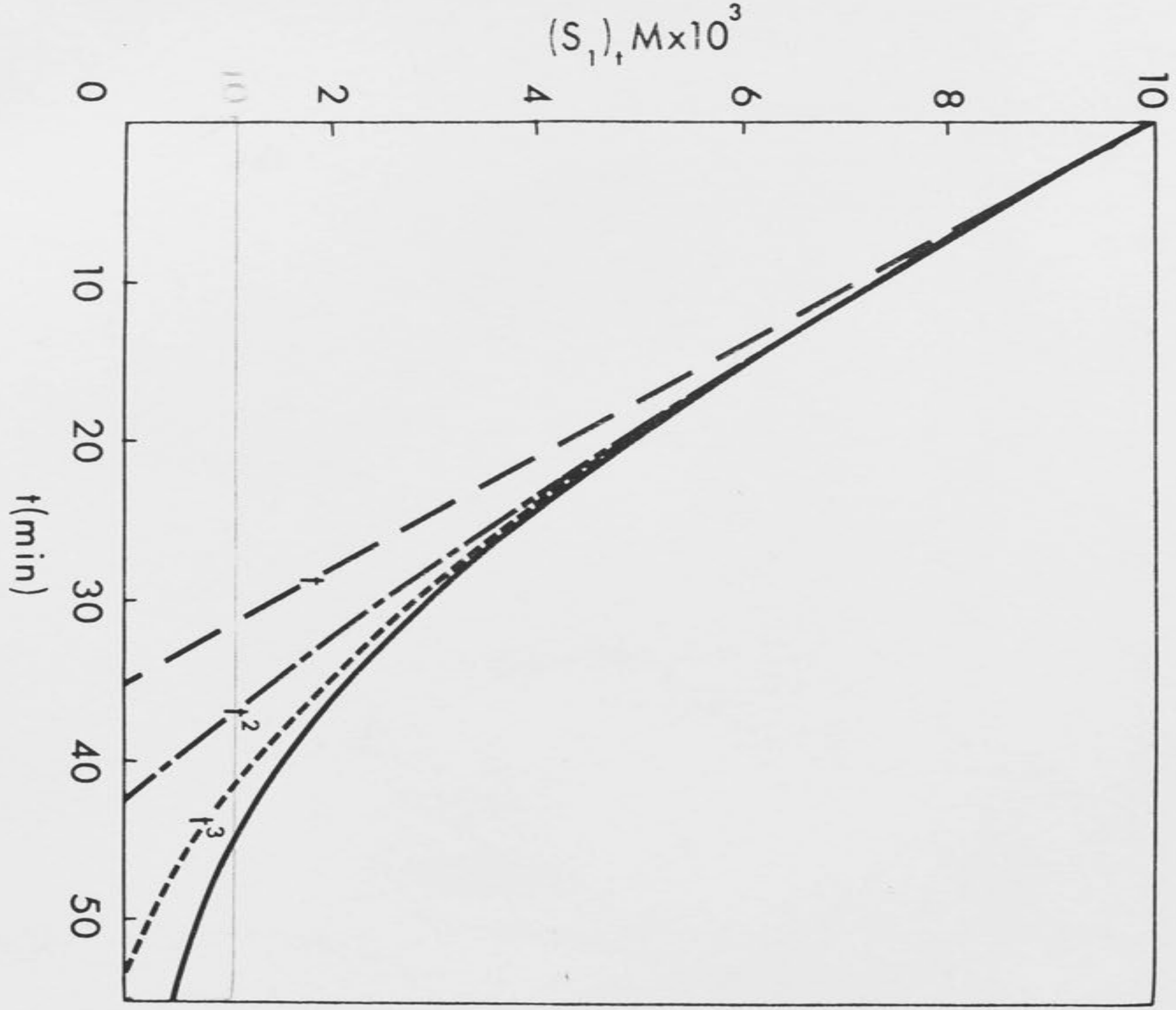

$(S_1)_t M \times 10^3$
0
2
4
6
8
10
0
10
20
30
40
50
t(min)
1
t^2
t^3

Figure II - 2

An illustration of the convergence of the alternating power series (Maclaurin polynomial) describing the time dependent depletion of initial substrate in a reaction of the Michaelis-Menten type. The solid line was computed using equation (II-3) with $[S_1]_o = 1x10^{-2}M$, $V_1 = 7.0x10^{-4}M\ min^{-1}$, and $K_1 = 5x10^{-3}M$. The broken lines were computed using equation (II-12) with the same parameters and with the progressive retention of the second (t), third (t^2), and fourth (t^3) terms.

computed on the basis of equation (II-3) for the parameters cited in the caption, while the broken lines were computed using equation (II-12) with retention of the t term (— — —), of the t^2 term (— - — -) and of the t^3 term (- - - - -). Clearly at early times even the retention of only the first two terms of equation (II-12) suffices to describe the time dependent depletion of substrate, while at longer times equation (II-12) continues to satisfactorily describe the situation, provided higher degree terms are included. Similarly it is possible to show, at least in terms of a numerical example, that equations (II-13) and (II-14) adequately describe the time dependence of $[S_2]_t$ and $[S_p]_t$ especially at early times. It suffices to illustrate this point in relation to the final product S_p and this is done in Figure (II-3). The solid line is computed using parameters cited in the caption, by numerical integration of equation (II-2) assuming a steady-state of both enzyme-substrate complexes. The dots denote values of $[S_p]$ computed by a fuller numerical integration procedure (Gear, 1969; see Chapter VI) in terms of the individual rate constants : its correspondence with the solid curve shows, as did Figure (II-1) for $[S_1]_t$, that the steady-state assumption is reasonable, for the parameters chosen, even for the coupled assay. The curve (— - — - —) is computed using equation (II-14) retaining all terms shown; it in fact describes closely the behaviour up to a point of 15% of total final product formation. Perhaps of greater interest is that equation (II-14) truncated after the t^2 term also describes the behaviour at very early times (- - -). This observation

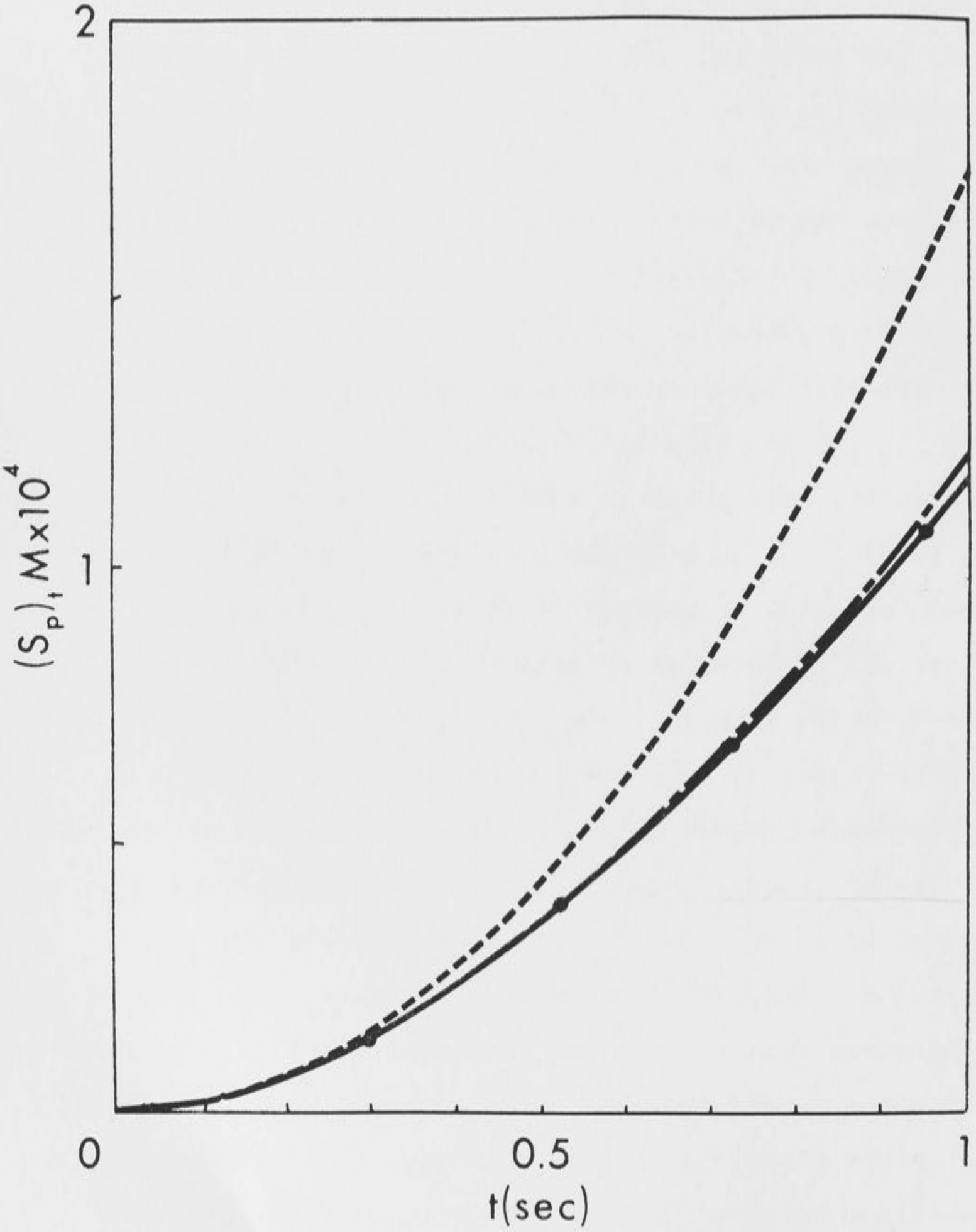

2
1
$(S_p)_t$, Mx10^4
0
0.5
1
t(sec)

Figure II - 3

The increase of final product concentration with time of a sequence of two reactions each catalyzed by a Michaelis-Menten enzyme. The solid curve was computed by numerically integrating equation (II-2) with the kinetic parameters ($V_2 = V_1$ and $K_2 = K_1$) reported for Figure (II-1). The solid points were obtained by numerically integrating the rate equations corresponding to equation (II-1) which does not assume a steady-state of the two enzyme-substrate complexes : values for the individual rate constants needed for this calculation were chosen to correspond to the reported Michaelis constants and maximal velocities. The broken lines refer to the Maclaurin polynomial (equation II-14) with retention of the t^2 term (- - -), and of all terms (— - — -).

will be used in the next Section.

(c) The practical use of equation (II-14)

Although equation (II-14) is in fact an extended power series in t it follows rigorously that,

$$\lim_{t \to o} \frac{[S_p]_t}{t^2} = \frac{V_2 V_1 [S_1]_o}{K_2 (K_1 + [S_1]_o) 2} = m \qquad (II - 15)$$

This suggests that a plot be constructed of $[S_p]_t$ versus t^2 and the slope be evaluated of the tangent to the curve drawn as t tends to zero; for then the slope (m) would equal the right hand side of equation (II-15) which is an explicit function of the initial substrate concentration $[S_1]_o$. The procedure is illustrated with the following numerical example, in which the enzyme E_1 is characterized by the parameters $K_1 = 4 \times 10^{-3}$M, $k_2 = 1 \times 10^{+5}$ min^{-1}, $[E_1]_o = 4 \times 10^{-9}$M, $V_1 = 4 \times 10^{-4}$M min^{-1}, and the second enzyme E_2 by the parameters $K_2 = 5 \times 10^{-3}$M, $k_2 = 2 \times 10^{+5} min^{-1}$, $[E_2]_o = .75 \times 10^{-9}$M, $V_2 = 1.5 \times 10^{-4}$M min^{-1}. These parameters having been chosen to closely resemble the literature values for arginase E_1 (Harell and Sokolovsky, 1972) and urease, E_2 (Reithel, 1971). Figure (II-4) shows the time course of the reaction for each reactant and was obtained by numerical integration of equation (II-2). Figure (II-5) shows the suggested plot of $[S_p]_t$ versus t^2 (solid line) and the limiting tangent (- - -) whose slope equals 4.29×10^{-6}M min^{-2}. The magnitude of this quantity and its dimensions are in agreement with the expression for m given by equation (II-15). The important point emerges

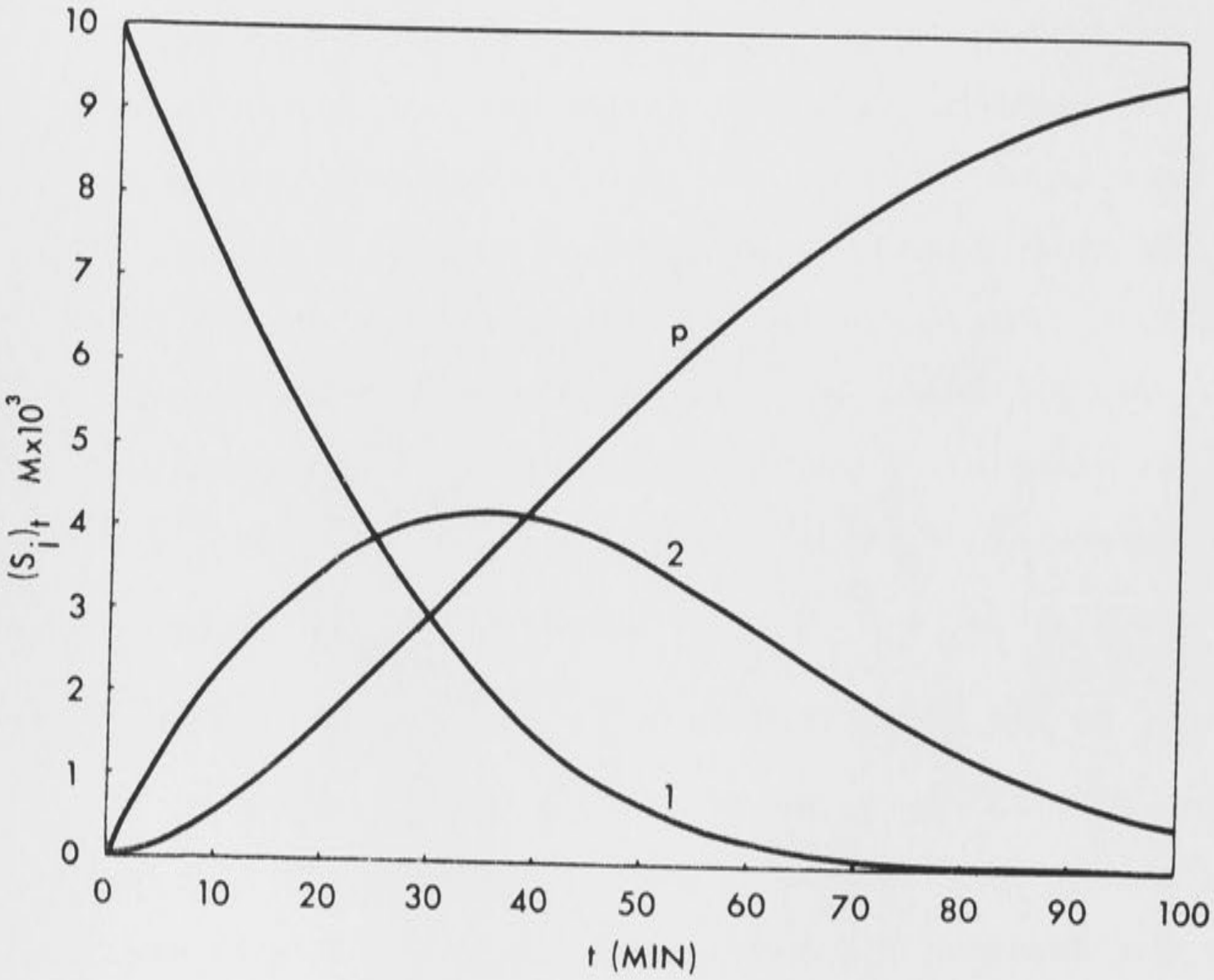

10
9
8
7
6
5
4
3
2
1
0
$(S_i)_t$ Mx10^3
p
2
1
0
10
20
30
40
50
60
70
80
90
100
t (MIN)

Figure II - 4

The time course of a coupled enzymic reaction, obtained by numerically integrating equation (II-2). The parameters utilized are as stated in the text. Curve 1 shows the decrease of $[S_1]_t$, curve 2 displays the rise and subsequent fall in the concentration of the intermediate compound S_2, and curve p displays the final product concentration.

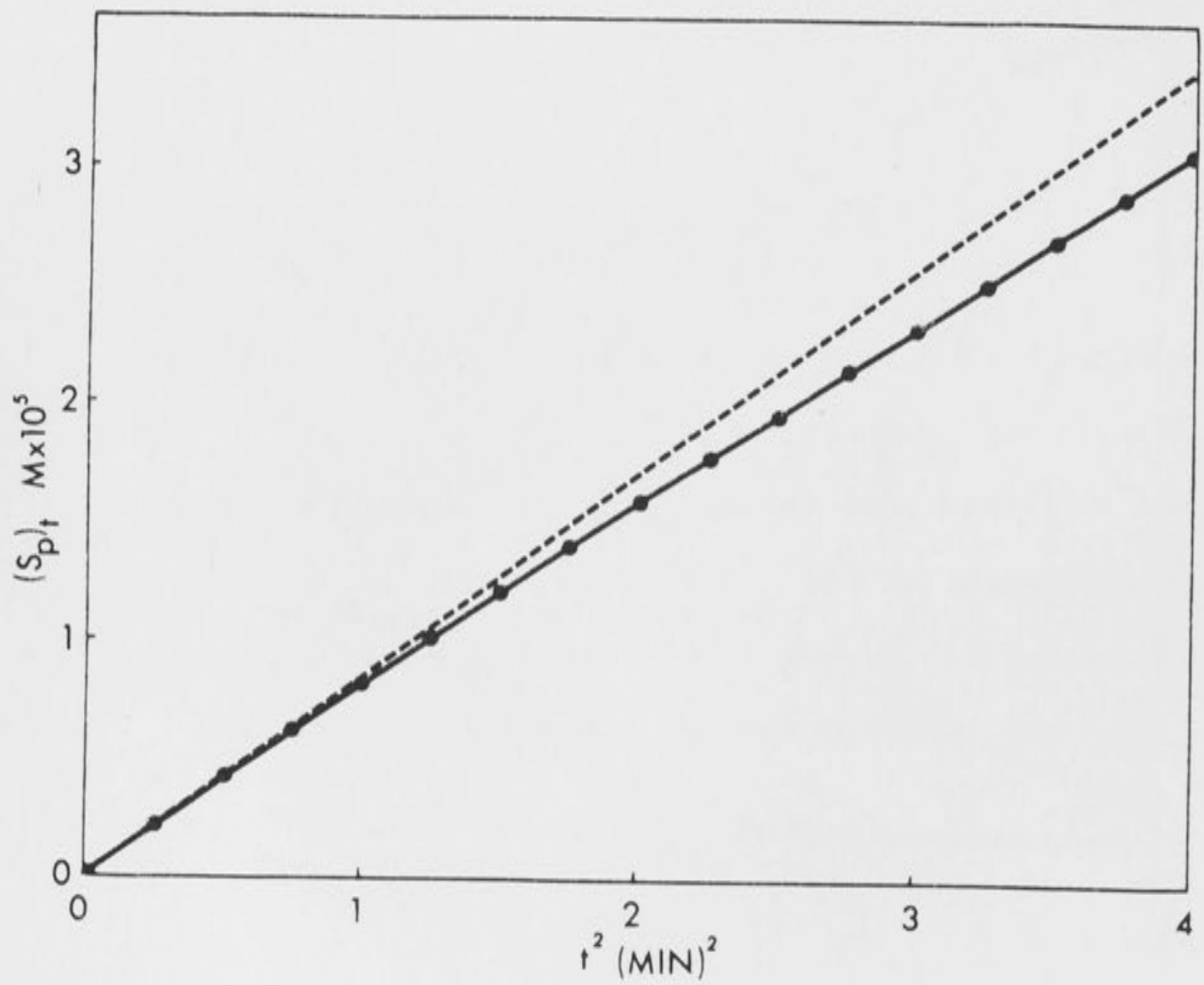

$(S_p)_t$ M×10^5
3
2
1
0
0
1
2
3
4
t^2 (MIN)^2

Figure II - 5

A plot of final product concentration (Figure II-4) versus the corresponding value of time squared. The points were calculated from Figure (II-4) and the solid line is an attempt to average these data. The broken line is the limiting tangent drawn as $t^2 \rightarrow o$.

that in practice values of m may be determined for a range of selected values of $[S_1]_o$ at fixed values of $[E_1]_o$ and $[E_2]_o$, whereupon values of the constants describing the form of the rectangular hyperbola obtained by plotting m versus $[S_1]_o$ may readily be obtained. For example a plot of 1/m versus $1/[S_1]_o$ would be linear with abscissa intercept $-1/K_1$ and ordinate intercept $K_2/2V_1V_2$. It follows that separate studies on E_2 alone, which is possible since S_p may be monitored, would yield values of V_2 and K_2 and hence the entire set of steady-state kinetic parameters.

It is noted that this procedure differs from the conventional determination and use of initial velocities which are inapplicable in the present context, since,

$$\lim_{t \to o} \quad \frac{d[S_p]_t}{dt} = o$$

It is also of interest to note that while $[S_2]_t$ is not in a steady-state over the time period used to evaluate m (Figure II-4) that this does not invalidate the assumption that $d[E_2S_2]_t/dt \simeq o$ since $[S_2]_t = 10^{-4}M$ at 1 minute in the example shown in Figure (II-5), which is 10^5 times the magnitude $[E_1]_o$.

(d) Correlation with previously described methods

Several workers (Easterby, 1973; McClure, 1969; Barwell and Hess, 1970; Bergmeyer, 1953) have examined the type of coupled enzyme system discussed above for the particular case of a large excess of second enzyme E_2. These works are typified by the recent formulation given

by Easterby (1973), which therefore suffices to illustrate this type of approach. Again the mass conservation condition is employed, which in differentiated form may be written as,

$$- \frac{d[S_1]_t}{dt} = \frac{d[S_2]_t}{dt} + \frac{d[S_p]_t}{dt} \qquad (II - 16)$$

Easterby (1973), reformulated equation (II-16) in the following way,

$$- \frac{d[S_1]_t}{dt} = v_o = \frac{d[S_2]_t}{dt} + \frac{V_2}{K_2} [S_2]_t \qquad (II - 17)$$

and noted that this equation could only be integrated provided v_o was assumed constant over the time domain of integration. With this assumption and using the integrating factor

$e^{V_2 t/K_2}$, Easterby (1973) in his equation (4) formulated the solution as,

$$[S_p]_t = v_o(t + \frac{K_2}{V_2} e^{-V_2 t/K_2} - \frac{K_2}{V_2}) \qquad (II - 18)$$

It follows that equation (II - 18) is based on two bold assumptions : (1) that v_o is a constant over the time domain of integration which cannot be strictly valid, and (2) that the concentration of S_2 is sufficiently large to permit a steady-state of $[E_2S_2]_t$ (and hence application of the Michaelis-Menten equation for the second enzyme E_2), but sufficiently small ($[S_2]_t << K_2$) to permit $d[S_2]_t/dt$ to be formulated as $V_2[S_2]_t/K_2$. Easterby (1973) now

argued that at relatively long times (strangely termed, $t \to \infty$) the exponential term in equation (II-18) could be neglected to give, for the appropriate time domain,

$$[S_p]_t = v_o(t - \frac{K_2}{V_2}) \qquad \text{(II - 19)}$$

In effect this result expresses the familiar observation that when E_2 is relatively large $d[S_p]_t/dt = -d[S_1]_t/dt$ since $d[S_2]_t/dt$ is effectively zero : but adds the observation that the abscissa intercept of the extrapolated linear portion of a plot of $[S_p]_t$ versus t will have the value K_2/V_2 (termed the transient time).

Of greater interest in relation to the present work is that equation (II-18) expresses $[S_p]_t$ as a function of t in a form which at first sight appears to be quite different from equation (II-14). It might be felt that this difference arises as a consequence of the different assumptions made by Easterby in formulating equation (II-18). However, the interesting point, not noticed by Easterby, emerges that these assumptions may in fact be reasonable for situations other than that involving a large excess of E_2. Thus if reference is made to Figure (II-4) it is seen that, at least at early times, $-(d[S_1]_t/dt)$ does approximate to a constant, that $d[S_2]_t/dt$ does not equal zero, and that $[S_2]_t$ is less than K_2, but as we have seen, sufficiently large to permit a steady-state of $[E_2S_2]_t$. It follows then that equation (II-18) might describe the true dependence of final product formation at early times, provided the exponential term is retained. That this is the case may be shown by expanding the exponential term as

an infinite series to give,

$$[S_p]_t = v_o \left\{ t + \frac{K_2}{V_2} \left[1 + \left(\frac{-V_2 t}{K_2} \right) + \left(\frac{-V_2 t}{K_2} \right)^2 \frac{1}{2} + \ldots \right.\right.$$

$$\left.\left. + \left(\frac{-V_2 t}{K_2} \right)^n \frac{1}{n!} + \ldots \right] - \frac{K_2}{V_2} \right\} \qquad \text{(II - 20)}$$

which in the limit as $t \to o$ is equation (II-15), where v_o has been replaced by the Michaelis-Menten expression.

While a correlation has been made between the Maclaurin polynomial approach and that described by Easterby (1973), it is reasserted that the Maclaurin polynomial approach is in fact more general for two reasons. Firstly, it is clear that equation (II-19) can only be valid over the time domain for which the assumptions implicit in equation (II-18) hold, whereas equation (II-20) is not so restricted. In other words both approaches give the same result as $t \to o$, but only the Maclaurin polynomial approach generates higher order terms which are likely to be valid at longer times. Secondly, whereas the approach by Easterby is readily applicable to a system of two enzymes acting consecutively it is not readily extended to more complicated systems. That such extensions may readily be made using the Maclaurin polynomial approach will become apparent in the next Section, Appendix I and Chapter IV.

2. <u>The general case of several enzymes of the Michaelis-Menten type acting consecutively</u>.

The basic set of differential rate equations for a system involving more than two enzymes of the simple Michaelis-Menten type may simply be written as an extension

of equation (II-2),

$$\frac{d[S_1]_t}{dt} = -\frac{V_1[S_1]_t}{(K_1+[S_1]_t)} \qquad \text{(II - 21a)}$$

$$\frac{d[S_i]_t}{dt} = +\frac{V_{i-1}\,[S_{i-1}]_t}{(K_{i-1}+[S_{i-1}]_t)} - \frac{V_i\,[S_i]_t}{(K_i+[S_i]_t)} \qquad \text{(II - 21b)}$$

$$\frac{d[S_p]_t}{dt} = +\frac{V_{p-1}\,[S_{p-1}]_t}{([S_{p-1}]_t + K_{p-1})} \qquad \text{(II - 21c)}$$

where (i = 1,2, ..., p-1). This set of equations may be used directly and in differential form, in conjunction with equation (II-9) to yield, as before, expressions for each $[S_i]_t$ and hence $[S_p]_t$ as a function of time. From an array of such expressions for $[S_p]_t$ (found with p = 3, 4 and 5) it was induced that the general expression for the first non-zero terms of the Maclaurin polynomials expressing $[S_p]_t$ as a function of time was given by,

$$\left\{ \prod_{k=2}^{p-1} \frac{V_k}{K_k} \right\} \frac{V_1[S_1]_o \; t^{p-1}}{(K_1+[S_1]_o)\,(p-1)!} \qquad \text{(II - 22a)}$$

and that rigorously,

$$\lim_{t\to o} \left(\frac{[S_p]_t}{t^{p-1}} \right) = \left\{ \prod_{k=2}^{p-1} \frac{V_k}{K_k} \right\} \frac{V_1[S_1]_o}{(K_1+[S_1]_o)\,(p-1)!}$$

(II - 22b)

It is evident that equation (II-22b) becomes equation (II-15) when p = 3.

Equation (II-22b) suggests that experimentally available results of $[S_p]_t$ versus time be plotted in the form $[S_p]_t$ versus t^{p-1} where (p-1) is the number of enzymes present. It would then be possible to plot the slopes of the limiting tangents so obtained against $[S_1]_o$ to obtain a rectangular hyperbola which could be used to define K_1 and the product of V_1 and the scaling factor $\left\{\prod_{k=2}^{p-1} \frac{V_k}{K_k}\right\}$. Again it is clear that the production of S_p depends upon the characteristic kinetic parameters of all enzymes present and that those of the succeeding enzymes (in terms of a single lumped parameter if desired) must be evaluated in separate studies if V_1 is to be uniquely determined. Some comment on the conventional method of determining slopes of tangents to the curves $[S_p]_t$ versus t seems appropriate. Clearly as $t \to o$ the slope so determined would also be zero, but if the slope were determined at a time in the domain of t over which the Maclaurin polynomial is well approximated by its first term, then the slope would equal t^{p-2} multiplied by the right hand side of equation (II-22b). The one reservation in the application of equation (II-22b) is that all enzyme-substrate complexes be in an approximate steady-state, a condition more difficult to achieve as the value of p increases.

In the formulation of (II-22) it was suggested that expressions for all $[S_i]_t$ based on equation (II-9) be written, and the expression for $[S_p]_t$ obtained by applying

the conservation of mass condition. This implies that all $[S_i]_o^{<n>}$ must be separately evaluated, which is indeed a laborious procedure. It is timely therefore to introduce a simpler formulation of $[S_p]_t$ based on equation (II-9) which in fact will be demonstrated to be applicable to more complicated mechanisms which are the concern of the rest of the Chapter. Direct application of equation (II-9) written for $[S_p]_t$ gives

$$[S_p]_t = [S_p]_o + [S_p]_o^{<1>} t + [S_p]_o^{<2>} \frac{t^2}{2} + \ldots + [S_p]_o^{<n>} \frac{t^n}{n!} + . \qquad \text{(II - 23)}$$

Since $[S_p]_t$ at t = o equals zero, and from equation (II-21c) $d[S_p]_t/dt$ is a function only of $[S_{p-1}]_t$ which also equals zero at t = o, equation (II-23) may be written as,

$$[S_p]_t = \sum_{n=2}^{n} [S_p]_o^{<n>} \frac{t^n}{n!} \qquad \text{(II - 24)}$$

It also follows from the chain rule of differentiation, and $[S_i]_t$ (i = 2,3,..., p) at t = o are all zero, that for n < p,

$$[S_p]_o^{<n>} = \left\{ \frac{d[S_{p-n+1}]_t}{dt} \right\}_o \left\{ \prod_{j=p-n+2}^{j=p} \frac{d\{d[S_j]_t/dt\}}{d[S_{j-1}]_t} \right\}_o \qquad \text{(II - 25)}$$

Since all $d[S_{p-n+1}]_t/dt$ = o at t = o except $d[S_1]_t/dt$ and $d[S_2]_t/dt$, it follows that for all n < p-1, $[S_p]_o^{<n>}$ = o. This explains why equation (II-14) commences at the t^2 term when p = 2, and why in general the series will commence at

the t^{p-1} term for all values of p. It also follows from equation (II-25) that for the case of two enzymes acting consecutively that

$$\lim_{t\to o} \frac{[S_p]_t}{t^2} = \frac{1}{2}\left\{\frac{d[S_2]_t}{dt}\right\}_o \left\{\frac{d\{d[S_2]_t/dt\}}{d[S_2]_t}\right\}_o = m \quad \text{(II - 26)}$$

The ease of formulating equation (II-15) by this more general approach is now apparent since $(d[S_2]_t/dt)_o$ follows directly from equation (II-2b) to be $V_1[S_1]_o/(K_1+[S_1]_o)$ and the second derivative in equation (II-25) follows directly from equation (II-2c) to be V_2/K_2.

3. Reversible product formation and product inhibition of Michaelis-Menten enzymes.

Thus far in this work only mechanisms of the irreversible Michaelis-Menten type have been considered and no inhibitory effects have been discussed. Since these are the major complications which might arise in the study of Michaelis-Menten enzymes in sequences, they will be explicitly considered in relation to the particular case p = 3. First, for reversible reactions of the type $E + S \rightleftharpoons ES \rightleftharpoons E + P$ we may write

$$\frac{d[S_1]_t}{dt} = -\frac{V_1[S_1]_t}{(K_1+[S_1]_t)} + \frac{V_1^R[S_2]_t}{(K_1^R+[S_2]_t)} \quad \text{(II - 27a)}$$

$$\frac{d[S_2]_t}{dt} = +\frac{V_1[S_1]_t}{(K_1+[S_1]_t)} - \frac{V_1^R[S_2]_t}{(K_1^R+[S_2]_t)} - \frac{V_2[S_2]_t}{(K_2+[S_2]_t)} + \frac{V_2^R[S_p]_t}{(K_2^R+[S_p]_t)} \quad \text{(II - 27b)}$$

$$\frac{d[S_p]_t}{dt} = +\frac{V_2[S_2]_t}{(K_2+[S_2]_t)} - \frac{V_2^R[S_p]_t}{(K_2^R+[S_p]_t)} \quad \text{(II - 27c)}$$

where the superscript R refers to the reverse reaction of product formation. Clearly the experimenter has the choice of initiating the reaction with either S_1 or S_p. Since the situation is symmetrical only one choice need be considered and it is taken that the only reactant present at t = o is S_1. It follows directly from equation (II-27c) that $[S_p]_o$ <1> is zero and therefore that the general equation (II-26) applies directly. From equation (II-27b), $\{d[S_2]_t/dt\}_o$ equals $V_1[S_1]_o/(K_1+[S_1]_o)$ and from equation (II-27c) the second derivative in equation (II-26) equals V_2/K_2 since $(d[S_p]_t/d[S_2]_t)$ is zero. This means that the slope, m, of the tangent to the curve, S_p versus t^2 is as given in equation (II-15), the complication of the reverse reactions being eliminated as $t \to o$. Therefore, in this study the experimenter could evaluate K_1 and V_1V_2/K_2, whereas, if varying concentrations of $[S_p]_o$ were employed in a set of experiments as the starting condition, the quantities K_2^R and $V_1^RV_2^R/K_1^R$ would become available, provided S_1 could be assayed. The solution would be complete in terms of the individual values for the eight constants if separate studies were done on the two enzymes using S_2 as the substrate in each case. While this may seem an unlikely system to study by means of a coupled assay the point of elaborating it was in fact to stress the consistency of the Maclaurin polynomial approach and the generality of equation (II-26).

It could also be noted that it is possible to generate higher order terms in the power series expressing $[S_p]_t$ as a function of time, which in contrast to those in equation (II-14) would contain parameters pertaining to

both the forward and reverse reactions. Since the procedure for obtaining such higher order terms has been amply demonstrated, it seems unnecessary to elaborate for each individual mechanism examined : it is of greater interest to examine alternate mechanisms in terms of the analogues to equation (II-15) when $t \to o$, and no direct consideration of the reversibility of product formation need be given.

In relation to inhibitory effects there are of course several types of possible inhibition, which may occur in a coupled assay, but it suffices in illustrating the application of equation (II-26) to consider only one type and thus, linear competitive inhibition of E_1 by S_2 is considered in an enzyme couple as described by,

$$\frac{d[S_1]_t}{dt} = - \frac{V_1[S_1]_t}{K_1\left(1+\frac{[S_2]_t}{K_I}\right) + [S_1]_t} \qquad \text{(II - 28a)}$$

$$\frac{d[S_2]_t}{dt} = + \frac{V_1[S_1]_t}{K_1\left(1+\frac{[S_2]_t}{K_I}\right) + [S_1]_t} - \frac{V_2[S_2]_t}{(K_2+[S_2]_t)} \qquad \text{(II - 28b)}$$

$$\frac{d[S_p]_t}{dt} = + \frac{V_2[S_2]_t}{(K_2 + [S_2]_t)} \qquad \text{(II - 28c)}$$

where K_I denotes the inhibition constant. Application of equation (II-26) to this set of differential equations leads again directly to equation (II-15) showing in accordance with expectation that the inhibition reaction fails to manifest itself in studies performed as $t \to o$. There are however cases when all $[S_i]_o$ do not equal zero and equation (II-15) does not apply. One example arises when an experimenter studying the type of system specified

by equation (II-28) introduces both S_1 and S_2 at time $t = o$, in an attempt to study explicitly the competitive inhibition of E_1 by S_2. In this case the conservation of mass condition must be formulated as,

$$[S_1]_o + [S_2]_o = [S_1]_t + [S_2]_t + [S_p]_t \qquad \text{(II - 29)}$$

whereupon application of equation (II-28), (II-29) and (II-9) yields,

$$[S_p]_t = \frac{V_2[S_2]_o t}{(K_2+[S_2]_o)} + \frac{V_2K_2}{(K_2+[S_2]_o)^2}\left\{\frac{V_1[S_1]_o}{K_1\left(1+\frac{[S_2]_o}{K_I}\right)+[S_1]_o} - \frac{V_2[S_2]_o}{(K_2+[S_2]_o)}\right\}\frac{t^2}{2} + \cdots \qquad \text{(II - 30)}$$

It will be noted that the first term in this series is in t, not t^2 as would be predicted from equation (II-26) which is applicable only when all $[S_i]_o$ ($i = 2, \ldots, p$) equal zero. Accordingly, in competitive inhibition studies a plot of $[S_p]_t$ versus t^2 is not appropriate since division of equation (II-30) by t^2 leads to a term in $\frac{1}{t}$ which is undefined in the limit as $t \to o$. It is however possible, from the experimental data to evaluate for each t, the first term on the right hand side of equation (II-30), provided V_2 and K_2 have been evaluated in separate studies on E_2. This permits construction of a plot of $[S_p]_t - V_2[S_2]_o\, t/(K_2+[S_2]_o)$ versus t^2. From the slope of the limiting tangent as $t \to o$ of the latter plot, values of $d[S_1]_t/dt$ (equation II-28a) may readily be obtained, and hence, from a set of experiments conducted with $[S_1]_o$ held constant and $[S_2]_o$ varied (or vice versa), the kinetic

parameters of E_1 (including the inhibition constant) could be evaluated in the conventional way (Dixon and Webb, 1958; Laidler and Bunting, 1973).

4. Coupled systems involving control enzymes.

Equations (II-2), (II-21), (II-27) and (II-28) have all been based on systems in which each individual enzyme if studied alone, would have led to a plot of initial velocity versus initial substrate concentration, of the form of a rectangular hyperbola. It is well known, however, from numerous studies on particular enzymes, that such plots may deviate from this form, the deviations frequently being associated with metabolic control effects (e.g. Stadtman, 1970). The deviations themselves may be of different forms, and those associated with negative cooperativity effects (Koshland and Neet, 1968) will be considered in Chapter V : for the present, attention is focused on the particular type of deviation which is manifested as a sigmoidal plot of initial velocity versus initial substrate concentration. The sigmoidal response has been described in various ways as: (1) a positive homotropic effect (Monod, Wyman and Changeux, 1965); (2) as one conforming to the requirement that $R_S < 81$ where R_S is the substrate concentration for which the initial velocity is 90% its maximal value, divided by that for which the initial velocity is 10% of its maximal velocity (Koshland, Némethy and Filmer, 1966); (3) as being associated with Scatchard (1949) plots exhibiting a maximum; and (4) as being associated with a double reciprocal plot exhibiting upward curvature (e.g. Nichol and Winzor,

1972). Likewise there have been various mechanisms proposed to explain the sigmoidal effect, and it is relevant now to discuss these briefly in order to formulate a basic rate equation which may be examined in relation to the application of the Maclaurin polynomial to systems where control enzymes act consecutively.

(a) A review of simple equations used to describe sigmoidal responses

One group of workers has approached the problem of the study of control enzymes by formulating binding equations which were then adapted to the kinetic situation. In relation to binding equations, two basic models have been proposed. The first involves preferential binding of ligand to one state of acceptor coexisting in equilibrium with at least one other state: the states may be isomeric (Monod, Wyman and Changeux, 1965); polymeric (Nichol, Jackson and Winzor, 1967; Frieden and Colman, 1967) or involve an enzyme bound and unbound to a cell-membrane structure (Masters, Sheedy, Winzor and Nichol, 1969). The treatment of this model may be exemplified by a system in which a monomeric form of the enzyme, A, coexists in equilibrium with its polymeric form C, and substrate S binds to p equivalent and independent sites on A with intrinsic association constant (Klotz, 1946), K_A and to q such sites on C, with intrinsic binding constant K_C. Nichol, Jackson and Winzor (1967) showed by simple application of the law of mass action that the binding function r_M, the moles of ligand bound per base-mole of acceptor, was related to the concentration of unbound substrate [S] by,

$$r_M = \frac{pK_A[A][S](1+K_A[S])^{p-1}+qK_C[C][S](1+K_C[S])^{q-1}}{[A](1+K_A[S])^p + n[C](1+K_C[S])^q}$$

(II - 31)

They noted that when $q = np$ and $K_A = K_C$ equation (II-31) simplified to the form of a rectangular hyperbola, thereby introducing the necessity of preferential binding to obtain a sigmoidal response. There is some experimental justification for this preferential binding model in that addition of certain competitive inhibitors to polymerizing enzyme systems has led to perturbation of the equilibrium in favour of one polymeric constituent (Frieden and Colman, 1967; Sophianopoulos, 1969; Howlett and Nichol, 1972b; Nichol, Jackson and Winzor, 1972). It is also clear that when $n = 1$, equation (II-31) becomes equation (2) of Monod, Wyman and Changeux (1965) who considered the special case of an isomerizing acceptor. The essential features of the sigmoidal response predicted by equation (II-31) can indeed be illustrated by rewriting equation (II-31) with $n = 1$, and the isomerization constant $X = [C]/[A]$, for the particular case that the isomer C is inactive (q and/or $K_C = o$),

$$r_M = \frac{pK_A[S](1+K_A[S])^{p-1}}{(1+K_A[S])^p + X} \qquad \text{(II - 32)}$$

First, it is noted that when $p = 1$ a plot of r_M versus $[S]$ assumes the form of a rectangular hyperbola even though an inactive isomer is present (Wyman, 1964). Secondly provided $p > 1$ this plot must deviate from the form of a rectangular hyperbola, the deviation (in the form of

sigmoidality) increasing with increasing values of X. Thirdly, it is clear that such plots will be independent of the value of the total acceptor concentration chosen, whereas for the polymerizing systems a concentration dependence is predicted, since in equation (II-31) r_M is an explicit function of [A] and [C]. It is a simple matter to transform an equation such as (II-32) to a corresponding kinetic equation by utilizing a relation first suggested by Frieden that $v/V = r_M/p$ where v is the initial velocity of the reaction and V the maximal velocity. Thus

$$v = \frac{VK_A[S](1+K_A[S])^{p-1}}{(1+K_A[S])^p + X} \qquad (II - 33)$$

There are assumptions implicit in writing equation (II-33) which were highlighted by Dalziel (1968). He considered a model exhibiting the essential features of that under discussion; namely that two isomeric states of the enzyme coexisted in rapid equilibrium, the binding of substrate to these individual forms being to equivalent and independent sites, such that the rate constants for the forward and reverse binding reactions could all be written, with the use of an appropriate statistical factor, in terms of the intrinsic rate constants k_1^A and k_{-1}^A for A. These statistical factors are combinations, as defined in factorial form by Nichol, Smith and Ogston (1969). Dalziel also assumed that the breakdown rate constants for each complex AS_i (i = 1, ..., p) could similarly be written in terms of a single intrinsic rate constant k_2^A. He applied a steady-state treatment (all $d[ES_i]_t/dt = o$) to obtain an expression entirely analogous with equation (II-32)

where V was identified as $pk_2[A]_o$ and K_A equals $k_1^A/(k_{-1}^A + k_2^A)$. In fact, Dalziel (1968) also considered the situation where the isomer C retained catalytic activity, a simple extension which need not be considered in detail in elaborating the general features of the model. The important features which emerge from the work of Dalziel (1968) are that, if an equation such as (II-32) is to be employed it must be recognized that: (1) the rate determining steps are assumed to be the breakdown of the AS_i complexes; (2) the use of intrinsic rate constants implies independence of sites both with regard to binding and breakdown; and (3) that the symbol K_A should properly be considered as the reciprocal of the conventional compound Michaelis constant rather than an equilibrium association constant. There is a distinct analogy between the extension made by Briggs and Haldane (1925) to the Michaelis and Menten (1913) derivation, and that made by Dalziel (1968) in elaborating on the Monod, Wyman and Changeux (1965) model, with the exception that Dalziel handled the multi active site problem in terms of intrinsic breakdown rate constants.

It is now timely to discuss the second basic model which has been used to explain sigmoidal binding or kinetic plots. Basically it involves a single form of acceptor in which the binding sites are dependent (mutually interacting) via a protein conformational change induced by ligand binding. Consider for example an acceptor A possessing two binding sites such that, according to Klotz (1953) notation, two 1:1 complexes may form, S-A- and -A-S. If the thermodynamic relation is written that [S-A-] = [-A-S] =

$K_1[A][S]$, then the assumption has been made that the two sites are equivalent, and therefore the K_A is an *intrinsic* binding constant. The complex S-A-S may form by two pathways, but as the final equilibrium concentration of this complex is independent of its pathway of formation, $[S\text{-}A\text{-}S] = K_1^2[A][S]^2$ (if the sites are not only equivalent but also independent) or $K_1K_2[A][S]^2$ (if the sites are equivalent and dependent). The former formulation was implicit in the derivation of the individual terms in the numerator and denominator of equation (II-31). The latter leads to the binding equation,

$$r_M = \frac{2K_1[S] + 2K_1K_2[S]^2}{1 + 2K_1[S] + K_1K_2[S]^2} \qquad \text{(II - 34)}$$

This type of formulation was originally used by Adair (1925) in discussing the binding of oxygen to haemoglobin, and may be readily extended to yield, for p equivalent and dependent sites,

$$r_M = \frac{\sum_{i=1}^{p} i\,[S]^i \binom{p}{i} \prod_{j=1}^{i} K_j}{1 + \sum_{i=1}^{p} [S]^i \binom{p}{i} \prod_{j=1}^{p} K_j} \qquad \text{(II - 35)}$$

where the K_j are the relevant binding constants. More recently Koshland, Némethy and Filmer (1966), Vassent (1974) and Ricard, Mouttet and Nari (1974) have adopted similar approaches, but have attempted to ascribe more detailed meaning to the thermodynamic parameters K_1 and K_2. For example, Koshland, Némethy and Filmer (1966) wrote for a

two site model,

$$r_M = \frac{2K_{XY}\ K_S\ K_T[S] + 2\ K_{XY}\ K_S^{\ 2}\ K_T^{\ 2}[S]^2}{1 + 2K_{XY}\ K_S\ K_T[S] + K_{YY}\ K_S^{\ 2}\ K_T^{\ 2}[S]^2} \qquad \text{(II - 36)}$$

where the product $K_S\ K_T$ was visualized as describing the binding of S to an isolated subunit comprising the oligomeric structure X, and the two "interaction constants" K_{XY} and K_{YY} were taken to reflect the respective stabilities of the interaction of the two forms of subunit X and Y, relative to the X - X interaction. The formal similarity between equations (II-34) and (II-36) is evident in that both are ratios of polynomials of the same degree in S. Moreover a similarity between these equations, and that formulated by Monod, Wyman and Changeux (1965), equation (II-31) with n = 1, is also obvious. Thus if the latter equation is written for the two site model, p = q = 2, it becomes,

$$r_M = \frac{2(K_A+XK_C)[S] \ + \ 2(K_A^{\ 2}+XK_C^{\ 2})\ [S]^2}{(1+X) + 2(K_A+XK_C)[S] + (K_A^{\ 2}+XK_C^{\ 2})\ [S]^2} \qquad \text{(II - 37)}$$

This is again a ratio of polynomials of the same degree, as observed in equations (II-34) and (II-36), and only the *ascribed* meanings of the constant coefficients differ: evidently the same conclusion would be reached if equation (II-32) were likewise expanded. It follows that thermodynamically obtained binding data cannot be used to distinguish between the alternate models, and that recourse must be made to experiments of different types reviewed by

Kirschner (1968). It is true that equations (II-34), (II-35) or (II-36) may, unlike equations (II-31) and (II-32), predict a negatively cooperative response, characterized by a double reciprocal plot exhibiting downward curvature, but it is noted in this connection such plots can be obtained in other situations, for example, when a rapidly established equilibrium does not exist between the two states A and C, or when either or both states possess non-equivalent but independent binding sites (Nichol and Winzor, 1972).

To this point it is clear that a selection of an equation such as equation (II-33) would be appropriate to the examination of a coupled system; for it typifies the form of a ratio of polynomials which is characteristic of all the models proposed. It is pertinent therefore to note that there may be a third explanation of a sigmoidal kinetic response which cannot be deduced from the consideration of binding. This third explanation involves consideration of systems typified by a situation in which a single state of an enzyme binds substrate at equivalent and independent sites and the breakdown of complexes so formed are governed by rate constants which cannot be formulated in terms of single intrinsic breakdown rate constants. This type of problem has been considered by various workers such as Sweeney and Fisher (1968) and Ricard, Mouttet and Nari (1974) who have derived kinetic equations which again involve ratios of polynomials in [S]. It may therefore be concluded, that while several different types of model pertain to the explanation of a sigmoidal

plot of r_M versus [S], and while it is impossible to utilize a single equation which encompasses them all, and accounts for all possible combinations of equivalence and non-equivalence, dependence and non-dependence, of both binding and breakdown constants, and for all possible states of an enzyme, that there is considerable justification in exploring the effect of a control enzyme in a coupled assay by choosing a particular equation which involves a ratio of polynomials. For simplicity in what follows, equation (II-33) is selected. It at least has the virtue that the parameters contained therein have been explicitly defined; X, as a thermodynamic isomerization constant, p as the number of active sites, and K_A as an intrinsic compound rate constant.

(b) <u>The application of Maclaurin polynomials to an analysis of the consecutive operation of control enzymes</u>

Based on equation (II-33), the general array of differential equations describing the rate of change of concentration of initial substrate, intermediate compounds, and final product in a linear sequence of catalyzed reactions may be written,

$$\frac{d[S_1]_t}{dt} = - \frac{V_1 K_1 [S_1]_t (1+K_1[S_1]_t)^{(y_1-1)}}{(1+K_1[S_1]_t)^{y_1} + X_1} \qquad \text{(II - 38a)}$$

$$- \quad - \quad -$$

$$\frac{d[S_i]_t}{dt} = + \frac{V_{i-1} K_{i-1} [S_{i-1}]_t (1+K_{i-1}[S_{i-1}]_t)^{(y_{i-1}-1)}}{(1+K_{i-1}[S_{i-1}]_t)^{y_{i-1}} + X_{i-1}}$$

$$- \frac{V_i K_i [S_i]_t (1+K_i[S_i]_t^{(y_i-1)}}{(1+K_i[S_i]_t)^{y_i} + X_i} \qquad \text{(II - 38b)}$$

$$\frac{d[S_p]_t}{dt} = + \frac{V_{p-1}K_{p-1}[S_{p-1}]_t(1+K_{p-1}[S_{p-1}]_t)^{(y_{p-1}-1)}}{(1+K_{p-1}[S_{p-1}]_t)^{y_{p-1}} + X_{p-1}} \qquad \text{(II - 38c)}$$

First, it is noted that equation (II-38a) may be integrated by separation of the variables to yield,

$$- V_1K_1t = K_1\{[S_1]_t - [S_1]_o\} + (1+X_1)\ln\left(\frac{[S_1]_t}{[S_1]_o}\right)$$

$$- \left\{X_1 \ln\left(\frac{1 + K_1[S_1]_t}{1 + K_1[S_1]_o}\right)\right\}$$

$$Y_1 \geqslant 2$$

$$+ X_1 \sum_{k=1}^{y_1-2} \frac{1}{k}\left\{\frac{1}{(1+K_1[S_1]_t)^k} - \frac{1}{(1+K_1[S_1]_o)^k}\right\}$$

$$Y_1 \geqslant 3$$

(II - 39)

where the first two terms apply when $y_1 = 1$; the first three when $y_1 = 2$; the first four when $y_1 = 3$ and so on. This appears to be the first time such an integrated expression pertaining to a control enzyme has been formulated. While equation (II-39) does not provide an explicit expression for $[S_1]_t$ it does provide a simple means of numerically simulating substrate depletion curves for comparison with those calculated on the basis of a Maclaurin polynomial now to be obtained.

Equation (II-38) which provides a direct means of evaluating the first derivatives $[S_j]_o^{<1>}$ in equation (II-9), contains only terms of the form,

$$V_j K_j [S_j]_t (1+K_j [S_j]_t)^{y_j-1} / \omega_j \qquad \text{(II - 40a)}$$

where $\omega_j = \{(1+K_j [S_j]_t)^{y_j} + X_j\}$, (II - 40b)

and thus expressions for the second and third derivatives involve only the respective terms

$$V_j K_j [S_j]_t^{<1>} (1+K_j [S_j]_t)^{2(y_j-1)} \{1+X_j (1+K_j [S_j]_t)^{-y_j} (1+y_j K_j [S_j]_t)\} / \omega_j^2 \qquad \text{(II - 41a)}$$

and

$$V_j K_j (1+K_j [S_j]_t)^{3(y_j-1)} \{[S_j]_t^{<2>} (1+K_j [S_j]_t+\Theta) - K_j ([S_j]_t^{<1>})^2 (2+\xi)\} / \omega_j^3 \qquad \text{(II - 41b)}$$

where

$$\Theta = X_j (1+K_j [S_j]_t)^{(1-y_j)} (2+y_j K_j [S_j]_t) + X_j^2 (1+y_j K_j [S_j]_t)(1+K_j [S_j]_t)^{(1-2y_j)}$$

$$\xi = - \frac{X_j \{2(y_j-2) - y_j K_j [S_j]_t (y_j+1)\}}{(1+K_j [S_j]_t)^{y_j}} - \frac{X_j^2 (y_j-1)(2+y_j K_j [S_j]_t)}{(1+K_j [S_j]_t)^{2y_j}} \qquad \text{(II - 41c)}$$

Use of these expressions in a successive substitution process leads to,

$$[S_1]_t = [S_1]_o - \frac{V_1 K_1 [S_1]_o (1+K_1 [S_1]_o)^{(y_1-1)} t}{(1+K_1 [S_1]_o)^{y_1} + X_1} + \frac{V_1^2 K_1^2 [S_1]_o (1+K_1 [S_1]_o)^{3(y_1-1)} \{1+X_1 (1+y_1 K_1 [S_1]_o)(1+K_1 [S_1]_o)^{-y_1}\} t^2}{2\{(1+K_1 [S_1]_o)^{y_1} + X_1\}^3} \qquad \text{(II - 41d)}$$

Numerical example has shown that equation (II-41d) which has been truncated after the third term, closely describes initial substrate depletion curves in the early stages of the reaction, calculated using equation (II-39).

Of greater interest in the present connection is the conclusion drawn from an array of such expressions that the first non-zero term in the polynomials of $[S_j]_t$ for $j \geqslant 2$ is given by,

$$\frac{[S_j]_o^{<j-1>}\, t^{(j-1)}}{(j-1)!} = \left\{ \prod_{k=3}^{j} \frac{V_{k-1}K_{k-1}}{1 + X_{k-1}} \right\} \frac{V_1K_1[S_1]_o(1+K_1[S_1]_o)^{y_1-1}\, t^{(j-1)}}{(j-1)!\ \{(1+K_1[S_1]_o)^{y_1} + X_1\}} \qquad (II - 42)$$

This equation also follows directly from equation (II-26) since $d[S_p]_t/dt$ is a function only of $[S_{p-1}]_t$ as is seen from equation (II-38c). For example, in the case of a coupled enzymic assay ($p = 3$) involving two control enzymes of the type specified, equation (II-42) may be used to write an expression for the concentration of final product

$$[S_p]_t = \frac{V_2K_2V_1K_1[S_1]_o(1+K_1[S_1]_o)^{(y_1-1)}\, t^2}{2(1+X_2)\ \{(1+K_1[S_1]_o)^{y_1} + X_1\}} \qquad (II - 43)$$

Although equation (II-43) is an approximation, it follows that,

$$\lim_{t\to o} \frac{[S_p]_t}{(t^2)} = \frac{V_2K_2V_1K_1[S_1]_o(1+K_1[S_1]_o)^{(y_1-1)}}{2(1+X_2)\ \{(1+K_1[S_1]_o)^{y_1} + X_1\}} = m \qquad (II - 44)$$

It is evident that when $X_1 = X_2 = o$ equation (II-44) becomes equation (II-15). This emphasizes the additional advantage of employing equation (II-33) as representative of rate

equations involving ratios of polynomials, in that by suitable selection of isomerization constants to equal zero various juxtapositions of control and Michaelis-Menten enzymes in the pathway may readily be examined. In relation to the use of equation (II-44), two cases are of particular interest. The first involves placing $X_1 = 0$ and $X_2 \neq 0$, which specifies that the first enzyme is of the Michaelis-Menten type while the second is a control enzyme. Equation (II-44) simplifies to the form of a rectangular hyperbola and from the corresponding linear double-reciprocal plot, values of $-K_1$ and $2(1+X_2)/V_2K_2V_1$ may be obtained from the abscissa and ordinate intercepts, respectively.

Independent studies with the second enzyme offer the opportunity of fitting the associated sigmoidal kinetic curve with values of the parameters X_2, V_2 and K_2. This could proceed in a manner analogous to that previously employed to obtain binding parameters relevant to a system (aspartate transcarbamylase-succinate) which exhibits the essential features of the model chosen to form equation (II-38) (Changeux and Rubin, 1968; Nichol, Smith and Winzor, 1969). It follows that values of V_1 and K_1 may be estimated. The second case of interest involves placing $X_1 \neq 0$ and $X_2 = 0$, specifying that a reaction catalyzed by a control enzyme is being monitored with the use of an enzyme of the Michaelis-Menten type. In this case equation (II-44) describes a sigmoidal curve ($y_1 > 1$ and X_1 large), which with independent knowledge of V_2 and K_2, permits application of the abovementioned fitting procedures to estimate X_1, V_1 and K_1.

5. Discussion.

No attempt has been made in this Chapter to be encyclopaedic with regard to the types of mechanisms which are encountered in enzyme catalysis, nor with regard to the possible complications such as enzyme-enzyme interaction which might be encountered in conducting a coupled assay : certain of these aspects are discussed in later Chapters. The basic aim of this Chapter was to introduce methods by which analytical expressions could be formulated for the time dependence of all major reactants in a linear sequence of enzyme catalyzed reactions. Of the various means examined, all led to a consistent result but application of the Maclaurin polynomial proved most tractable. Thus the application of Maclaurin polynomials led to a two dimensional array of expressions.

In the horizontal dimension, it has been shown, by equations (II-12), (II-13) and (II-14), for example, that it is possible to generate higher degree terms in t which may be useful in attempting to interpret experimentally observed concentrations of species at relatively long times. In this connection, it is noted that it would not be impossible to generate even higher degree terms than those shown if these were needed. It is however recognized that such terms generated for a particular enzyme model may have limited use in practice since it is in the domain of long times that complications such as pH changes, or protein denaturation may be encountered. Emphasis has therefore been placed,in relation to experimental interpretation, on

information obtained at relatively early times. Thus, by examining the first non-zero term of the array in a vertical dimension it has been possible to arrive at the relatively general expressions given in equations (II-15), (II-22b) and (II-44). In each case these equations suggest that the experimenter should construct a plot of $[S_p]_t$ versus t^{p-1}, which is perhaps an unfamiliar format for workers engaged in initial velocity steady-state kinetic studies. The reason for making the suggestion is that the slope of the limiting tangent to this plot, as $t \to o$, may not only readily be obtained (e.g. Figure II-5) ; but also it is explicitly related to the concentration of initial substrate by an expression which involves the steady-state kinetic parameters for each of the enzymes in the sequence. It is important to note such an analytical expression is not available from particular numerical examples constructed by numerically integrating the basic differential rate equations as is illustrated in Figure (II-3). The use of numerical integrations in interpreting experimental results is not denied; but it is felt that they assume greatest value when used in conjunction with the analytical expression, and such a joint use is illustrated in Chapter IV. The analytical expression for the slope is given by the initial velocity expression for the first enzyme multiplied by an ordinate scaling factor, which involves the kinetic parameters characterizing the succeeding enzymes. For example, from equation (II-15) the ordinate scaling factor is $V_2/2K_2$, where V_2/K_2 is the first order rate constant for E_2. Four points worthy of discussion emerge from this observation.

First, it suggests a simple "rule of thumb" for writing an expression for the slope m: the expression for $-d[S_1]_t/dt$ is written and multiplied by the product of the first order rate (with respect to substrate) constants of the succeeding enzymes and divided by (p-1)! where (p-1) is the number of enzymes in the sequence. Secondly, it becomes clear that the kinetic parameters characterizing the first enzyme may only be uniquely determined if the kinetic parameters of the succeeding enzymes can be evaluated from separate studies, for otherwise only the Michaelis constant for the first enzyme, and a value for the ordinate scaling factor multiplied by V_1 are available. Thirdly, it is implicit that a plot of m versus $[S_1]_o$ must assume the *form* appropriate to the first enzyme in the sequence : in particular, spurious sigmoidality cannot be introduced by monitoring an enzyme of the Michaelis-Menten type with a control enzyme, nor can sigmoidality be totally obscured when the reverse situation is encountered. This may be of some reassurance to workers who are forced to characterize an enzyme of interest by means of a coupled assay. On the other hand, it must be emphasized that such studies are likely to be less accurate than those conducted with an enzyme alone, since errors involved in the evaluation of the ordinate scaling factor are also introduced. Fourthly, it is clear that the expression for m will be valid provided all enzyme substrate complexes are in an approximate steady-state in the early time domain. While this has been shown for a particular numerical example, Figure (II-5), it remains to establish its validity in experimental situations.

This is the subject of Chapter IV which is concerned with the arginase-urease couple. Further consideration is also given in Chapter IV to systems where a large excess of the second enzyme is employed in a coupled assay, ($p = 3$). It appears from Section (II-1.(d)) that the particular development formulated for this case by Easterby (1973) is encompassed by the more general expressions derived in this Chapter since these were based on a Maclaurin polynomial which made no explicit assumption as to the ratio of $[E_1]_o/[E_2]_o$. This tentative conclusion is explored further in Chapter IV, utilizing experimental results presented by Easterby and paying particular attention to the possibility of evaluating m from a plot of $[S_p]_t$ versus t^2. In relation to the major studies to be presented on arginase and urease in Chapter IV, it is noted that it is important not only to evaluate the kinetic parameters for each of these enzymes studied alone, but also to have an understanding of their physico-chemical behaviour in terms of possible dissociation reactions and heterogeneous associations at concentration levels to be used in their coupled assay. This involves application of physico-chemical methods which are discussed in the beginning of the next Chapter, prior to their application to the enzymes under study.

CHAPTER III

ISOLATION AND PHYSICO-CHEMICAL CHARACTERIZATION OF ARGINASE

In this Chapter the evaluation of the characteristic parameters for arginase such as molecular weight, partial specific volume and isoelectric point is discussed. Parameters of this type suffice to characterize a simple non-interacting protein. However, many proteins are known to exist in solution as a mixture of various polymeric states (Nichol, Bethune, Kegeles and Hess, 1964; Frieden, 1971) and thus the characterization would not be complete without investigation of whether such equilibria operated in the arginase system, particularly in environments and at concentration levels where kinetic experiments are to be performed. Also, in relation to the coupled assays to be presented it is important to establish whether complex formation occurs between arginase and urease. The determination of the basic characteristic parameters and the exploration of the possible existence of interactions (both polymerizations and heterogeneous associations) is effected by the application of a range of physico-chemical techniques which can be divided into two broad categories. The first involves mass migration of the solute species under the influence of an applied potential gradient and encompasses the techniques of electrophoresis (Bier, 1962), frontal gel chromatography, (Winzor and Scheraga, 1963) and sedimentation velocity, (Svedberg and Pedersen, 1940). The second category involves the equilibrium methods of sedimentation equilibrium (Fujita, 1962) light scattering, (Stacey, 1956) and osmotic pressure (Kupke, 1960).

This Chapter commences with a review of the basic theory pertaining to the methods which were employed in this study. It emphasizes those aspects relating to the

behaviour of a single non-interacting solute, the situation which pertains to arginase; but includes sufficient theory of interactions to give meaning to the design of experiments used to explore the possibility of such interactions. It is also noted that this background theory will be of use in the discussion of the properties of argininosuccinase to be presented in Chapter V.

1. A review of theory pertaining to the relevant physico-chemical methods.

(a) Mass migration methods

The behaviour in a mass migration experiment of a single or multi-solute system may be described on the basis of continuity equations which express the conservation of mass of the migrating solutes. Such equations have been discussed by many workers, both with regard to their differential form (e.g. Gilbert, 1959; Gilbert and Jenkins, 1959; Nichol, Ogston and Rescigno, 1970), and their integrated forms (e.g. Tiselius, 1930; Johnson, 1946; Nichol and Ogston, 1965a). The differential forms offer the advantage that they permit comment on the shapes of boundaries which arise as a consequence of mass migration, but they are complicated by the necessity to include second order differential terms to account for diffusional spreading. While this aspect will be discussed later, it is noted that certain basic relations relevant to this study emerge more simply by considering first the integrated forms of the basic continuity equations.

(i) Basic continuity equations in integrated form pertaining to single solutes. Consider first a single solute system which comprises a solvent (buffer) and

either an unreacting solute species or a series of polymeric species in rapid equilibrium. In the latter situation the total weight-concentration at each point in the cell is given by $\bar{c}(x) = \sum_{i=1}^{n} c_i(x)$ where c_i is the weight-concentration of each polymer (including monomer) present, and x is the distance from a reference point in the cell. Reference is now made to Figure (III-1) which represents the amount of solute (A), used to fill a cell of unit and uniform cross-sectional area, by the area ABCD, where AD is given by the initial loading concentration $\bar{c}^{o}$ and AB denotes the cell length. After application of an homogeneous potential gradient for a time t, a boundary denoted by XJY is formed between a solvent plateau (β phase) and the original solution plateau (α phase) where the concentration $\bar{c}^{o}$ (= $\bar{c}_A^{\alpha}$) is preserved. Even for a polymerizing system, a single boundary in constituent concentration must arise since equilibrium between the species is considered to be maintained at all points in the cell where the concentration is finite. The form of the boundary depends on several factors including diffusional spreading and chemical interaction, and thus its representation in Figure (III-1) is merely schematic. Regardless of the form of the boundary it is always possible to construct a median bisector IO, such that the area YIJ equals the area JXO. It follows that the area ABCD equals the area IBCO plus the amount of material which has piled up at the cell base, and which is represented by the area BKLC. In other words the statement of mass conservation may be written; the area ABCD equals the area IKLO, or since the area IBCO is common, the area

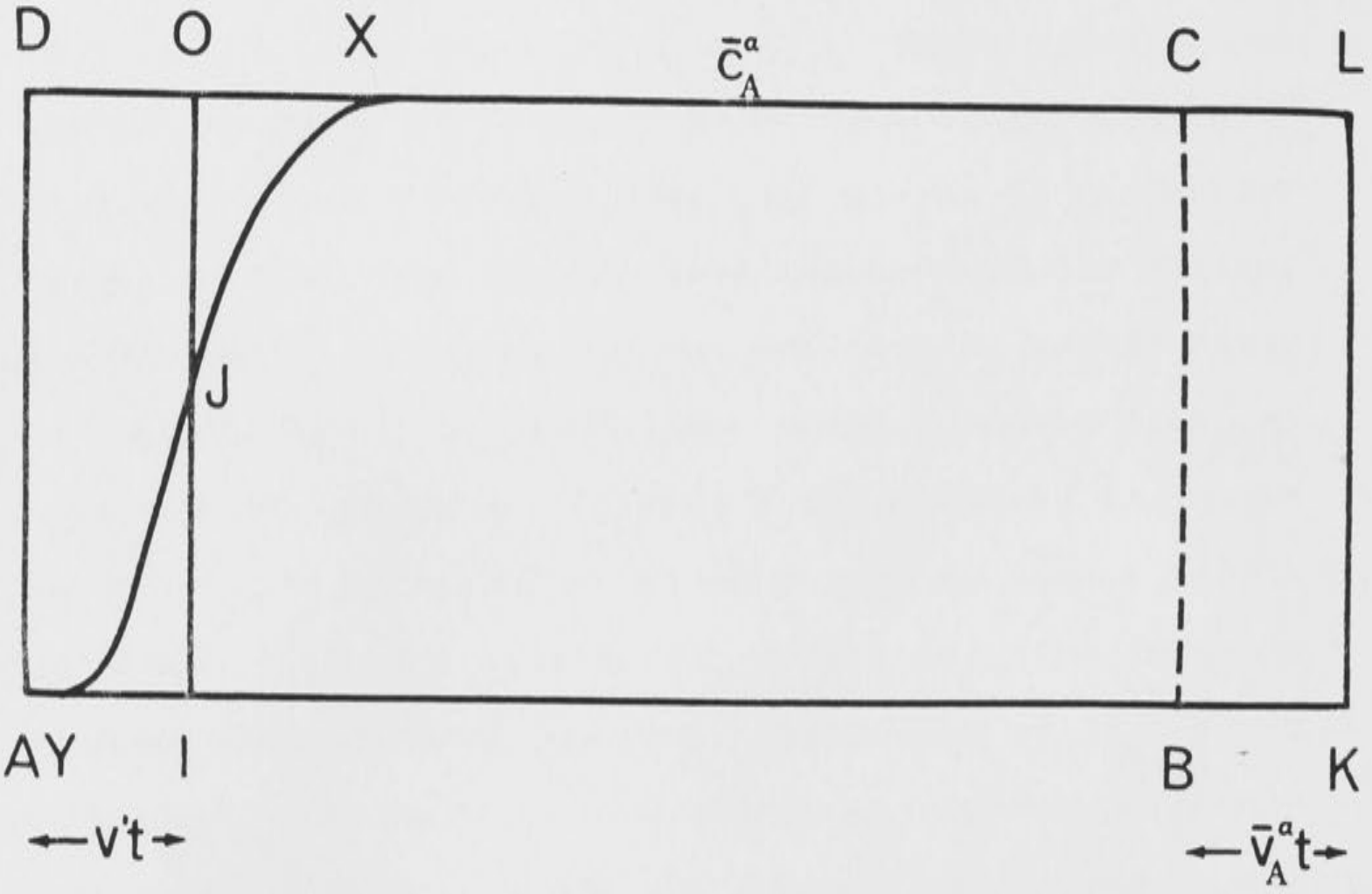


Figure III - 1

A schematic representation of the mass migration of a single solute A in a uniform cell and under the influence of a uniform potential gradient. The original plateau concentration (on a weight basis of A) $\bar{c}_A^{\alpha}$ is represented by the vertical magnitude of AD, and the area ABCD represents the total mass of A in the cell.

AIOD equals the area BKLC. Since AD = IO = KL = $\bar{c}^{o}$ ($=\bar{c}_{A}{}^{\alpha}$) and AI = v't, where v' is the velocity of the median bisector IO, and BK = $\bar{v}_{A}{}^{\alpha}t$, where $\bar{v}_{A}{}^{\alpha}$ is the constituent or weight-average velocity of the solute in the original solution plateau, it follows that,

$$v't\,\bar{c}^{o} = \bar{v}_{A}{}^{\alpha}t\,\bar{c}^{o} \qquad \text{(III - 1a)}$$

$$\text{or,}\quad v' = \bar{v}_{A}{}^{\alpha} = \frac{\sum_{i=1}^{n} v_i c_i}{\sum_{i=1}^{n} c_i} \qquad \text{(III - 1b)}$$

This geometric argument may appear simple, but it leads to the valuable result, which may not be immediately obvious, that the weight-average velocity pertaining to the particular concentration $\bar{c}^{o}$ may be obtained by measuring the velocity of the median bisector of the observable gradient of constituent concentration.

In sedimentation velocity, the situation described in relation to Figure (III-1) basically pertains, with the exception that the applied potential gradient is not uniform, and the cell is not of uniform cross-sectional area, but rather is sector shaped to avoid convective disturbance when migration proceeds along radii extending from the axis of rotation. Goldberg (1953) has shown that the movement of the median bisector (termed the first moment of the boundary) no longer gives the weight-average velocity of solute in the original solution plateau; the correct position is the square root of the second moment. The position, in a boundary, of the square root of the second moment is given by,

$$\bar{x} = \left\{ \frac{\int_{x_X}^{x_Y} x^2 \, \partial c/\partial x \, dx}{\int_{x_X}^{x_Y} \partial c/\partial x \, dx} \right\}^{\frac{1}{2}} \qquad \text{(III - 2a)}$$

where x_X and x_Y denote the positions of onset of the solvent and solution plateaux respectively, and x is a radial distance parameter, with $\partial c/\partial x$ the corresponding gradient of concentration which is measured, in schlieren optics, as the directly proportional quantity, the gradient of refractive index (or the height of the peak above the base line). The weight-average sedimentation coefficient (the velocity per unit gravitational field), is determined by use of the definition,

$$\bar{s} = \frac{d\bar{x}/dt}{\bar{x}\,\omega^2} = \frac{d\ln\bar{x}/dt}{\omega^2} \qquad \text{(III - 2b)}$$

where ω is the angular velocity. It is therefore possible to perform a series of experiments in which the value of $\bar{c}^o$ is varied, and to construct a plot of $\bar{s}$ versus $\bar{c}^o$. In the case of non-polymerizing globular proteins, such plots are frequently linear with negative slope and are described by,

$$s = s_o(1 - kc^o) \qquad \text{(III - 3)}$$

where the bar superscript has been omitted to emphasize that only one oligomeric form is being considered, and k is the coefficient of concentration dependence. It is also noted that in these cases, the observed schlieren boundary is symmetrical (Fujita, 1962) and the position of the maximum ordinate corresponds to the use of $\bar{x}$ in determining

s. This type of concentration dependence arises as a consequence of frictional interactions, and depends on factors such as the viscosity of the medium, the back-flow of solvent, and the density of the solution (Schachman, 1959): in this sense, equation (III-3) must be regarded as empirical. In the case of a polymerizing solute, $\bar{s}$ determined from equation (III-2b) will be a non-linear function of $\bar{c}^o$ and in fact will have an initial positive slope, since by Ostwald's Dilution Law, dilution must favour an increase in the relative proportions of the lower molecular weight species. Indeed from equation (III-1b) it follows that at infinite dilution, $\bar{s}$ equals the sedimentation coefficient of monomer. As in the case of the polymerizing α-chymotrypsin system (Schwert, 1949), this may provide a valuable diagnostic test for the operation of a self interaction provided a concentration range may be investigated where the relative proportions of polymeric species become a sensitive function of total concentration. In this connection it is noted that in high concentration ranges where higher polymeric forms dominate, the continual operation of frictional effects may well lead to a negative slope of the $\bar{s}$ versus $\bar{c}^o$ plot. With conventional schlieren optics the lowest value of $\bar{c}^o$ which may reasonably be measured is 0.1 g dl^{-1} and this evidently is a restrictive condition if it is desired to investigate the possible operation of polymerization equilibria at concentrations generally employed in conducting enzyme kinetic assays: this situation may be improved with the use of absorption optical systems, but not necessarily to the extent that extremely low

concentration ranges may be reliably investigated. In this connection, the method of frontal gel chromatography offers promise.

In frontal gel chromatography a column of uniform cross-sectional area packed with a gel, such as Sephadex, is pre-equilibrated with buffer and a sufficiently large volume of solution is applied to ensure that a plateau of original concentration $\bar{c}^o$ is maintained in the mobile phase of the column (Nichol and Winzor, 1972) and in the elution profile. Once this condition has been ensured the material is eluted with buffer, to obtain a trailing elution profile described by Figure (III-1) where the abscissa now represents elution volume rather than distance. In this case there is no pile up at a cell base, and LK may be regarded as the median bisector of the advancing front which is also observable experimentally. Therefore the geometrical argument used previously is directly applicable to this context, with the area ABCD being the product of the loading volume and $\bar{c}^o$, and it shows that V' determined from the median bisector of the advancing front (or the difference between the median bisector of the trailing front and the loading volume) is a correct measure of the weight-average elution volume.

$$\bar{V} = \left\{ \frac{\int_o^{\bar{c}^o} V dc}{\int_o^{\bar{c}^o} dc} \right\} = \left\{ \frac{\sum_{i=1}^{n} c_i V_i}{\sum_{i=1}^{n} c_i} \right\} \qquad \text{(III - 4)}$$

The first equality of equation (III-4) defines the means by which the median bisector may be determined from the experimental result, while the second equality shows that

$\bar{V}$ is a direct analogue of equation (III-1b) with elution volumes replacing velocities (Gilbert, 1966; Nichol, Ogston and Winzor, 1967). In the case of non-polymerizing globular proteins it has been observed experimentally (Winzor and Nichol, 1965; Gilbert and Kellet, 1971) that $\bar{V}$ is a linear function of $\bar{c}^{o}$ with positive slope, an effect which has recently been ascribed to be osmotic in origin (Edmond, Farquhar, Dunstone and Ogston, 1968; Nichol, Janado and Winzor, 1973), and therefore of little significance in studies performed with extremely low values of $\bar{c}^{o}$. In the case of a polymerizing system, re-equilibration effects following dilution will predominate and the plot of $\bar{V}$ versus $\bar{c}^{o}$ will exhibit upward curvature as $\bar{c}^{o}$ decreases since, again by Ostwald's Dilution Law, lower molecular weight polymeric species with larger elution volumes will predominate. A series of experiments is therefore suggested in which the plateau concentration $\bar{c}^{o}$ is progressively lowered. Two points must be stressed in relation to such experiments. First, it is inappropriate to employ zonal analysis for the study of interacting systems since zones must necessarily dilute on passage through the column and the advantage that a rigorously defined weight-average elution volume which may be related to a corresponding $\bar{c}^{o}$, is lost; indeed Winzor (1966) has shown that the study of polymerizing systems by zonal analysis may well result in an observed elution volume corresponding closely to that of the monomer. Secondly, in the frontal analysis experiments, values of $\bar{c}^{o}$ may be reasonably determined by means of serial dilution even at very low levels, and the experimenter is merely required to determine from the

collected fractions the elution volume at which the protein emerges. This is aided by the observation, that for a polymerizing system the advancing front tends to be hypersharp (Gilbert, 1959; Nichol and Ogston, 1965a) in that chemical re-equilibration effects oppose the tendency of the front to spread by dispersion. Moreover the method of detecting protein in the collected fractions is a matter of choice for the experimenter and bears no relation to the behaviour of the protein on the column. For example, if an enzyme is being studied it would be appropriate to add substrate to each of the collected fractions and thereby determine by enzymic assay the sharply defined elution volume at which the enzyme appears: no explicit assumption is made with regard to the dependence of enzyme activity on concentration, and the $\bar{V}$ obtained is a function of protein concentration only. In this respect it differs from a zonal sedimentation velocity technique which detects the rate of migration of the enzyme by following the product formation via absorption optics, as it passes through its substrate (Cohen and Mire, 1971a,b). The frontal analysis method is exemplified by the work of Nichol and Roy (1965) who showed that arylsulphatase A continued to exist as a monomer of molecular weight 100,000 at pH 7.5 at concentrations as low as 0.00003 g dl^{-1}, but that at pH 5, where it existed as a tetramer in a concentration of 0.1 g dl^{-1}, dilution to the above concentration resulted in dissociation toward the monomer, as seen by an increasing $\bar{V}$ with decreasing $\bar{c}^{o}$.

Finally it is noted that with systems where monomer

coexists in rapid equilibrium with a single higher polymer ($nA \rightleftharpoons C$) the evaluation of $\bar{s}$ or $\bar{V}$ as a function of $\bar{c}^o$ may be used to evaluate the equilibrium constant describing the interaction, ($K = c_C/c_A^n$). Thus combination of this definition with equation (III-1b) gives,

$$K = \frac{\bar{c}^{o(1-n)} \quad (s_A - s_C)^{n-1} \quad (s_A - \bar{s})}{(\bar{s} - s_C)^n} \qquad (III - 5)$$

An entirely analogous expression with elution volumes replacing sedimentation coefficients may be derived on the basis of equation (III-4). In both cases it is required to obtain the sedimentation coefficients or elution volumes of A and C alone which is not a simple procedure since at finite concentration only constituent concentration gradients are observed. Various methods which may be employed including extrapolation of $\bar{V}$ or $\bar{s}$ to infinite dilution, to obtain respectively s_A and V_A and the use of ƶ-average elution volumes (Winzor, Loke and Nichol, 1967) have recently been reviewed (Baghurst, Nichol and Sawyer, 1972). It seems unwarranted to discuss these in detail since equation (III-5) and its elution volume analogue have not been used in this work.

(ii) Integrated continuity equations for a system comprising two solutes. Up to this point consideration has only been given to mass migration experiments in which a single boundary of constituent concentration arises. It is relevant to extend the treatment to systems where two discrete boundaries will be evident in the mass migration process since these encompass the

following situations; (1) a protein migrating in the presence of a macromolecular contaminant with which it does not interact; (2) a system in which a monomer coexists in equilibrium with a single higher polymer and the rate of equilibrium is slow compared with their separation under the influence of the applied potential gradient; and (3) the migration of a mixture of two proteins such as arginase and urease, which is performed with the deliberate intention of detecting the presence or absence of an interaction between them. The two distinct species specified in each case will be termed A and B and considered to move with individual velocities v_A and v_B, such that $v_A > v_B$. Reference is now made to Figure (III-2) which is more simply discussed in terms of sedimentation velocity, although as before the conclusions that are drawn, are readily applicable to the chromatographic case. The cell represented by ABCD, of unit cross-sectional area, is filled with a solution of composition defined by specifying the total weight-concentrations of A and B, $\bar{c}_A^{\alpha}$ and $\bar{c}_B^{\alpha}$ respectively. If A and B do not chemically interact, or are very slowly equilibrating polymers (B a monomer of A, say), application of the centrifugal field for a time t will result in partial separation of A and B. Thus a boundary between the solvent plateau, and a plateau of B alone, c_B^{β} will be generated. This plateau will in general be terminated at the position where the concentration of A increases with radial distance from zero to its original plateau concentration of $\bar{c}_A^{\alpha}$. As the concentration of A increases with distance, three possibilities arise in relation to the

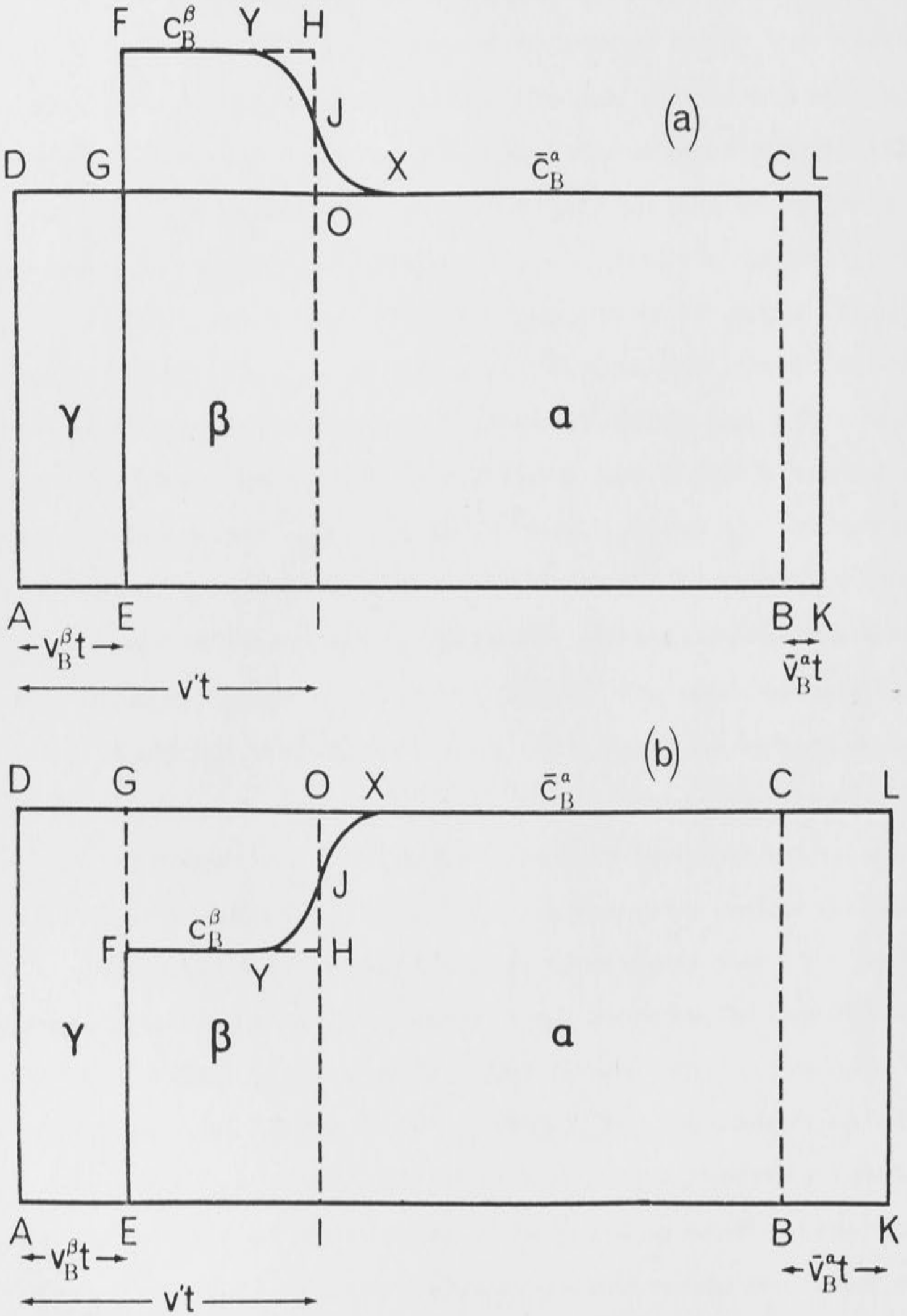

F
C_B^β
Y
H
J
(a)
D
G
X
$\bar{C}_B^\alpha$
C
L
O
γ
β
α
A
E
I
B
K
$v_B^\beta t$
$v't$
$\bar{v}_B^\alpha t$
(b)
D
G
O
X
$\bar{C}_B^\alpha$
C
L
J
C_B^β
F
H
Y
γ
β
α
A
E
I
B
K
$v_B^\beta t$
$v't$
$\bar{v}_B^\alpha t$

Figure III - 2

Schematic representations of the mass migration of a mixture of two solutes A and B in a cell of uniform cross-section. The α-phase is that of the original solution, the β-phase contains B alone, and the γ-phase is solvent. Two possible situations are depicted:

(a) $c_B^{\beta} > \bar{c}_B^{\alpha}$, which is shown in the text to pertain when frictional interactions cause a composition dependence on the constituent velocity of B.

(b) $c_B^{\beta} < \bar{c}_B^{\alpha}$, which is shown in the text to pertain when chemical interaction of the type $A + B \rightleftharpoons C$ causes a composition dependence of the constituent velocity of B.

concentration of B; (1) it could remain constant whereupon it would be required that c_B^{β} equals $\bar{c}_B^{\alpha}$; (2) it could decrease as is illustrated in Figure (III-2a) from c_B^{β} to $\bar{c}_B^{\alpha}$ or; (3) it could increase to $\bar{c}_B^{\alpha}$ as illustrated in Figure (III-2b). In the latter two cases it would be appropriate to term the second boundary observable by the experimenter, a *reaction boundary* because both of the concentrations of A and B change coextensively. If A and B chemically interact according to the scheme $A + B \rightleftharpoons C$, it is appropriate to define a constituent concentration of B as $\bar{c}_B = c_B + \frac{M_B}{M_C} c_C$, (where M_B and M_C are the molecular weights of B and C respectively), whereupon the same three possibilities as discussed above pertain. Thus it is entirely possible that a boundary of pure B will be left near the meniscus (Figure 1(b) of Gilbert and Jenkins, 1959), and a reaction boundary across which the constituent concentration of B remains constant, increases (Figure III-2a) or decreases (Figure III-2b), separates the plateau of B alone, c_B^{β}, and the original solution plateau, where the constituent concentration of B is $\bar{c}_B^{\alpha}$.

The following geometrical argument applies to both Figures (III-2a and b) and to all the two-solute systems under discussion whether they are interacting or not. Three constructions have been made in Figure (III-2); (1) the line FE which represents the median bisector of the pure B gradient which in practice will be spread by diffusion; (2) the line OH, which is the median bisector of the B constituent gradient separating the α and β plateaux, and; (3) the area BKLC is drawn to represent the amount of material piled up at the cell base BC. Thus,

the area ABCD = area EKLXYF (III - 6a)

area EKLXYF = area EIHF + area IBCO + area BKLC (III - 6b)

area ABCD = area AEGD + area IBCO + area EIOG (III - 6c)

Combination of these equalities yields,

area EIHF + area BKLC = area AEGD + area EIOG (III - 6d)

In addition the following relations pertain;

$AE = v_B^{\beta}t$, $EI = (v' - v_B^{\beta})t$, $BK = \bar{v}_B^{\alpha}t$, $HI = c_B^{\beta}$ and $AD = LK = \bar{c}_B^{\alpha}$ where v' is the velocity of the median bisector OH, and $\bar{v}_B^{\alpha}$ is, for the chemically non-interacting case, the velocity of B migrating in the presence of A, and for the chemically interacting case, the constituent velocity $(v_B^{\alpha}c_B^{\alpha} + v_C^{\alpha}c_C^{\alpha})/\bar{c}_B^{\alpha}$. It follows from equation (III-6d) that, $v_B^{\beta}t\,\bar{c}_B^{\alpha} + (v' - v_B^{\beta})t\,\bar{c}_B^{\alpha} = (v' - v_B^{\beta})t\,c_B^{\beta} + \bar{v}_B^{\alpha}t\,\bar{c}_B^{\alpha}$ (III - 7)

which upon rearrangement gives,

$$c_B^{\beta} = \frac{\bar{c}_B^{\alpha}(v' - \bar{v}_B^{\alpha})}{(v' - v_B^{\beta})} \qquad \text{(III - 8)}$$

From equation (III-8) it follows that if $\bar{v}_B^{\alpha} < v_B^{\beta}$ then $c_B^{\beta} > \bar{c}_B^{\alpha}$ and Figure (III-2a) pertains. This is the situation encountered where A and B interact frictionally but not chemically, and in this context equation (III-8) is recognised as the Johnston-Ogston equation (Johnston and Ogston, 1946). It is of some interest to note that in their original formulation, Johnston and Ogston did not realize that v' was the median bisector of the B constituent gradient across which the A constituent disappears (Nichol and Ogston, 1965a). In the case of chemical interaction it

is clear from the definition of the constituent velocity $\bar{v}_B^{\alpha}$ that $\bar{v}_B^{\alpha} > v_B^{\beta}$, and therefore from equation (III-8) that $c_B^{\beta} < \bar{c}_B^{\alpha}$ as illustrated in Figure (III-2b). In this context equation (III-8) has been termed *the moving boundary equation* (Longsworth, 1959), and it permits the estimation of $\bar{v}_B^{\alpha}$ from the available experimental results and hence the estimation of the association equilibrium constant for the reaction $A + B \rightleftharpoons C$. In the experiments to be reported, on mixtures of arginase and urease, and arginase and argininosuccinase, use will be made of these observations in comparing observable values of c_B^{β} with the value of $\bar{c}_B^{\alpha}$ selected to construct these mixtures. It could be noted that when chemically interacting systems are studied, frictional interactions almost certainly operate and the possibility arises therefore that two opposing effects may result in c_B^{β} equalling $\bar{c}_B^{\alpha}$: this however is an unlikely situation since one effect will almost certainly predominate.

(iii) The continuity equations in differential form: boundary shapes. Consider first the sedimentation velocity of a single non-interacting solute. Even for this simple case the shape of the sedimenting boundary is a function of several factors including diffusional spreading and concentration dependence effects. It follows that analysis of this boundary shape is potentially capable of providing more information pertaining to the system than is obtainable from determining weight-average velocities alone. Lamm (1929) formulated the differential equation which describes the flux of a single solute in a radially divergent gravitational field,

$$\frac{\partial c}{\partial t} = \frac{1}{x} \frac{\partial}{\partial x} \left\{ x \left(D \frac{\partial c}{\partial x} - s \, x \, \omega^2 \, c \right) \right\} \qquad \text{(III - 9)}$$

Equation (III-9) is a non-linear partial differential equation of order two, since a term describing diffusion in terms of the coefficient D, is included. The integration of equation (III-9) provides a functional relationship between c and x which describes the boundary shape. Such solutions in approximate form were first obtained by Faxén (1929) and by Archibald (1942), assuming that both s and D were concentration independent, and are therefore of limited applicability to the description of boundary shapes pertaining to real systems. Fujita (1956) reconsidered the integration problem, incorporating a linear concentration dependence of s, described by equation (III-3), but ignoring the concentration dependence of D, since diffusional spreading itself is a second order effect depending on the square root of time (Crank, 1970). Fujita showed that equation (III-9) could be written in the form of a linear partial differential equation of order two which was capable of solution by standard means to yield an infinite series, each term of which was itself an infinite series multiplied by an exponential term. He pointed out that this solution was of little direct use in relation to experimental results particularly as the alternating series possesses undefined convergence characteristics. Fortunately, a simpler formulation of equation (III-9) in analogous form to the familiar unidimensional diffusion equation of Fick (second law) was possible in cases where the experimental parameters conform to the relation,

$(ye^{-\tau})/\varepsilon(1-e^{-\tau}) >> 1$, where $y = (x/x_m)^2$, x_m being the radial position of the meniscus; $\tau = 2\omega^2 s_o t$ and $\varepsilon = 2D/\omega^2 s_o x_m^2$. The advantage of this formulation was not only that many experimental systems conform to this condition, but also that the solution of the Fick equation in terms of the probability integral was available. In these terms, the experimental sedimentation velocity pattern is analyzed in the following way (Baldwin, 1957). A series of experiments performed at different plateau concentrations together with the application of equation (III-3) permits the evaluation of k and s_o, whereupon τ is defined for each value of t, the time from the commencement of the experiment to the photographic recording of a particular schlieren peak. It follows that corresponding values of λ and ξ_M may be determined where,

$$\lambda = kc^o(1 - e^{-\tau}) \qquad \text{(III - 10a)}$$

$$\xi_M = \left\{ \frac{\omega^2 s_o x_m^2 kc^o}{2D} \right\}^{\frac{1}{2}} \left\{ \frac{\lambda^{\frac{1}{2}}}{1 + (1 - \lambda)^{\frac{1}{2}}} \right\} \qquad \text{(III - 10b)}$$

provided a value of D is assumed. The probability integral of ξ_M, $\Phi(\xi_M)$ and its first derivative $\Phi'(\xi_M)$, the error function, may be obtained from standard tables (U.S. Department of Commerce, National Bureau of Standards. Applied Mathematics Series, 1954), whereupon the following function may be evaluated for each value of t.

$$F(\xi_M) = \frac{\Phi'(\xi_M)}{1 + \Phi(\xi_M)} + 2\xi_M \qquad \text{(III - 10c)}$$

Fujita (1956) showed that the final solution of the approximated differential equation could be written in a form which related $F(\xi_M)$ to the area A of the schlieren peak and the height H of its maximum ordinate, by,

$$\left\{\frac{A\ F\ (\xi_M)}{H}\right\}^2 = \frac{2D}{\omega^2 s_o}\ (e^T-1)\left[1 + (1-\lambda)^{\frac{1}{2}}\right]^2 \qquad \text{(III - 11)}$$

The ratio, A/H, implicitly involves consideration of boundary shape, but of more importance, equation (III-11) as written suggests a plot of $(AF(\xi_M)/H)^2$ versus $(e^T-1)\left[1 + (1-\lambda)^{\frac{1}{2}}\right]^2$ which permits determination of D on the basis that the plot must be linear when the correct value of D is initially chosen. Modifications of this procedure have more recently been suggested by Van Holde (1960) and Fujita (1959), but the basic information available is similar. It is also noted that the procedure of analysis not only provides a means of estimating the diffusion coefficient, but also provides a test of the homogeneity of the sample, since the suggested plot will be curvilinear regardless of the original value of D selected if the sedimenting boundary comprises more than one species.

It is perhaps self-evident from the above review of theory pertaining to the migration of a single non-interacting solute, that the analysis of experimental data obtained with a polymerizing, or a two solute system in similar terms is prohibitively difficult. Accordingly in recent efforts (Cann, 1970; Cox, 1969; Gilbert and Gilbert, 1973) an entirely different approach has been employed in which the original differential equation expressing

conservation of mass is numerically integrated by finite difference methods. The procedure necessitates assigning values to relevant parameters including sedimentation coefficients of the solute species present, corresponding diffusion coefficients and concentration dependence terms, and equilibrium constants if applicable. The computer simulated boundary shape is then compared with the experimental pattern in order to refine the choice of these parameters. Considerable success has been achieved by this procedure in analyzing relatively simple systems, such as the polymerizing β-A-lactoglobulin system (Gilbert and Gilbert, 1973) where reliable values for many of the required parameters are available from other experimental sources. These refined calculations have confirmed earlier predictions made by Gilbert (1955, 1959) on the behaviour of polymerizing systems in mass migration: these earlier predictions were based on integrations of the differential continuity equations neglecting diffusional spreading and accordingly refer to asymptotic solutions at infinite time. In summary the important features which characterize a rapidly polymerizing system are, (1) that a monomer-dimer system will sediment as a unimodal boundary which may be skewed by chemical reaction, (2) that an $nA \rightleftharpoons C$ system ($n > 2$) will exhibit a bimodal reaction boundary in sedimentation velocity, the area of the back peak remaining constant as $\bar{c}^{o}$ decreases provided $\bar{c}^{o}$ is sufficiently large to ensure that a minimum is observed, and (3) that the distinct bimodality will be obscured by the presence of appreciable amounts of polymers of size intermediate between A and C.

(b) Sedimentation equilibrium

Of the previously cited equilibrium methods which may be used to obtain fundamental information on a macromolecular system interacting or not, that of sedimentation equilibrium was employed in this work. In this procedure a solution of macromolecular species is introduced into an ultracentrifuge cell and the rotor spun at constant angular velocity and temperature until the total potential of each solute component is a constant independent of radial distance. In effect, the centrifugal potential acting on each solute species has been counterbalanced by the tendency of the solute to diffuse in the centripetal direction under the influence of a chemical potential gradient. The value of ω is selected such that a distribution (a plot of total concentration versus radial distance) in terms of Rayleigh interference fringes, is measurable at equilibrium where the concentration distribution is independent of time. For a single non-interacting solute whose activity coefficient is unity it may be readily shown by a rigorous thermodynamic argument (Fujita, 1962) that the equation which describes this distribution is given by,

$$c(x_1) = c(x_2)e^{\phi M (x_1^2 - x_2^2)} \qquad \text{(III - 12)}$$

where $\phi = (1 - \bar{v}\rho)\omega^2/2RT$, and x_1 and x_2 are any radial distances at or between the meniscus and base x_m and x_b respectively; $\bar{v}$ is the partial specific volume of the solute and ρ the density of the solution. Equation (III-12) is also derivable from equation (III-9) by placing $\partial c/\partial t$ equal to zero, the statement of sedimentation equilibrium in this instance, and the integration of the result, leads by this

pathway to the ratio s/D in the exponential term which may be replaced by $M(1 - \bar{v}\rho)/RT$; the Svedberg molecular weight expression, whereupon equation (III-12) is obtained. The equivalent differential form of equation (III-12) is,

$$\frac{d\ln c(x)}{d(x^2)} = \phi M \qquad \text{(III - 13)}$$

which shows that the slope of a plot of $\ln c(x)$ versus x^2 will yield the molecular weight provided $\bar{v}$ is determined by independent difference density measurements.

Two cases where the experimentally available plot of $\ln c(x)$ versus x^2 will not be linear are worthy of comment. First, if a mixture of species of different molecular weight (albeit they be in chemical equilibrium or not) is subjected to sedimentation equilibrium, it has been shown that each species distributes individually according to equation (III-12) (Adams and Fujita, 1963; Nichol and Ogston, 1965b). In this case equation (III-13) written in terms of the experimentally observable total weight-concentration $\bar{c}(x)$ becomes

$$\frac{d\ln \bar{c}(x)}{d(x^2)} = \phi \frac{\sum_{i=1}^{n} M_i c_i}{\sum_{i=1}^{n} c_i} = \phi \bar{M} \qquad \text{(III - 14)}$$

where $\bar{M}$ is the weight-average molecular weight and all $\bar{v}_i$ are identical. Since $\bar{M}$ must increase with $\bar{c}$ (that is with x) for both a chemically reacting system (Ostwald's Dilution Law) or with a heterogeneous system, due to preferential depletion of the higher molecular weight forms

from the meniscus, it follows for these systems that $\ln\bar{c}(x)$ versus x^2 plots exhibit upward curvature for increasing values of x^2. Methods for analyzing this type of data in terms of the composition of a non-interacting heterogeneous mixture (Jeffrey and Pont, 1969), or in terms of the equilibrium constants governing the composition of an interacting mixture (Steiner, 1952) have been presented. The second case involves the consideration of thermodynamic non-ideality which in the present context will be discussed only in terms of a single non-interacting solute: equation (III-13) applies except that c(x) is replaced by a(x) the product of c(x) and the activity coefficient $\gamma(x)$. Simple rearrangement leads to (Nichol, Ogston and Preston, 1967),

$$\frac{d\ln c(x)}{d(x^2)} = \frac{\phi M}{1 + \left(\frac{d\ln\gamma(x)}{d\ln c(x)}\right)} \qquad \text{(III - 15)}$$

It is generally assumed that $\ln\gamma(x)$ may be written as a power series in total concentration whereupon equation (III-15) becomes,

$$\frac{d\ln c(x)}{d(x^2)} = \frac{\phi M}{1 + BMc + CMc^2 + \ldots} \qquad \text{(III - 16)}$$

where B and C are non-ideality coefficients corresponding to the conventional second and third virial coefficients. Clearly if the non-ideality coefficients do not equal zero the plot of $\ln c(x)$ versus (x^2) will deviate from linearity in a way which depends on their sign and magnitudes.

(c) The determination of partial specific volume

Basic to the determination of the molecular weight of a species, either by the calculation of sedimentation and diffusion coefficients estimated separately via the Svedberg (1925) molecular weight expression or from an analysis of sedimentation equilibrium results, is the requirement that the partial specific volume of the solute be known. This thermodynamic parameter is given by

$$\left(\frac{\partial V}{\partial g_i}\right)_{T,P,g_j,j\neq i},$$

and can not be determined operationally. Its experimental counterpart is the apparent specific volume which can be written as

$$(\Phi v_2) = \frac{V - V_1}{g_2} = \frac{\Delta V}{\Delta g_2} \qquad \text{(III - 17)}$$

Where V and V_1 are the volumes of solution and solvent respectively, and g_2 is the number of grams of solute in the solution of the given volume. Moreover $V_1 = g_1 V_1^{\circ}$ where g_1 is the number of grams of solvent and V_1° is the volume of solvent per gram. In these terms equation (III-17) becomes,

$$V = g_2(\Phi v_2) + g_1 V_1^{\circ} \qquad \text{(III - 18)}$$

which can be differentiated with respect to g_2 to give,

$$\left(\frac{\partial V}{\partial g_2}\right)_{T,P,g_1} = \bar{v}_2 = (\Phi v_2) + g_2 \frac{\partial (\Phi v_2)}{\partial g_2} \qquad \text{(III - 19)}$$

Thus provided the apparent specific volume is independent of g_2 then it equals the partial specific volume $\bar{v}_2$; a condition that will be assumed in this work. In practice,

equation (III-19) is not used directly to estimate $\bar{v}_2$, but rather density measurements are made. In these terms equation (III-17) becomes,

$$(\Phi v_2) = \frac{1}{\rho}\left\{\frac{\rho_1 - \rho}{c} + 1\right\} \qquad \text{(III - 20)}$$

where ρ is the solution density, ρ_1 that of the solvent, and c the concentration of the solution. Thus the apparent specific volume can be estimated by means of a precision differential density measurement (as discussed in Chapter VI, Methods Section) and a knowledge of c. If this concentration, albeit measured indirectly by refractometry or spectroscopy, fundamentally refers to a dry-weight concentration, then the use of the $\bar{v}$ so determined leads to a value of the unhydrated molecular weight (Oncley, 1941; Casassa and Eisenberg, 1964).

(d) The correlation of determined parameters

Einstein (1905) showed, from the thermodynamics of irreversible processes, that the diffusion coefficient of a single solute could be related to its frictional resistance per molecule (f) by,

$$D = \frac{RT}{Nf}\left\{1 + \frac{d\ln\gamma}{d\ln c}\right\} \qquad \text{(III - 21)}$$

where N is Avogadro's number. The existence of the non-ideality term in this equation is a basic reason why the Svedberg molecular weight expression is more correctly formulated in terms of a ratio s_o/D_o, for in the limit of infinite dilution the non-ideality term tends to zero. It follows from equation (III-21), for an ideal solute, that f

may be evaluated provided D may be estimated by, (1) a free diffusion experiment (Gosting, 1956), (2) a sedimentation velocity experiment in which boundary analysis is performed, (equation III-11), or (3) by a combination of the molecular weight determined by sedimentation equilibrium, and a sedimentation coefficient determined by sedimentation velocity. It is now possible to calculate, from Stokes Law (Stokes, 1856) the frictional resistance f_o of a sphere whose unhydrated partial specific volume and molecular weight are that of the solute under discussion.

$$f_o = 6\pi\eta \left\{ \frac{3 M \bar{v}}{4\pi N} \right\}^{\frac{1}{3}} \qquad \text{(III - 22)}$$

where η is the viscosity of the medium through which the solute migrates. This permits the direct evaluation of the frictional coefficient f/f_o which equals unity only if the molecule is actually spherical and unhydrated, otherwise the ratio must be greater than unity. It is possible to derive, from an f/f_o greater than one, the value of the axial ratio of an assumed ellipsoid (prolate or oblate) provided a degree of hydration is also assumed (Perrin, 1936; Oncley, 1941).

2. The purification of arginase.

(a) Introduction

The first partial success with the isolation of arginase was achieved by Richards and Hellerman (1940) who obtained a fifty-fold purification of the enzyme from beef liver extracts. The enzyme was known to be activated by various metal ions, Mn^{++}, Co^{++} and Fe^{++}, (Hellerman and

Perkins, 1935) but it was Van Slyke and Archibald (1942) who discovered the stabilizing effect of manganese salts. Later workers (Hunter and Downs, 1944; Mohamed and Greenberg, 1945) utilized manganese or cobalt ions in their preparative steps and obtained samples which ranged in purity from ∿35% to ∿50%. The final product of these earlier preparations was always tainted yellow by liver pigments and a method was developed for their removal (Thompson, 1948), but was found to be impractical by most workers (Mohamed, 1949). In 1946 Bach succeeded in obtaining hexagonal protein crystals from a beef liver arginase preparation after treating the final solution with ammonium sulphate, acetone or isopropanol. However, the method was not reproducible and doubt was even cast as to whether the structures were crystals (Mohamed, 1949). With a better understanding of manganese activation effects, preparations of arginase of greater purity were obtained (Roche, Thoai and Verrier, 1953; Greenberg, Bagot and Roholt, 1956) and more reliable methods of removing liver pigments involving the use of pyridine and zinc sulphate were developed (Bach and Killip, 1958). Although these earlier preparations differed in detail, they showed the common features that after acetone precipitation of liver homogenate, a heat step at 60°C was employed as a valuable purification procedure. Bach and Killip (1958, 1961) summarized earlier findings and presented a detailed preparative procedure for beef liver arginase based on them, which was not only reproducible but also applicable to the isolation of the sheep and horse liver enzymes. These latter enzymes

exhibited similar kinetic properties and maximum specific activity to the beef liver enzyme, although the crystal morphologies in each case were different.

The next major advance in the preparation of arginase samples involved the use of chromatographic steps in the purification procedures. It was typified by the work of Schimke (1964) and Hirsch-Kolb and Greenberg (1968) who employed carboxy-methyl cellulose cation exchange columns and gel filtration on Sephadex G100 in their preparations of the rat liver enzyme. It was clear from ultracentrifuge studies that these chromatographic steps were valuable in improving the homogeneity of samples obtained. In 1972 Harell and Sokolovsky adapted the new step of Sakai and Murachi (1969) to the preparation of beef liver arginase, but added an anion exchange chromatographic step and achieved final purification by means of isoelectric focusing. Their homogeneous product represented an 800-fold purification from liver supernatant, similar to that reported by Bach and Killip (1958, 1961). Since the physico-chemical characterization reported by the former workers was more extensive than that performed by the latter group and showed that a sample homogeneous with respect to molecular weight was obtained, it was decided to employ the method described by Harell and Sokolovsky (1972) in the present work. Details of this procedure, together with modifications made to it are summarized in the next Section.

(b) <u>The preparative method employed</u>

The steps involved in the preparative procedure are summarized in Table (III-1) and will be discussed in detail using the same numbering. Table (III-1) also summarizes the

TABLE III - 1

Summary of arginase purification.

STEP	VOLUME	MASS OF PROTEIN	SPECIFIC ACTIVITY ($U\ ml^{-1}$) AVE.	SPECIFIC ACTIVITY ($U\ ml^{-1}$) MAX.	TOTAL ACTIVITY (U)	YIELD (% of original U)
1. EXTRACTION a. Homogenate of 2kg of Calf liver	~6ℓ	-	-	-	200,000	100
1. EXTRACTION b. Supernatant	3.8ℓ	-	-	-	200,000	100
2. ACETONE FRACTIONATION	1ℓ	390 g	0.5	-	182,000	91
3. HEAT TREATMENT	970ml	32 g	5.3	-	172,000	86
4. DE52 CHROMATOGRAPHY	64ml	7.3 g	19	35	136,000	68
5. SEPHADEX G100 CHROMATOGRAPHY	20ml	0.8 g	125	300	100,000	50
6. SP SEPHADEX C50 CHROMATOGRAPHY	28ml	150 mg	400	600	60,000	33
7. ISOELECTRIC FOCUSING	-	33 mg	600	720	20,000	10

relative efficiency of each step in terms of the specific activity and the yield. For this purpose activities were determined by a method suggested by Ward and Srere (1967) in which the disappearance of the substrate, arginine, is measured spectrophotometrically at 205.7nm at 25°C (Chapter VI), and the initial velocity of disappearance estimated. It is recognized that this is an approximate method when used in relation to impure enzyme samples, but it is rapid and simple, and therefore suitable in the present context: alternative assay procedures for the elucidation of kinetic parameters of the purified enzyme are discussed in the next Chapter. One unit of arginase activity is defined as that amount of enzyme that will convert in 1 min one micro mole of arginine at a concentration of 1.6mM in water, adjusted to pH 9.5, (with HCl) and at 25°C.

Step 1: Two batches of non-pathological calf liver, each of 1kg, were minced and then homogenized for 45 sec at the medium speed setting in a Waring blender in 2 ℓ each of 0.01M tris-HCl pH 7.5, 0.01M KCl, and 0.05M $MnCl_2$ at 4°C. The homogenate (6 ℓ) was centrifuged at 0°C for 15 min at 16,000g in teflon lined buckets, to yield 4 ℓ of supernatant which retained the total initial activity.

Step 2: The pooled supernatant was cooled to -8°C in a stainless steel container and 1.5 volumes of acetone (L.R. grade) at -15°C was slowly added to the continuously stirred mixture, the addition taking ∿1h. The precipitate which formed was collected by centrifugation at -10°C for 5 min at 16,000g, the yellow supernatant being discarded.

The red-brown precipitate was placed in a tared crystallizing dish, and the acetone partially removed by placing the dish in a desiccator at 25°C maintained at low pressure with a water pump. The precipitate aggregates were broken up three times during the 1.5h evaporation step. The resulting 390g of fine brown powder, retaining a residual smell of acetone, was placed in 1ℓ of the tris-HCl buffer (pH 7.5) containing 0.05M $MnCl_2$ at 4°C and homogenized in the Waring blender at medium speed for 1 min, employing a plate in the chamber to minimize frothing. The mixture was again centrifuged at 0°C for 10 min, the ∿50g of solid being discarded and the clear brown-green supernatant being retained. The 1ℓ of supernatant was divided into 70 ml aliquots and dialyzed (Visking 23/32 tubing) at 4°C for 16 h against 10 volumes of the pH 7.5 tris-HCl buffer with continuous stirring, and a change of buffer after 6 h. It is noted that the dialysis buffer did not contain $MnCl_2$ since it was required to remove this salt prior to Step 3 conducted at pH 7.8 where precipitation of MnO_2 complicates the column procedure. The omission of $MnCl_2$ in the dialysis step did not result in any substantial loss of activity in accordance with the observation originally made by Harell and Sokolovsky (1972). Step 2 was routinely completed within 24 h of the start of the preparation.

Step 3: The pH of the combined dialysate (∿pH 6.0) was adjusted to pH 7.5 with 1M KOH, and the solution heated to 60°C with constant stirring in a conical flask immersed in a water bath, the temperature being maintained for 20 min. The flask was then cooled to 5°C in an ice bath and the

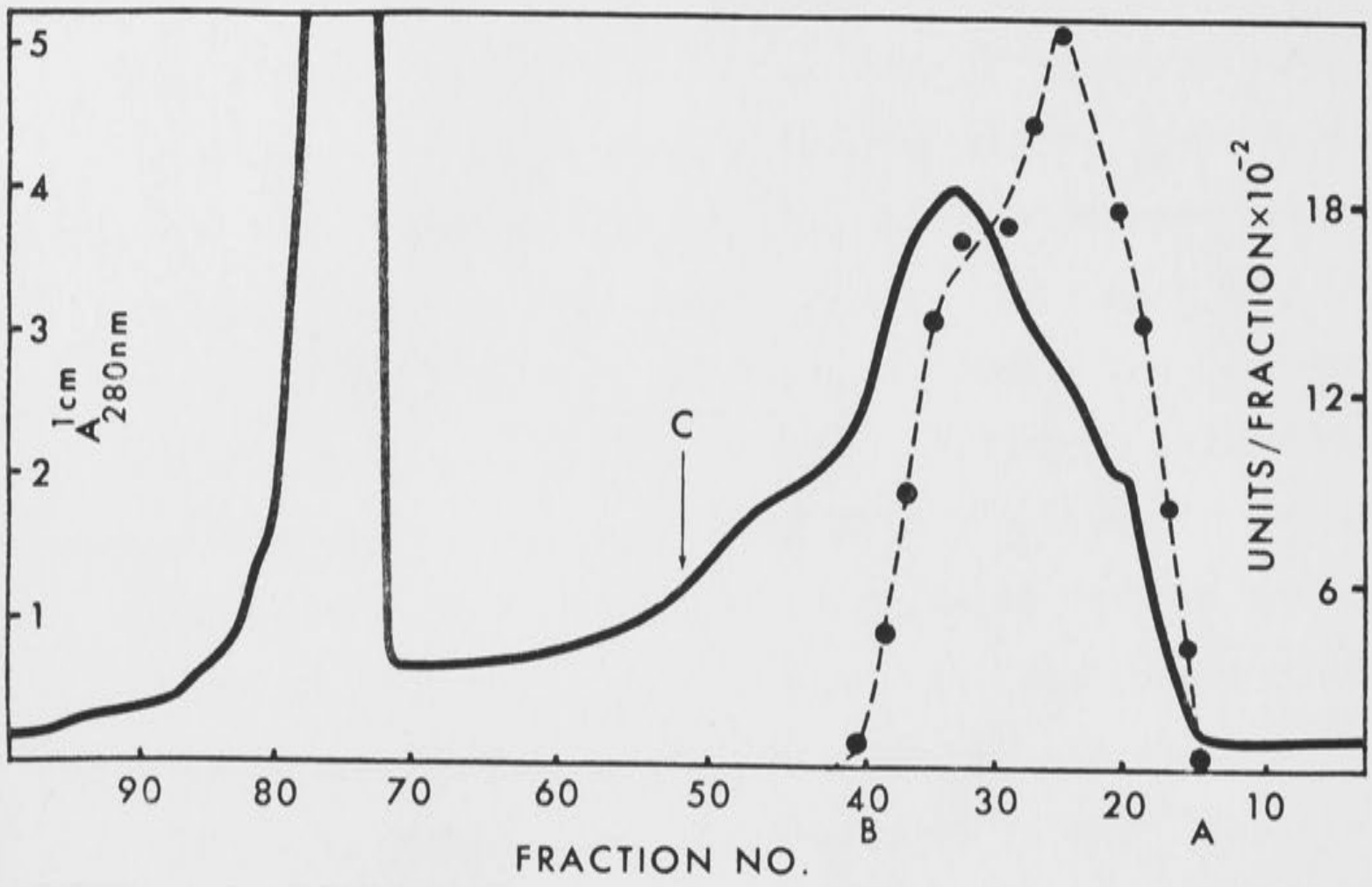


Figure III - 3

Elution profile of protein, in the beef liver arginase isolation procedure, obtained from a column of DEAE - cellulose DE52 (4 x 50cm). The solid line is the absorbance of eluant at 280nm, and the dotted line that of total number of arginase units (as defined in the text) per 13ml fraction. A flow rate of 100ml h^{-1} was employed and the column was maintained at 4°C. The fractions that were collected for further processing lay between the points A and B on the abscissa. The point C marks the point at which 130ml of 0.25M tris-HCl pH 7.8 buffer was added to elute the second large peak.

flocculated protein separated from the clear yellow-brown solution by centrifugation at 0°C for 15 min at 16,000g. The pH of the supernatant was adjusted to pH 7.8 with 0.5 M tris-HCl pH 7.8 at 4°C, so that the final concentration of tris was 0.05 M.

Step 4: A 4 x 50cm column of Whatman DE52 microgranular (pre-swollen) cellulose was pre-equilibrated at 4°C with 0.05M tris-HCl pH 7.8 buffer, the flow rate being 250ml h^{-1}. The 970ml of solution obtained from step 3 was divided into 4 equal portions each of which was chromatographed using the equilibration buffer for elution at a flow rate of 100ml h^{-1}. The eluant from each experiment was collected in 13ml fractions using an Isco (Golden Retriever) fraction collector and continuously monitored spectrophotometrically at a wavelength of 280 nm. Arginase activity appeared in the break-through fractions of the elution pattern as shown in Figure (III-3): the fractions containing more than a total of 100 units of arginase activity were pooled, and concentrated $MnCl_2$ solution was added to a final concentration of 0.05 M. Each of the four DE52 runs took 12 h for completion, the column being washed between each experiment, first, with 130 ml of 0.25M tris-HCl pH 7.8 buffer (to elute a dark band of protein near the top of the anion exchanger) and then with 1ℓ of equilibration buffer. Two points merit comment in relation to this column procedure. First, it was found that use of DEAE-Sephadex A50, or BioRad Celex D in place of DE52 cellulose led to less effective separations. Secondly, the loaded mass of protein used with the cellulose column was three times

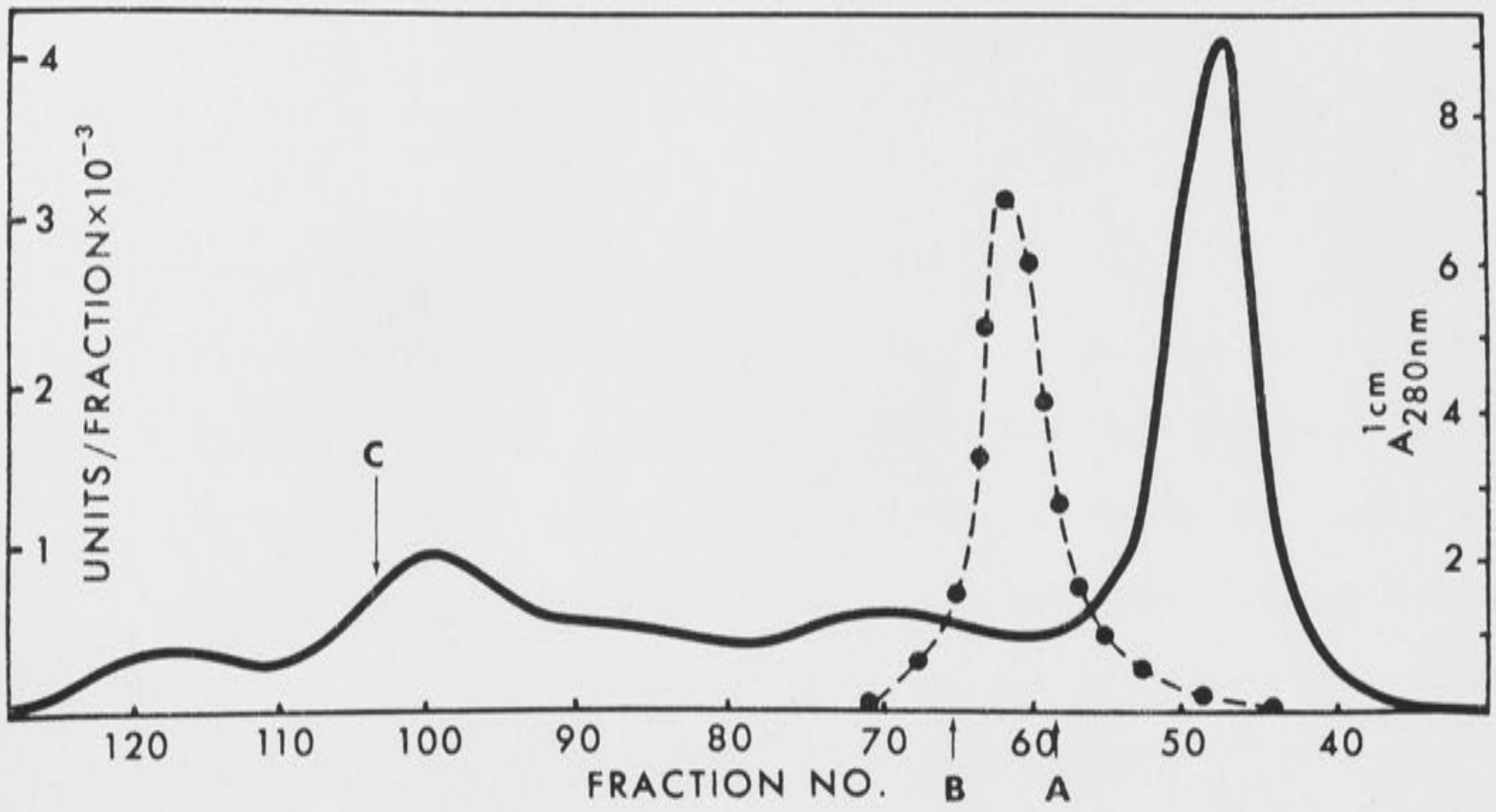


Figure III - 4

A typical elution profile of beef liver arginase from a column of Sephadex G100 (4 x 100cm). The protein was eluted with 0.01M tris-HCl pH 7.5 containing 0.05M $MnCl_2$, at a flow rate of 40ml h^{-1}. Fractions of 13ml were collected; the solid line is eluant absorbance at 280_{nm}; the dashed line is arginase activity per fraction. The fractions between the points A and B on the abscissa were pooled for further processing.

greater than that employed by Harell and Sokolovsky (1972), but with a column twice the volume.

The pooled column fractions from the four experiments were concentrated in batches of 400 ml in an ultrafiltration cell using a UM10 membrane and a pressure of 40 lb $inch^{-2}$ (2.8×10^6 dyne cm^{-2}) maintained by pure nitrogen, to yield a final volume of 64 ml of translucent pink solution.

Step 5: In this step the previous workers (Harell and Sokolovsky, 1972) employed Biogel P-150, but it was found that equally effective separation could be achieved with the use of Sephadex G100 which has superior flow characteristics. A 4 x 100 cm column was pre-equilibrated at 4°C with 0.01M tris-HCl pH 7.5 buffer containing 0.05M $MnCl_2$. Eight ml of the concentrate from Step 4 was applied and eluted with the same tris buffer at a flow rate of 40 ml h^{-1}. Figure (III-4) shows a typical elution profile obtained with 13 ml fractions. The first 6 fractions of the profile were opaque white, and gave a positive Molisch test characteristic of glycogen. Fractions 44 to 55 were brown-red in colour and showed only slight arginase activity while fractions 58 to 65 lying in the trough between the two peaks evident in Figure (III-4) routinely exhibited the highest arginase activity and were pooled. This column procedure was repeated to treat the remainder of the concentrate from Step 4, the pooled fractions being concentrated by ultrafiltration, as in Step 4, to yield a final volume, from all experiments, of 45 ml.

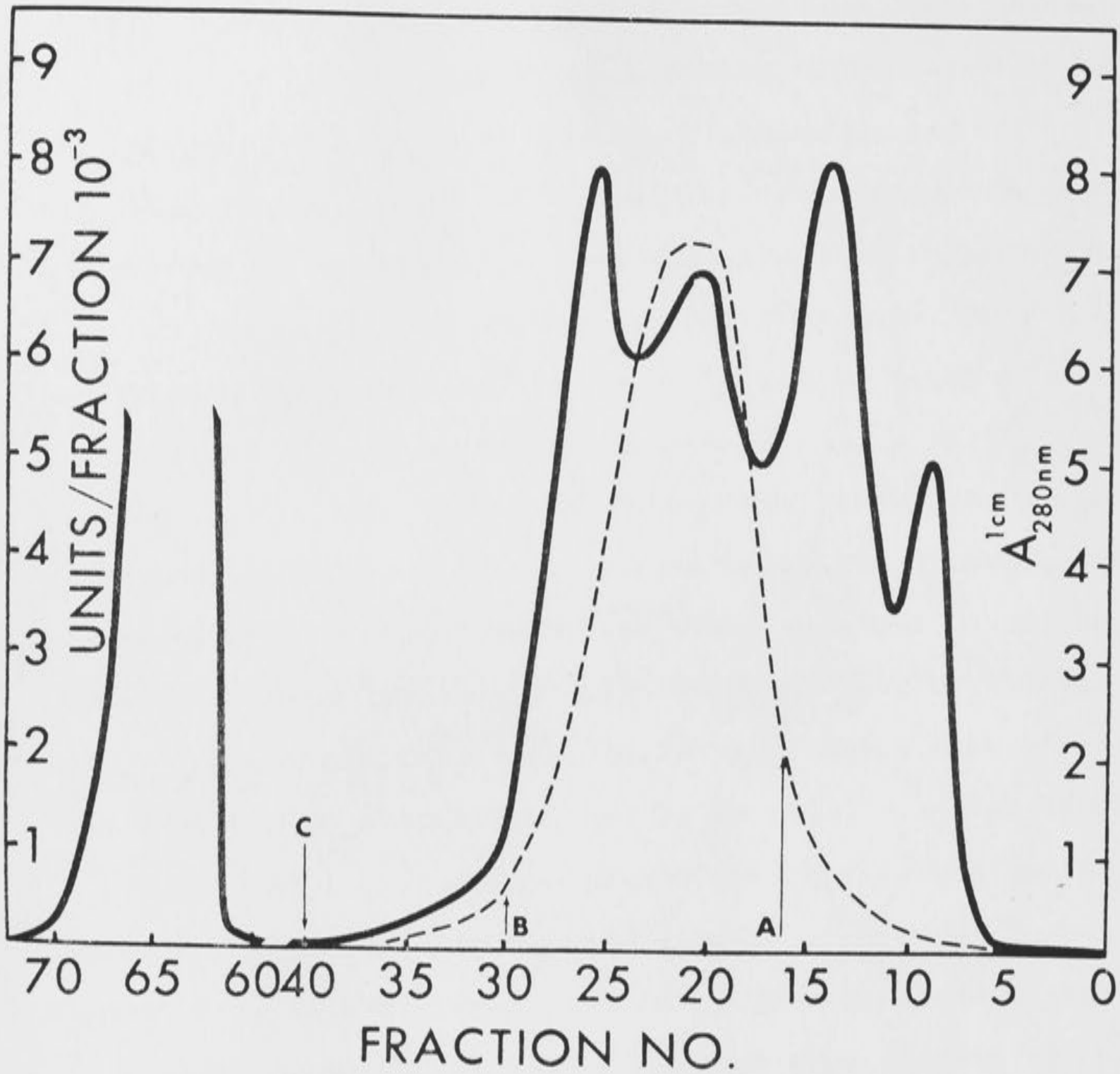


Figure III - 5

Elution profile of beef liver arginase from a column of SP-Sephadex C50 (4 x 37cm). The protein was eluted with 0.01M tris-acetate pH 6.3 at 4°C, at a flow rate of 20ml h^{-1}, and collected in 13ml fractions. The solid line is that of absorbance of eluant at 280nm, the broken line indicates the arginase activity per fraction. The points A and B mark the limits of the fractions pooled for further processing, and C is the point at which 1M NaCl was pumped onto the column.

Step 6: The pH of the concentrated sample from Step 5 (pH 7.5) was adjusted to pH 6.3 by alternate addition of 0.01 M tris-acetate pH 6.3 at 4°C, and ultrafiltration using a UM10 membrane, the final volume being 25 ml. This volume was loaded onto a 4 x 37 cm column of SP Sephadex C50 pre-equilibrated with the pH 6.3 buffer and eluted with this buffer at a flow rate of 20 ml h^{-1}. This step was the most variable in terms of obtaining a reproducible elution profile, and great care had to be exercised in the preparation of the gel and its pre-equilibration in the column. It will also be noted that no $MnCl_2$ was used in the buffer as this adversely affected the separation characteristics of the gel. It was therefore necessary to complete this Step rapidly in order to avoid excessive activity losses. The arginase activity emerged with the break-through peaks as shown in Figure (III-5), and those tubes containing the enzyme were pooled and adjusted to pH 7.5, with 0.05M tris-HCl pH 7.5 at 4°C, and $MnCl_2$ was added to a final concentration of 0.05 M. This sample was concentrated to 28 ml by ultrafiltration and stored at 4°C in sterilized sealed tubes, the enzymic activity being fully retained for several weeks under these conditions.

The sample at this stage of the preparation was characterized by a polyacrylamide electrophoretogram showing one major band and one or two minor components (Figure III-6a) and by sedimentation velocity patterns exhibiting a major peak of s equal to 6S, but with an asymmetric trailing edge extending to the meniscus.

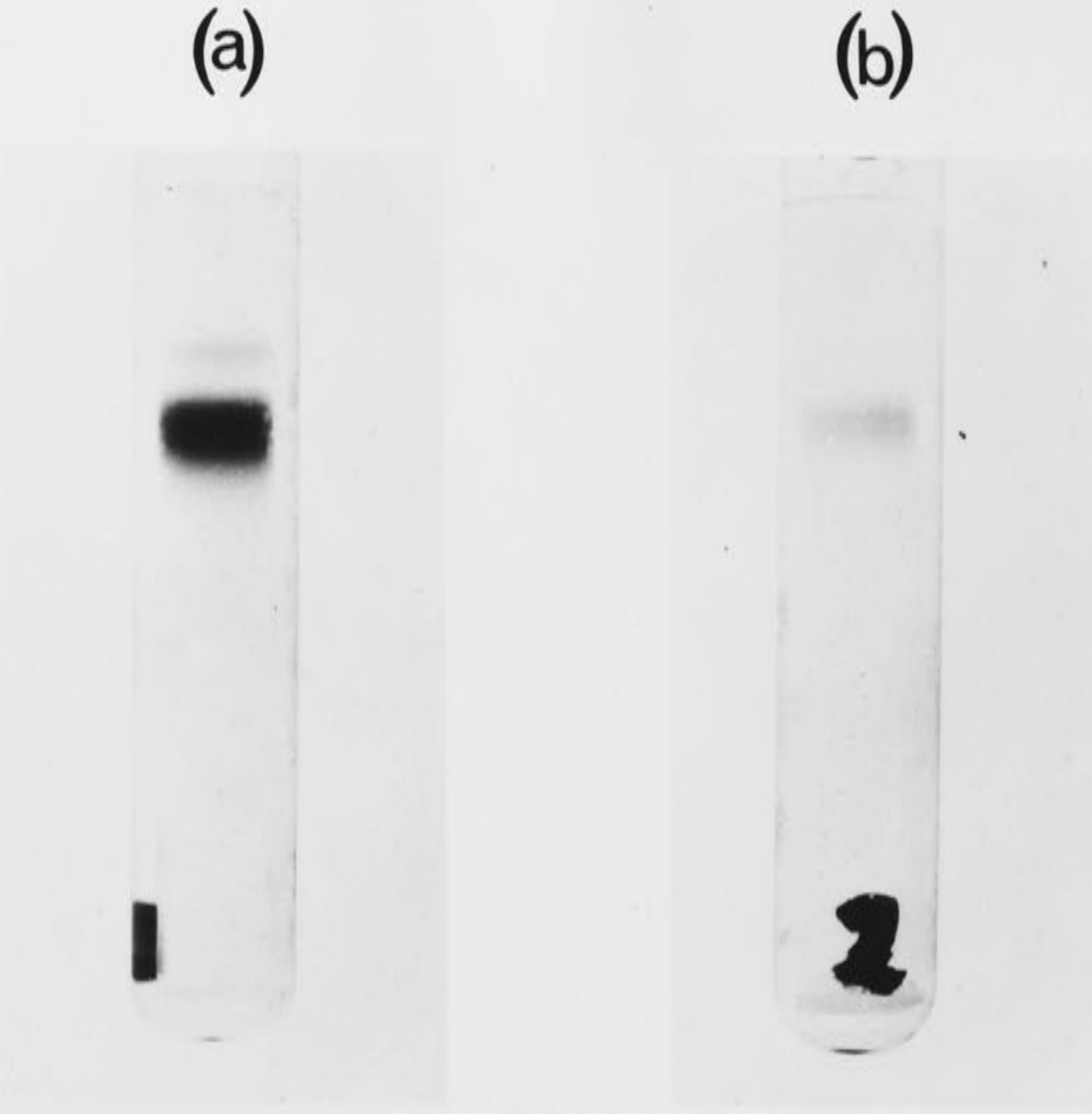


Figure III - 6

Electrophoretograms obtained with samples of arginase in polyacrylamide gel electrophoresis (7.5% acrylamide) conducted at 25°C in 0.5 x 7.5cm tubes employing 0.006M tris - 0.037M glycine pH 8.3 as electrode buffer and a constant current of 5mA for 30 min.

(a) An arginase sample from step 6 of the isolation procedure: loading mass ∿100µg.

(b) Arginase further purified by isoelectric focusing: loading mass ∿30µg.

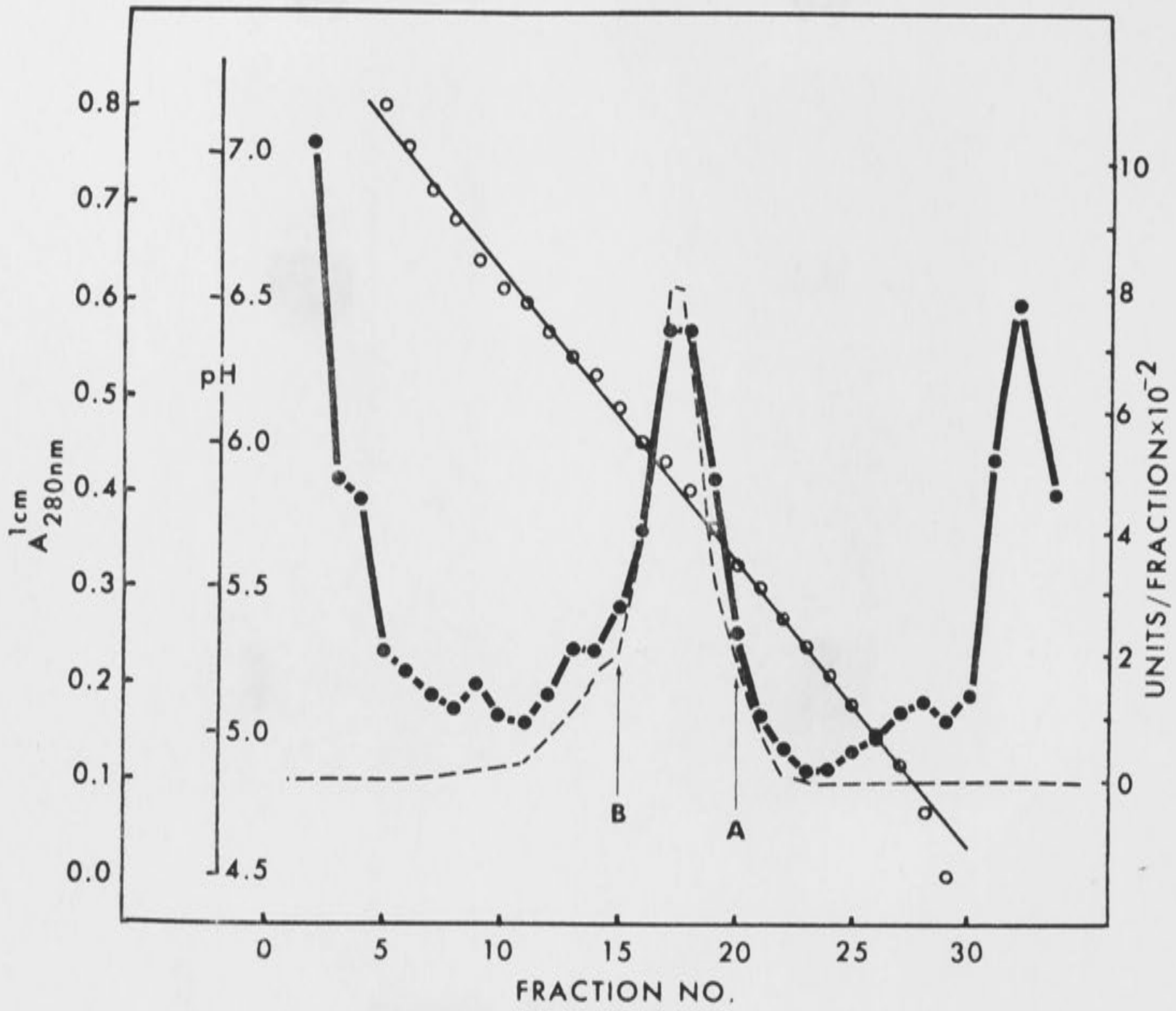


Figure III - 7

Profile of the absorption (solid thick line) at 280nm of the eluant from a 110ml isoelectric focusing column which was run for 70 h at 4°C with 20mg of beef liver arginase obtained from Step 6 of the preparation. The hollow circles give the pH gradient and the dotted line the arginase activity per 3.5ml fraction. The points A and B delimit the final retained fractions.

Step 7: The minor components evident in Figure (III-6a) were removed by isoelectric focusing using the method of Vesterberg and Svensson (1966) and employing carrier ampholytes in the pH range 5 - 7. A sample of 20 mg of protein was loaded in the centre of the 110 ml column, stabilized by a sucrose density gradient (Chapter VI), and after application of the potential for 70 h the profile shown in Figure (III-7) was obtained by monitoring fractions (3.5 ml) collected drop-wise from the base of the column. It is evident that the isoelectric point of the protein is pH 5.9 $\pm$ 0.05 in contrast to the value of pH 6.6 found by Van Slyke and Archibald (1942), but in agreement with that reported by Harell and Sokolovsky (1972). In the construction of Figure (III-7) enzymic activity measurements which followed pH and absorbance determinations were performed as follows. To each 3.5 ml fraction was added 3 ml of 0.1M HCl-tris pH 7.5, 0.05M $MnCl_2$ at 4°C, and the mixture heated to 60°C prior to cooling to 4°C and determination of enzymic activity. It can also be seen from the activity profile in Figure (III-7) that most of the activity resided in 6 fractions. These were pooled and contained approximately 5 mg of protein.

Repetition of the isoelectric focusing procedure utilizing the entire concentrate from Step 6 therefore yielded approximately 33 mg of colourless enzyme with specific activity of 600 - 720U mg^{-1}, the highest specific activity reported in Table (III-1). The final step prior to physico-chemical characterization involved the removal of the ampholyte and sucrose, which was achieved by ultrafiltration, with repeated flushing over 24 h with

0.05M HCl-tris, 0.02M NaCl, 0.01 M $MnCl_2$ pH 7.5 at 4°C. This method proved to be superior to the other earlier attempts which involved chromatography on Biogel P4 or Sephadex G50. It was found that the specific activity of the sample was unchanged on removal of the ampholyte, and that the sample could be stored for several weeks at 4°C, in sealed tubes, without loss of activity. Finally it is noted from Table (III-1) that the isoelectric focusing step leads to a considerable decrease in the yield, in agreement with the findings of Harell and Sokolovsky (1972). This loss may well be due to the partial separation of manganese ions from the protein by the electric field, but it is nevertheless an important purification step if the enzyme of highest specific activity, free of minor contaminants, is to be obtained. That the minor contaminants have been removed by this step is shown by the polyacrylamide gel electrophoresis pattern shown in Figure (III-6b). A total of five successful preparations were performed over the period of investigation for this thesis, each yielding the same final product of similar specific activity.

3. The physico-chemical characterization of arginase.

(a) Amino acid analysis and partial specific volume

Later in this Section it will be demonstrated that the arginase fractions from isoelectric focusing (Step 7) are homogeneous and characterized by a molecular weight of 114,000. The amino acid composition, determined with a Beckman Unichrome amino acid analyzer, of such a salt free sample based on a 24 h hydrolysis (6N HCl) is reported in Table (III-2) with the content of each amino acid averaged from duplicate experiments and corrected to the nearest

TABLE III - 2

Amino acid composition of beef liver arginase.

Amino acid residue	Nearest integral residues per 114,000g	Nearest integral residues per 114,000g (Harell and Sokolovksy, 1972).
Lysine	80	84
Histidine	23	29
Arginine	39	39
Aspartic acid	93	89
Threonine [a]	64	57
Serine [a]	61	46
Glutamic acid	97	91
Proline	71	68
Glycine	111	103
Alanine	64	57
Half-cystine [b]	17	17
Valine	81	86
Methionine	15	15
Isoleucine	57	61
Leucine	108	103
Tyrosine	27	33
Phenylalanine [a]	35	34
Tryptophan [a]	-	10

[a] Values in this work were not extrapolated

[b] Determined as cysteic acid on a performic acid oxidized sample.

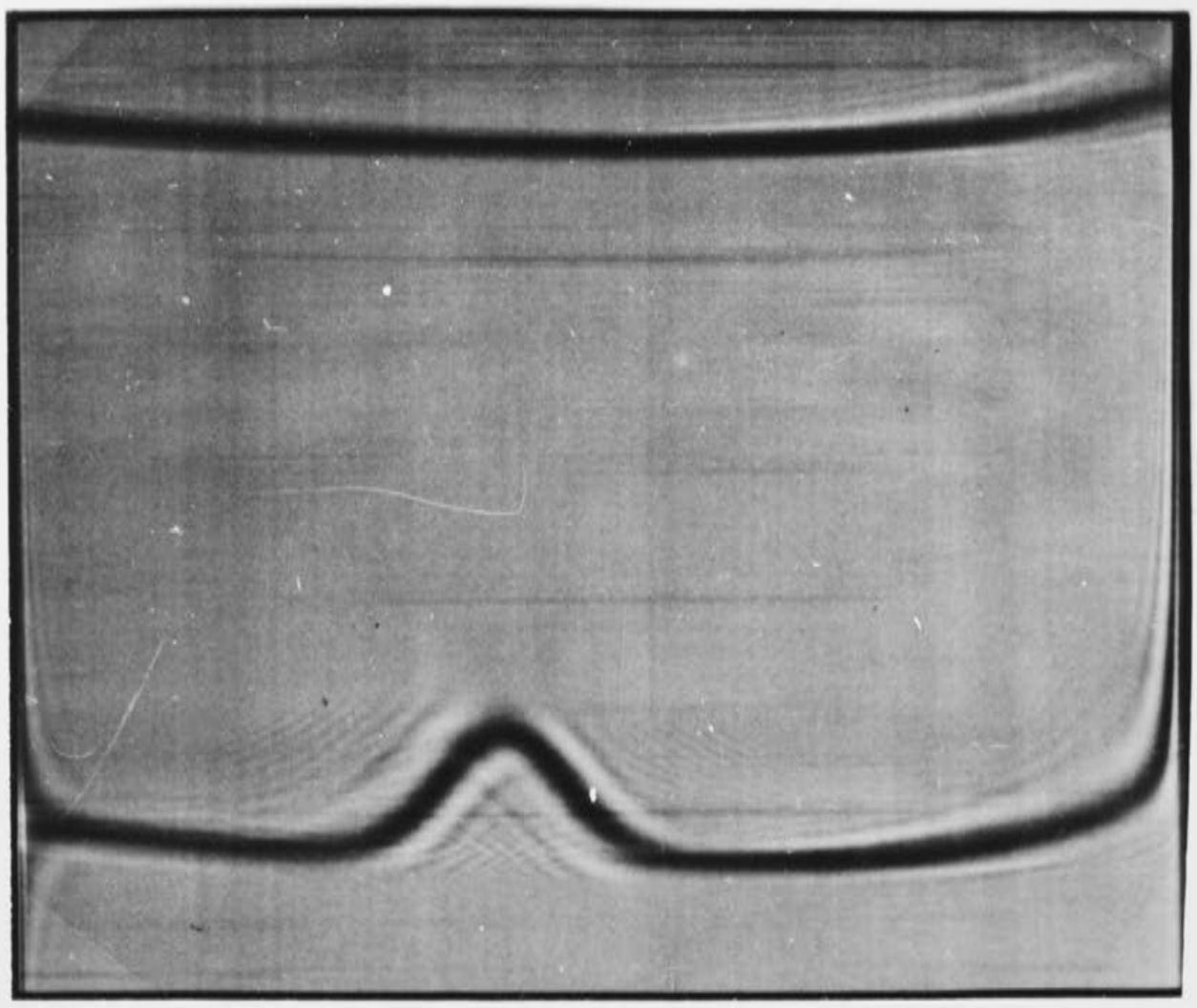
integer, expressed per 114,000 g. These values are compared with those found by Harell and Sokolovsky (1972) expressed on the same basis. With the exception of serine, it is seen that the composition is similar to that reported previously (Harell and Sokolovsky, 1972) and that the beef liver enzyme contains both methionine and proline in contrast to reported results with the horse liver enzyme (Grassmann, Hormann and Janowsky, 1958; Greenberg, 1960).

From the partial specific volume of each constituent amino acid (Cohn and Edsall, 1943) it is possible to calculate from Table (III-2) the apparent specific volume of the enzyme as a weighted sum: the value obtained from the present results by this means was 0.739 $g^{-1}ml$. As previously noted a more reliable estimate of this parameter may be obtained from differential density measurements. A solution of arginase was dialyzed against 0.01M HCl-tris, 0.02M NaCl, 0.01M $MnCl_2$ pH 7.5 at 20°C for 24 h with three changes of buffer, and the protein concentration was estimated, following dialysis, to be 0.199 g dl^{-1} by use of the extinction coefficient of 0.96 for a 1 mg ml^{-1} solution, at a wavelength of 278 nm (Harell and Sokolovsky, 1972). The dialyzed solution was used to fill the oscillator tube of an Anton Paar DMA 02C Digital Precision Density Meter, which measures the time taken for the tube to complete 10^4 oscillations when vibration is initiated by a controlled electrical impulse. This period (which was averaged from 30 readings in order to minimize error due to thermal fluctuations) may be directly related to the density of the solution by calibrating the instrument with fluids of

known density. The concentration and density values so determined were directly substituted into equation (III-20) to obtain a value of the apparent specific volume of $0.734 \pm 0.003\ g^{-1}ml$ (measured at $20 \pm 0.01°C$). The value is in satisfactory agreement with that of $0.739 g^{-1}ml$ calculated from the amino acid compositions given in Table (III-1), the former value being more reliable.

(b) The molecular weight of arginase

Samples of arginase from each complete preparation were routinely subjected to sedimentation velocity analysis in 0.05M HCl-tris, 0.02M NaCl, 0.01M $MnCl_2$ pH 7.5 at 20°C following the step involving isoelectric focusing. A typical result is shown in Figure (III-8a) where the upper pattern records the buffer baseline obtained in a separate cell. It is clear that in the cell containing the arginase solution (lower pattern) there is a single reasonably symmetrical boundary sedimenting with a sedimentation coefficient of 6.0S. It is hazardous to ascribe meaning to the boundary shape without performing an extensive boundary analysis, and as a prelude to this it is necessary to determine the concentration dependence of the sedimentation coefficient. The results of a series of experiments employing the pH 7.5 buffer and plateau concentrations in the range $0.15g\ dl^{-1}$ to $1.0g\ dl^{-1}$ are shown in Figure (III-8b). Values of s found at 20°C and referring to buffer were determined from the rate of movement of the maximum ordinate and were, within experimental error, the same as those determined by employing equation (III-2b), based on



(a)

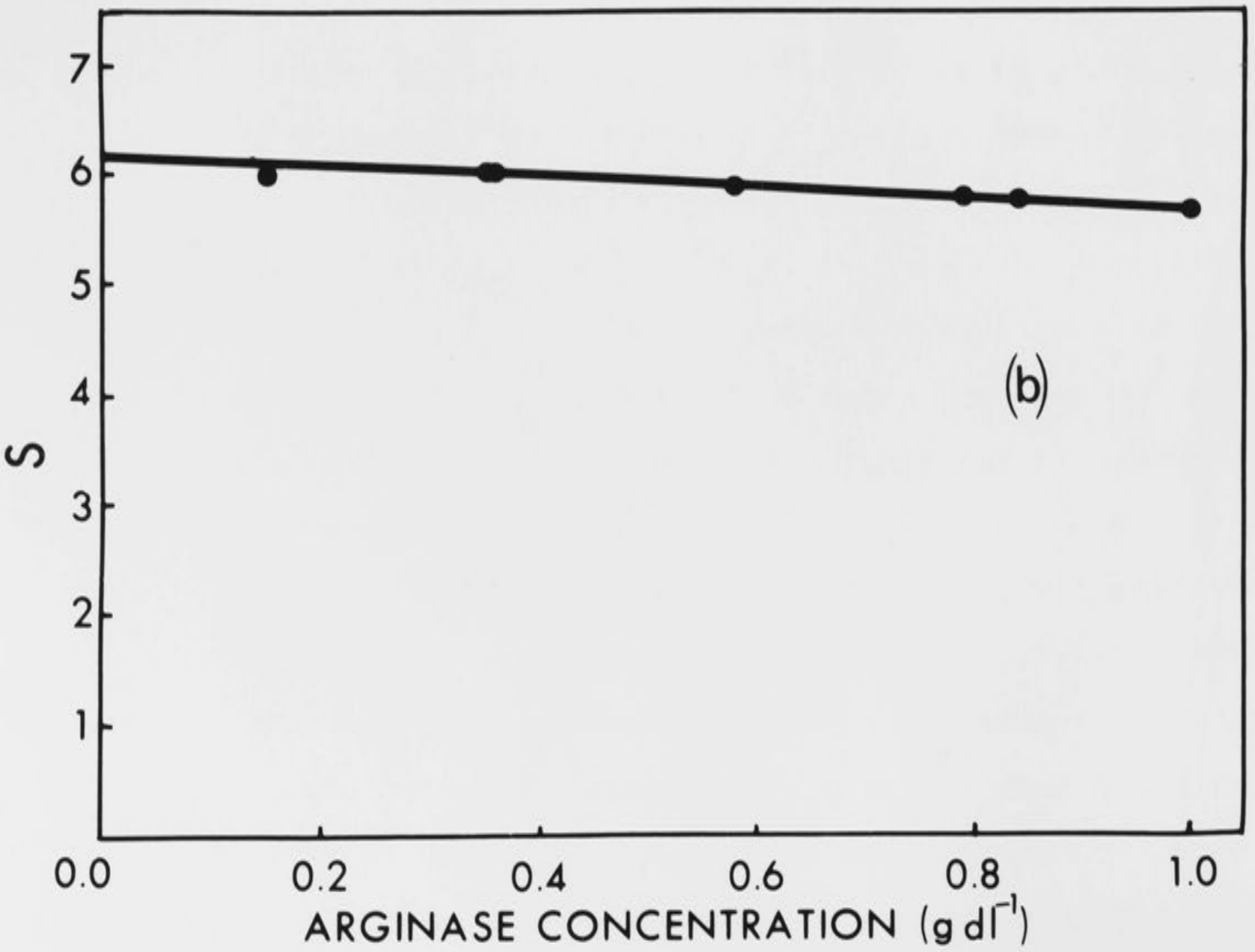


Figure III - 8

(a) Sedimentation velocity pattern obtained with beef liver arginase (0.3g dl^{-1}) in 0.05M HCl-tris, 0.02M NaCl, 0.01M $MnCl_2$ pH 7.5 at 20°C. The angular velocity was 60,000 rpm and the pattern was recorded using schlieren optics with a phase plate angle of 70° at a time of approximately 50 min from the commencement of the run. The upper pattern shows the distribution of buffer in a separate cell.

(b) The concentration dependence of the sedimentation coefficient of beef liver arginase in 0.05M HCl-tris, 0.02M NaCl, 0.01M $MnCl_2$ pH 7.5 at 20°C.

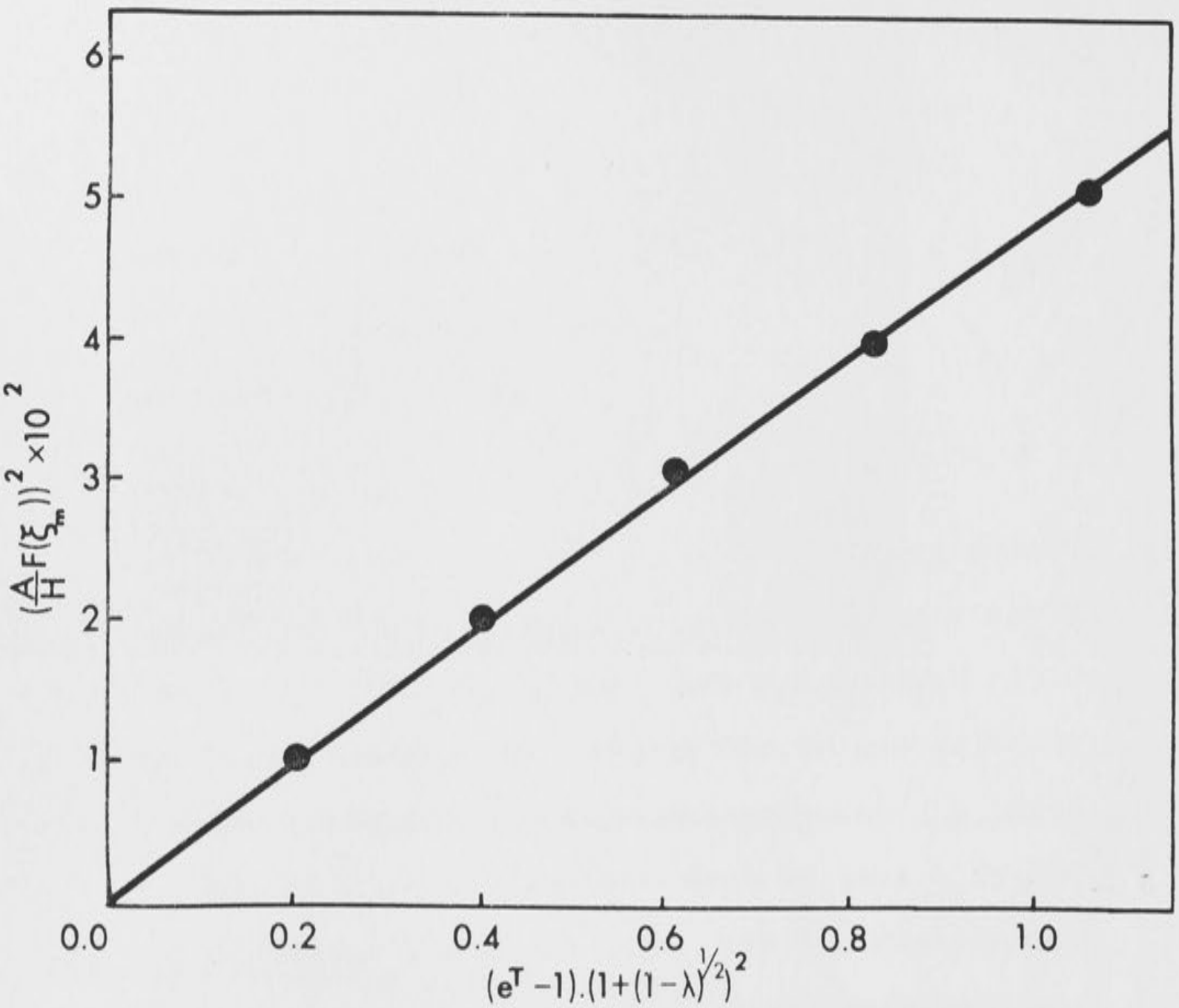


Figure III - 9

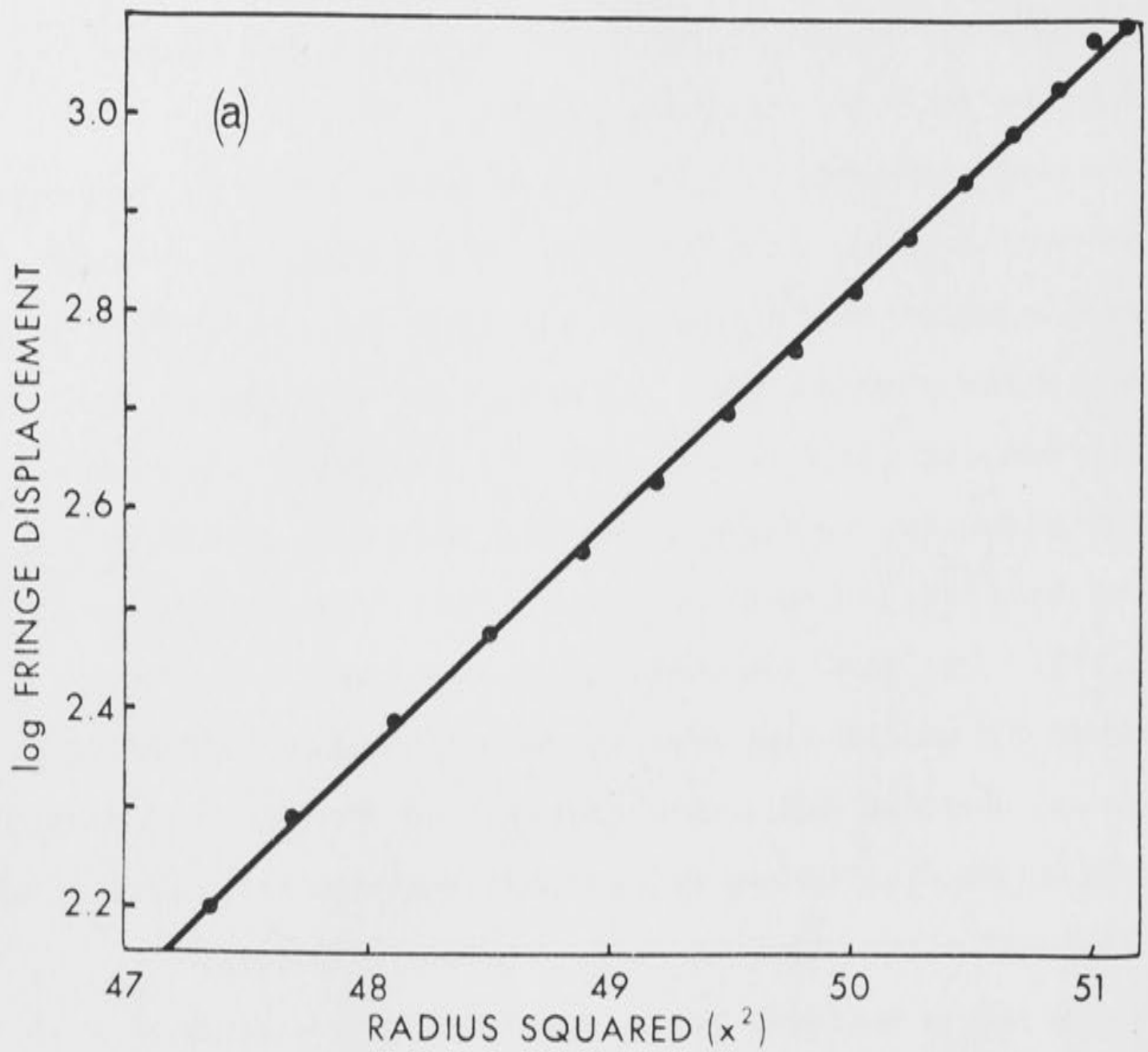
Boundary analysis of schlieren patterns obtained from a sedimentation velocity experiment on purified beef liver arginase. The plotted parameters are those suggested by equation (III - 11).

the position of the square root of the second moment. Clearly the results are described by equation (III-3), the least squares estimates of s_o and k being 6.15S and $0.069 \pm .005 g^{-1} dl$ respectively.

It is now possible to employ equations (III-10) and (III-11) which refer to the analysis of the boundary shape shown in Figure (III-8a), and recorded at different times, to obtain the plot shown in Figure (III-9). In these calculations the required values of A and H were found using a two dimensional comparator and the values t were estimated by a linear extrapolation of log $\bar{x}$ versus the time interval of photography to log $\bar{x}_m$. The linearity of the plot shown in Figure (III-9) indicates that the material is homogeneous. The value of the apparent diffusion coefficient calculated from the slope of the line in Figure (III-9) is 5.9×10^{-7} cm^2 sec^{-1} which was the value used to calculate ξ_M in the construction of the plot. Use of this value of D together with $\bar{v} = 0.734$ and $\bar{s}_o = 6.15S$ in the Svedberg molecular weight expression leads to an estimate of the molecular weight of arginase, in the cited environment, of 96,000. This value must be regarded as approximate for two reasons. First, it has been implicitly assumed in the formulation of equation (III-10), that the diffusion coefficient is concentration independent and that the activity coefficient of the species is unity. Secondly, while Figure (III-9) provides a reliable indication of the homogeneity of the sample it is not suggested that the value of D derived from it is an accurate

value since it relies on the measurement of A/H values which are subject to considerable error.

In order to obtain more reliable estimates of molecular weight over a wider concentration range, arginase samples dialyzed against the same pH 7.5 buffer were subjected to sedimentation equilibrium. In the first experiment the initial loading concentration was 0.41 g dl^{-1}, the final angular velocity was 6,000 rpm, the column height 3 mm, and the temperature controlled with the RTIC unit was 20 $\pm$0.1°C. The time required to attain equilibrium was shortened by use of the over-speeding technique described by Hexner, Radford and Beams (1961), and Howlett and Nichol (1972a); an angular velocity of 8,000 rpm was initially selected and maintained for 3.6 h before adjustment to 6,000 rpm for a further 3.5 h, by which time, according to theoretical calculation, equilibrium should have been attained to within experimental error. In practice the experiment was allowed to continue for a total of 14 h and it was shown by measurement of interferograms recorded at 11 h and 14 h that the distribution was indeed time invariant. The measurements from the final pattern recorded were analyzed with the use of a computer programme developed by Baghurst (1972), which permits the construction of the plot shown in Figure (III-10a) together with the estimation of the molecular weight based on equation (III-13). It is clear from the linearity of the plot shown in Figure (III-10a) that the material is homogeneous and that terms describing non-ideality effects (equation III-16) are of negligible magnitude in the concentration range studied.


(a)
3.0
2.8
2.6
2.4
2.2
log FRINGE DISPLACEMENT
47
48
49
50
51
RADIUS SQUARED (x²)

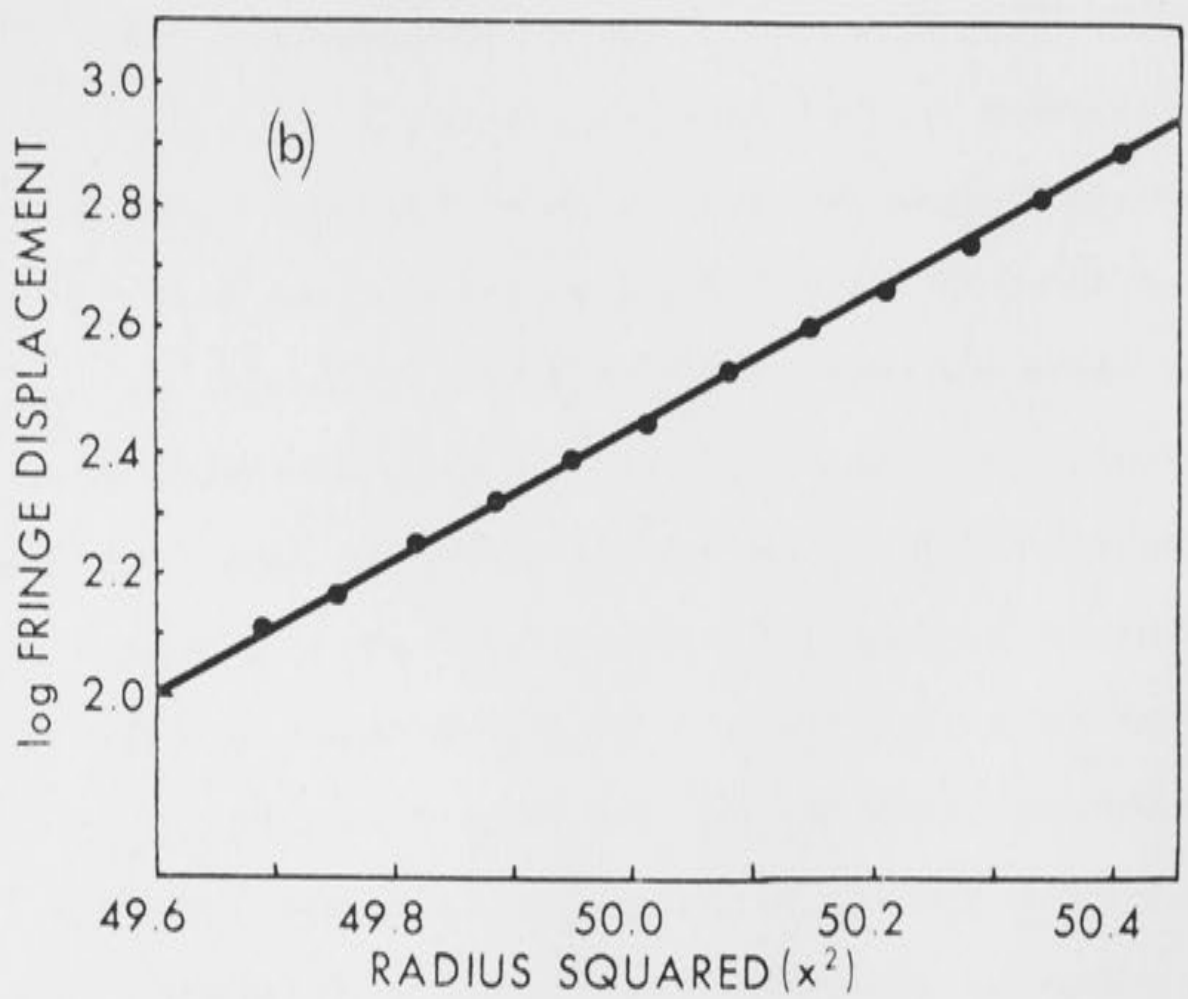

(b)
3.0
2.8
2.6
2.4
2.2
2.0
log FRINGE DISPLACEMENT
49.6
49.8
50.0
50.2
50.4
RADIUS SQUARED (x²)

Figure III - 10

(a) Sedimentation equilibrium results for pure beef liver arginase; ω = 6,000 rpm, T = 20°C. The slope of the plot in conjunction with equation (III - 13) yields a molecular weight for arginase of 114,000 $\pm$ 3,000 over a concentration range of 0.014 to 0.11g dl^{-1}.

(b) High speed sedimentation equilibrium of pure beef liver arginase; ω = 20,000 rpm, T = 20°C, in the buffer 0.05M HCl-tris, 0.02M NaCl, 0.01M $MnCl_2$ pH 7.5. The initial loading concentration of arginase was 0.07g dl^{-1} and the concentration domain scanned equals 0.01g dl^{-1} to 0.08g dl^{-1}.

The value of the molecular weight obtained from the slope of the line in Figure (III-10a) was 114,000 ± 3,000 and is seen to apply in the concentration range 0.014g dl^{-1} to 0.11g dl^{-1} (utilizing the relation that one fringe displacement corresponds to 0.025g dl^{-1}, Van Holde, 1967). This concentration range extends to lower concentration values, the range shown in Figure (III-8b), and clearly indicates that the approximate molecular weight of 96,000 is an underestimate. It is noted in this connection that if dissociation was occurring, the concentration dependence of the observed molecular weight would decrease with decreasing concentration (equation III-14), in contrast to the behaviour observed. This does not exclude the possibility that dissociation effects may become apparent at concentrations below 0.014g dl^{-1}. In order to explore a lower concentration range a second sedimentation equilibrium experiment was performed in the same environment using an initial loading concentration, of dialyzed arginase solution, of 0.07g dl^{-1} and a fixed angular velocity of 20,000 rpm. This value of ω was computed on the basis that $\sigma = \omega^2 M(1-\bar{v}\rho)RT = \omega^2 s/D \sim 5cm^{-2}$ (utilizing the value of D estimated from boundary analysis), and accordingly the design is the meniscus depletion experiment described by Yphantis (1964). In accordance with the suggestion of Teller, Horbett, Richards and Schachman (1969) the over-speeding technique was not employed, but the final distribution recorded at 11 h was shown to be time invariant as before. Other details of the experiment are reported in the caption to Figure (III-10b) which by its linearity shows, according

to equation (III-13), that arginase is a single non-interacting species of molecular weight 114,000 ± 5,000 down to a concentration of 0.01g dl^{-1}, corresponding to a net fringe displacement of approximately 100 microns, the lower limit of acceptable measurement (Yphantis, 1964).

Although the limits of the available optical system in the ultracentrifuge prohibit exploration of the concentration range below 0.01g dl^{-1}, it is possible to explore this range with the use of frontal gel chromatography experiments designed to ensure a plateau of original concentration in the elution profile. Five separate experiments were performed using plateau concentrations of 0.01, 0.005, 0.001, 0.0005 and 0.00005g dl^{-1} on Sephadex G100 equilibrated with the same pH 7.5 HCl-tris-NaCl - $MnCl_2$ buffer. The elution profiles were monitored by means of enzymic activity using the spectrophotometric procedure previously described. The exception was the analysis performed in relation to the experiment conducted at the lowest plateau concentration, where the enzymic assay was performed in the following way: 0.5 ml of the ∿0.8 ml fractions (the exact volume being determined accurately by weight) was added to 2 ml of 2.5 mM arginine pH 7.5 in the buffer employed for the chromatography, and the mixture incubated at 25°C for 2 h after which time 1 ml of 2M HCl was added to stop the reaction. The urea content of the incubation medium was assayed by the diacetyl monoxime method described in Chapters IV and VI. The chromophore was developed for 30 min in a light-free chamber at 100°C, and after cooling the A^{1cm}_{460nm} was measured for each fraction.

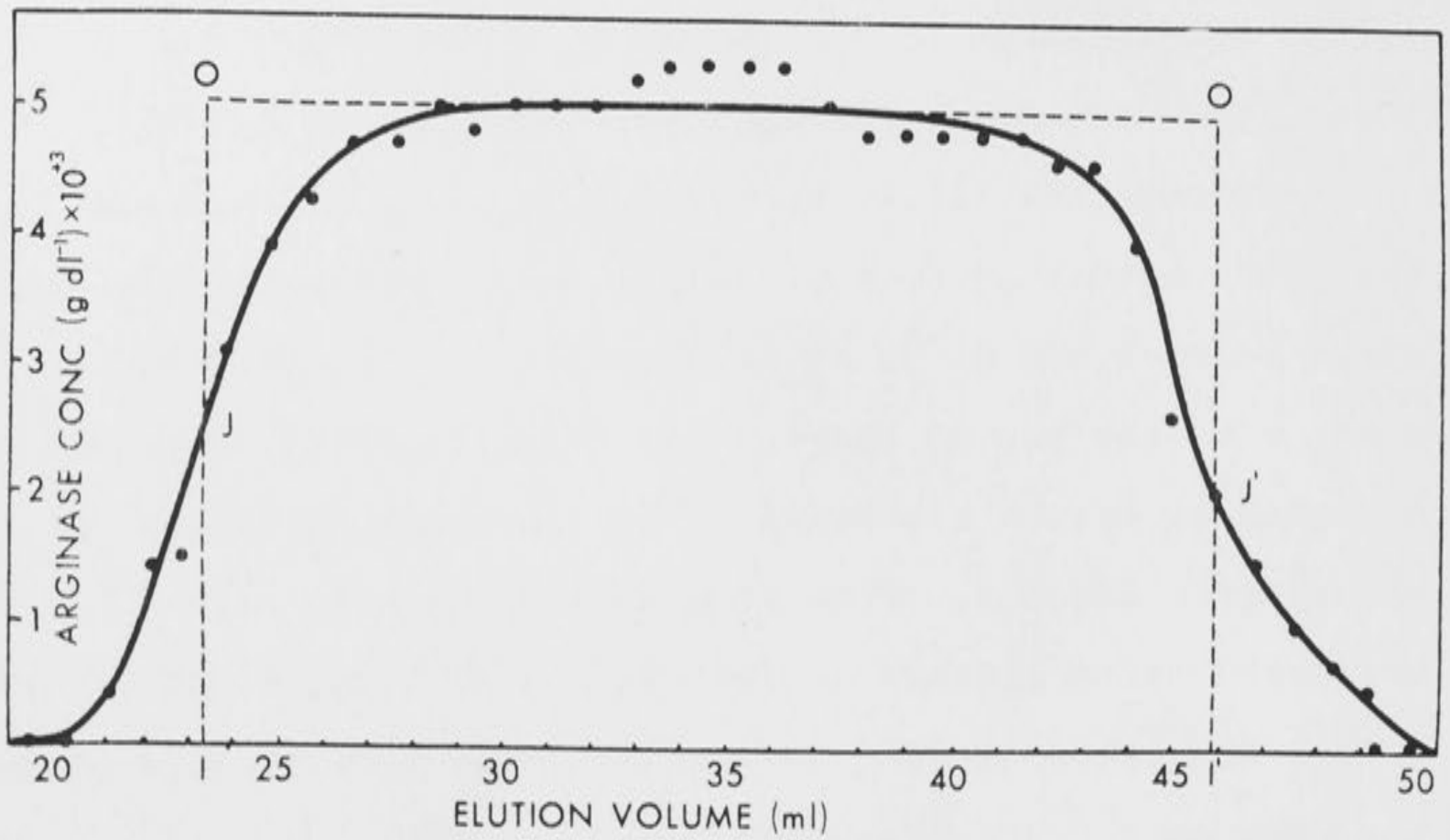


Figure III - 11

Elution profile of beef liver arginase from a frontal gel chromatography experiment on a column of Sephadex G100 (1.27 x 46.5cm). The solid line represents arginase concentration estimated by enzymic assay. The lines IO, and I'O' are the median bisectors of the leading and trailing edges respectively; T = 20°C, plateau concentration of arginase = 0.005g dl^{-1}; buffer = 0.05M HCl-tris, 0.02M NaCl, 0.01M $MnCl_2$ pH 7.5 (I.S. = 0.1); flow rate = 30ml h^{-1}.

A typical elution profile is shown in Figure (III-11) where it is seen that a plateau of the applied concentration 0.005g dl^{-1} is observed in the elution profile: the experimental scatter is reasonable in view of errors involved in the spectrophotometric assay as performed for these experiments. Also in the Figure is shown the construction of the median bisector of the advancing front, OJI, which corresponds to an elution volume of 23.4 ml. This correspondence, together with the observed plateau concentration shows that arginase does not bind irreversibly to Sephadex G100, although in the experiment conducted at 0.0005g dl^{-1} it was observed that the plateau concentration, estimated by enzymic activity measurements, was approximately 20% lower than that applied, perhaps due to an inactivation effect on the column (Nichol and Roy, 1965). The value of 23.4 ml agrees with that corresponding to the median bisector of the trailing edge, O'J'I', 45.8 ml minus the loading volume of 22.40 ml. The elution volume of 23.4 ml found from Figure (III-11) is, according to equation (III-4), a weight-average elution volume, corresponding to the plateau concentration, 0.005g dl^{-1}, a quantity which retains meaning even for a single non-reacting solute when $\bar{V} = V_1$. Values of $\bar{V}$ so obtained were transformed to corresponding K_{AV} values by the definition given by Laurent and Killander (1964),

$$K_{AV} = \frac{\bar{V} - V_o}{V_t - V_o} \qquad \text{(III - 23)}$$

where V_o is the void volume of the column determined using Blue Dextran 2000, and V_1 is the total bed volume determined

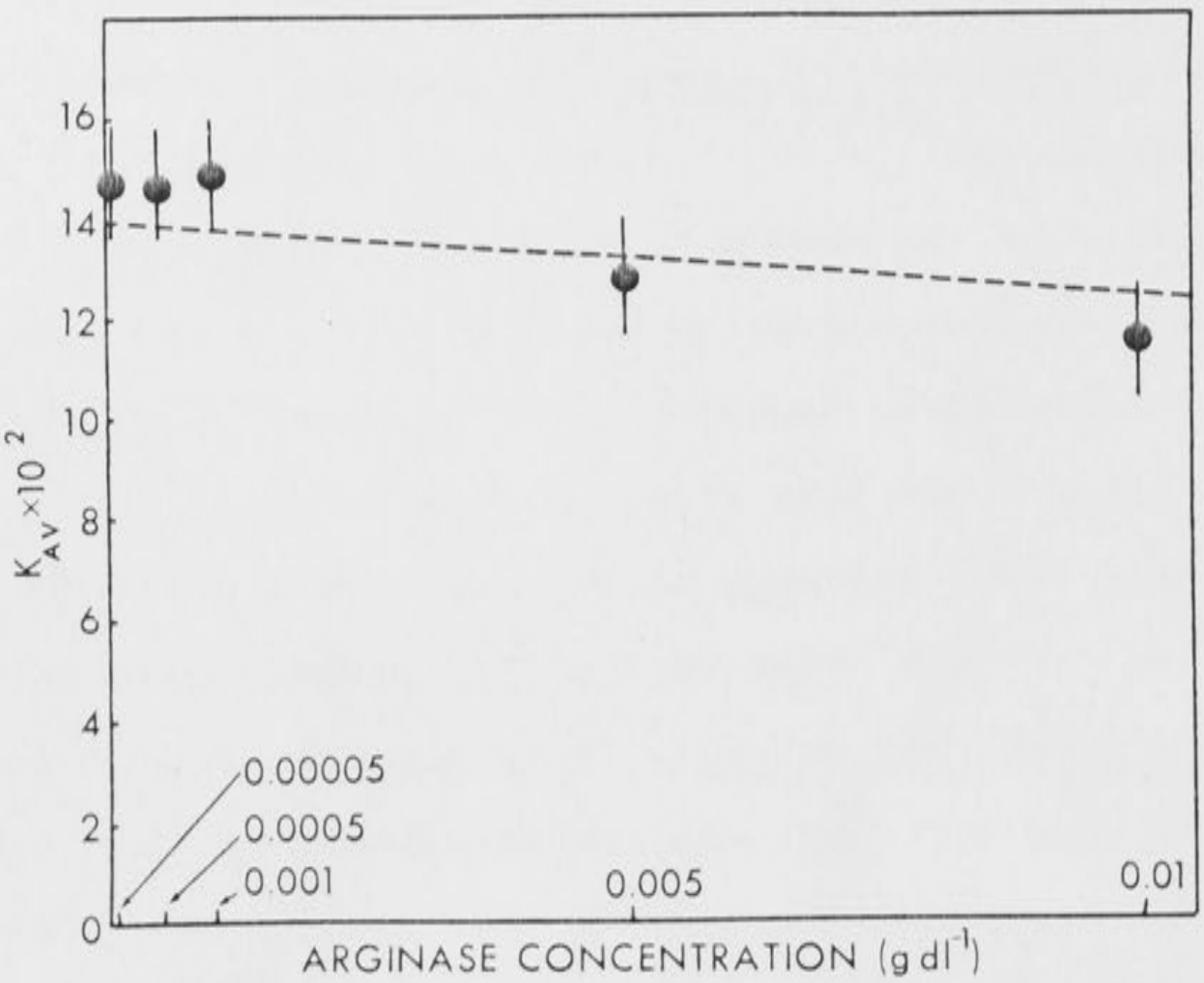


Figure III - 12

Plot of K_{AV} (as defined in equation III - 23) versus arginase plateau concentration for the series of frontal gel chromatography experiments, employing purified beef liver arginase on Sephadex G100, described in the text.

from the geometry of the column. This transformation permits comparison of results obtained with columns of different sizes. This distribution coefficient corresponds closely to the conventional K_d but omits consideration of the actual volume of the gel which is generally of small relative magnitude. Values of K_{AV} obtained are plotted against their corresponding plateau concentrations in Figure (III-12) where the indicated error bars correspond to an estimated 2.5% error in the ordinate values.

It is apparent that no concentration dependence of K_{AV} (or $\bar{V}$) is observed, within experimental error. Accordingly it is concluded that in the pH 7.5 buffer employed at 20°C, arginase exists in solution as a non-reacting species of molecular weight 114,000 $\pm$ 3,000 over the concentration range 0.00005g dl^{-1} (4.4×10^{-9}M) to 1.0g dl^{-1} (8.8×10^{-5}M), a range which encompasses that employed in enzyme kinetic studies to be reported in the next Chapter. It is also of interest to note that the average value of K_{AV} found from Figure (III-12) leads by interpolation (denoted by A) in a standard curve (Andrews, 1966), to a molecular weight of 100,000 $\pm$ 10,000 as shown in Figure (III-13). It is also noted in relation to the design of the frontal analysis experiments, that had the arginase dissociated into half-units its K_{AV}, denoted by B in Figure (III-13), would have been at least 0.22 and thus would have manifested itself in the experiments performed, as an increase of at least 3 ml in the elution volume; a value readily detectable when it is considered that ~0.8ml fractions were collected.

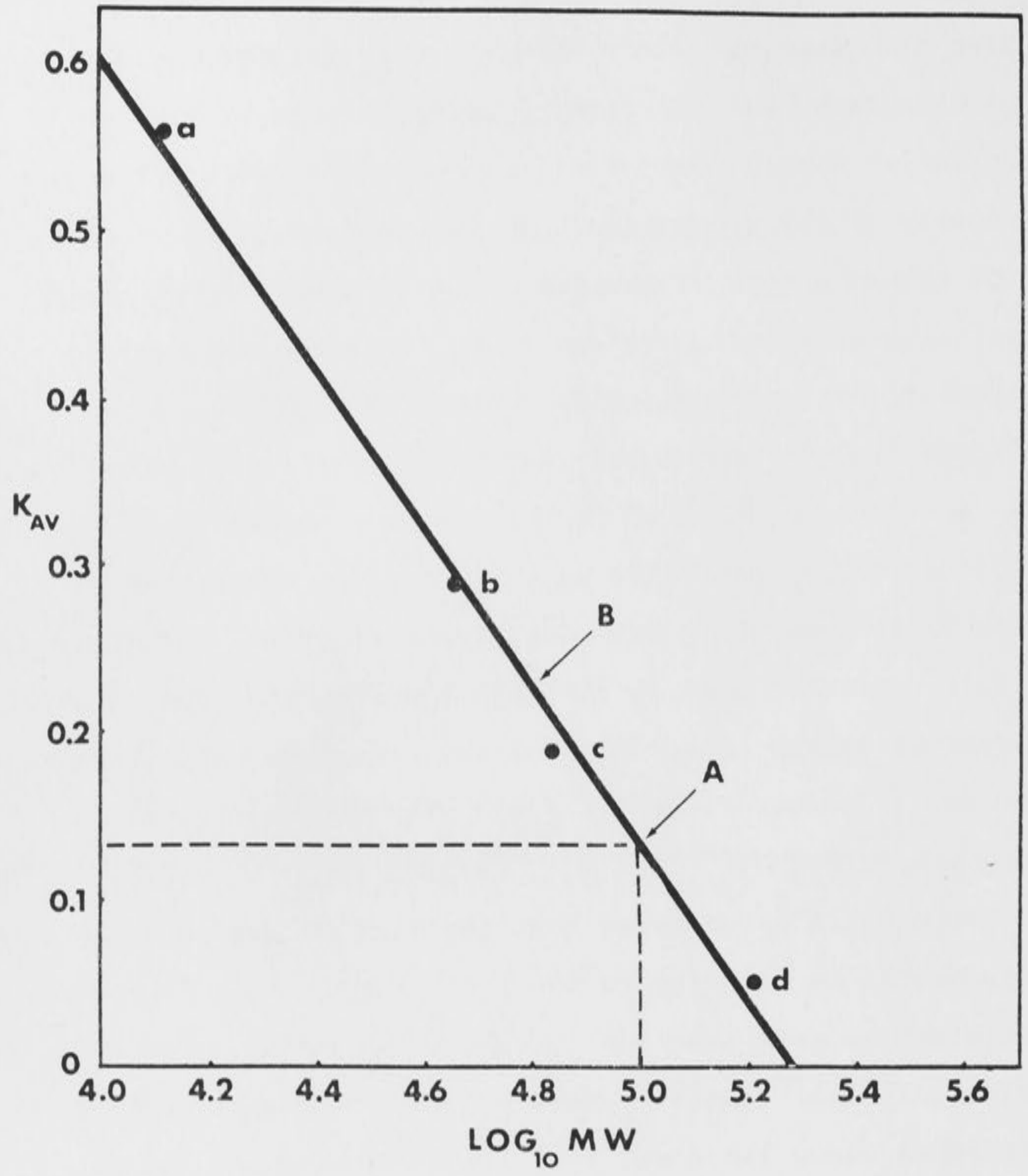


Figure III - 13

K_{AV} for Sephadex G100, versus $\log_{10}$ molecular weight (MW) of four different proteins; a, ribonuclease (13,600); b, ovalbumin (45,000); c, serum albumin (68,000); and d, γ-globulin (160,000). A, corresponds to the K_{AV} for arginase giving a $\log_{10}$ MW of 5 (i.e. MW = 100,000). The K_{AV} corresponding to a MW of 50,000 is shown by the point B.

(c) The frictional coefficient of arginase

The most reliable estimate of molecular weight reported in the previous Section is that obtained from the sedimentation equilibrium experiments and it is for this reason that the molecular weight of 114,000 was employed in the calculations of Table (III-2), and will be employed in the remainder of this study. The value together with a partial specific volume equal to 0.734 $g^{-1}ml$ leads to a revised estimate of the diffusion coefficient of $4.94 \times 10^{-7} cm^2 sec^{-1}$. With the knowledge that non-ideality effects are small it is possible to calculate the frictional resistance per molecule employing equation (III-21). This leads to a value of f of 8.19×10^{-8} dynes per molecule. The value of f_o calculated from equation (III-22) is 6.07×10^{-8} dynes per particle, which leads to a frictional ratio f/f_o of 1.3. The Stokes radius $(3M\bar{v}/4\pi N)^{1/3}$ was calculated to be 3.21nm. It must be emphasized that the value of the frictional coefficient which is greater than unity, strongly suggests that the molecule is not simply an hydrated sphere but is most probably an hydrated ellipsoid. Indeed the value of 1.35 is compatible with the molecule having the shape of an ellipsoid of axial ratio 1:5.6 if the degree of hydration is 0.2g of water per gram of protein; of axial ratio 1:4 if the hydration is 0.4g H_2O/g protein; and of axial ratio 1:2 if the hydration is 0.87g H_2O/g protein (Cohn and Edsall, 1943; Oncley, 1941). It is noted that the characteristic parameters reported for arginase in this Section are in realistic ranges as judged by reported values for other proteins. For example, the protein canavalin from jack bean meal is characterized by quite

similar parameters, namely M = 113,000; $\bar{v}$ = 0.73; s_o = 6.4S; D = 5.1 x $10^{-7}cm^2sec^{-1}$ and $f/f_o = 1.3_1$ (Svedberg and Pedersen, 1940).

(d) The properties of arginase in other experimental environments

Sedimentation velocity experiments were conducted in different buffered environments in order to ascertain the effects of variation of pH, ionic strength, and $MnCl_2$ content, on the properties of arginase samples. All experiments were conducted at 20°C and the results are summarized in Table (III-3), which merits several comments. First, at pH 7.5 the value used in all molecular weight determinations, it is seen that the arginase retains a sedimentation coefficient value of 6S in the ionic strength range 0.025 - 0.16 and with a ten-fold variation of $MnCl_2$ concentration. Secondly, this value of the sedimentation coefficient pertains at all pH values in the range 5.0 - 9.5 regardless of the variation of ionic strength, indicating that the 114,000 species is stable in these environments. Thirdly, the interesting result emerges that at pH 4.0 slower sedimenting species of proportions indicated replace the 6S species even though $MnCl_2$ is present to the extent of 0.01M. A possible explanation of this finding follows from an observation, made with concanavalin A, that certain metal ions, including transition metal ions, important in maintaining the structural integrity of the molecule, are dissociated at acid pH values (Kalb and Levitzki, 1968). It is possible, although not proven, that a similar removal of manganese ions may result in the

Table III - 3

The sedimentation velocity behaviour of Arginase (∿ 0.5g dl^{-1}) at 20°C in various buffered environments.

Buffer	pH	Ionic strength	Sedimentation coefficient (S)
0.08M NaAc, 0.08M HAc 0.05M NaCl, 0.01M $MnCl_2$	4.0	0.16	3.2 (60%), 4.2 (40%)
0.07M NaAc, 0.03M HAc 0.01M $MnCl_2$	5.0	0.10	6.0
0.01M tris-HAc, 0.001M $MnCl_2$	6.3	0.013	6.0
0.01M tris-HCl, 0.005M $MnCl_2$	7.5	0.025	6.2
0.05M HCl-tris, 0.02M NaCl 0.01M $MnCl_2$	7.5	0.10	6.0
0.01M tris-HCl, 0.05M $MnCl_2$	7.5	0.16	6.0
0.03M glycine, 0.023M NaOH 0.037 NaCl	9.5	0.09	6.15

dissociation of the 6S material at pH 4.0. It would be tempting to postulate that the species with s $\approx$ 4S was a half-subunit of the species of molecular weight 114,000 and that with s $\approx$ 3S a quarter-subunit of the species, since these values are in approximate accord with the general relation that $s_M = s_{M/n}\, n^{2/3}$ where s_M = 6S and n is the subunit order. However caution must be exercised, since in the acid pH range it is entirely possible that unfolding as well as dissociation may occur reflecting on the frictional coefficients of the species present, and hence on the sedimentation coefficients. Two further observations implicate the importance of bound manganese in maintaining the structural integrity of the 114,000 molecular weight unit. First, when the sample in the pH 6.3 buffer specified in Table (III-3) was dialyzed against buffer of the same type not containing $MnCl_2$ for 48 h the resulting sedimentation velocity pattern revealed the presence of the 6S material and a slower sedimenting species with s$\approx$4S. Secondly, when a sample in 0.05M HCl-tris, 0.02M NaCl, 0.01M $MnCl_2$ pH 7.5 was introduced as a zone onto a Sephadex G100 column pre-equilibrated with the same buffer not containing $MnCl_2$, it was found that the elution profile exhibited a small leading asymmetry associated with enzymic activity and a major peak of inactive material: evidently the differential migration rate of the protein and $MnCl_2$, in the zonal experiment, down the column, had resulted in partial separation of the protein from the salt and subsequent dissociation and loss of activity. These results are consistent with those of Hirsch-Kolb, Heine, Kolb and Greenberg (1970) who noted that the molecular weight of beef

liver arginase determined by gel filtration on a column not containing manganese at pH 7.5 in 0.01M tris-HCl, was 30,000, in contrast to the value of 120,000 obtained for the rat liver enzyme (Hirsch-Kolb and Greenberg, 1968). The 30,000 unit was inactive, and probably represented one subunit of four which constitute the quaternary structure of active arginase, as was found for rat liver arginase (Hirsch-Kolb and Greenberg, 1968). Thirdly, the total activity of the dialyzed sample was less than the original and this arginase activity could not be regained by the addition of $MnCl_2$ with attempted heat activation.

Clearly several questions remain, in relation to the stabilizing effect of bound manganese, on the arginase quaternary structure. For example, it is interesting to note in Table (III-3) that at pH 9.5 the structural integrity was maintained even though the external buffer contained no manganese ions (a requirement necessitated by the hydrolysis reaction and subsequent precipitation as MnO_2). It would have been possible to explore the phenomenon further by, for example, studying the time dependence of optical rotatory dispersion properties of the protein when manganese was removed by various procedures (acid treatment, dialysis, or gel filtration). However it was decided that this type of endeavour would not contribute to the main theme of this thesis. The importance of the results in Table (III-3), in relation to the present work, was that they dictated that all future studies be performed near neutral pH values and, where possible, in the presence of excess manganese ions. These were the conditions used in the molecular weight determinations and were sufficient

to ensure that a well defined and enzymically active species was being studied.

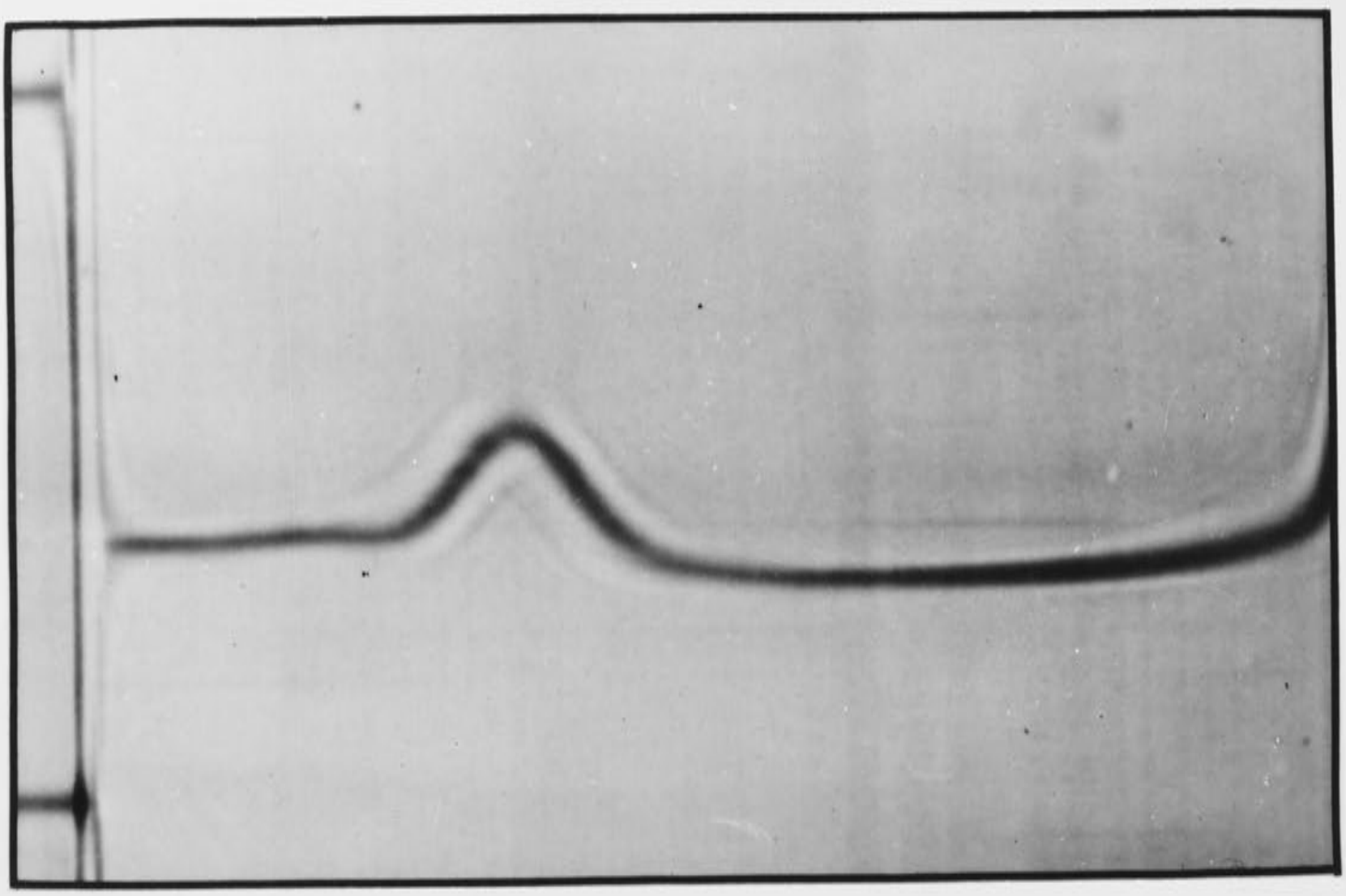
(e) Studies on mixtures of arginase and urease

Since the next Chapter is concerned with a coupled assay involving arginase and urease it is timely in the present context to comment on the interaction, or lack of it, between these macromolecular species. In these studies commercially available urease (Sigma jack bean highly purified type VII powder batch no. 101C-5040, 110,000 Sumner units g^{-1}) was employed. The sedimentation velocity behaviour of which, at pH 7.5, was in accord with the finding of Creeth and Nichol (1960) in that 3 discrete peaks with sedimentation coefficients of 19S (75%), 27S (20%) and 35S (5%) were observed. The latter species were identified by Creeth and Nichol (1960) to be the dimer and trimer of the 19S species formed by inter-molecular disulphide cross linkages, the polymeric species not being in rapid equilibrium. These workers routinely found that storage of urease solutions under these conditions ultimately led to precipitation, and that this was accelerated in the presence of metal ions and oxygen. It was similarly found in this study that urease in high concentration could not be stored in buffer containing $MnCl_2$ without precipitating. Accordingly the procedure of Nichol and Creeth (1963) was employed to modify at least some of the external sulphydryl groups on the native urease molecule to the sulphenyl-sulphite form by the exhaustive dialysis of urease solutions against buffer containing sodium sulphite (0.05M HCl-tris, 0.02 M NaCl, 0.03M Na_2SO_3, pH 7.5); in

this medium the inter-molecular disulphide bonds are heterolytically cleaved and a cyclic mechanism operates (Nichol and Creeth, 1963) to effect the chemical modification of the sulphydryl groups. After this treatment the solution was dialyzed free of sulphite ions against 0.05M HCl-tris, 0.05M NaCl, (I.S. = 0.1), pH 7.5, and subjected to sedimentation velocity. There were small amounts, in total approximately 10%, of slower and faster sedimenting material, indicating as Nichol and Creeth (1960 and 1963) observed, that the modification was not entirely effective in preventing dimer (27S) formation and that small amounts of urease subunits (12S) were formed by this treatment. On the other hand such modified samples remained in clear solution for long periods when stored in the absence of oxygen. Unfortunately turbidity developed in such solutions on the addition of 0.01M $MnCl_2$.

It was required therefore to perform the sedimentation velocity studies on mixtures of arginase and urease in the presence of considerably reduced $MnCl_2$ concentrations. As a control experiment arginase stored in 0.05M HCl-tris, 0.02M NaCl, 0.01M $MnCl_2$ pH 7.5 was dialyzed against the buffer in which the $MnCl_2$ was replaced by a further 0.03M NaCl, for a period of 1.5 h in a rapid dialysis apparatus (Englander and Crowe, 1965) and immediately subjected to sedimentation velocity: the result is shown in Figure (III-14a) where a major peak with a sedimentation coefficient of 6.1S is evident, together with an immeasurably small amount of slower sedimenting material. Evidently, this procedure, with

(a)



(b)

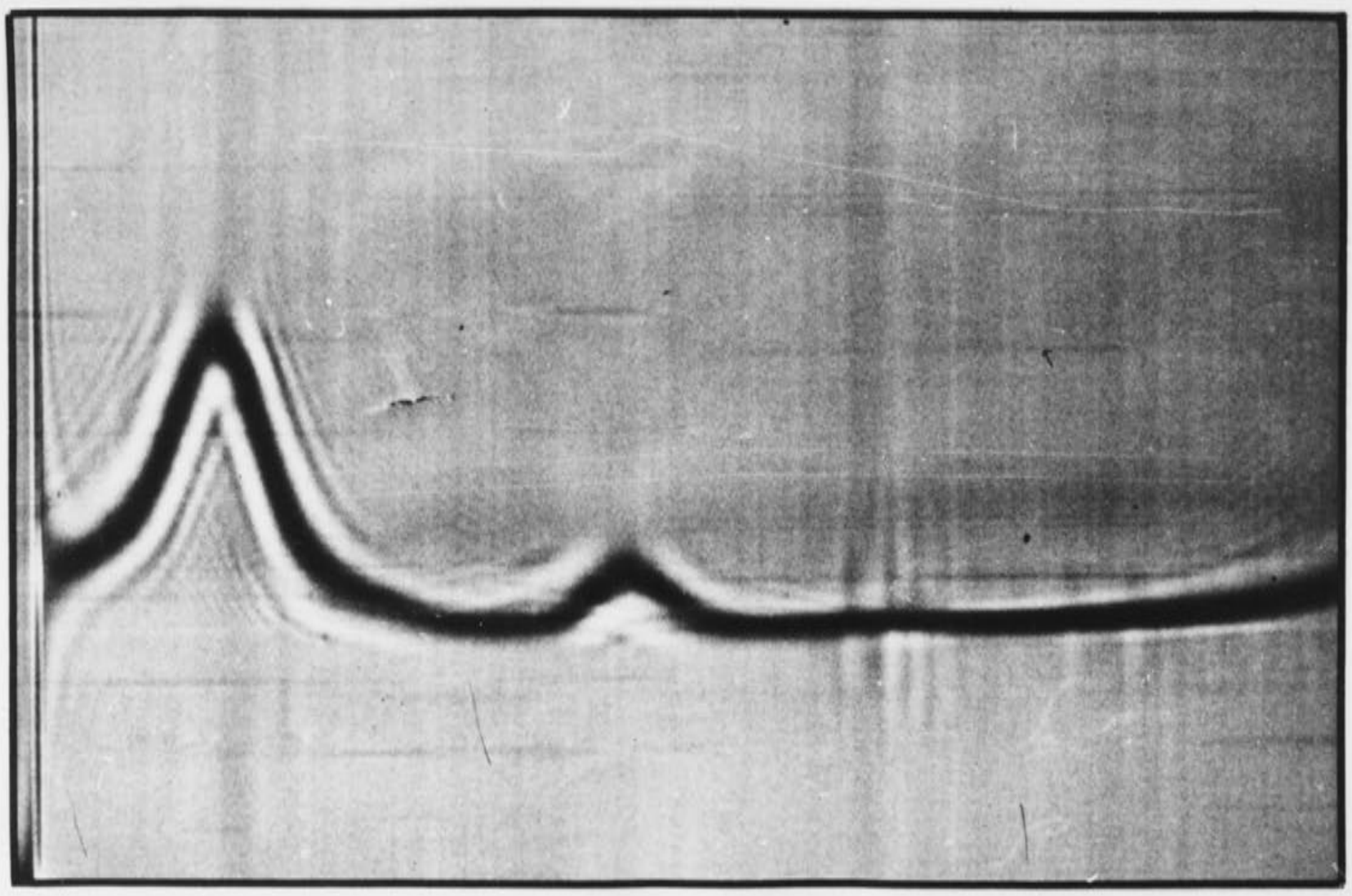


Figure III - 14

(a) Schlieren pattern, obtained ∿45 min after the commencement of a sedimentation velocity experiment at 60,000 rpm and 20°C, of beef liver arginase which had been rapidly depleted of manganese ions by rapid dialysis for 1.5 h against 0.05M HCl-tris, 0.05M NaCl pH 7.5. Phase plate angle = 70°.

(b) Schlieren pattern, obtained ∿28 min after the commencement of a sedimentation velocity experiment at 60,000 rpm and 20°C, of a mixture of beef liver arginase and sulphite modified jack bean urease in 0.05M HCl-tris, 0.05M NaCl pH 7.5. Phase plate angle = 70°.

respect to time and method of removing manganese ions, is less effective than the zonal Sephadex G100 column treatment, discussed above, with the fortunate result that the structural integrity of arginase is largely maintained in a medium in which the sulphite modified urease remains soluble. The results of sedimenting a mixture of arginase and urease of composition 0.1g dl^{-1} and 0.68g dl^{-1} arginase are shown in Figure (III-14b). This pattern shows the features described above. The slight asymmetry of the main peak near the meniscus is due to the small proportion of arginase subunit evident in Figure (III-14a). The major peak moves with a sedimentation coefficient of 6.1S, characteristic of intact arginase. The slight asymmetry of the leading edge of this main peak is due to the small amount of urease subunit. The well defined leading peak is characterized by s = 19S, that of urease monomer, with evidence for a small amount of 27S (urease dimer) material moving foremost. Two major points emerge: (1) the identity of the sedimentation coefficients of the major peaks evident in Figure (III-14b) with those obtained in the control experiment performed with urease and arginase alone, when account is taken of concentration dependence, provides the first indication that no chemical interaction has occured between the species, and (2) the area of the peak with s = 6.1S corresponds to an arginase concentration of 0.68g dl^{-1} which is in accord with equation (III-9) with $v_B^{\beta} \approx \bar{v}_B^{\alpha}$. It may be computed from Figure (III-14b) that if a Johnston-Ogston effect operated alone in this mixture

the magnitude of the relative velocity term in equation (III-9) would be 1.004 and therefore within experimental error that $c_B^{\beta} = \bar{c}_B^{\alpha}$ as observed. This observation therefore supports the claim that no interactions occur between urease and arginase in this environment, since clearly c_B^{β} is not less than $\bar{c}_B^{\alpha}$ and v_B^{β} is not less than $\bar{v}_B^{\alpha}$. It is true that the mixing concentrations used in relation to Figure (III-14b) are much larger than those which will be encountered in future kinetic studies, but consideration of Ostwald's Dilution Law suggests that since interaction does not occur at the higher concentrations, it cannot arise in the lower concentration range. In the next Chapter additional evidence will be presented to support this view and also to explore the possibility of a ligand mediated interaction between the enzymes.

4. Discussion.

It is clear that the preparative method described in detail in this Chapter leads to samples of beef liver arginase which may be judged homogeneous with respect to electrophoretic mobility (Figure III-6b) with respect to sedimentation coefficient (Figures III-8b and III-9) and with respect to molecular weight (Figure III-10a and b).

The most recent report on the physico-chemical properties of beef liver arginase is that given by Harell and Sokolovsky (1972) with which the present findings will now be compared. These workers reported that the isoelectric point, established by isoelectric focusing of the protein, was pH 5.9, in complete agreement with the present findings. This means that the arginase bears a net negative charge at

pH 7.5, the prime pH value selected for studies reported in this and later Chapters. Since the isoelectric point of unmodified urease is pH 5.0 $\pm$ 0.05(Creeth and Nichol, 1960) it follows that urease also bears a net negative charge at pH 7.5; and hence net electrostatic repulsion between the two enzymes is likely to operate at this pH, preventing interaction between the species. It is noted in this connection that the result in Figure (III-14b) shows only that there is no interaction between arginase and chemically modified urease which bears additional net negative charges at pH 7.5 (Nichol and Creeth, 1963).

There is also agreement between the values of the sedimentation coefficient and of the molecular weight of beef liver arginase found in this work and as reported earlier. Thus Harell and Sokolovsky (1972) found in 0.01M tris-acetate pH 6.3 (presumably in the presence of excess manganese ions) values of 6.1S and 113,000 $\pm$ 5,000 respectively, while the corresponding values in the present study, found at pH 7.5, were 6.15S and 114,000 $\pm$ 3,000. The additional information has been provided in the present study that the concentration dependence term of equation (III-3) is $0.069 g^{-1} dl$, that the measured partial specific volume is $0.734 \pm 0.003 g\ dl^{-1}$ and that the molecule has a Stokes radius of 3.21nm with frictional coefficient 1.35. Moreover it is now established by Figures (III-11) and (III-12) that this unit continues to exist even at high dilution with a K_{AV} of 0.13 on Sephadex G100. As noted earlier the detailed quaternary structure of the 114,000 unit has not been explored in this study, although in agreement with Harell and Sokolovsky (1972) slow sedimenting species

were observed on acid treatment (Table III-3). It does appear that four moles of manganese are bound per 114,000 unit, an atomic absorption spectroscopy finding of the previous workers, and that removal of some or all of these manganese ions results in a loss of stability of this unit. The situation is similar to that found with rat liver arginase (Hirsch-Kolb, Kolb and Greenberg, 1971) where nuclear magnetic resonance (NMR) studies have revealed that four moles of manganese are bound per mole of fully activated arginase. These workers found that the binding affinities of the metal ions were not identical. Extensive dialysis, for example, removed only 50% of the bound manganese, with a corresponding loss of enzymic activity. No report was given of the molecular characteristics of the protein so depleted in manganese content. In the case of rabbit liver arginase (Vielle-Breitburd and Orth, 1972) the 110,000 unit is converted into an estimated four non-identical subunits by addition of sodium dodecyl sulphate (SDS) or acid treatment, and again manganese ions were implicated in the retention of enzymic activity.

It may be concluded from these findings that provided kinetic experiments may be designed in which arginase exists in the presence of excess $MnCl_2$ at pH 7.5 a well defined molecular entity will be operative. The additional finding that urease is unlikely to interact with arginase at pH 7.5 suggests that this couple is particularly suited for the experimental test of the theory presented in Chapter II.

CHAPTER IV

THE KINETICS OF THE COUPLED REACTION CATALYZED BY ARGINASE AND UREASE

A study of the kinetics of the coupled reaction catalyzed by arginase and urease is reported in this Chapter. As a preliminary to these studies it was necessary to explore the behaviour of both enzymes individually in relation to their substrates, and then in the presence of the compounds that they were to encounter in a coupled assay. The buffer employed in these studies was the same as that used for the physico-chemical characterization of arginase, 0.05M HCl-tris, 0.02M NaCl, 0.01M $MnCl_2$, pH 7.5, and the assays were performed at 25°C. This buffer was selected on the basis of the known pH dependence of activity of both enzymes. Thus urease exhibits a reasonably sharply defined pH optimum at pH 7.5 in tris buffer (Lynn 1967), while the pH activity curve for arginase is broad with its maximum around pH 9.5 (Greenberg, 1960), overlapping that of urease at pH 7.5 with retention of 60% of the maximal activity. Other possible buffer types, such as citrate and borate were not chosen on the grounds that they inhibit arginase and complex manganese ions (possibly related effects). It is true that tris buffer also complexes manganese ions, but as was shown in control experiments, not to an extent which affects arginase activity, provided the Mn^{++} concentration is in excess (10mM), a condition also required for structural stability of the enzyme at pH 7.5 (Chapter III).

1. The steady-state kinetics of arginase.

(a) Methods of following the reaction

It is of particular interest in relation to the present work that as early as 1924 arginase was assayed by the use of urease, the ammonium ions produced as a final product being estimated colourimetrically (Hunter and Dauphinee, 1924), titrimetrically (Hunter and Dauphinee, 1929) or alternatively the carbon dioxide was measured manometrically (Wiel and Russell, 1934; Hunter and Pettigrew, 1936; Van Slyke and Archibald, 1946). However it is evident that in the present initial studies it is necessary to establish methods of assaying arginase without recourse to the coupled assay. In the early work of Kossel and Dakin (1904a, b) arginase activity was estimated by noting the elevation, in a solution of arginine, in the amount of nitrogen not precipitated by phosphotungstic acid. The first quantitative procedure was that of formol titration in which the amount of acid titrated in order to attain a given end-point increased with the release of extra amino groups on the formation of ornithine from arginine (Edlbacher, 1917). It is also possible to monitor the reaction by estimating the concentrations of either arginine or urea. In the former instance the method devised by Rosenberg, Ennor and Morrison (1956), a modification of the Sakaguchi reaction, using α-naphthol, may be employed; a less sensitive but easier method of estimating arginine is the spectrophotometric procedure described by Ward and Srere (1967), and outlined in Chapter III. In the latter instance urea may be estimated by either the precipitation as xanthylurea with xanthydrol, or the colourimetric methods

using diacetyl monoxime (Fearon, 1939; Ceriotti and Spandrio, 1963) or α-isonitrosopropiophenone (Van Slyke and Archibald, 1946). In this study the spectrophotometric method for estimating arginine, and the colourimetric method employing diacetyl monoxime to estimate urea were selected.

(b) The dependence of initial velocity on initial substrate concentration

(i) Non-continuous assay. The format for these experiments was as follows: 1 ml of arginine solution of known concentration was introduced into a 10ml Quick-fit test tube with ground glass stopper, and 1 ml of arginase solution, also pre-equilibrated at 25°C was added. The reaction was terminated at known times by the addition of 0.5 ml of 2M HCl, blanks being obtained by reversing the order of addition of acid and enzyme. The urea produced was estimated by diluting 1 ml of incubation mixture with 1 ml of H_2O, and addition of 1 ml of Solution I (0.25% w/v diacetyl monoxime in 3% v/v aqueous acetic acid) and 1 ml of Solution II (0.8% w/v antipyrine in 80% sulphuric acid). This mixture was stirred using a Vortimix, care being taken to avoid layering of the sulphuric acid. The chromophore was developed by incubating the tubes at 100°C for 30 min in a closed copper boiler since the chromophore is photo-sensitive especially at high temperatures. This also necessitated flushing the system after the 30 min incubation period with cold water, prior to the removal of the tubes. The absorbance of the contents of each tube was measured at 460nm in 1cm cuvettes employing a Ziess (model PMQ II) spectrophotometer. The corresponding

concentration of urea was obtained by interpolation from a standard line constructed using urea solutions of known concentration. This plot was linear over the urea concentration range in the cuvette of 2×10^{-6}M to 2×10^{-5}M and exhibited a slope of $3.8 \pm 0.1 \times 10^{4}$ over an absorbance range of 0 to 0.8. It is noteworthy in relation to this relatively large value of the slope that, since the average wet thumb-print contains 0.47μM of urea (Hamilton, 1965), if a moist stopper is handled without caution, the concentration of urea in the assay tube may be enhanced to give a resultant increase in the absorbance of the solution of 0.5. Thus considerable care must be exercised in washing and handling incubation and assay tubes. For similar reasons, the buffered solutions were transferred with 1 ml and 0.5 ml Carlsberg pipettes fitted with a bulb device used for withdrawing and dispelling solutions. It could also be noted that prior to interpolation in the standard curve, absorbance readings were corrected using blanks, which exhibited colour due to the formation of chromophore from arginine. This procedure is valid because the absorbance of the arginine-chromophore at 460nm is only 1% of that of the urea-chromophore and because the concentration of arginine in the blank closely approximated the residual arginine in the reaction mixture since times were examined corresponding to only a maximum of 10% depletion of substrate. It would perhaps have been preferable to isolate the arginine from the incubation mixture prior to the development of the urea-chromophore, but attempts to employ cation exchange resins for this purpose were unsuccessful in that the separation of arginine

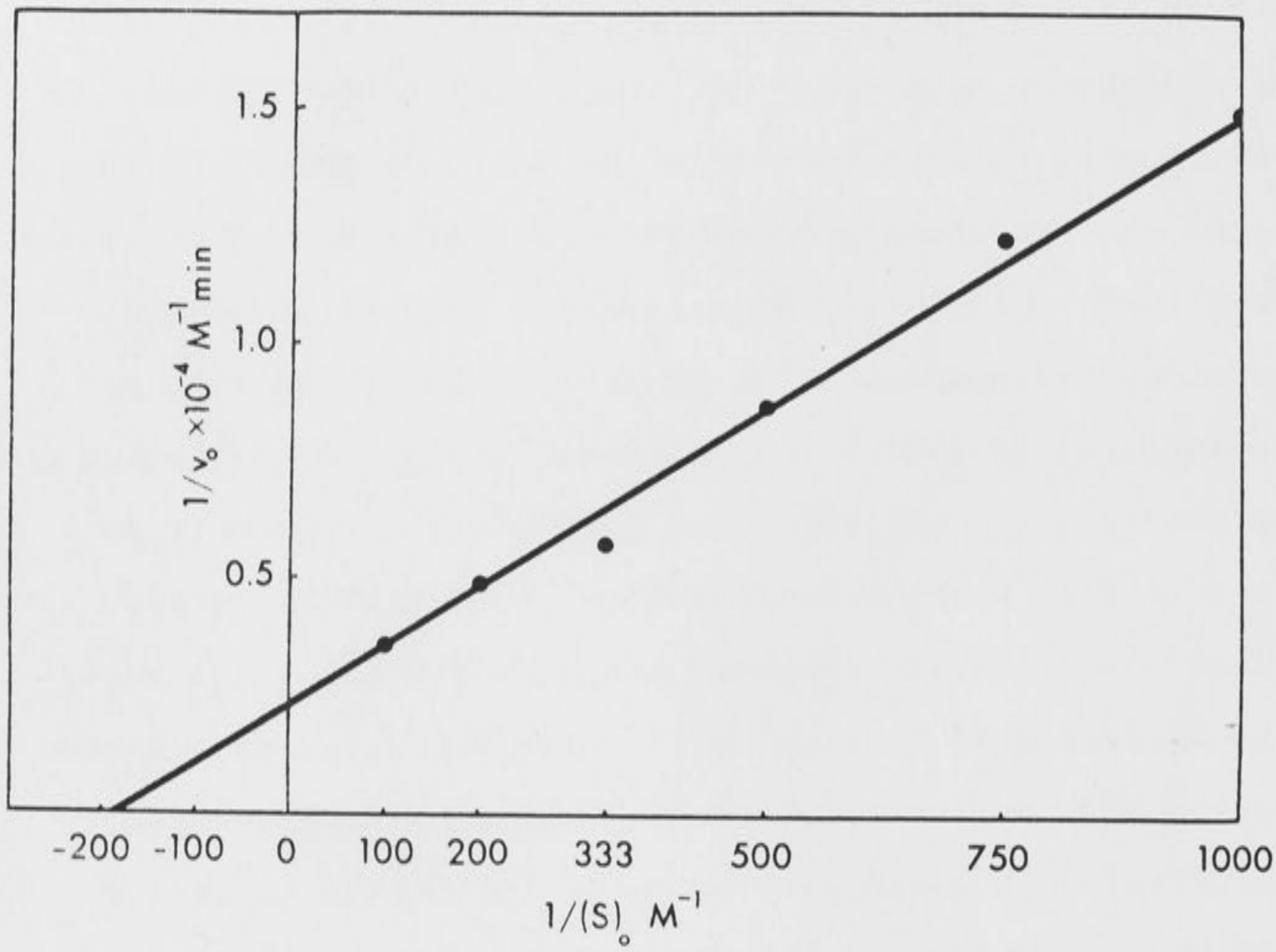


FIGURE IV - 1

A double reciprocal plot of data from initial velocity studies on beef liver arginase in 0.05M HCl-tris, 0.02M NaCl, 0.01M $MnCl_2$ pH 7.5 at 25°C. The solid line is that fitted by weighted least squares linear regression.

from the urea resulted in excessive dilution of the urea eluant and was too tedious for efficient use in the multiple assays employed in any given experiment.

From plots of urea concentration versus time, initial velocities of the reactions were measured and plotted against the corresponding initial substrate concentration. Typical results, in double reciprocal form are shown in Figure (IV-1) where the solid line was obtained by fitting the experimental points by the method of weighted linear least squares regression. The results are in accord with the conventional Michaelis-Menten equation (equation II-2a) with values of K_m equal to 6.0 $\pm$ 1.2mM and V_{max} 4.2 $\pm$ 0.5 x 10^{-4}M min^{-1}. The latter value corresponds to an overall breakdown rate constant of 8.2 x 10^4 min^{-1} on the basis of an enzyme concentration of 5.12 x 10^{-9}M, determined by dilution of a stock solution whose concentration was measured spectrophotometrically employing the extinction coefficient, $E^{1mg\ ml^{-1}}_{278nm}$ = 0.96 (Harell and Sokolovsky, 1972), and the molecular weight of 114,000. Similar sets of experiments were performed employing different preparations of arginase of slightly different concentrations and in each case analysis of the double reciprocal plots led to the values of the kinetic parameters which were in basic agreement with the values reported above. All findings are summarized in Table (IV-1) together with associated estimated standard deviations.

(ii) The flow-assay. In order to study more readily the kinetics of arginase using small increments of substrate concentration, the flow apparatus depicted schematically in Figure (IV-2) was constructed. It consisted

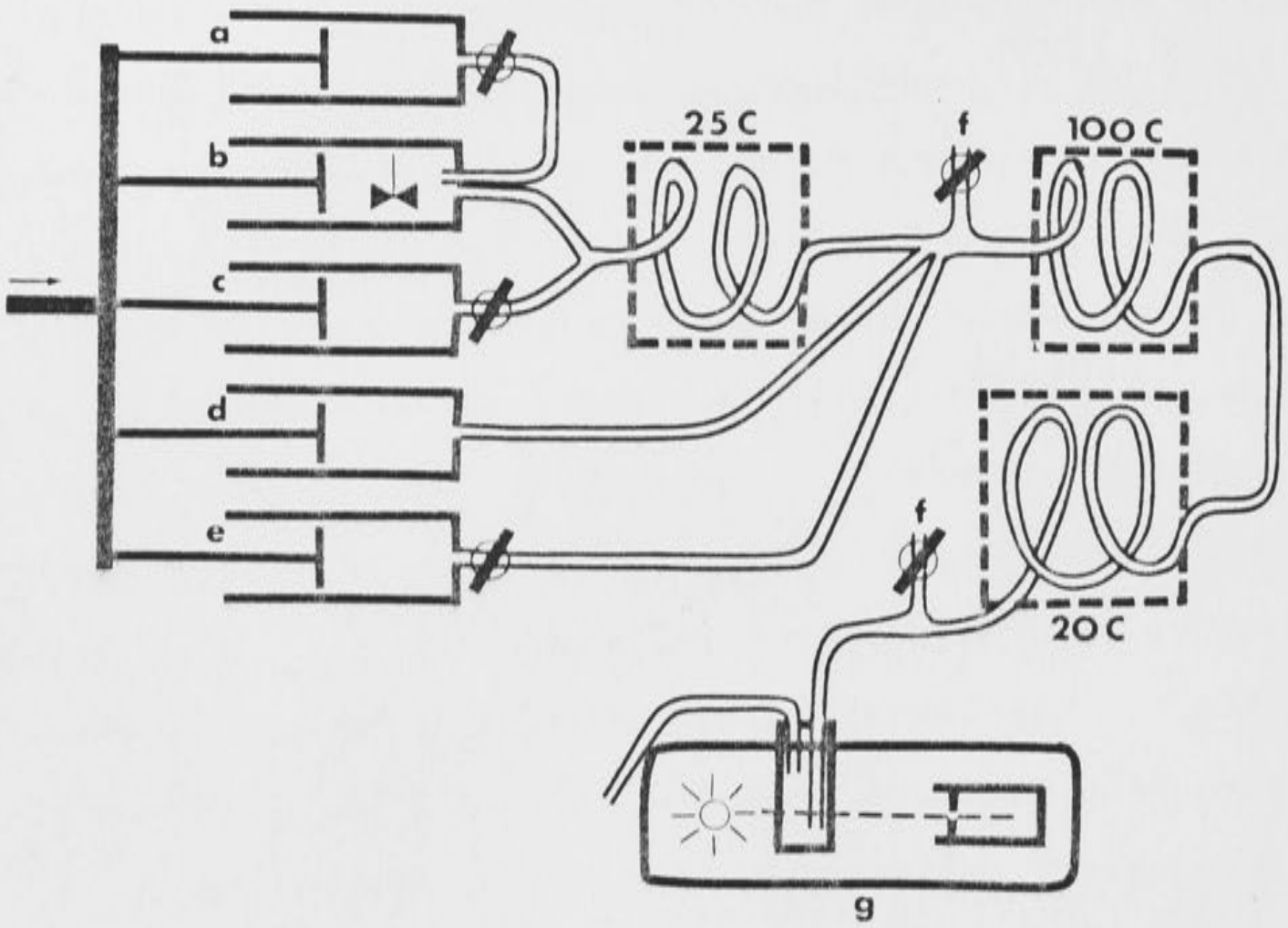


FIGURE IV - 2

A schematic representation of the continuous flow assay device used for the study of the initial time kinetics of arginase. The device consisted of a Braun UNITA I six syringe infusion apparatus; the syringes are marked *a*, *b*, *c*, *d* and *e*. The mixing chamber was syringe *b* and stirring was effected by a magnetic follower. The enzyme reaction coil was maintained at 25°C, the chromophore development coil at 100°C, and the cooling coil at 20°C. The two taps (*f*) were for venting bubbles from the reaction coils prior to the commencement of an experiment. The spectrophotometer is represented by section *g*.

of a Braun UNITA I six syringe infusion apparatus in which the two syringes denoted by *a* and *b* were used to form an accurate linear concentration gradient of the substrate arginine, which was mixed with an arginase solution delivered from syringe *c*, prior to incubation at 25°C. The arginase while in the coil acted on the substrate for 1.6 min (a value dependent on the flow rate and tube dimensions). Reagent solutions I and II of the diacetyl monoxime urea assay, delivered from syringes *d* and *e* respectively, were then added. This mixture flowed through a light free chamber maintained at 100°C, the internal tubing being sufficiently extensive to ensure a period of 15 min for the development of the chromophore. The outflow was cooled in jacketed tubing maintained at 20°C by tap water prior to its passage through a small volume (1cm) flow cell situated in the spectrophotometer *g* (wavelength 460nm), which was coupled to a Rikadenki chart recorder. To avert the formation of bubbles in the reaction lines, which resulted in great disturbance of the spectrophotometer trace, all solutions were degassed by twice freezing and thawing in ethanol dry ice mixtures under vacuum. The enzyme solution was constituted from concentrated stocks and diluted in degassed buffer.

A representative recording trace (a plot of absorbance versus time) is shown by the solid line in Figure (IV-3). The value of 22 min, where it is seen that the trace first rises above the base line, was determined in a separate experiment as the time taken for a methylene blue solution to appear in the spectrophotometer cell from its initial starting position in syringe *b*. The time axis shown in

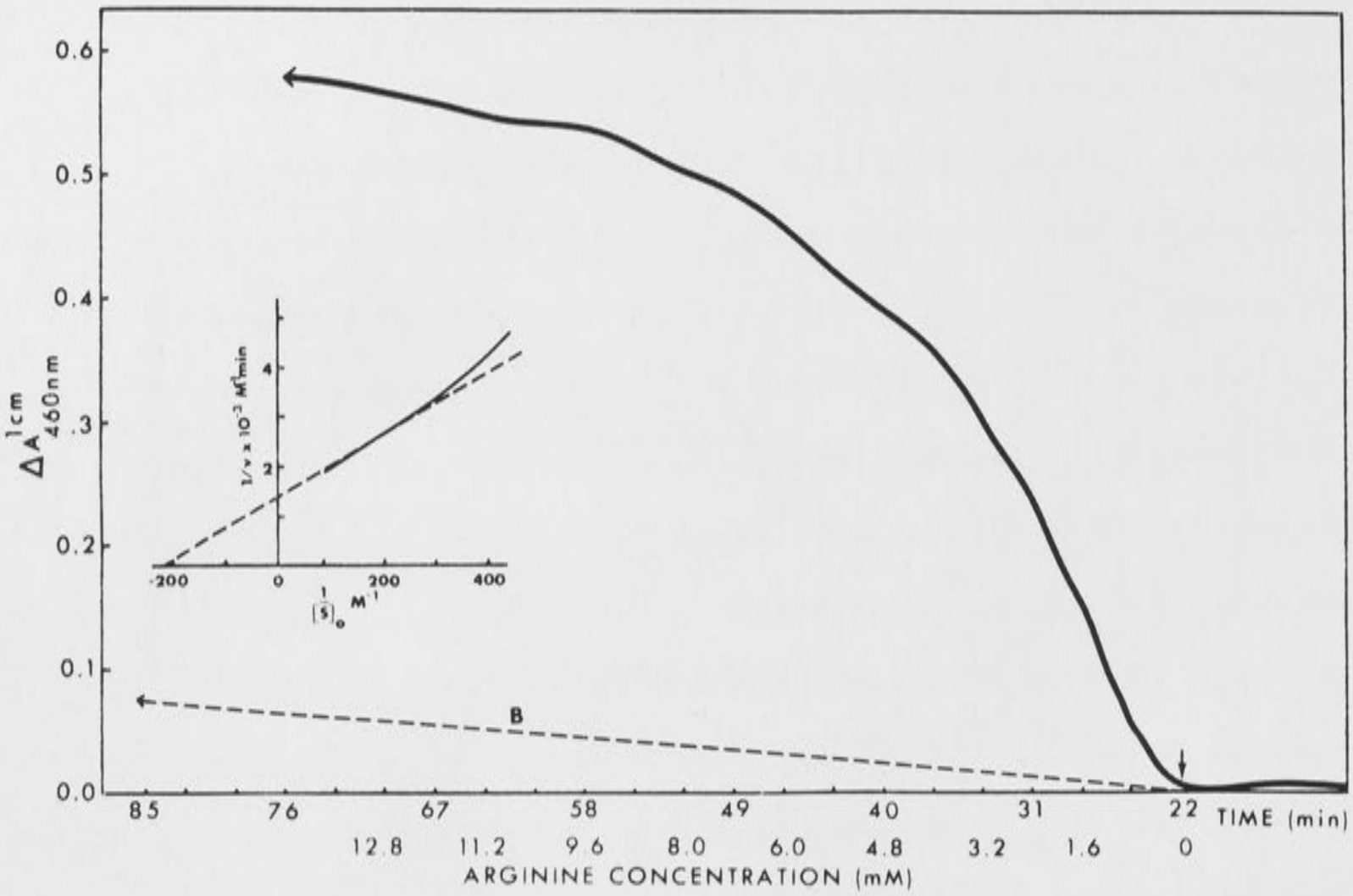


FIGURE IV - 3

A spectrophotometric trace (solid line) obtained at 460nm from a continuous flow assay of arginase. The abscissa is calibrated in minutes which in turn (according to equation IV - 1) corresponds to initial arginine concentration for the enzyme catalyzed reaction at that time. The narrow broken line (B) is the blank absorbance due to the arginine-chromophore. Inset is a double reciprocal plot of the data (———) and the weighted least squares fit to them (– – –).

Figure (IV-3) is readily transformed into the corresponding substrate concentration axis in the following way. The initial concentration of arginine which flows from syringe *b*, containing initially only buffer, is zero, corresponding to the 22 min time period defined above. As the arginine solution from syringe *a* moves into the buffer in syringe *b* the emergent arginine concentration from syringe *b* must steadily increase from zero, and may be calculated from the equation,

$$C_t = C_o(1 - V_t/V_o) \qquad \text{(IV - 1)}$$

where C_o is the initial concentration of arginine in syringe *a*, V_o is the initial volume in syringe *a* (equal to that of buffer in syringe *b*) and V_t is the new volume in syringe *a* (or *b*) following the operation of the infusion apparatus for time t. Thus to generate a concentration gradient from 0 to 15mM with an initial volume of 20ml in syringe *a* (and *b*) the initial arginine concentration in syringe *a* was 60mM. The dotted line B in Figure (IV-3) was obtained in control experiments performed in the absence of enzyme using a buffer solution alone in syringe *c*. The difference between the absorbance readings shown by the solid and broken lines is proportional to the concentration of urea produced in the 1.6 min incubation period spent in the chamber at 25°C, the proportionality constant being determined from a standard urea line constructed employing urea solutions initially placed in syringe *a*. It was assumed that the concentrations of urea so determined from Figure (IV-3) divided by 1.6 min represented the initial velocity of the reaction: certainly with the arginase concentrations

employed an arginine solution of concentration 5mM would have been less than 6% depleted. These initial velocities are plotted against the corresponding initial substrate concentrations in double reciprocal form shown in the inset of Figure (IV-3) as a continuous solid line. This solid curve exhibits slight upward curvature which might at first sight suggest a positively cooperative effect (Koshland and Neet, 1968).

There are two other possible reasons for the departure from linearity observed at high $1/[S]_o$ values (low initial substrate concentrations). For these values, the percentage depletion of substrate in 1.6 min is considerably larger than 6% (in fact of the order of 20% for 1mM arginine) and this raises the related question of whether the corresponding v values are true initial velocity values. To explore this possibility equation (II-2a) was numerically integrated using a 4^{th} order Runge-Kutta method to obtain plots of product concentration versus time for the range of initial substrate concentration presently under study. From these plots apparent initial velocities were obtained by dividing the concentration obtained at 1.6 min by 1.6: these apparent values when plotted in double reciprocal form against the initial substrate concentration did indeed show upward curvature even though the mechanism considered in the simulation was Michaelis-Menten. At first sight, therefore, it might appear that the deviation from linearity observed in the inset to Figure (IV-3) could be attributed to the failure of obtaining true initial velocities at low initial substrate concentrations. However, the magnitude of the simulated effect indicated that this was not the sole

cause in relation to the present experimental results. A second contributing cause for the deviation is almost certainly the forward mixing effect discussed in detail by Illingworth and Tipton (1969) who worked with a flow apparatus of similar design to that shown in Figure (IV-2). In view of this, of the result shown in Figure (IV-1), and the results to be presented, it was judged that the deviation could not be attributed to positive cooperativity. Therefore the results were analyzed by the weighted least squares method to give the broken line in the inset of Figure (IV-3). A value of 4.8 $\pm$ 0.3mM for the K_m was found by this procedure as reported in Table (IV-1).

Possibly the greatest value of the flow assays is the demonstration of the absence of intermediary plateaux in the v versus $[S]_o$ plot as found with other enzyme systems, (Teipel and Koshland, 1969). It is also noted that the detailed description of the flow assay presented here will be of use in discussion of arginosuccinase kinetics discussed in the next Chapter.

(iii) Spectrophotometric assay. For these experiments a Carey 14 spectrophotometer set at a wavelength of 205.7nm was used with a slit setting of 0.8mm, dynode setting 2 and chart speed usually adjusted to yield an initial slope in the substrate depletion trace of 45°. By use of standard solutions, the molar extinction coefficients of ornithine, arginine and urea, at 205.7nm were determined to be 0.15×10^3, 1.10×10^3, 0.020×10^3, respectively. These values correspond to a molar extinction coefficient for the disappearance of arginine in this assay of 0.98×10^3 in agreement with the value obtained by Ward and Srere (1967).

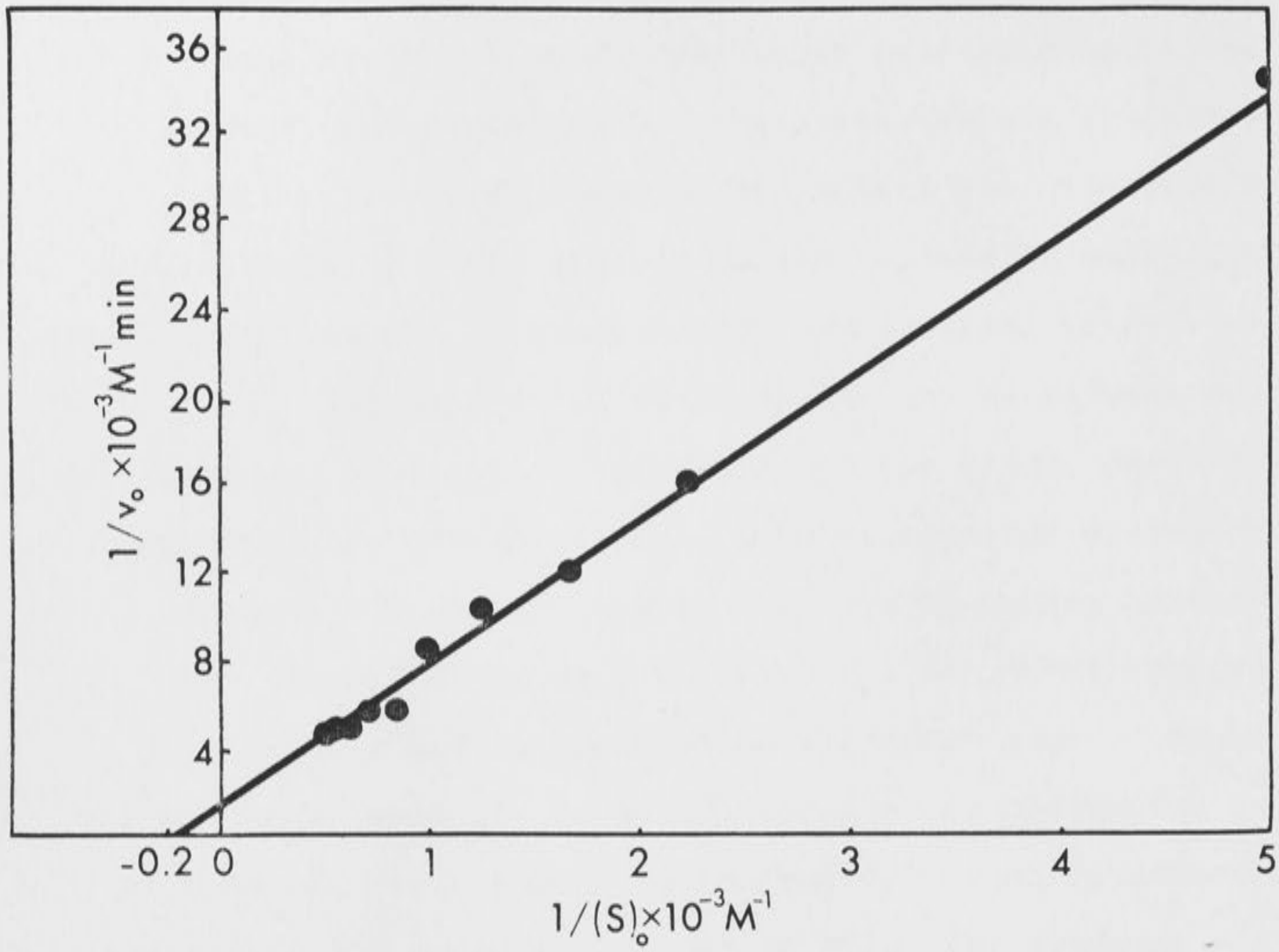


FIGURE IV - 4

A double reciprocal plot of initial velocity data (●) taken from continuous traces of the spectrophotometric assay for beef liver arginase. The solid line was fitted by weighted least squares linear regression.

TABLE IV - 1

The steady state kinetic parameters of arginase and urease

ARGINASE

Assay type	$[E]_o$ (M) x 10^9	K_m (M) x 10^3	V_{max} (M min^{-1}) x 10^4
Diacetyl monoxime	2.1	4.0 ± 0.6	1.72 ± 0.16
"	2.9	3.0 ± 1.5	2.3 ± 0.6
"	1.3	4.3 ± 1.2	1.1 ± 0.2
"	5.1	6.0 ± 1.2	4.2 ± 0.5
Flow-diacetyl monoxime	-	4.8 ± 0.3	-
Spectrophoto-metric	11.3	5.9 ± 2.0	9.3 ± 0.4
"	2.2	6.4 ± 0.6	3.7 ± 0.2

AVERAGE 5.0 ± 1

UREASE

Conway micro-diffusion	3.1	5.0 ± 0.4	1.04 ± 0.05
"	1.71	5.6 ± 1.0	2.84 ± 0.33
"	1.1	2.9 ± 0.3	1.8 ± 0.1
"	1.0	2.9 ± 0.3	1.7 ± 0.1
"	9.45	5.1 ± 1.2	1.6 ± 0.2

AVERAGE 4.2 ± 1

The range of absorbance of an arginine solution over which the Beer-Lambert Law held was 0 to 3. The assays in arginine concentrations below 2mM were performed in 1cm quartz cuvettes, whereas the higher concentrations up to 10mM were studied in 1 or 2mm cuvettes, the cuvettes being jacketed and thermostated at 25°C. Typical results of initial velocity studies conducted in this way are shown in Figure (IV-4) from which the value of 5.9 ± 2.0mM for K_m was derived together with a value of 9.3 ± 0.4 x 10^{-4}M min^{-1} for the maximal velocity.

In summary, it may be concluded from the three different types of assay that the arginase catalyzed hydrolysis of arginine, in the environment selected for study, conforms to the conventional Michaelis-Menten scheme, and that the values of the kinetic parameters reported in Table (IV-1) are consistent with the average value of K_m of 5.0 ± 1mM and a breakdown rate constant of 8.2 x 10^4 min^{-1}. It was also observed that the initial velocity of the reaction at various substrate concentrations was linearly dependent on enzyme concentration, suggesting in accord with the results shown in Figure (III-12) that arginase acts as a non-dissociating unit of molecular weight 114,000.

(c) The inhibitors of arginase

Arginase is inhibited by many compounds, as discussed in the reviews by Greenberg (1951, 1960), but in the present context it is only important to elucidate the possible inhibitory or activating action of the various species which will appear in the coupled assay. By use of the spectrophotometric assay, arginase activity was estimated in various arginine concentrations in the presence of urease,

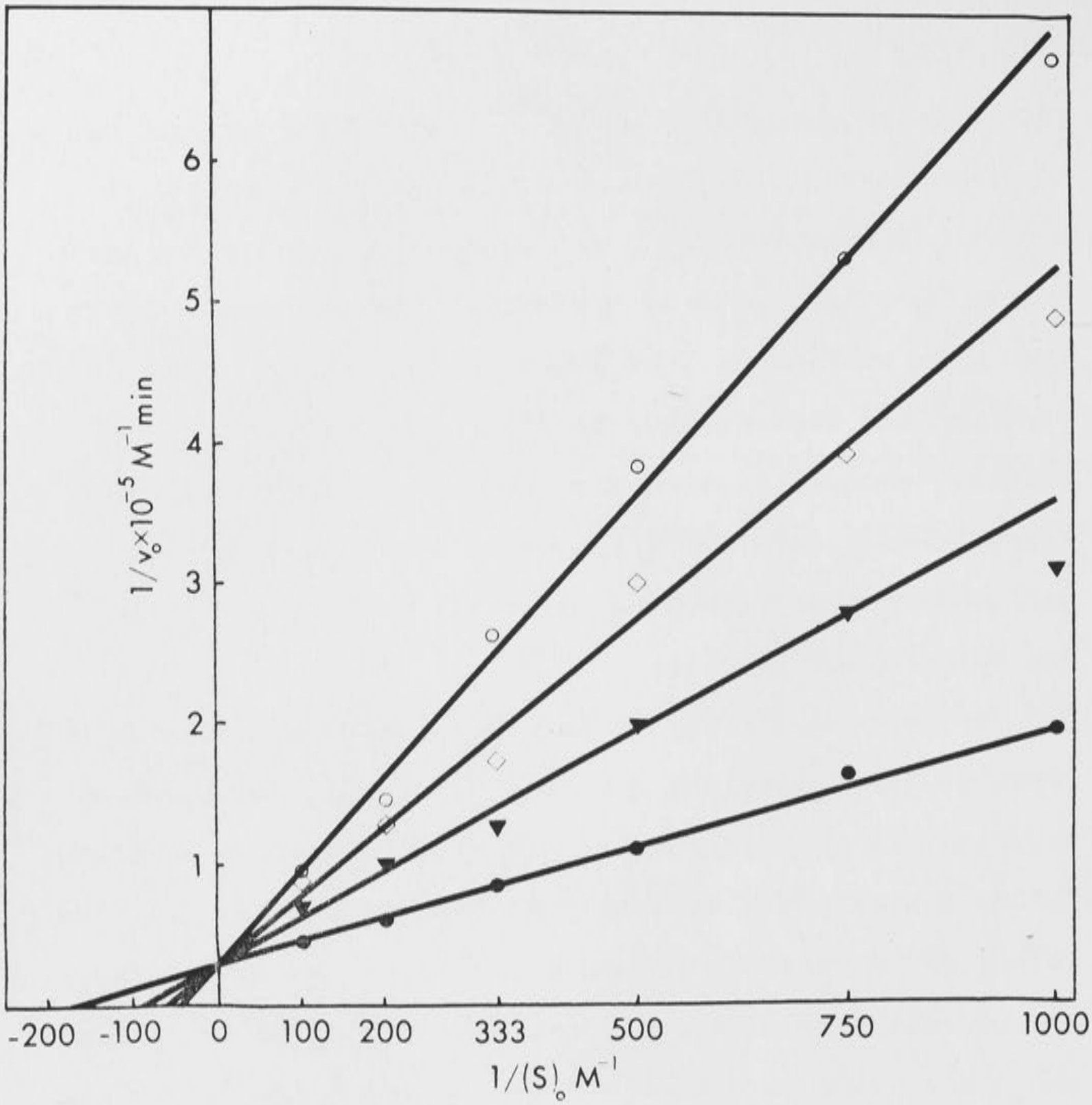


FIGURE IV - 5

The linear competitive inhibition of arginase by ornithine. The points were obtained from initial velocity studies conducted with the spectrophotometric assay utilizing the indicated values of initial arginine concentration $[S]_o$. The values of ornithine concentration employed were as follows: 0mM (●), 5mM (▼), 10mM (◇), and 15mM (○).

and concentrations of ammonium ions and urea ranging from 0 to 10mM. The values of the Michaelis constant and maximal velocity were unchanged, showing that these materials exert no inhibition or activation effects at the concentrations investigated. The remaining compound of interest is ornithine, and this was shown by all three different types of assay procedures described above, to be a linear competitive inhibitor. This effect is illustrated by the results shown in Figure (IV-5) which were further analyzed by conventional means to yield an inhibition constant of 3 ± 1mM. The inhibitory effect of ornithine was investigated in another way. The solid points in Figure (IV-6) show the depletion of arginine from an initial value of 5mM, as a function of time, as monitored continuously in the spectrophotometric assay. Curve d in this Figure was compiled by means of numerical integration of the differential equation describing linear competitive end product inhibition (equation II-28a), employing the reported average value of K_m (5mM) and a V_{max} measured at the time of the experiment, of 1.4×10^{-3}M min^{-1}, with K_I equal to 10^6M (i.e. effectively no product inhibition). Curves b and c were similarly computed with progressively decreasing values of K_I. Clearly the curves are converging on the experimental results with decreasing values of K_I and indeed, curve a is seen to fit the results extremely well, confirming that the inhibition constant is 3 ± 0.2mM. It is possible, though not strictly necessary, to analyze the experimental results presented in Figure (IV-6) and those similar to them, in an alternate way. The integrated form of the differential rate equation describing linear competitive inhibition by

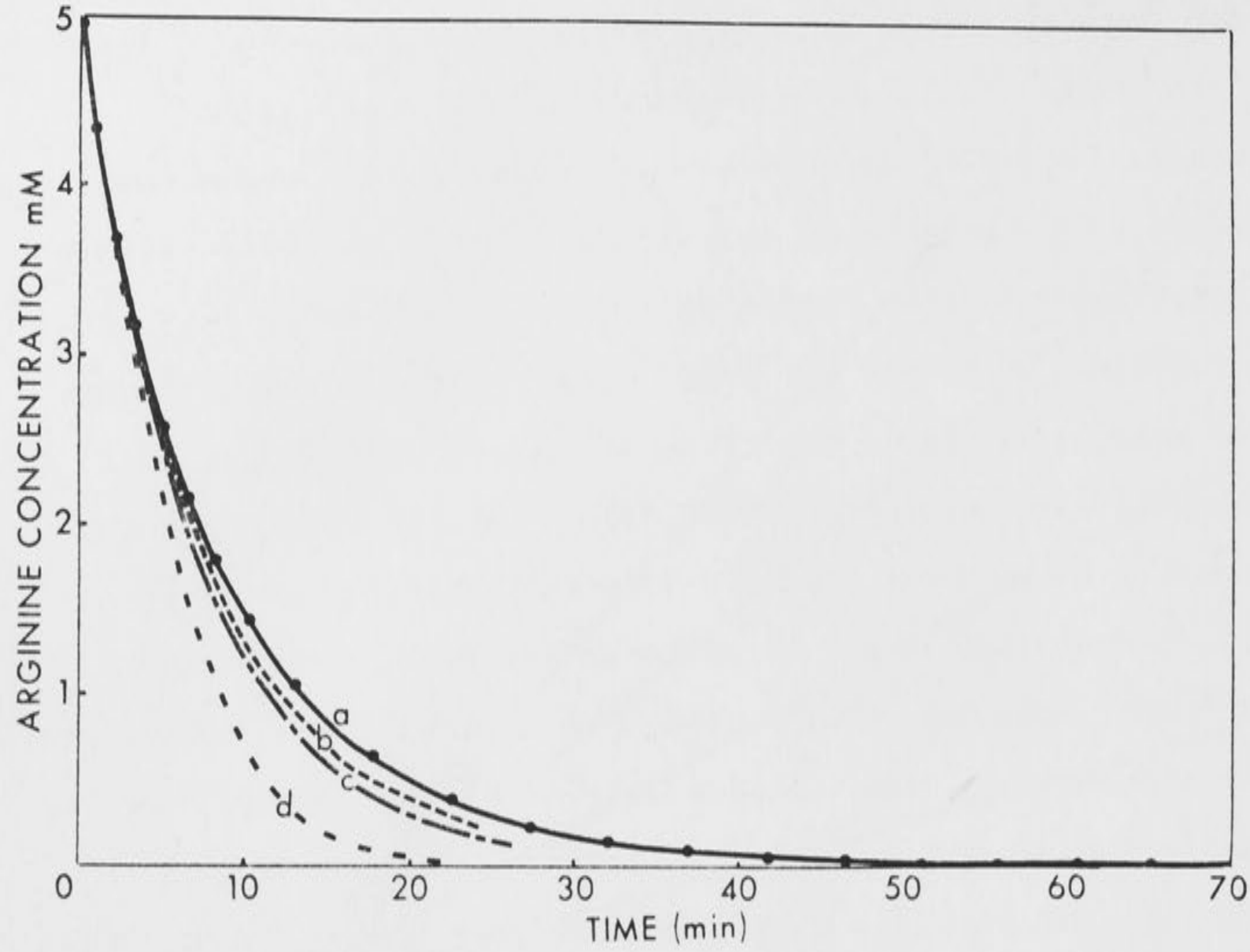


FIGURE IV - 6

The time dependent depletion of arginine (initially 5mM) at 25°C (●); measured by the spectrophotometric assay. The lines were obtained by numerically integrating equation (II - 28a) employing $K_m = 5 \times 10^{-3}$M; $V_{max} = 1.4 \times 10^{-3}$M min^{-1}; with $K_I = 10^6$M, (d); 5×10^{-3}M, (c); 4×10^{-3}M, (b); and 3×10^{-3}M, (a).

the product of the reaction is,

$$V_{max}t = K_m\left\{1 + \frac{[S]_o}{K_I}\right\} \ln \frac{[S]_o}{[S]_t} + \left\{1 - \frac{K_m}{K_I}\right\} ([S]_o - [S]_t) \qquad (IV - 2)$$

Thus as Foster and Niemann(1953) first noted, a plot of $([S]_o - [S]_t)/t$ versus $(1/t)\ln[S]_o/[S]_t$ is linear. In the present case this line has a positive slope and intersects the $([S]_o - [S]_t)/t$ axis via the fourth quadrant in contrast to the illustration of such plots obtained with α-chymotrypsin (Foster and Niemann, 1953) where the lines lay in the first quadrant with negative slope. The difference arises because the affinity of arginase for ornithine is greater than the apparent affinity for its substrate arginine. It is of interest in connection with equation (IV-2) that in the special case that K_m equals K_I (a situation not far removed for the arginase system under study), the equation may be directly re-arranged to give

$$[S]_t = [S]_o \exp\left(\frac{-V_{max}}{K_m + [S]_o}\right)t \qquad (IV - 3)$$

This suggests that the depletion of substrate with time in the arginase system, under study, approximates to a simple first order exponential decay.

2. The steady state kinetics of urease.

(a) The dependence of initial velocity on initial substrate concentration

In these studies commercially available urease (Sigma jack bean highly purified type VII powder, batch no. 101C-5040,

110,000 Sumner units g^{-1}) was studied in HCl-tris buffer pH 7.5 as previously specified, without sulphite modification of the enzyme. The hydrolysis reactions were conducted in sealed tubes at 25°C, by the addition of 0.5ml of enzyme solution in the buffer to 1.5ml of urea in the same buffer, the reactions being terminated at various times by the addition of 0.5ml of 2M HCl. Ammonia-free H_2O was used throughout these experiments. It is possible to monitor this reaction in several ways including the estimation of urea concentrations by, for example, the diacetyl monoxime method; but in this study, direct estimation of the ammonium ions produced as one of the products was selected.

In preliminary studies ammonium ions were estimated by addition of Nessler's solution (Kistiakowsky, Mangelsdorf, Rosenberg and Shaw, 1952; Creeth and Nichol, 1960), the spectrophotometrically measured absorbance of the brown-yellow colour which developes being measured together with a standard curve to estimate the required concentration. As the previous workers noted, this procedure requires the preliminary isolation of ammonium ions, since other compounds in the incubation mixture (including manganese ions) interfere with the quantitative colour estimation. The isolation was effected by passing the incubation mixture down a Dowex 50 column of bed volume 2ml: ammonium ions and manganese ions adsorb strongly to this cation exchange resin, the remaining components of the mixture being washed free of the column with water. Elution of the

column with 1M KOH quantitatively eluted the ammonium ions and hydrolyzed the freed manganese ions to an insoluble form which did not complicate the subsequent Nesslerization. Despite the success of this method, an alternative procedure was sought for two reasons. First, the ion exchange chromatography step resulted in considerable dilution and was laborious in relation to analysis of many incubation mixtures. Secondly, the procedure is inapplicable in studies (to be reported later), where arginine is included in the reaction mixture, for this too, is adsorbed by Dowex 50, eluted with 1M KOH and forms a colour with Nessler's solution. The alternative procedure was the Conway micro-diffusion analysis method (Conway, 1939, 1947) which is a well established procedure, and an extremely sensitive one. The Conway unit comprises two concentric glass compartments, and an etched glass plate lid, fitted to the unit with specially prepared ammonium ion-free grease (Conway, 1939, 1947). A Carlsberg pipette was used to introduce 1ml of incubation mixture containing ammonium ions to the outer compartment of the unit, and 1ml of 2% w/v boric acid solution, containing the mixed indicator methylene blue (0.0001% w/v) and methyl red (0.0004% w/v) (McKenzie and Murphy, 1971) was placed in the inner compartment. Prior to its addition to the unit, a 100ml volume of the solution for the inner compartment was adjusted to the recognizable grey-blue colour of the indicator with a few drops of 0.1M KOH. 1ml of 40% w/v KOH was added to the outer compartment in order to liberate ammonia, the unit being quickly sealed with the lid, and stacked with 9 others

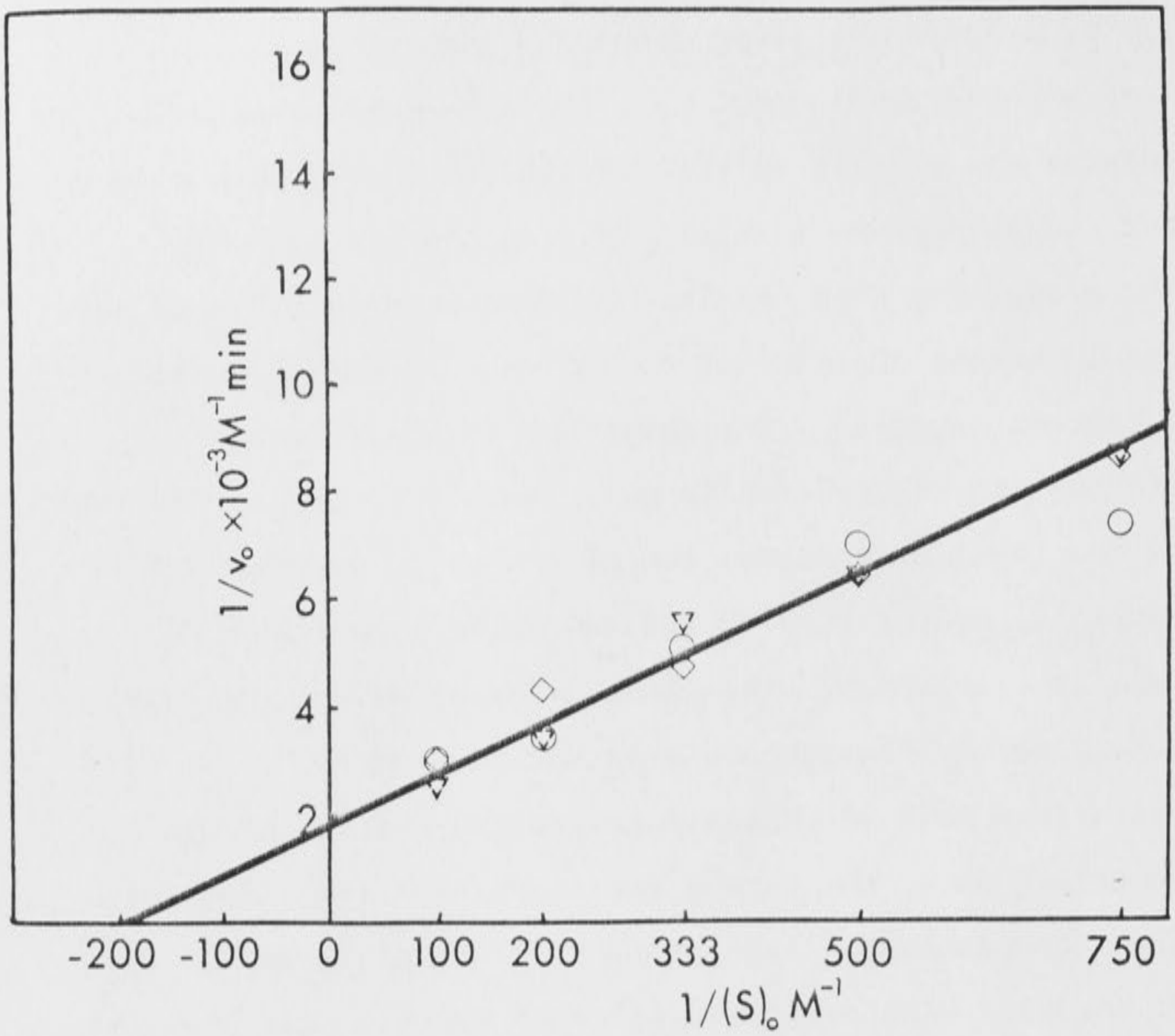


FIGURE IV - 7

A double reciprocal plot of data from an initial velocity study of jack bean urease in the presence and absence of arginine at 25°C. The ammonia produced by the reaction was estimated titrimetrically after employing a Conway micro-diffusion method. The following arginine concentrations were employed, 0mM (▽), 5mM (O), and 10mM (◇).

under a compressing weight. The transferral of ammonia from the outer compartment to the inner solution was shown by control experiments to be virtually complete in 2 h, at which time the inner solution was titrated with the standard acid, potassium biiodate, contained in a micro-burette, until the blue-grey end-point was again achieved: during the titration the solution was stirred with a bent glass rod, and the tip of the micro-burette was immersed in the solution.

The double reciprocal plot shown in Figure (IV-7) is typical of the results obtained with urease and was used in a weighted least squares procedure to obtain values of K_m equal to 5.6 ± 1.0mM and the breakdown rate constant of 1.66×10^5 min^{-1} at an enzyme concentration of 1.71×10^{-9}M. Other estimations obtained in entirely similar experiments are summarized in Table (IV-1) from which it may be seen that the average K_m value has been assessed as 4.2 ± 1mM. Several points in relation to these results merit comment. First, in agreement with several other workers it appears that urease kinetics (at least at substrate concentrations reported in Figure IV-5), follow the simple Michaelis-Menten mechanism, (Kistiakowsky and Rosenberg, 1952; Wall and Laidler, 1953; Lynn, 1967). Secondly, the reported average value of K_m is in accord with that cited by Lynn (1967) of 4mM in the same buffer type at pH 7.4. Thirdly, it is noted that samples of urease used almost certainly contained a mixture of polymeric forms (Chapter III), but in this connection it is noted that kinetic behaviour of

the mixture will be that of the monomeric form (the 488,000 unit), provided polymer formation affects neither the number of active sites nor their catalytic activity. All previous workers (Nichol and Creeth, 1963; Lynn, 1967), who have commented on the polymerization phenomenon, have basically agreed that the kinetic parameters of urease polymers are indeed closely similar to, if not identical with, that of the monomer. It may be that slight deviations from linearity of the double reciprocal plot (in the form of "negative cooperativity"), are to be expected if the polymers have slightly different activities than the monomer (Nichol and Winzor, 1972), but it is evident from Figure (IV-7), that such a deviation, if it exists, is undetectable within experimental error. It therefore follows that the coexistence of polymers in urease solutions is of little concern in relation to the present results. Fourthly, it is noted that the results were obtained in the presence of 0.01M $MnCl_2$, an environment which resulted in precipitation problems in approximately 0.5g dl^{-1} solutions of urease (Chapter III), but in which urease at the low concentration levels used in enzymic assay, approximately 8×10^{-9}M, evidently remained in solution. Further comment on the effect of manganese ions is reported in the next Section.

(b) Studies on the components of the coupled assay mixture as possible inhibitors of urease

A series of experiments was performed as described above in which various concentrations of arginine, ornithine and arginase were introduced into the initial reaction mixture. In all cases these compounds were found to have

no effect on the urease kinetic results, a typical result, that for arginine, being shown in Figure (IV-7). Similarly the literature on urease shows that the products of the urea hydrolysis and urea itself exhibit no marked inhibitory effects at the concentrations encountered herein. Inhibition of urease due to excess substrate occurs with urea concentration above 0.4M (Wall and Laidler, 1953), a concentration much larger than those employed in the present studies. It remains therefore to make more detailed comment on the effect of $MnCl_2$, a necessary constituent of the coupled assay. In a first series of experiments the % of activity of a urease solution was measured as a function of the time of storage in the presence and absence of $MnCl_2$. In the absence of $MnCl_2$ a linear decrease of activity was observed, 85% of the original activity being retained after a period of 275 min. In the presence of $MnCl_2$ the % activity reduced to 80% of its original value after 90 min, but thereafter remained fixed up to a time period of 275 min. In view of the existence of this reproducible plateau of urease activity in the presence of $MnCl_2$, it was decided to incubate all solutions of urease in the presence of $MnCl_2$ for 90 min prior to their use in any kinetic experiment: this procedure for example was followed in obtaining the results in Figure (IV-7).

The results of a second series of experiments in which the urease was incubated for 90 min in the presence of the indicated concentrations of $MnCl_2$ prior to its assay at various urea concentrations, in which the $MnCl_2$ concentration

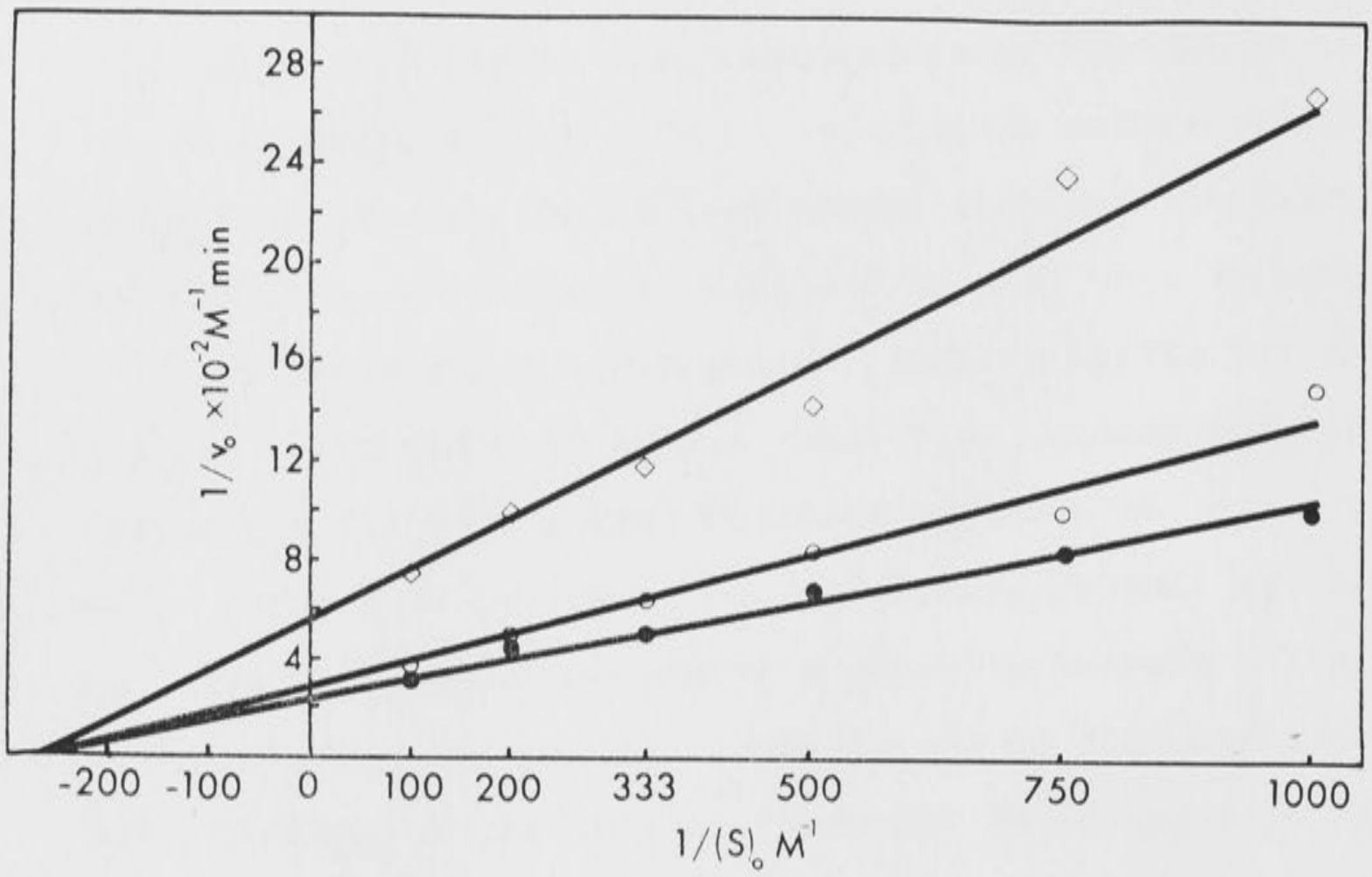


FIGURE IV - 8

Initial velocity data from urease kinetic studies plotted in double reciprocal form. The three sets represent studies in the presence of 10mM (●), 20mM (o) and 100mM (◇), $MnCl_2$.

was held fixed at the indicated values, is shown in Figure (IV-8). Clearly $MnCl_2$ acts as a non-competitive inhibitor, the K_m value of 4.0 ± 1.0mM being within experimental error, that found from Figure (IV-7), but with the apparent maximal velocity varying with the $MnCl_2$ content of the solution. Secondary plots which in principle permit the evaluation of the non-competitive inhibition constant have not been constructed since weak complexing of manganese ions by the tris buffer prevents explicit determination of the actual initial concentration. The important point in relation to coupled assays emerges however, that provided the apparent maximal velocity of urease appropriate to the $MnCl_2$ concentration employed is used in the calculations, and pre-incubation is allowed for 90 min, that analysis may proceed in terms of a system with well defined parameters including a K_m which is unaffected by the presence of $MnCl_2$. It is also noteworthy that while a complexing of manganese by arginine may be postulated, the results in Figure (IV-7) show that removal of manganese ions by this means does not significantly alter the apparent maximal velocity in solutions containing 0.01M $MnCl_2$.

3. Results of the coupled assay.

(a) Initial velocity studies

The conduct of arginase-urease assays was as follows. A solution containing both enzymes was prepared in the cited buffer and incubated at 25°C for 90 min prior to use. One ml of this solution was added to 1 ml of arginine solution in the same buffer, and the reaction terminated at various times by the addition of 0.5 ml of 2M HCl. The ammonium ions produced were analyzed by the Conway micro-diffusion

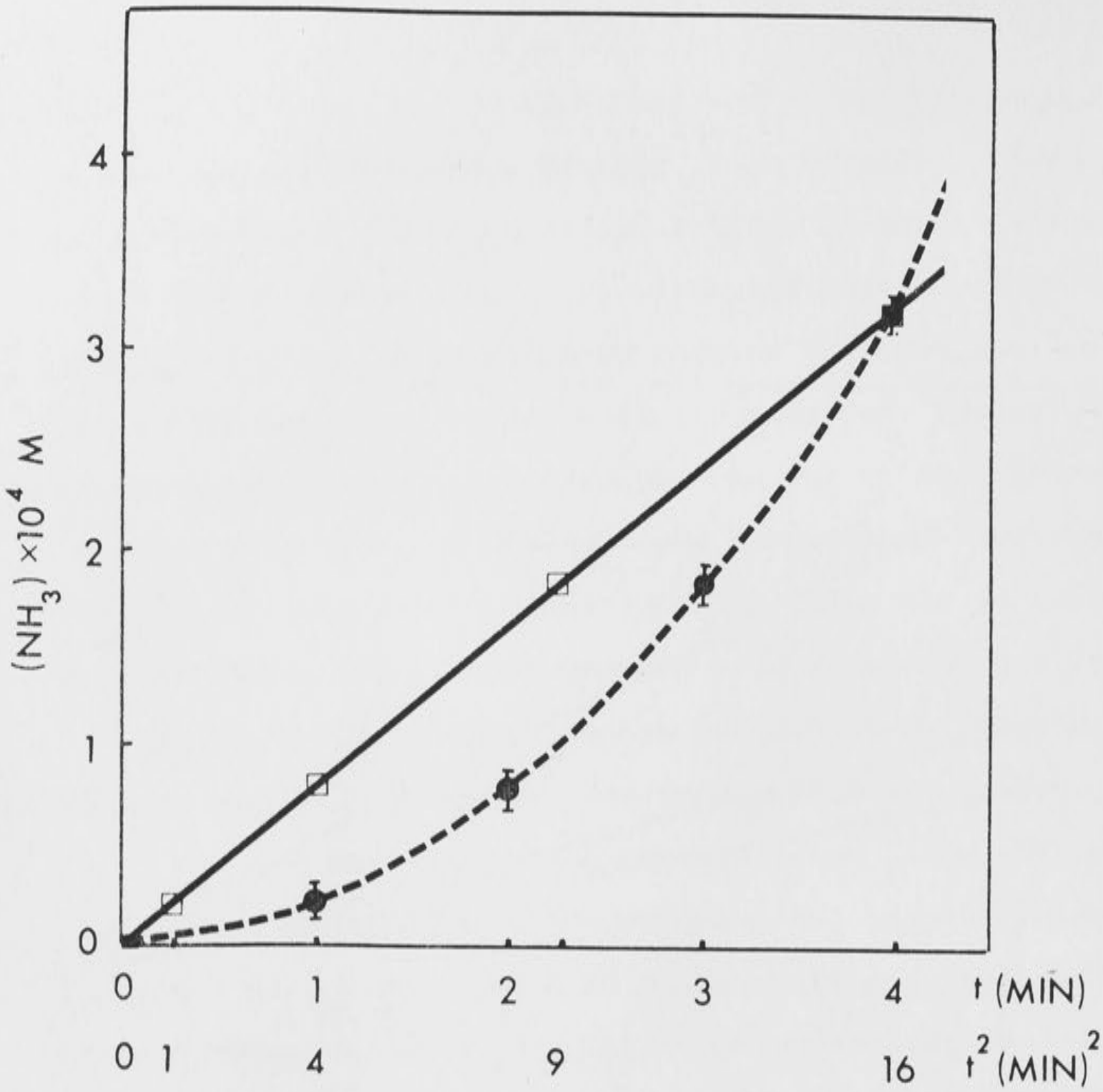


FIGURE IV - 9

Final ammonia concentration as a function of time obtained from a coupled arginase - urease catalyzed reaction with initial arginine concentration of 10mM. The NH_3 was estimated by the micro-diffusion method of Conway. The broken line is an attempt to average these data, and the solid straight line is drawn through the same data when plotted versus time squared according to equation (II - 15).

method described above, with the following two modifications. First, a 1% solution of boric acid was used in the inner compartment in the absence of any indicator, the absorbed ammonium ions being estimated by direct Nesslerization (a procedure which was found in later studies to be even more sensitive than the titration procedure employed previously). Secondly, in order to utilize this Nesslerization procedure, it was required to liberate the ammonia from the incubation mixture contained in the outer compartment with 1 ml of 60% w/v potassium metaborate rather than the previously employed 40% w/v KOH, because this latter reagent was found to liberate from tris buffer containing $MnCl_2$, a diffusible component which reacts with Nessler's solution. Control experiments showed that very little of such complicating reactant was released when potassium metaborate was utilized as the liberating agent.

Although several coupled assay experiments were performed (with arginine concentration the variable in any given set) it is convenient first, for the sake of illustration, to discuss the results of a single study. In Figure (IV-9) the points on the broken line are the experimentally obtained values of ammonium ion concentration produced at the indicated times in a solution containing 5.6×10^{-9}M arginase, 1.81×10^{-9}M urease and an initial substrate concentration of 10mM arginine. The broken curve which is an attempt to average these results is similar in form to the computed product formation curve shown in Figure (II-3): it clearly emphasizes the futility of attempting to define an initial velocity in the conventional manner, for indeed the slope of the tangent to the broken curve as $t \rightarrow 0$ is zero. The

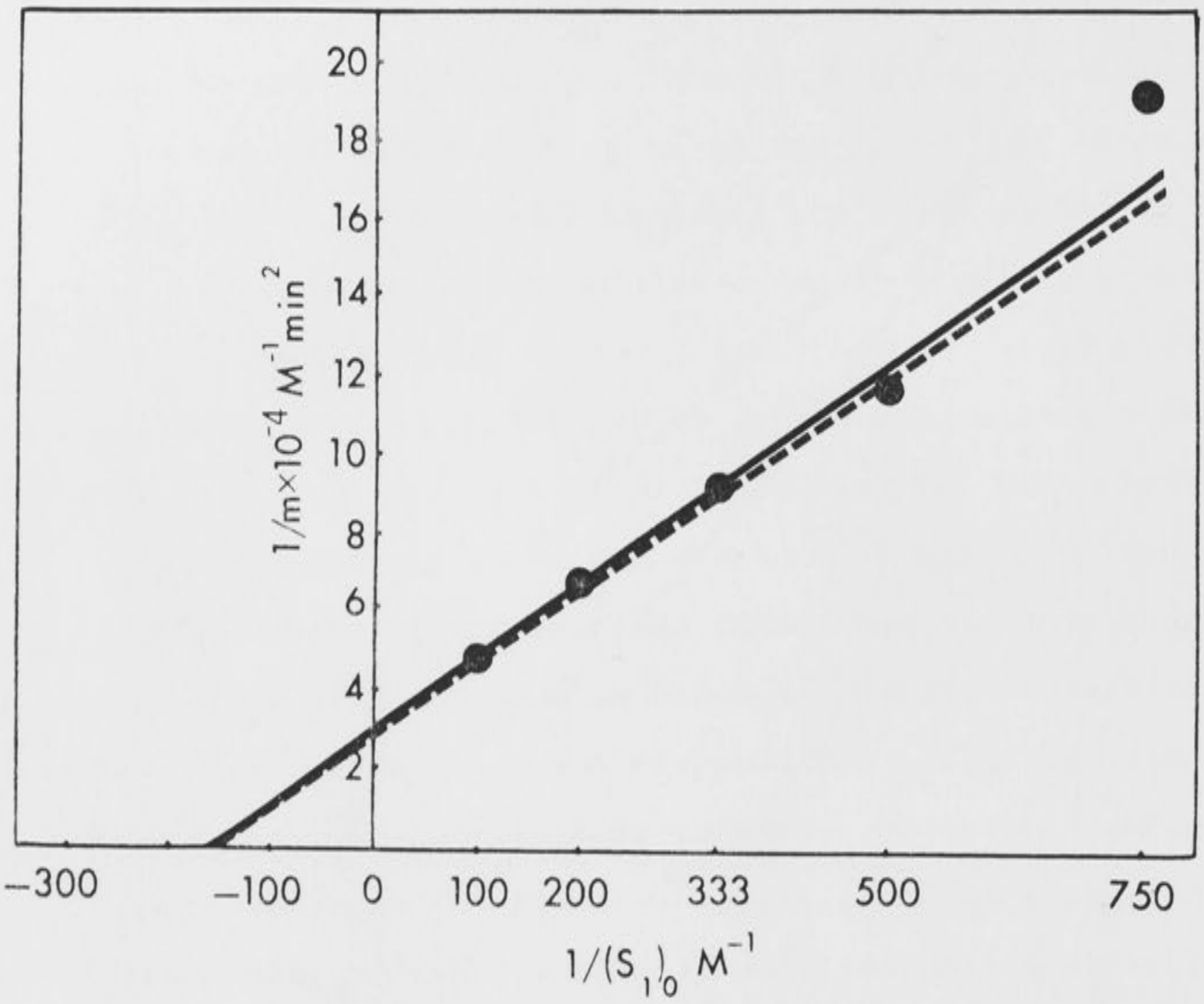


FIGURE IV - 10

Double reciprocal plot of values of 1/m (as defined in equation II-15 and IV-4) versus the initial arginine concentration, obtained from coupled kinetic studies on the arginase - urease system. The solid line is the weighted least squares fit to the experimental points (●), and the broken line is the plot of equation (IV-4) in double reciprocal form employing parameters obtained from each enzyme studied independently (Table IV-1).

essence of the theory presented in Chapter II and embodied in equation (II-15) suggests that the result for such a system be plotted as function of t^2. Such a plot is shown by the solid line in Figure (IV-9) and is seen to be linear in the time domain indicated. The slope of this line (2.0×10^{-5}M min^{-2}) is given by equation (II-15) rewritten here for convenience,

$$m = \frac{V_1 V_2 [S_1]_o}{2K_2(K_1 + [S_1]_o)} \qquad (IV - 4)$$

provided V_2 refers to the maximal velocity of ammonia production. Similar analysis of experiments conducted with different initial arginine concentrations permitted construction of a plot of 1/m versus $1/[S_1]_o$ which is shown in Figure (IV-10), where the solid line was determined from these data by the method of weighted linear least squares regression, and yields from the abscissa and ordinate intercepts values of K_1 = 6.0 ± 1.0mM and $V_1V_2/2K_2$ of $3.22 \pm 0.27 \times 10^{-5}$M min^{-2}. It is now possible to employ the value of K_2 = 4.2mM (Table IV-1) and $V_2/2 = 3.0 \times 10^{-4}$M min^{-1} (footnote, *), to calculate that $V_1 = 4.5 \times 10^{-4}$M min^{-1} corresponding to a breakdown rate constant of the first reaction of 8.02×10^4 min^{-1}.

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* Since 1 mole of urea produces 2 moles of ammonia, then in equation (II-2), $|-d[S_2]_t/dt| = 0.5\ d[S_p]_t/dt$. This stoicheiometric constant is simply accounted for by substituting, for $V_2/2$ in equation (IV-4), the maximal velocity found in studies with urease alone which is given by the product of the breakdown rate constant ($1.66 \times 10^5 min^{-1}$) and the initial enzyme concentration (1.81×10^{-9}M).

The effectiveness of the theoretical analysis suggested in Chapter II and illustrated in Figures (IV-9) and (IV-10) may be judged by making two comparisons. First the derived value of K_1 for arginase of 6.0 ± 1mM is compared with the average value reported in Table (IV-1) of 5.0 ± 1mM; a satisfactory agreement in view of the reported standard deviations and the scatter of the mean values reported in Table (IV-1). The agreement is recognized as being even more satisfactory when it is noted that the K_m value found with the particular arginase sample used in the coupled assay, when studied alone prior to the coupled assay, was 6.0 ± 1.2mM. Secondly, a comparison may be made with the values of the breakdown rate constant derived from the coupled assay of 8.02×10^4 min^{-1} and that found by the independent studies on arginase of 8.2×10^4 min^{-1}. Certainly there are error bars associated with each of these quantities but they are more difficult to assign than those for K_m values since an enzyme concentration term is involved. Nevertheless the agreement is eminently satisfactory. Finally, the overall agreement with theoretical prediction and experimental results may be displayed visually by employing equation (IV-4) together with the kinetic parameters obtained in the study of the individual enzymes, to construct the broken line shown in Figure (IV-10). The agreement is within experimental error.

The coupled assay experiment was repeated twice with similar composition of original mixed enzyme solutions and yielded K_1 for arginase of 6.0 ± 1mM and 5.0 ± 1mM. There was also agreement between the derived V_1 values, and those

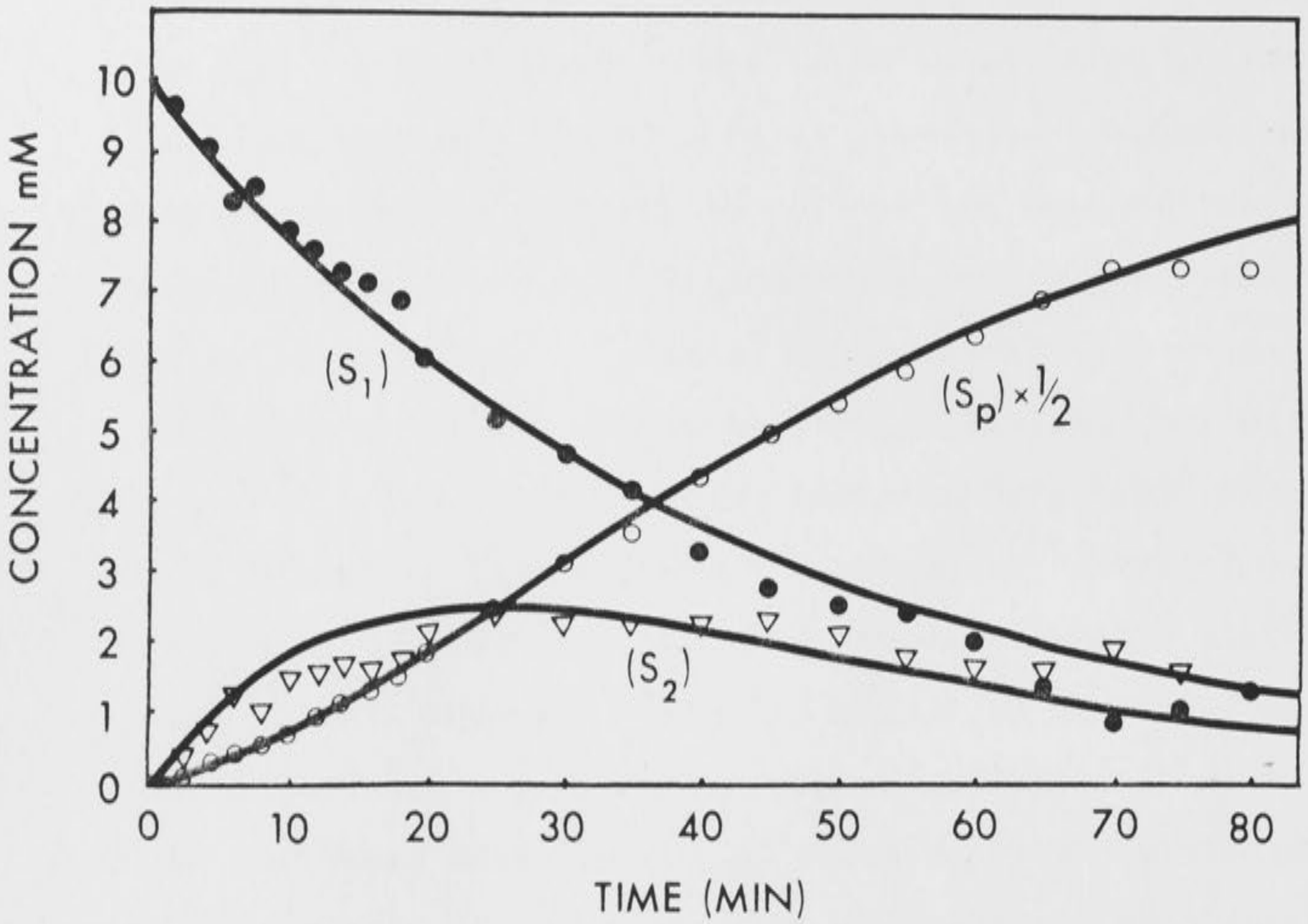


FIGURE IV - 11

The time course of a coupled enzymic reaction catalyzed by beef liver arginase and jack bean urease employing an initial arginine concentration of 10mM; (●), $[S_1]$, arginine; (∇), $[S_2]$, urea; (o), $[S_p]$, ammonia.

obtained in separate studies on arginase alone.

(b) The arginase-urease system studied at long times

A reaction mixture of the same composition as that reported in relation to Figure (IV-10) was thermostated at 25°C, and at chosen time intervals aliquots of this solution were taken and added to 2M HCl to stop the reaction. Each mixture was analyzed in aliquots separately for urea (the diacetyl monoxime method) and for ammonium ions (the Conway micro-diffusion method with biiodate titration). The results are plotted as points in Figure (IV-11), those referring to arginine depletion being obtained by difference according to the conservation of mass requirement. The purposes of obtaining such results were two-fold. First, it was desired to explore whether the simple Michaelis-Menten mechanisms would continue to apply at long times provided account was taken of the competitive inhibition of arginase by ornithine (Figures IV-5 and IV-6). Secondly, it was desired to illustrate the usefulness of interplaying computer-based integration procedures, together with the method described in the previous Section for analyzing experimental results, in determining the kinetic parameters governing the operative consecutive reactions. Accordingly the kinetic parameters obtained from the analysis of the coupled assay result, summarized previously, and the inhibition constant of 4mM similar to that employed in Figure (IV-6) were used in the integration of equation (II-28). In this instance the integration was performed with a general purpose electronic analogue computer (EAI PACE TR-48) details of the circuit being given in Chapter VI: this was

used in preference to the digital computer programmed with a 4^{th} order Runge-Kutta integration routine which would have taken at least 10 times as long to complete a numerical integration. The results of the analogue computer simulation are shown by the solid lines in Figure (IV-11). It is immediately apparent that the proposed mechanism has continued to operate even at long times, and that the kinetic parameters derived from the coupled kinetic analysis successfully describe this set of results also. It is also of interest that the inhibition constant of ornithine for arginase could have been derived solely on the basis of the results shown in Figure (IV-11). Thus once the values of V_1, V_2, K_1 and K_2 have been found by the other kinetic methods, the remaining parameter in equation (II-28) is this inhibition constant. In such cases where a single variable requires specification, the procedure of computer integration and comparison with experimental results provides a particularly efficient method of evaluating the parameter . In the present example it was shown quite rapidly with the analogue computer that the value of K_I equal to 4 ± 1mM gave the best fit of many assigned values in this range. It is also noted that considerably less certainty in this value would arise if it were required to fit the experimental data to the five parameters V_1, V_2, K_1, K_2 and K_I.

4. A discussion of the effects on the kinetics of coupled assays of interaction between the enzymes.

At the outset, in this Section it should be stressed that the results in Figures (IV-9), (IV-10) and (IV-11)

strongly support the contention that no interaction occurs between arginase and urease in the chosen environment, which includes all substrates and products involved in the coupled assay. This conclusion supports and extends the less direct evidence (found with a sulphite modified derivative of urease and in the absence of reaction mixture ligands) discussed in relation to Figure (III-14). It is nevertheless interesting to enquire in a more general framework whether an interaction between enzymes would be manifested in the type of plots thus far suggested for the interpretation of the results of a coupled assay. The question is particularly pertinent since suggestions have been made that certain enzymes in the same metabolic pathway may form complexes (Lynen, 1964; Messenguy, Penninckx and Wiame, 1971; Hess and Boiteux, 1972; Kirschner and Wiskocil, 1972; Fahien and Smith, 1974), a type of reaction which is extremely difficult to characterize at low concentration levels.

Consider a system in which two enzymes A (= E_1) and B (= E_2) associate to form a complex C (= AB) in solution, the equilibrium being rapidly established and governed by the equilibrium constant,

$$X = [C] / [A]\,[B] \qquad (IV - 5)$$

The basic set of differential rate equations which govern the conversion $S_1 \rightarrow S_2 \rightarrow S_p$ may be formulated on the basis of the assumption that all possible enzyme-substrate complexes are in a steady-state, following a procedure outlined by Dalziel (1968). It is however possible to obtain more simply the same *form* of the desired equations by applying equilibrium binding equations: it is then merely

required to reconsider the meaning of constants introduced, in relation to assumptions employed, in order to coalesce completely the different approaches of derivation.

Accordingly, it is considered that when the compound S_1 is added to the equilibrium mixture of A, B and C that it binds to p equivalent and independent sites on A and to q such sites on C with intrinsic association constants K_A and K_C, respectively. At equilibrium, the molar concentration of the A constituent, comprising unbound A and the complexes $A(S_1)_i$ $(i = 1,2,\ldots,p)$ is given by (Klotz, 1946),

$$[\bar{A}] = [A]\ (1 + K_A[S_1])^p \qquad \text{(IV - 6a)}$$

Similarly, the molar concentration of the C constituent, comprising unbound C and the complexes $C(S_1)_j$ $(j = 1,2,\ldots,q)$ may be written

$$[\bar{C}] = [C]\ (1 + K_C[S_1])^q \qquad \text{(IV - 6b)}$$

Since each mole of C contains one mole of A, it follows that the total weight-concentration of the A enzyme in all forms divided by the molecular weight of A is simply expressed as

$$[A]_T = [\bar{A}] + [\bar{C}] \qquad \text{(IV - 6c)}$$

The moles of ligand bound by the A constituent is given by $pK_A[A][S_1](1 + K_A[S_1])^{p-1}$ and to the C constituent by $qK_C[C][S_1](1 + K_C[S_1])^{q-1}$. Thus constituent molar binding functions may be formulated as,

$$\bar{r}_A = pK_A[A][S_1](1 + K_A[S_1])^{p-1}/[A]_T \qquad \text{(IV - 7a)}$$

$$\bar{r}_C = qK_C[C][S_1](1 + K_C[S_1])^{q-1}/[A]_T \qquad \text{(IV - 7b)}$$

If subsequent to the binding of S_1 to A, each complex breaks down to form product S_2 with the same intrinsic rate constant k_2, i.e.

$$A(S_1)_i \xrightarrow{k_2} A(S_1)_{i-1} + S_2,$$

then the rate of depletion of S_1 from this source, v_1, may be deduced from equations (IV-6) and (IV-7a) to be,

$$v_1 = - \frac{V_1 K_A[A][S_1](1 + K_A[S_1])^{p-1}}{[A](1 + K_A[S_1])^p + [C](1 + K_C[S_1])^q} \qquad \text{(IV - 8)}$$

This formulation is based on the relationship suggested by Frieden (1967) that $v_1/V_1 = - \bar{r}_A/p$, where the maximal velocity $V_1 = pk_2[A]_T$. There is an additional assumption implicit in equation (IV-8), inherent on its derivation from equilibrium binding equations. This may be seen most easily for the case X = 0, [C] = 0 (no interaction between A and B) when equation (IV-8) simplifies to the Michaelis-Menten equation with K_A identified as k_1^A / k_{-1}^A, where k_1^A and k_{-1}^A are microscopic (intrinsic) rate constants as defined by Dalziel (1968). The assumption implicit in equation (IV-8) is then that k_2 is relatively small. As noted previously an equation of identical form arises if it is assumed that all enzyme-substrate complexes are in a steady-state, with K_A now being identified as $k_1^A / (k_{-1}^A + k_2)$.

When $X \neq 0$, S_1 is also depleted by the breakdown of complexes $C(S_1)_j \xrightarrow{k_3} C(S_1)_{j-1} + S_2$ and the rate of its depletion from this source may be deduced from equations (IV-6) and (IV-7a) to be,

$$v_2 = - \frac{V_2 K_C [C][S_1](1 + K_C[S_1])^{q-1}}{[A](1 + K_A[S_1])^p + [C](1 + K_C[S_1])^q} \qquad (IV - 9)$$

where $V_2 = qk_3[A]_T$ and $K_C = k_1^C / k_{-1}^C$ (if k_3 is small) or $K_C = k_1^C / (k_{-1}^C + k_3)$, if a steady-state of all $C(S_1)_j$ is assumed. In either case, the denominators of equations (IV-8) and (IV-9) equal $[A]_T$, which is invariant with time. Since the overall depletion of S_1 is given by the sum $v_1 + v_2$, it follows that,

$$d[S_1]/dt = -\{V_1 K_A [A][S_1](1+K_A[S_1])^{p-1} + V_2 K_C [C][S_1](1+K_C[S_1])^{q-1}\} / [A]_T \qquad (IV - 10)$$

If instead of S_1, the substrate S_2 was added to the equilibrium mixture $A + B \rightleftharpoons C$ and it bound to y equivalent and independent sites on B and to w such sites on C, each complex breaking down to form product S_3, then by entirely analogous reasoning, the overall rate of production of S_3 may be written

$$d[S_3]/dt = \{V_3 N_B [B][S_2](1+N_B[S_2])^{y-1} + V_4 N_C [C][S_2](1+N_C[S_2])^{w-1}\} / [B]_T \qquad (IV - 11a)$$

where $$[B]_T = [B](1+N_B[S_2])^y + [C](1+N_C[S_2])^w \qquad (IV - 11b)$$

and N_B and N_C are the respective intrinsic binding constants

(or reciprocal microscopic Michaelis constants) pertaining to the interactions of S_2 with B and C. In a coupled sequence of reactions ($S_1 \rightarrow S_2 \rightarrow S_3$), therefore, the basic set of differential rate equations is given by equations (IV-10) and (IV-11a and b) and the negative values of their sum,

$$d[S_2]/dt = + \{V_1K_A[A][S_1](1+K_A[S_1])^{p-1} + V_2K_C[C][S_1](1+K_C[S_1])^{q-1}\}/[A]_T - \{V_3N_B[B][S_2](1+N_B[S_2])^{y-1} + V_4N_C[C][S_2](1+N_C[S_2])^{w-1}\}/[B]_T \qquad (IV - 11c)$$

Equation (IV-11c) follows from the condition of mass conservation assuming that the amounts of substrates bound is small. It could also be noted that the set of rate equations assumes equilibrium (or steady) states of all enzyme-substrate complexes but not necessarily of the intermediate compound S_2.

The required expression for the dependence of $[S_p]_t$ on time is a Maclaurin polynomial, the first term of which is given by equation (II-26). This requires evaluation of equation (IV-11c) at t = o and the differentiation of equation (IV-11a) with respect to S_o to yield,

$$[S_3]_t = \frac{(V_3N_B[B]_o+V_4N_C[C]_o)(V_1K_A[A]_o[S_1]_o\alpha^{p-1}+V_2K_C[C]_o[S_1]_o\beta^{q-1})t^2}{2[A]_T[B]_T} \qquad (IV - 12)$$

where $\alpha = (1+K_A[S_1]_o)$, $\beta = (1+K_C[S_1]_o)$ and $[A]_o$, $[B]_o$ and $[C]_o$ are molar concentrations of *unbound* A, B and C, respectively, at t = o. To obtain explicit expressions for these

quantities, equations (IV-5) and (IV-6) are rewritten as,

$$[C]_o = X[A]_o[B]_o \qquad \text{(IV - 13a)}$$

$$[A]_o = [A]_T/(\alpha^p + X\beta^q[B]_o) \qquad \text{(IV - 13b)}$$

and with the use of equation (IV-11b) written as $[B]_T = [B]_o + [C]_o$, the simultaneous solution is completed by,

$$[B]_o = -\{\alpha^p + X[A]_T - X\beta^q[B]_T - \sqrt{\Delta}\}/2X\beta^q \qquad \text{(IV - 13c)}$$

$$\Delta = \{\alpha^p + X[A]_T - X\beta^q[B]_T\}^2 + 4X\beta^q\, p[B]_T \qquad \text{(IV - 13d)}$$

The negative root of equation (IV-13c) is excluded since $[B]_o > 0$. Although the right hand side of equation (IV-12) is the first non-zero term of a power series in time, it follows from equation (II-26) that the slope of the limiting tangent (m) of the plot of $[S_p]_t$ versus t^2 is given by the coefficient in equation (IV-12). It is therefore possible from a set of coupled assay experiments to construct a plot of m versus $[S_1]_o$. It was shown in Chapter II, that if there is no enzyme-enzyme interaction between enzymes of the Michaelis-Menten type in a coupled assay, the result of such a plot is a rectangular hyperbola. There is one situation in relation to the model of interaction under discussion, where identical behaviour would be observed even though interaction does occur: it is when the complex C possesses the same activity as A towards S_1 ($q = p$, $K_A = K_C$, $V_1 = V_2$) and the same activity as B towards S_2 ($y = w$, $N_B = N_C$, $V_3 = V_4$). With these equalities equations (IV-13a and b) yield $[A]_o + [C]_o = [A]_T / \alpha^p$ and it follows from

equation (IV-12) that,

$$m = V_3 N_B V_1 K_A [S_1]_o / 2(1+K_A[S_1]_o) \qquad \text{(IV - 14)}$$

which is idential with equation (II-15) when account is taken of the use of dissociation constants in the equation. Except for this particular case, interaction between A and B in a coupled assay must give rise to deviations from the rectangular hyperbola described by equation (IV-14) even though separate studies on A and B have revealed that each is of the Michaelis-Menten type. Such separate studies permit the use of equation (IV-14) in the construction of a theoretical reference curve with which to compare the results of the coupled assay.

The type of deviation from the reference curve, which arises as a consequence of interaction between A and B leading to a complex with different activity toward S_1 and/or S_2, may be examined with the aid of equations (IV-12) and (IV-13). While these equations are general in that they would permit examination of any changes in the numbers of binding sites, intrinsic binding constants or break-down rate constants inherent on the formation of C, it suffices to illustrate the behaviour of one system and the following is chosen. A is enzymically active toward S_1 but C is not (q or $K_C = 0$), while B and C are equally active toward S_2 ($y = w$, $N_B = N_C$, $V_3 = V_4$). For this case,

$$m = \frac{V_3 N_B V_1 K_A [S_1]_o \ (1 + K_A[S_1]_o)^{p-1}}{(1+K_A[S_1]_o)^p + X([B]_T - [A]_T) + \sqrt{\Delta}} \qquad \text{(IV - 15a)}$$

$$\Delta = \{(1+K_A[S_1]_o)^p + X([A]_T-[B]_T)\}^2 + 4X[B]_T(1+K_A[S_1]_o)^p$$

(IV - 15b)

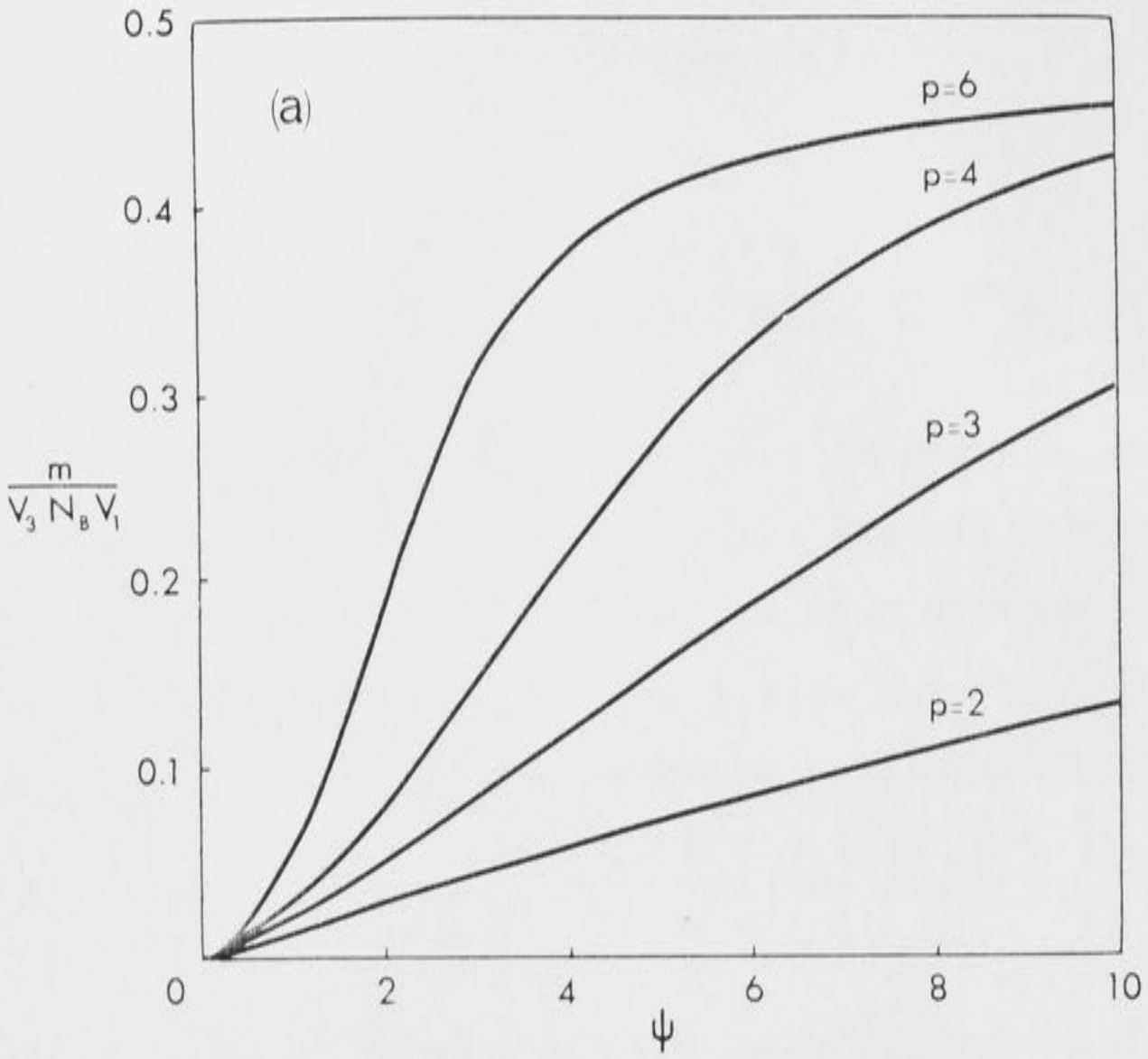
Figures (IV-12a and b) present plots of equation (IV-15) for the case $[A]_T = [B]_T$, when equation (IV-15) may be written with $\psi = K_A[S_1]_o$ as,

$$\frac{m}{V_3 N_B V_1} = \frac{\psi\,(1+\psi)^{p-1}}{(1+\psi)^p + \{(1+\psi)^{2p} + 4X[B]_T(1+\psi)^p\}^{0.5}} \qquad \text{(IV - 16)}$$

which is seen to require examination of two variables p and the dimensionless product $X[B]_T$. Figure (IV-12a) illustrates the effect of varying p at a fixed value of $X[B]_T = 10^3$, while in Figure (IV-12b) p = 4 and the variation of $X[B]_T$ is examined. It follows from equation (IV-16) that

$$\lim_{X[B]_T \to 0} \quad m = \frac{V_3 N_B V_1 \psi}{2(1+\psi)} \qquad \text{(IV - 17)}$$

which shows that the rectangular hyperbola in Figure (IV-12b) ($X[B]_T = 0$) is the reference curve described by equation (IV-14). It is clear from Figure (IV-12b) that deviations from the reference curve do indeed increase as $X[B]_T$ increases, the deviations being in the form of sigmoidality for all values of p examined (Figure IV-12a). In fact, in form, this behaviour arising as a sole consequence of enzyme-enzyme interaction is indistinguishable from the behaviour in a coupled assay of a control enzyme being monitored by but not interacting with an enzyme of the Michaelis-Menten type. Since the plot basically reflects the time dependence of final product formation as a function of $[S_1]_o$ (equation IV-12), the basic finding emerges that interaction between enzymes of the non-control type provides an alternate means of obtaining a control response in a consecutive series of reactions. In addition it is now clear


(a)
p=6
p=4
p=3
p=2
0.5
0.4
0.3
0.2
0.1
0
2
4
6
8
10
ψ
m / V3 NB V1

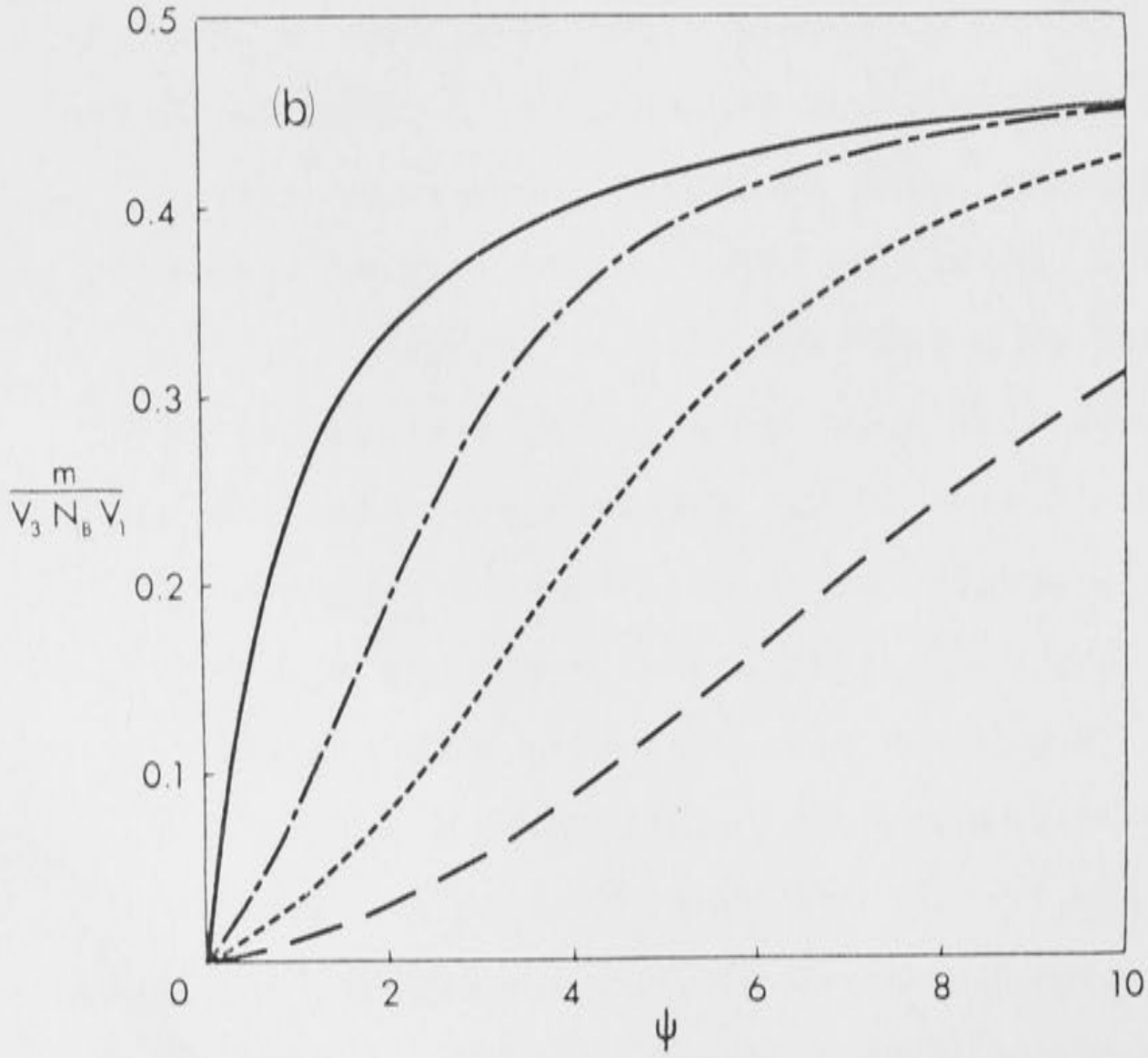

(b)
0.5
0.4
0.3
0.2
0.1
0
2
4
6
8
10
ψ
m / V3 NB V1

FIGURE IV - 12

Numerical examples illustrating the effect of interaction between two enzymes ($A + B \rightleftharpoons C$) catalyzing consecutive reactions ($S_1 \rightarrow S_2 \rightarrow S_3$). A is enzymically active toward S_1 but C is not, while B and C are equally active toward S_2. Abscissa ψ equals the dimensionless product $K_A[S_1]_o$, where K_A is the intrinsic association constant for the interaction of S_1 with A and $[S_1]_o$ is the initial concentration of S_1. The ordinate is also dimensionless and equals $m/V_3N_BV_1$ where m is the initial limiting slope of the plot of $[S_3]_t$ *vs.* t^2, V_1 and V_3 are the respective maximal velocities of the first and second reaction and N_B is the intrinsic binding constant of S_2 to B or C.

(a) The effect of varying p, the number of binding sites on A for S_1, at fixed $X[B]_T = 10^3$.

(b) The effect of varying $X[B]_T$ with p = 4 where X is the equilibrium constant [C]/[A][B] and $[B]_T$ is the total molar concentration of B. ——, $X[B]_T = 0$, — - — - , $X[B]_T = 10^2$; — — —, $X[B]_T = 10^3$; — — —, $X[B]_T = 10^4$.

that the existence of such interactions (or their absence) may be tested experimentally at low enzymic concentration levels for any selected pair of enzymes simply by performing steady-state kinetic studies on the enzymes separately and in a coupled assay. Thus in the case of interaction, it is noted that the present treatment has provided explicit expressions describing the deviations to be expected from the reference curve (equation IV-14). Moreover, the treatment may readily be extended to cases where one or both of the enzymes studied separately is of the control type. For these cases, the reference curve may itself be sigmoidal; but interaction leading to a complex with different activity would act to change the extent of this sigmoidality.

5. Discussion.

The major aim of this Chapter was to explore, with a model system (the arginase-urease couple) the application and validity of the theoretical equations developed in Chapter II. The summarized results exemplified by Figures (IV-9), (IV-10) and (IV-11) indeed show that the developed theory is useful in analyzing the results of a coupled assay. It is recognized that the theory has only been applied to one experimental system and it is therefore relevant to comment briefly on its application to results obtained with an entirely different system.

A situation of particular interest is one where the experimenter has chosen to employ a large excess of the second enzyme because this is a frequently employed experimental design. Reference is therefore made to the

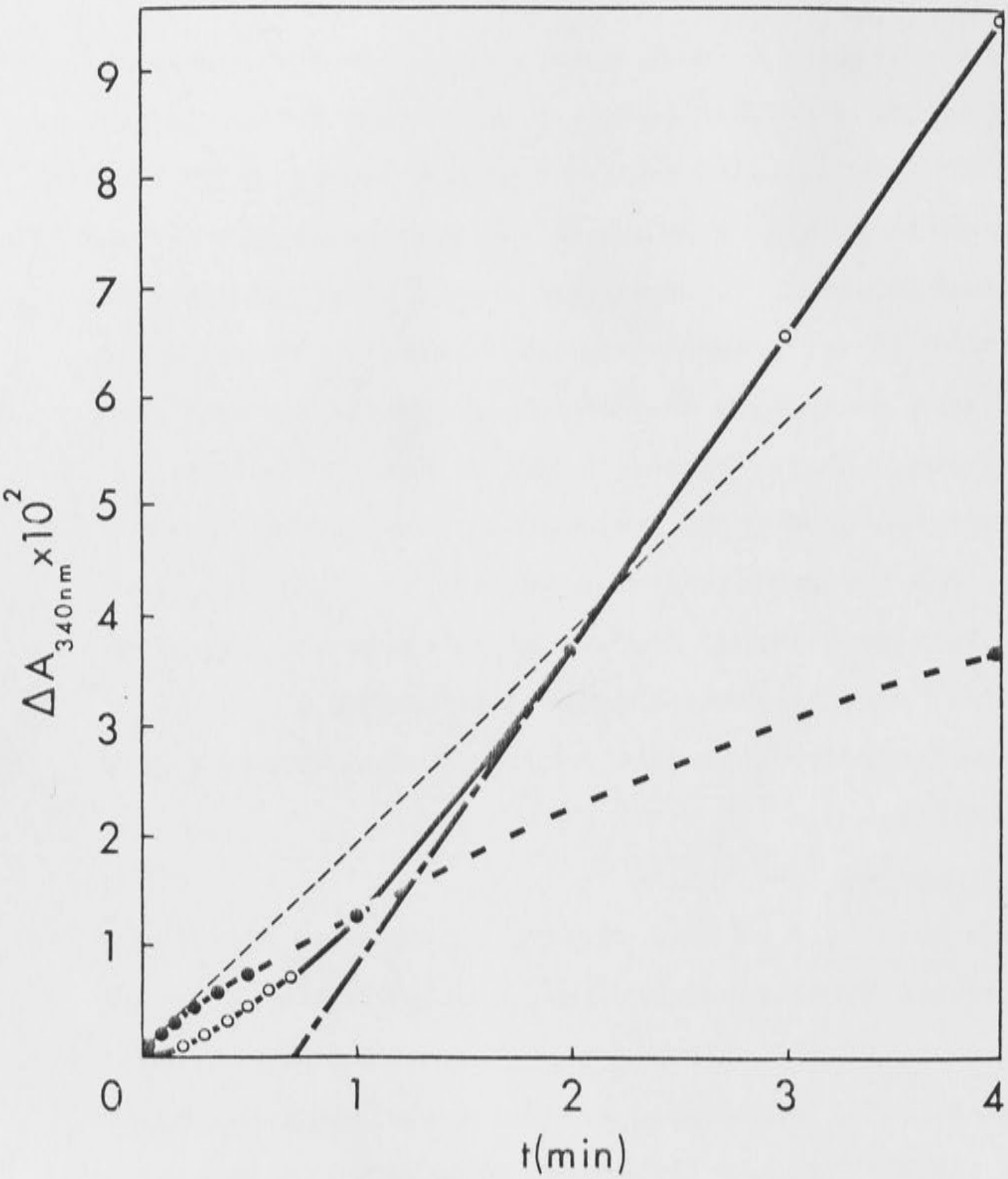
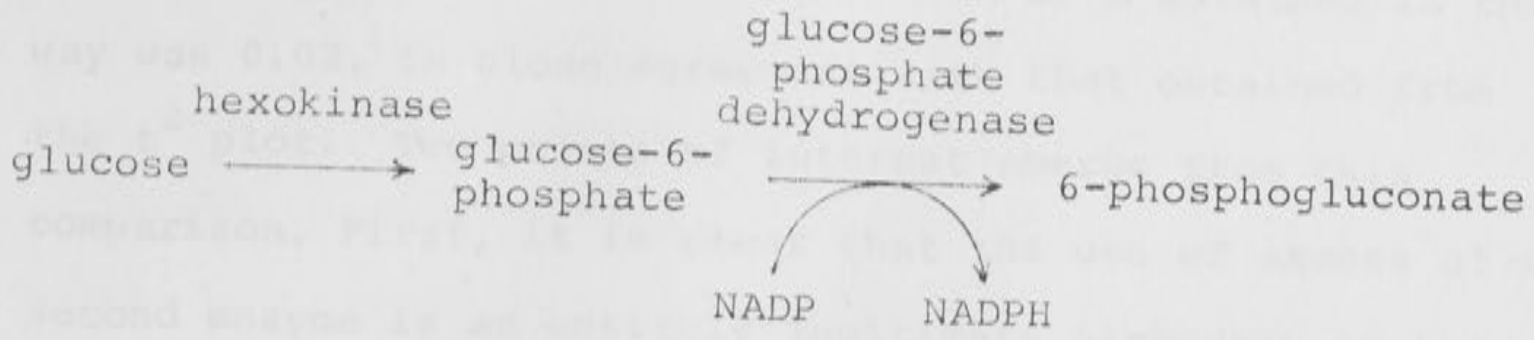


FIGURE IV - 13

A replot of the data of Easterby (1973) for the coupled enzyme system involving hexokinase and glucose-6-phosphate dehydrogenase. The ordinate axis is ΔA^{1cm}_{340nm} which gives a measure of the NADPH concentration. The solid line is an attempt to average the original data (o), and the linear extrapolation (— - —— -) is that suggested by equation (II - 19). The data plotted in t^2 format (●) has the limiting tangent (— — —).

study made by Easterby (1973) on the system,



Using a large activity excess of the second enzyme, Easterby (1973) obtained, for a particular initial substrate concentration (20mM), the concentration of final product (NADPH) as a function of time, data which are reproduced as open circles in Figure (IV-13). According to the present theoretical development this plot should be transformed to one with t^2 as the abscissa (since two enzymes are involved) and the data so transformed are shown as the solid circles in Figure (IV-13). The tangent to this latter curve, as $t \to o$, is drawn as the broken line, and it is immediately noted by comparison with Figure (IV-9) that the determination of this tangent is made more difficult, but nevertheless possible, in the present instance as a consequence of the use of a high concentration of the second enzyme. A desirable practice would be to plot the slope of this tangent, m = 0.019, against the corresponding initial substrate concentration as illustrated in Figure (IV-10), but unfortunately results at other initial substrate concentrations are not available. It is however possible to show the consistency of the present approach with that used by Easterby based on equation (II-19). From this equation it follows that the slope of the line (drawn in Figure (IV-13) as — - — -) divided by twice the abscissa intercept τ equals $m = v_o V_2/2K_2$ where v_o is the initial velocity of the first

enzyme catalyzed reaction. The value of m obtained in this way was 0.02, in close agreement with that obtained from the t^2 plot. Two points of interest emerge from this comparison. First, it is clear that the use of excess of the second enzyme is an entirely legitimate procedure in the determination of v_o from the slope of the tangent illustrated by (— - — -) in Figure (IV-13), which is reassuring since many coupled assays have been performed in this way. Secondly, the use of a t^2 plot based on the Maclaurin polynomial formulation provides an alternative method of analyzing the same results obtained when the second enzyme is in excess. In this connection it should be emphasized that the t^2 method possesses the decided advantage that it is applicable even when the second enzyme is not in large excess, a situation which may be encountered in experimental practice either unwittingly, or by necessity, as in the case of a protein possessing two active sites each capable of catalyzing consecutive reactions (e.g.chorismate mutase - prephenate dehydrogenase).

An additional advantage of treating the problem with the use of Maclaurin polynomials is that it has permitted explicit comment on enzyme-enzyme interactions. In this context it is noted that if an excess of the second enzyme was employed and an interaction occurred, that the equilibrium defined by equation (IV-5) could be shifted to favour strongly the complex C. In this instance the interaction might be detectable by an enzyme composition dependence of τ (Hess and Wurster, 1970; Barwell and Hess, 1970; Wurster and Hess, 1970; Hess and Boiteux, 1972) but the procedure could be insensitive and the results may not be readily

interpretable in terms of interaction and kinetic parameters. In contrast, the use of the t^2 method permits the construction of plots such as shown in Figures (IV-12a and b) which may be compared with corresponding plots calculated on the basis of no interaction using kinetic parameters obtained in studies of the enzymes individually. In this case then not only may the interaction be detected, but also explicit expressions are available for further quantitative interpretation. It is true that the expressions given in equations (IV-12) and (IV-13) are based on a particular model involving equivalent and independent sites on each individual acceptor entity but alternative formulations are possible if more complicated models are indicated. It is not suggested that kinetic experiments alone would be capable of elucidating all parameters governing a coupled system in which interaction occurs between the enzymes, but it does appear that they may provide a valuable adjunct to physico-chemical studies such as sedimentation equilibrium and velocity in the characterization. The joint use of sedimentation velocity studies and kinetic studies on individual enzymes, and in a couple, has been used in the present work to show that arginase and urease do not interact either in the presence or absence of the various substrates and products encountered in the coupled assay.

In relation to the urea cycle it would be of interest, while continuing the theme of studies on arginase, to explore the coupled kinetics of it with argininosuccinase and ornithine transcarbamylase. It was not possible with the time available to study in detail both systems, and the decision was made to explore the argininosuccinase-arginase

couple. The choice of argininosuccinase was partly dictated by the knowledge that it is a reasonably well characterized cytoplasmic enzyme which may be assayed by a simple procedure. The findings with this system in relation to the urea cycle form the contents of the next Chapter.

CHAPTER V

STUDIES ON ARGININOSUCCINASE IN RELATION TO ARGINASE AND THE UREA CYCLE

1. A review of the physico-chemical properties of argininosuccinase.

Argininosuccinase may be prepared in homogeneous form from beef liver utilizing a procedure which involves $(NH_4)_2SO_4$ fractionation, a heat step, and ion exchange chromatography, prior to crystallization (Ratner, Anslow and Petrack, 1953; Havir, Tamir, Ratner and Warner, 1965). The most recent physico-chemical information on the enzyme comes from the work of Lusty and Ratner (1972) and Ratner (1973), which will now be summarized. Sedimentation velocity analysis in 0.1M phosphate buffer pH 7.5 revealed a single symmetrical peak sedimenting with an $s_{20,w}$ of 9.3S which pertained over the temperature range 0 - 38°C. Sedimentation equilibrium studies in the same environment showed that this material was homogeneous with molecular weight 202,000. This unit appears to be comprised of four identical subunits each of molecular weight 50,000: the evidence for this conclusion includes molecular weight determinations in the presence of SDS, 6M guanidine hydrochloride or 8M urea, peptide mapping and electron microscopy (Lusty and Ratner, 1972). No metal ions are implicated in maintaining the structural stability or the catalytic activity of the 202,000 unit.

It is relevant in relation to the selection of buffer types to be used in this work to comment on factors affecting the structural stability of the 202,000 molecular weight species, in the absence of urea or SDS. The previous workers found that dissociation into the catalytically

inactive half-units (molecular weight 100,000) was effected in either tris or imidazole buffer at pH values near 8.3 at 20°C, whereas in the same buffers at pH 7.5 no dissociation of the enzyme was observed until the temperature was lowered to approximately 4°C. This temperature dependence of the extent of dissociation, suggests that hydrophobic interactions may contribute to the formation of the 202,000 unit from its half-subunits (Kauzmann, 1959). Under conditions where dissociation occurred, the reaction appeared to be relatively slow (and irreversible) in that association to the 202,000 unit could only be achieved by changing the temperature or the pH of the environment. It was also found that in 0.1M phosphate buffer pH 7.5 no dissociation occurred even at low temperatures. In this work basically three sets of environmental conditions are employed, namely 0.05M HCl-tris, 0.05M NaCl pH 7.5 at ∿20°C, 0.066M phosphate buffer pH 7.5 at ∿20°C and 0.1M phosphate buffer pH 7.5 at ∿20°C: in all environments the results of the previous work suggest that the operational unit of the enzyme is of molecular weight 202,000, at least at the concentrations above 0.1g dl^{-1} used in the previous studies. It is also noteworthy that the dissociation reaction at 4°C in imidazole or tris buffer is prevented by addition of 2mM argininosuccinate or arginine, but not by fumarate. Further comment on the subunit structure of the enzyme and on its catalysis of the reaction, argininosuccinate $\rightleftharpoons$ arginine + fumarate will be made later.

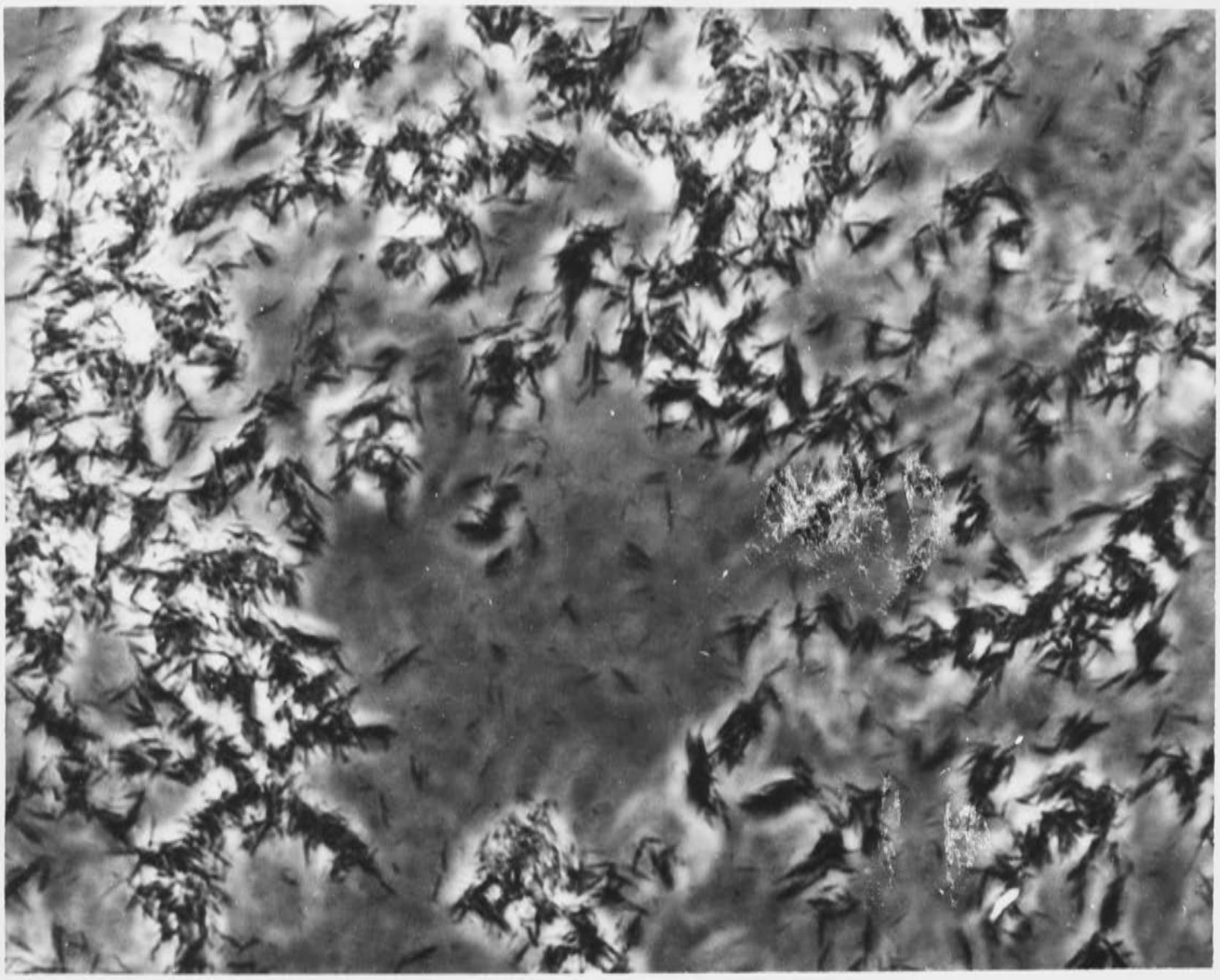


FIGURE V - 1

A photomicrograph (microscope magnification 1000x, using an oil immersion lens) of crystals of beef liver argininosuccinase prepared as detailed in Chapter VI. The specific activity of the crystals was 1400 RU mg^{-1} (1 RU = 1 μmole of argininosuccinate at saturating levels for the enzyme, converted per hour at 38°C in the buffers specified in the text).

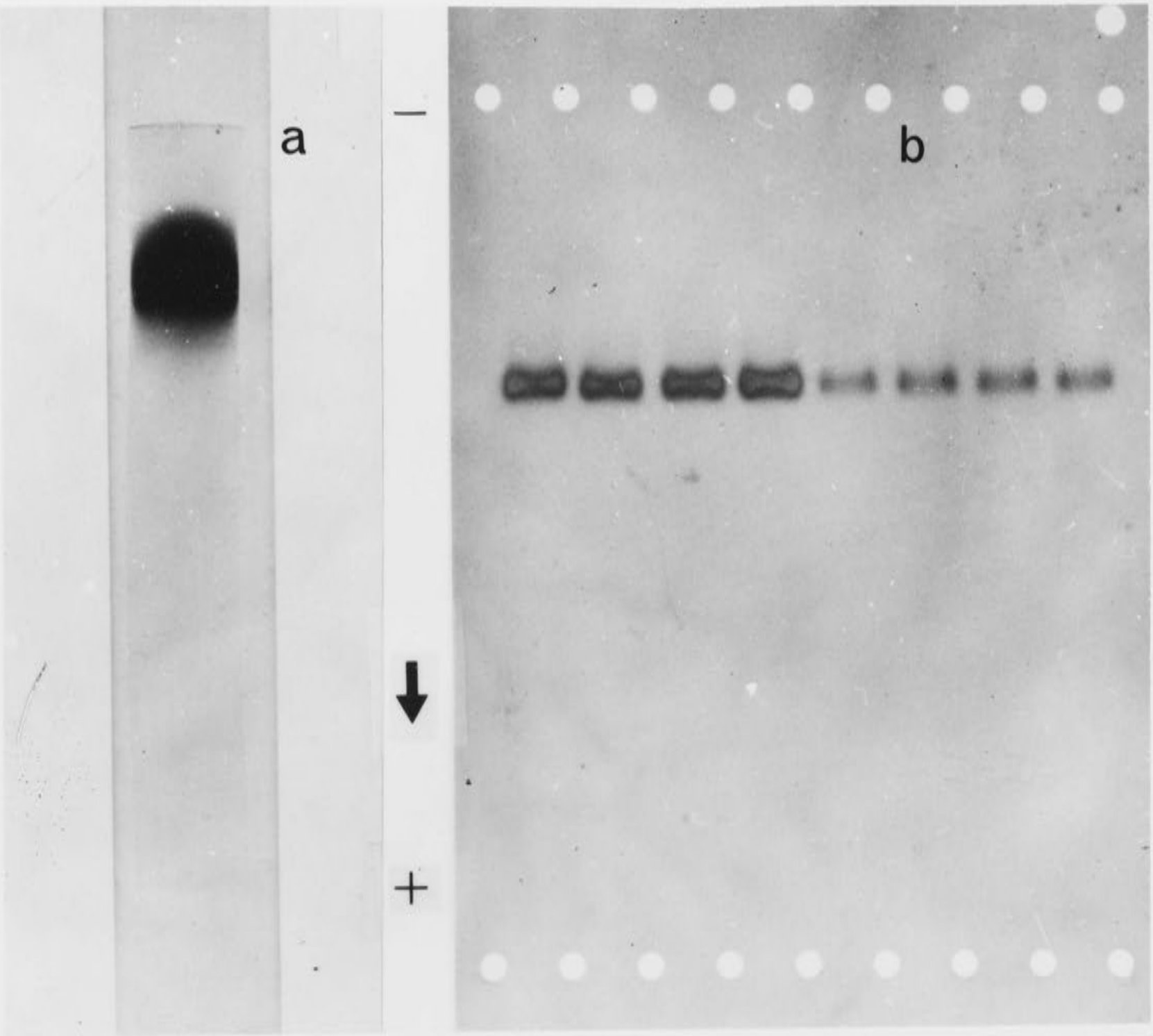
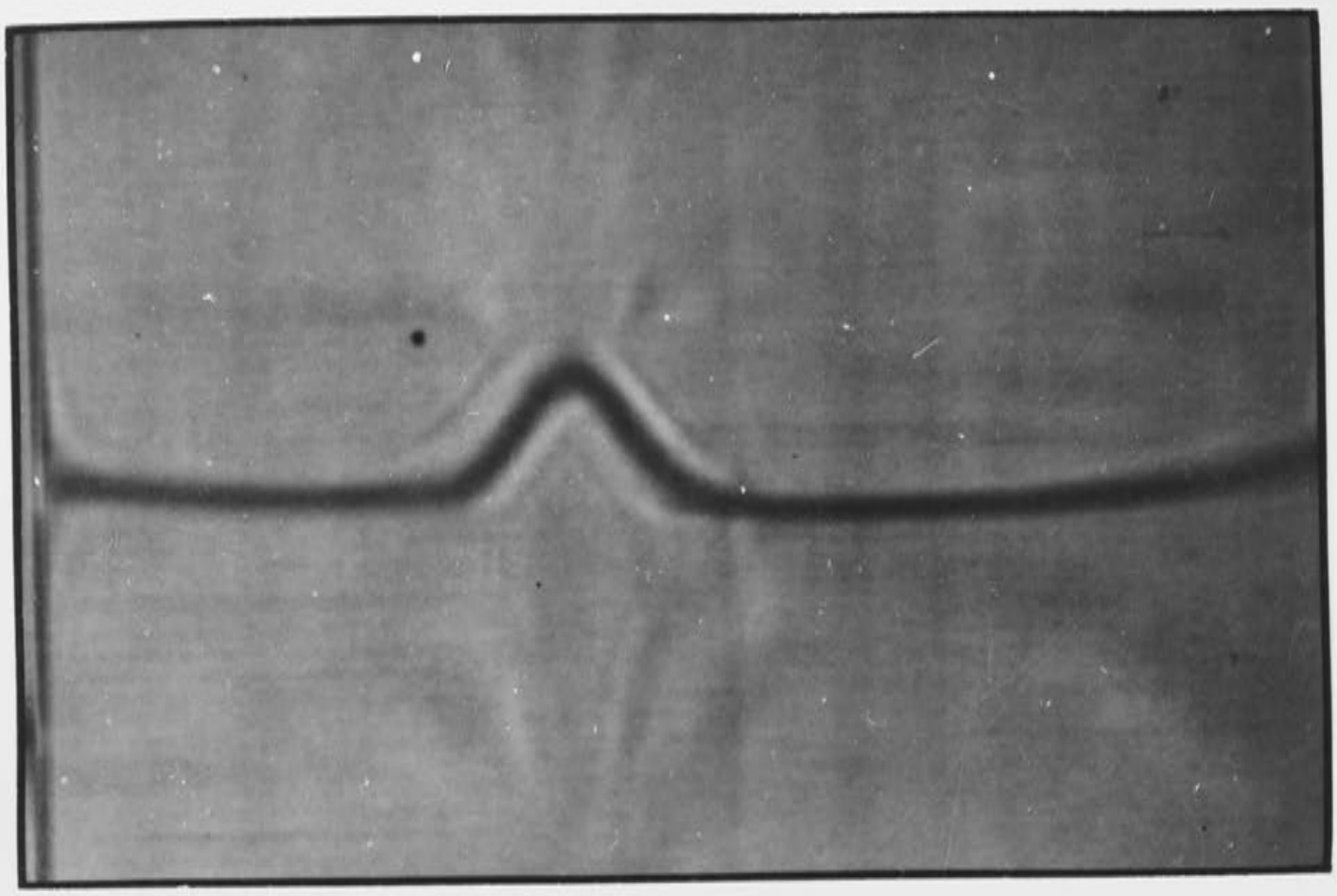


FIGURE V - 2

Electrophoretograms of argininosuccinase on;

(a) polyacrylamide (7.5%) at pH 8.3 at 25°C employing the method described in Chapter VI; the loading mass of protein was ∿ 100 µg.

(b) cellulose acetate at pH 8.5 and 25°C performed as described in Chapter VI; the loading mass of protein was approximately 10 µg and 40 µg.



(a)

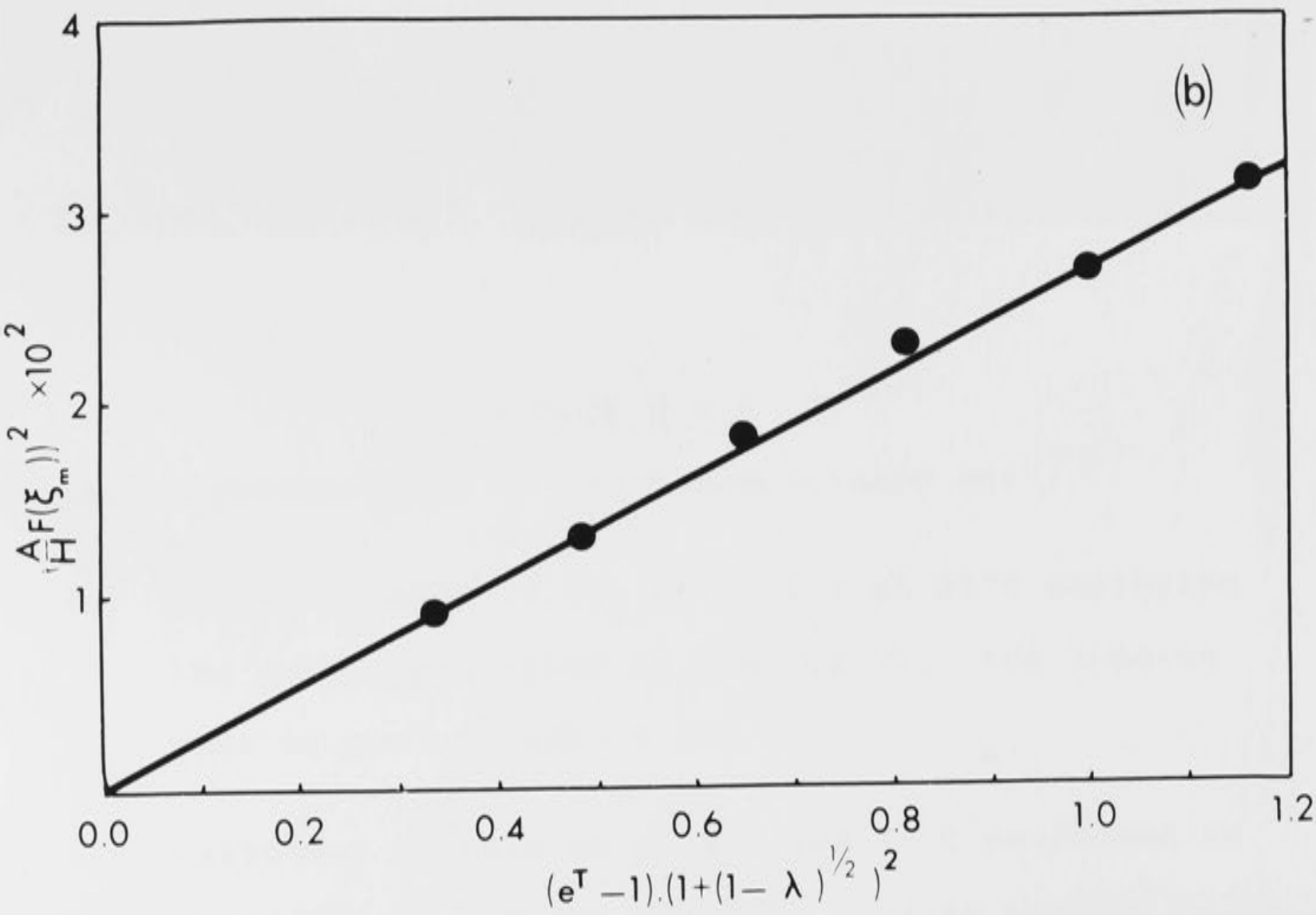

(b)
4
3
2
1
$\frac{A_F(\xi_m)}{H}^2 \times 10^2$
0.0
0.2
0.4
0.6
0.8
1.0
1.2
$(e^T - 1).(1+(1-\lambda)^{1/2})^2$

FIGURE V - 3

(a) A schlieren pattern obtained after approximately 30 min from the commencement of a sedimentation velocity experiment at 60,000 rpm, on beef liver argininosuccinase (0.25g dl^{-1}) in 0.05M HCl-tris, 0.05M NaCl pH 7.5 at 20°C, with a phase plate angle of 70°.

(b) A plot of equation (III-11) pertinent to the analysis of the shape of the boundary shown in Figure (V-3a). The following parameters were employed in the calculation;

c^o = 0.25g dl^{-1}; k = 0.04$g^{-1}dl$;

ω = 60,000 rpm; s_o = 9.3S; D = 4.9 x 10^{-7} cm^2 sec^{-1}.

TABLE V - 1

A summary of the physico-chemical parameters of argininosuccinase

Environment	Value of parameter	Comment
0.05 M HCl-tris, pH7.5, 20°C 0.05 M NaCl	Sedimentation coefficient, $s_{20,w}$ 9.4 ± 0.1S	Maximum ordinate values found in sedimentation velocity experiments conducted at 20°C employing an enzyme concentration of 0.2 to 0.45g dl^{-1}.
0.1 M phosphate, pH7.5, 20°C	9.6 ± 0.1S	
0.1 M phosphate, pH7.5, 4°C	9.8S	Found at 4°C and corrected to s at 20°C in water.
0.05 M HCl-tris, pH7.5, 20°C 0.05 M NaCl	App. Diffusion coefficient $D_{20,w} = 4.9 \times 10^{-7} cm^2 sec^{-1}$	Found as illustrated in Figure (V-3) by the boundary analysis procedure encompassed by equations (III-10) to (III-12).
0.05 M HCl-tris, pH7.5, 20°C 0.05 M NaCl	App. Specific volume $\bar{v} = 0.732\ g^{-1}\ ml$	Found using equation (III-20) using density measurements and confirmed by the amino acid analysis reported in Table (V-2).
0.05 M HCl-tris, pH7.5, 20°C 0.05 M NaCl	Molecular weight 180,000	Obtained using the Svedberg molecular weight expression and the value of the apparent diffusion coefficient found by the boundary analysis procedure.
0.066 M phosphate, pH7.5 20°C	203,000	Found by sedimentation equilibrium. Figure (V-4) and equation (III-13).
0.066 M phosphate, pH7.5, 20°C	Frictional ratio $f/f_o = 1.27$ Stokes radius r = 3.90nm	Calculated by employing equations (III-21) and (III-22). $r = (3 M \bar{v} / 4 \pi N)^{1/3}$

2. Results of the physico-chemical investigation of argininosuccinase.

The enzyme was prepared by the method described by Schulze, Lusty and Ratner (1970), and Ratner (1970), details of which are given in Chapter VI. Figure (V-1) shows a photograph of the crystals obtained. These had a specific activity of 1400 RU mg^{-1} (Chapter VI), as obtained by the above workers: corresponding to a k_{cat} of 4.2×10^3 min^{-1} at 38°C.

As described in Chapter III, mass migration methods were used in initial attempts to characterize the homogeneity of the preparation. A single migrating band was observed in electrophoresis at pH 8.3 at 25°C on both polyacrylamide gels (7.5% acrylamide) and cellulose acetate, as shown in Figure (V-2). It would appear from these results that the dissociation into half-subunits, observed in other buffer systems at the same pH value, has not proceeded appreciably within the time of duration of the experiments, approximately 30 min. A more critical test of homogeneity follows from the analysis of the shape of the boundary observed in sedimentation velocity, conducted at 20°C, in 0.05M HCl-tris, 0.05M NaCl pH 7.5, where no dissociation is expected. Figure (V-3a) shows the sedimenting boundary obtained, and Figure (V-3b) a plot of equation (III-11): the linearity of the latter plot provides the crucial observation on which homogeneity with respect to sedimentation coefficient is claimed. The corresponding values of the sedimentation coefficient, diffusion coefficient and molecular weight are summarized in Table (V-1). The value of

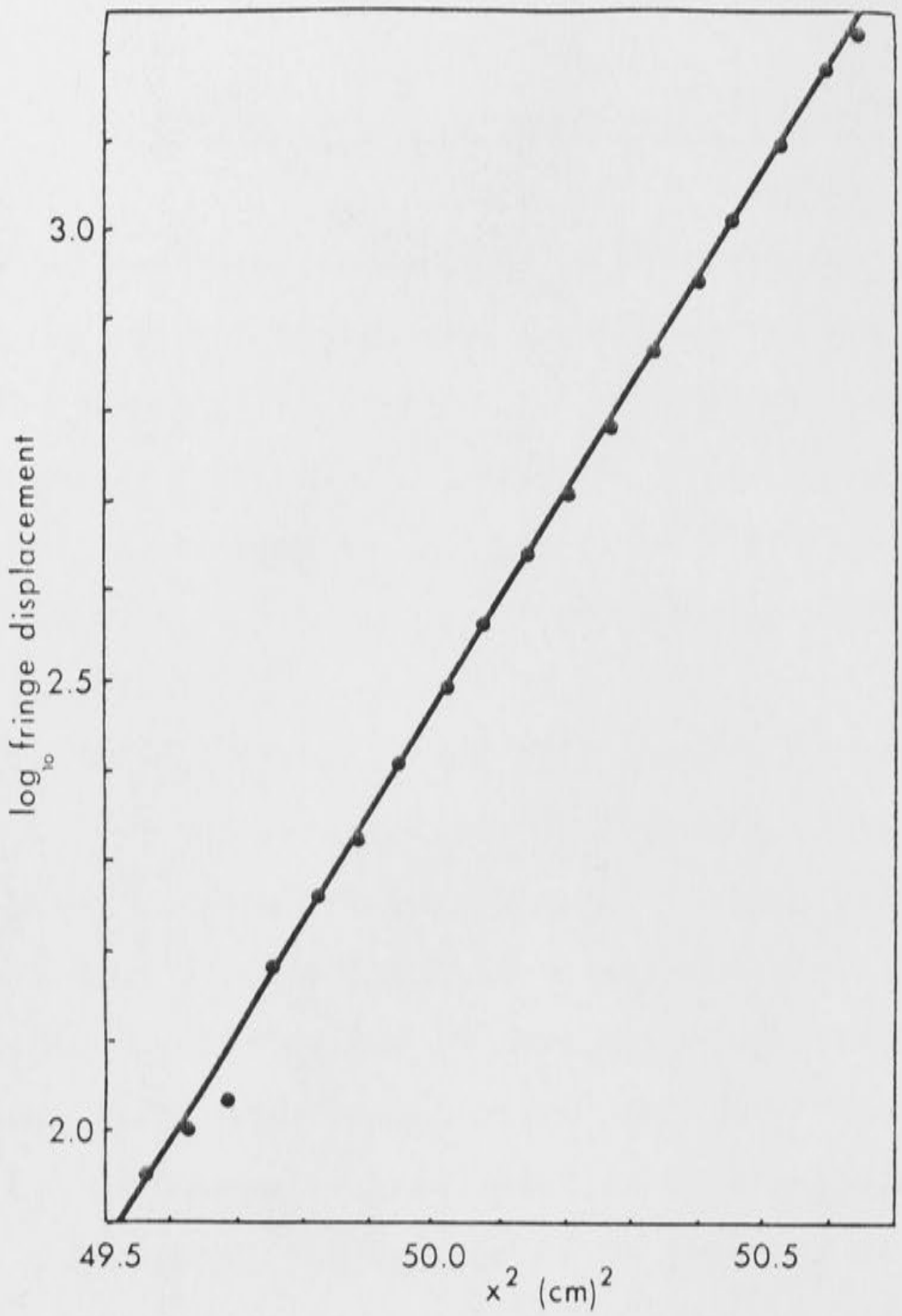


FIGURE V - 4

A plot of $\log_{10}$ fringe displacement versus radial distance squared with Rayleigh interference data obtained from a high speed sedimentation equilibrium experiment (ω = 15,000 rpm) on beef liver argininosuccinase in 0.066 M phosphate buffer pH 7.5 at 20°C. The starting concentration was 0.04g dl^{-1} and the concentration range scanned by this plot is 0.08 to 0.132g dl^{-1}, the former corresponding to a net fringe displacement of $\sim$ 100μ.

$s_{20,w}$ = 9.4 ± 0.1S is an average value from different experiments and is in reasonable agreement with the corresponding value of 9.3S obtained by the previous workers. Sedimentation velocity experiments were also conducted in 0.1M phosphate buffer pH 7.5 as reported in Table (V-1). The values of the sedimentation coefficients found from experiments conducted at 4°C and 20°C are in reasonable agreement when account is taken of the large and essentially empirical correction factor (Svedberg and Pedersen, 1940) needed to compare the results: certainly no dissociation is indicated at the lower temperature in this phosphate medium. These findings confirm earlier observations (Ratner, 1972) that dissociation into half-subunits does not occur in these environments. Further support of the latter observation, and a more reliable estimate of the molecular weight, was obtained by conducting a sedimentation equilibrium experiment in 0.066M phosphate buffer pH 7.5. Figure (V-4) shows a plot of the sedimentation equilibrium distribution, in the form suggested by equation (III-13): the linearity of the plot confirms that the sample is homogeneous with respect to molecular weight, and that terms involving the non-ideality coefficients are of negligible magnitude in the concentration range investigated (equation III-16). The value of the molecular weight (203,000) derived from the slope of the plot shown in Figure (V-4) is reported in Table (V-1), and is seen to be in good agreement with the value of 202,000 reported by the previous workers. It could also be noted that the value of the molecular weight

TABLE V - 2

Amino acid composition of beef liver argininosuccinase.

Amino acid residue	Nearest integer residues per 203,000g	Nearest integer residues per 202,000g from (Bray & Ratner, 1971)
Lysine	81	82
Histidine	48	51
Arginine	121	125
Aspartic acid	160	149
Threonine [a]	101	102
Serine [a]	143	150
Glutamic acid	226	206
Proline	58	66
Glycine	134	130
Alanine	161	163
Half-Cystine [b]	9	17
Valine	105	113
Methionine	51	55
Isoleucine	60	64
Leucine	233	218
Tyrosine	39	39
Phenylalanine [a]	56	55
Tryptophan	-	38

[a] Values in this work were not extrapolated

[b] Determined as cysteic acid on a performic acid oxidized sample.

estimated, utilizing the diffusion coefficient derived from boundary analysis is an underestimate by approximately 11%, a result similar to that found in the investigations with arginase (Chapter III). In these calculations a partial specific volume of $0.732 g^{-1}$ ml was employed, the value being obtained from the amino acid analysis of argininosuccinase reported in Table (V-2), and confirmed by density measurements. Little comment need be made on the amino acid composition reported in Table (V-2) which evidently basically agrees with that found earlier (Bray and Ratner, 1971), except to note that in the present analysis the individual half-cystine content is lower even though performic acid oxidation was performed prior to the analysis.

Since argininosuccinase is capable of dissociation into half-subunits, it is particularly relevant to perform a frontal Sephadex chromatography study analogous to that illustrated in Figure (III-12) to enquire whether dissociation may be effected by dilution in the environment to be used for enzyme kinetic studies. Figure (V-5a) shows an elution profile of the enzyme obtained employing Sephadex G200 and a plateau concentration of $0.01 g\ dl^{-1}$. The median bisector of the advancing front (OIJ) occurs at an elution volume of 35.4ml which corresponds to a K_{AV} of 0.21 and to a molecular weight of $\sim$ 200,000 (Laurent and Killander, 1964). This was the value obtained in the sedimentation equilibrium experiment conducted in the same environment where a concentration range of 0.08 to $0.132 g\ dl^{-1}$ (Figure V-4) was scanned. Figure (V-5b) pertains to a similar experiment conducted with a plateau concentration of $0.0001 g\ dl^{-1}$ of argininosuccinase, and from the information reported in the

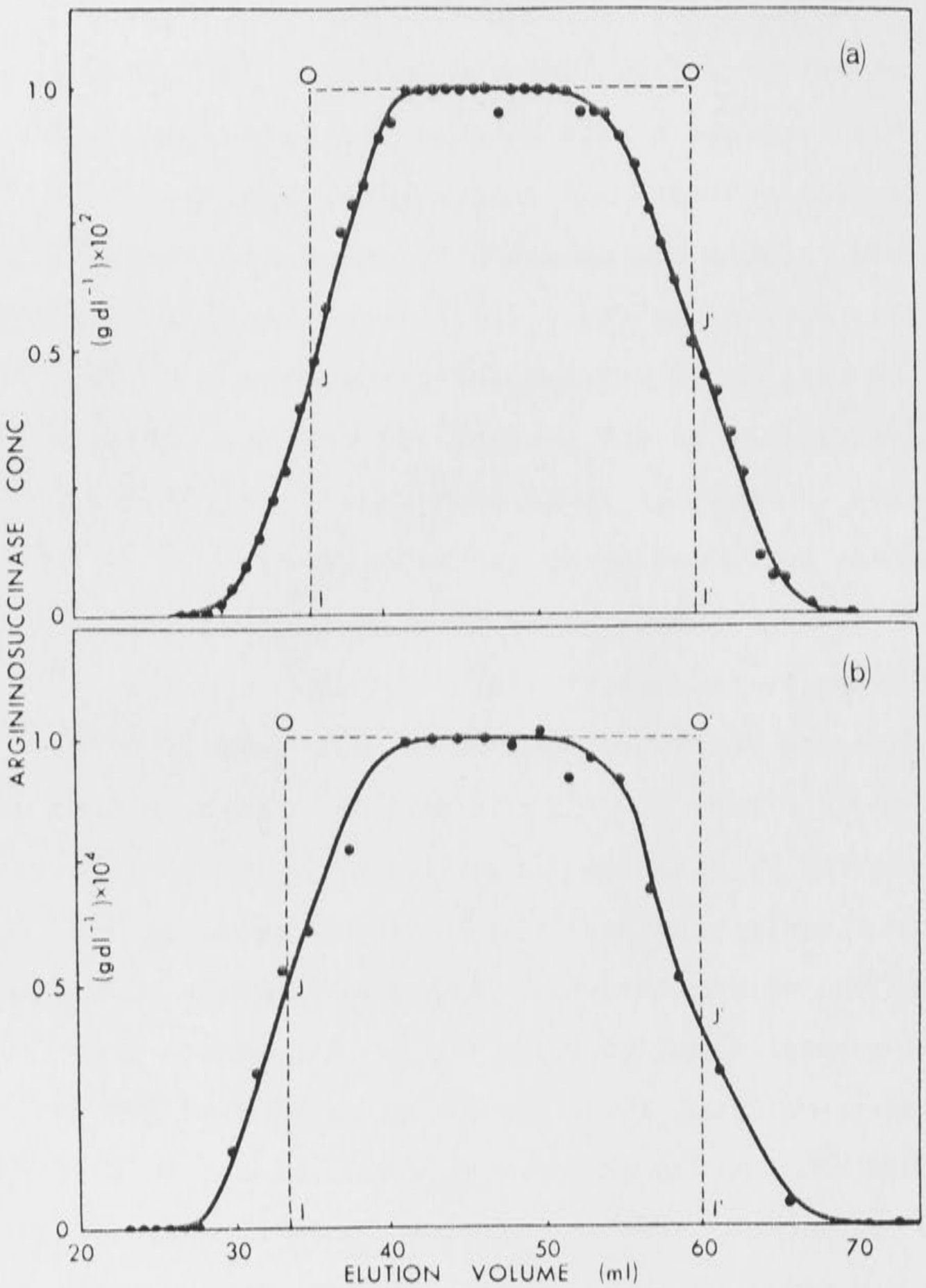

(a)
O
O'
J
J'
I
I'
1.0
0.5
0
ARGININOSUCCINASE CONC.
(g dl⁻¹)×10²
(b)
O
O'
J
J'
I
I'
1.0
0.5
0
(g dl⁻¹)×10⁴
20
30
40
50
60
70
ELUTION VOLUME (ml)

FIGURE V - 5

Elution profiles obtained from a frontal gel chromatography experiment on Sephadex G200 with argininosuccinase in 0.066 M phosphate buffer pH 7.5 at 20°C. Enzyme concentration in each fraction was measured by use of the spectrophotometric assay for argininosuccinase in 1mM argininosuccinate.

(a) 24.5 ml of 0.01g dl^{-1} enzyme solution was applied to a column of bed volume 63.4 ml and eluted at a flow rate of 15 ml h^{-1}. The position of the median bisectors OIJ and O'I'J' both correspond to an elution volume of 35.4 ± 0.1 ml. The void volume of the column determined with Blue Dextran 2000 was 27.7 ml.

(b) 27.3 ml of 0.0001g dl^{-1} of enzyme solution was applied to a column of bed volume 60.4 ml and eluted at a flow rate of 15 ml h^{-1}. The positions of the median bisectors OIJ and O'I'J' both correspond to an elution volume of 33.6 ± 0.1 ml. The void volume of the column determined with Blue Dextran 2000 was 26.4 ml.

caption leads to a value for K_{AV} of 0.21. It may be concluded that no dissociation of the enzyme is indicated in the concentration range 0.45g dl^{-1} to 0.0001g dl^{-1} in the specified environment.

It is now possible to correlate the findings reported in Table (V-1) with the physico-chemical parameters reported for the subunits of argininosuccinase (Lusty and Ratner, 1972), to comment (albeit tentatively) on a possible arrangment of the subunits in the 203,000 molecular weight unit. First, it is noted that the sedimentation coefficient reported for the half-subunit was 5.6S, from which the frictional ratio of this unit may be calculated as 1.36. From the data of Oncley (1941), this frictional ratio could be taken to correspond to a prolate ellipsoid, of axial ratio approximately 4:1 when a degree of hydration of 0.3g H_2O per g protein is assumed. It is now required to show that this description of the half-subunit is consistent with the value of 1.27 for the frictional ratio of the 203,000 unit (Table V-1). This may be attempted by utilizing the relation suggested by Bloomfield,Dalton and Van Holde (1967) which permits the calculation of the frictional resistance of a molecule based on an assumed model. The steps involved are as follows: (1) the prolate ellipse of revolution of axial ratio 4:1 is assumed to comprise four spheres each of radius r, these spheres having no physical significance; (2) the half-subunit of molecular weight 101,000 (i.e. the prolate ellipsoid) is now assumed to dimerize to form the 203,000 unit in a tetrahedral arrangement (with D_2 symmetry) of the eight hypothetical spheres of radius r. A linear, or cyclic

arrangment of the eight spheres (with C_4 symmetry) seems less probable, since different bonding domains are evidently involved in dimer and tetramer formation. Additional evidence which suggests a tetrahedral arrangement comes from the appearance of the electron micrographs of the 203,000 unit (Lusty and Ratner, 1972); (3) the equation of Bloomfield, Dalton and Van Holde (1967) is now applied,

$$f = \frac{6 \pi \eta N r}{1 + \frac{r}{N} \sum_{\ell=1}^{N} \left(\sum_{s=1}^{N} \langle R_{\ell s} \rangle^{-1} \right)} \qquad (V - 1)$$

Where N is the number of hypothetical spheres, in this case eight, and $R_{\ell s}$ is the centre to centre distance from any given sphere to any other sphere in the model. This procedure leads to a value of f, for the 203,000 unit of 11.9×10^{-8} dynes per molecule, and hence to a frictional ratio of 1.60 which may be compared with the experimentally obtained value of 1.27. The agreement is not unreasonable in view of the assumptions made, and is certainly better than that which is obtained if either a linear or cyclic arrangement within the 203,000 unit is assumed. Two interesting points emerge. First, the basic tetrahedral arrangement of subunits in the 203,000 molecular weight unit of argininosuccinase as visualized by Lusty and Ratner (1972) appears to be basically correct. Secondly, the present work suggests that the quarter-subunits of molecular weight $\sim$ 50,000 are not spherical as depicted by Lusty and Ratner (1972), but rather themselves approximate prolate ellipsoidsof axial ratio 2:1.

This hypothesis is somewhat tentative as implicit assumptions involving degrees of hydration have been made and is not subject to ready experimental test, since the sedimentation velocity behaviour of the quarter-subunit may only be observed after treatment with urea or SDS (Lusty and Ratner, 1972), denaturing agents which clearly alter the frictional ratio of these subunits.

3. Enzyme kinetic studies with argininosuccinase.

Argininosuccinase catalyzes the reversible reaction, argininosuccinate $\rightleftharpoons$ fumarate + arginine, and in the present work initial velocity studies were performed utilizing argininosuccinate as the initial substrate, (prepared enzymically as described in Chapter VI). A convenient method for following the reaction was to monitor the appearance of fumarate spectrophotometrically at a wavelength of 240nm where neither arginine nor argininosuccinate appreciably absorb light (Havir, Tamir, Ratner and Warner, 1965). In detail a typical protocol for an experiment was as follows. The enzyme, stored at -12°C in phosphate buffer, was diluted in 0.066M phosphate pH 7.5 to an appropriate concentration level, and incubated in this buffer at 25°C for several hours. Two ml of the phosphate buffer was introduced into a 1cm quartz cuvette, followed by 0.25ml of the enzyme solution, and 0.25ml of argininosuccinate solution of known concentration, mixing being effected with the use of a teflon cuvette plunger. Recording was commenced within 10-15 sec of the initiation of the reaction, with the chart speed previously set to yield an initial slope of approximately 45° on the tracing. Initial velocities (v_o) were determined from the recordings,

which in no instance exhibited a "lag" period. Each assay was performed in duplicate or triplicate, the average value of the initial velocity being determined. A minor difficulty arises in estimating corresponding values of the initial substrate concentration, since in solution, argininosuccinate cyclizes to five and six membered ring anhydrides (Ratner, Petrack and Rochovansky, 1953; Ratner and Kunkemueller, 1966) which are not substrates or inhibitors of argininosuccinase. The difficulty was overcome by allowing selected assays to proceed to equilibrium whereupon the operational initial argininosuccinate concentration could be readily calculated by estimating the equilibrium concentration of fumarate, [F], and utilizing the equilibrium constant, K_{eq}, for the reaction. Thus,

$$[\text{argininosuccinate}]_{\text{operational}} = [F]^2/K_{eq} + [F] \qquad (V - 2a)$$

K_{eq} (3.22×10^{-3}M) was determined in separate experiments by permitting solutions of fumarate and arginine of known solution composition to proceed to equilibrium and by determining again the equilibrium concentration of fumarate. Thus,

$$K_{eq} = \frac{[F]\{[\bar{A}]_o - [\bar{F}]_o + [F]\}}{[\bar{F}]_o - [F]} \qquad (V - 2b)$$

where $[\bar{F}]_o$ and $[\bar{A}]_o$ are respectively, the molar concentrations of fumarate and arginine initially selected. In practice the correction was small, since cyclization proceeded to the extent of only $\sim$ 2% when a solution of argininosuccinate was stored for a period of 7 days at 4°C.

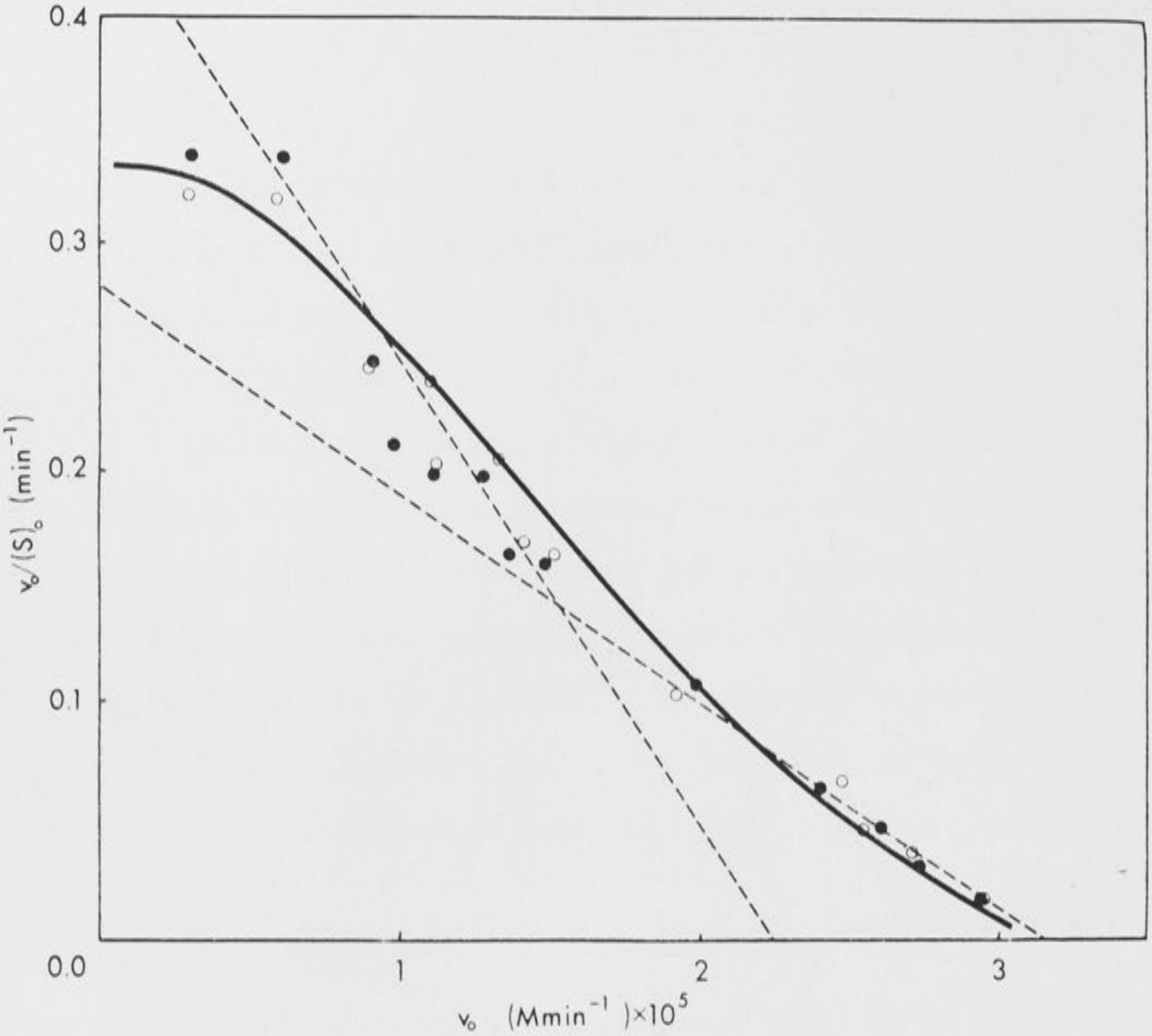


FIGURE V - 6

Data from an initial velocity study of argininosuccinase plotted in the Eadie-Hofstee format. The assays were performed at 25°C in 0.066 M phosphate buffer pH 7.5 following the protocol described in the text. The solid line was fitted according to equation (V-3) and also by use of the rapid equilibrium model discussed in the text. The relevant parameters used for the fitting are given in Section (V-3). The dotted lines have slopes which correspond to Michaelis constants of 5×10^{-5}M and 1.1×10^{-4}M.

It is possible to plot corresponding values of v_o and the corrected initial substrate concentration $[S]_o$ in various ways: the method selected in this instance, to best display the important features was a plot of $v_o/[S]_o$ versus v_o; the Eadie-Hofstee (Eadie, 1942; Hofstee, 1952) plot, which is the enzyme kinetic analogue of the Scatchard plot (Scatchard, 1949) used for the analysis of binding data. This is shown in Figure (V-6) where the solid points represent measurements found in a set of experiments in which the value of $[S]_o$ was progressively decreased. The open circles are also measurements found using the same enzyme preparation and progressively increasing the initial substrate concentration. The scatter between the two sets of points is within experimental error, an observation which shows that the enzyme in solution has not undergone time dependent changes which affect its catalytic activity during the course of the experiment. The broken lines are attempts to average these data assuming a linear relationship between $v_o/[S]_o$ and v_o in two domains of initial substrate concentration, the slopes corresponding to apparent K_m values of 5×10^{-4}M and 1.1×10^{-4}M (footnote *) for the lower and higher concentration

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* It is of interest to note that when a plot of $v_o/[S]_o$ versus v_o was constructed employing experimental data obtained using a continuous flow assay similar to that depicted in Figure (IV-2) that a linear relationship (with $K_m = 1.7 \times 10^{-4}$M) was indeed indicated. This is similar to the value of 1.6×10^{-4}M, reported by Bray and Ratner (1971). However the accessible range of initial substrate concentration which could be studied by this method was only 0.1 to 3mM which corresponds in Figure (V-6) to an ordinate range of 0 to 1.6 min^{-1}.

domains respectively. It is however immediately apparent that the broken lines are a poor representation of the experimental points which tend to exhibit, in a continuous curve, a maximum at low v_o, and a continuously decreasing slope in the high v_o range. To be sure, experimental scatter makes a definite description of the form of the curve difficult but as these essential features were exhibited in studies performed with a separate enzyme preparation in the same environment, and in 0.05M HCl-tris 0.05M NaCl pH 7.5, it seems reasonable to assess them as realdeviations.

There are several possible models which might explain the deviations from linearity observable in Figure (V-6), but for the sake of simplicity only two basic models will be considered which may be taken as representative of other models which differ from them only in detail. The first model assumes that the enzyme exists in solution, in the absence of substrate, as a single state, and indeed the physico-chemical evidence summarized in Figures (V-2), (V-3), (V-4) and (V-5) support this contention. In view of the subunit structure including four subunits, it is reasonable to suppose that on addition of ligand (substrate) four catalytic sites are encountered, and indeed this finds support in equilibrium binding studies of arginine to the enzyme (Ratner, 1972). It is a simple matter to derive a binding equation for a four site model in which the sites are equivalent but dependent (i.e. mutually interacting or cooperative) : it is merely required to define a single intrinsic association constant K_1 which describes the binding of one ligand molecule to any of the four sites

and then to introduce the different association equilibrium constants K_2, K_3 and K_4, to describe the formation of higher order complexes. The appropriate binding equation is the Adair equation (equation II-35) with n = 4. If the bold assumption is made that the intrinsic breakdown rate constants are all identical, the transformation suggested by Frieden (1967), and discussed in Chapter II, may be used to write the binding equation in a kinetic format as follows,

$$v = \frac{V}{n}\left\{\frac{\alpha_1[S] + \alpha_2[S]^2 + \alpha_3[S]^3 + \alpha_4[S]^4}{1+\alpha_1[S] + (\alpha_2/2)[S]^2 + (\alpha_3/3)[S]^3 + (\alpha_4/4)[S]^4}\right\} \qquad (V - 3)$$

where, $\alpha_1 = 4K_1$; $\alpha_2 = 12K_1K_2$; $\alpha_3 = 12K_1K_2K_3$; $\alpha_4 = 4K_1K_2K_3K_4$; n = 4 (the number of catalytic sites) and V the maximal velocity (3.2×10^{-5}M min^{-1} in relation to Figure V-6). Extensive numerical computation utilizing equation (V-3) resulted in the solid line shown in Figure (V-6) which corresponds to $K_1 = 1.04 \times 10^4$; $K_2 = 1.48 \times 10^4$; $K_3 = 9.0 \times 10^3$; $K_4 = 7.14 \times 10^3$. It is not suggested that this is a unique set which describes the experimental data, but it serves to illustrate that deviations from linearity such as is seen in Figure (V-6) may arise from a combination of positive cooperative effects ($K_2 > K_3 > K_4$) and negative cooperative effects ($K_1 < K_2$). Such a combination of effects mediated by undefined conformational changes of the protein, inherent on ligand binding, has previously been suggested in relation to binding studies performed on glyceraldehyde-3-phosphate dehydrogenase (Cook and Koshland, 1970). While

the possible operation of such an allosteric mechanism is not denied it is an unfortunate feature of such a proposal that it could be used to describe a kinetic curve of virtually any form which deviated from classical behaviour, as in the present instance. It also suffers from the additional disadvantage that it is difficult to envisage conformational changes affecting subunit-subunit interactions which operated asymmetrically within a structure which has tetrahedral (D2) symmetry.

The second model to be considered is a modification of the basic system envisaged by Monod, Wyman and Changeux (1965) which involves two isomeric states of the enzyme, coexisting in rapid equilibrium prior to the addition of the substrate. While the available physico-chemical evidence definitely eliminates the possibility that two or more *polymeric* states coexist in an equilibrium, it is compatible with this isomerization hypothesis. Thus, for $A \rightleftharpoons C$ where $M_A = M_C$, it is seen from equation (III-14) that $\bar{M} = M_A = M_C$ and that a plot of log $\bar{c}(x)$ versus x^2 obtained from a sedimentation equilibrium experiment will be linear as observed in Figure (V-4). For an entirely analogous reason the weight-average elution volume from a frontal gel chromatography experiment, would only be a function of $\bar{c}$ if V_A differed significantly from V_C which is unlikely: the result in Figure (V-5) is therefore also compatible with the isomerization hypothesis. Finally, mass migration in electrophoresis of a rapidly equilibrating isomerizing system can only produce a single band, even if the electrophoretic

mobilities of A and C are different (Nichol and Winzor, 1972), in accord with Figure (V-2). A simple model is therefore proposed in which the half-subunit of argininosuccinase, in the A isomer, possesses two catalytic sites which are non-equivalent and therefore related to the different intrinsic association constants K_1 and K_2: the whole unit of molecular weight 203,000 therefore possesses two classes of non-equivalent binding sites. No assumption will be made that any of these four sites are mutually interacting. The A isomer is assumed to coexist in rapid equilibrium with the C isomer which for the sake of simplicity will be taken to be completely catalytically inactive (although theory pertaining to more complicated models of the same type may be readily written). The system is therefore defined in terms of three equilibrium constants K_1, K_2 and $X = [C]/[A]$, the isomerization constant. Formulation of the binding equation for this system, and transformation to the kinetic format, again using the transformation of Frieden (1967), leads to equation (V-3) where, in this case, the coefficients are identified as,

$$\alpha_1 = 2(K_1 + K_2)/(1 + X)$$

$$\alpha_2 = 2(4K_1K_2 + K_1^2 + K_2^2)/(1 + X)$$

$$\alpha_3 = 6K_1K_2(K_1 + K_2)/(1 + X)$$

$$\alpha_4 = 4K_1^2K_2^2/(1 + X)$$

With $K_1 = 3.33 \times 10^4$; $K_2 = 4 \times 10^3$; and $X = 0.8$, these relations, used in conjunction with equation (V-3), also yield the solid line shown in Figure (V-6). Three points emerge from this analysis. First, it is not possible to

distinguish, on the basis of Figure (V-6) between the two models proposed. Secondly, it is of interest that the deviations from linearity may find explanation, without in any way involving substrate induced cooperativity between the catalytic sites on a particular isomeric acceptor entity. Thirdly, regardless of which particular mechanism is operating, in causing the deviations from linearity observed in Figure (V-6), equation (V-3) with $\alpha_1 = 4.15 \times 10^4$, $\alpha_2 = 1.85 \times 10^9$, $\alpha_3 = 1.66 \times 10^{13}$ and $\alpha_4 = 3.95 \times 10^{16}$ provides a suitable phenomenological description of the initial velocity of the reaction under discussion which may be useful in future simulations of reactions in which argininosuccinase is involved.

4. <u>An investigation of the possible interaction between argininosuccinase and arginase</u>.

An estimate of the isoelectric point of argininosuccinase was obtained by subjecting a 20mg sample of the purified material to isoelectric focusing for a period of 72 h at 4°C. Some flocculation was observed but the majority of the protein banded in the final pH gradient as a symmetrical zone with maximum located at pH 6.1: the procedure is not recommended as a routine purification step, however, since it resulted in a considerable loss of activity. The result implies that at pH values greater than 6.1 both argininosuccinase and arginase bear a net negative charge, but this does not *a priori* exclude the possibility of a specific interaction between the enzymes. This possibility was investigated in two ways.

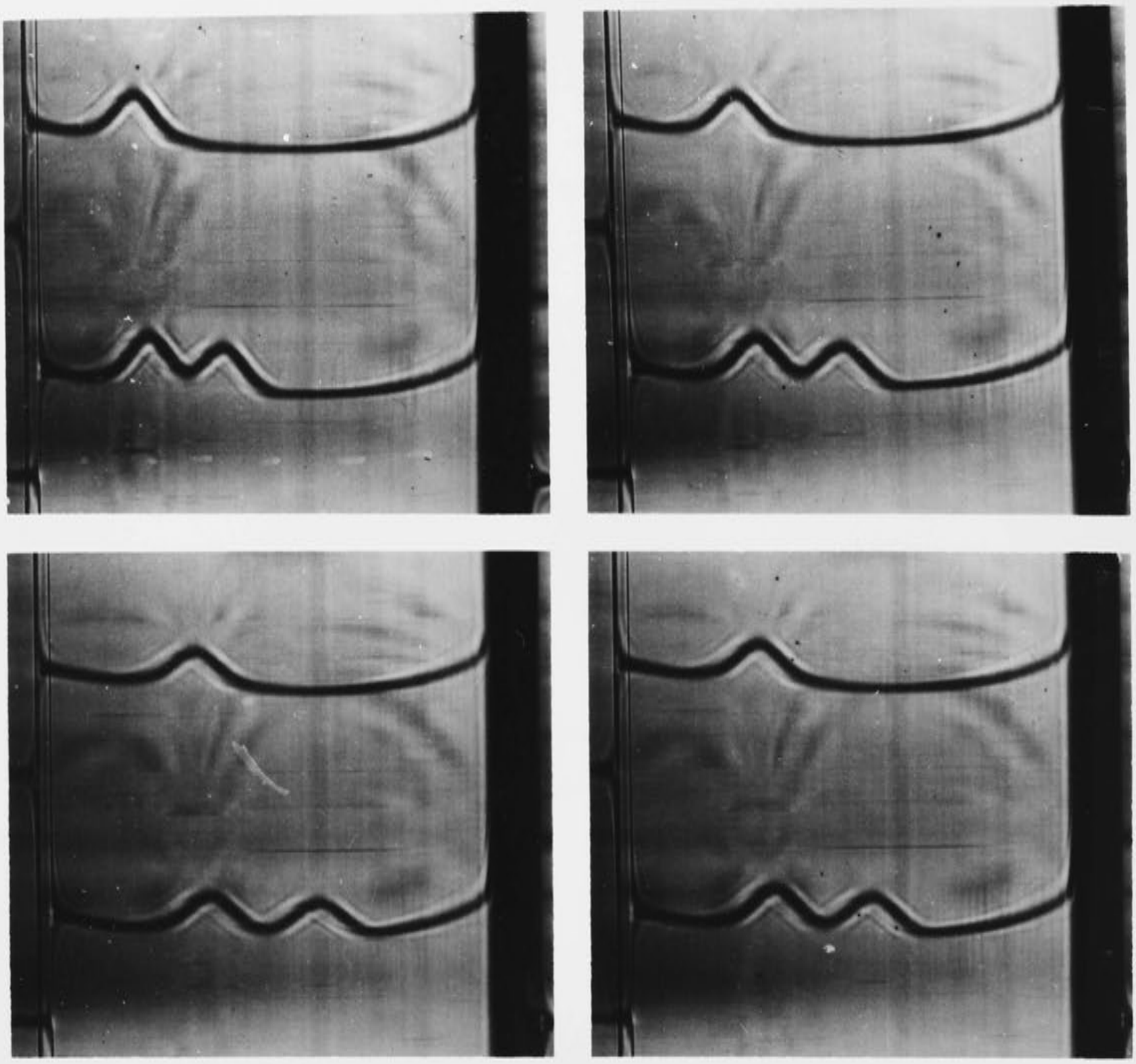


FIGURE V - 7

A series of schlieren patterns obtained from a sedimentation velocity experiment on arginase alone (upper pattern) and in the presence of argininosuccinase (lower pattern) in 0.1 M phosphate buffer pH 7.5 at 4°C. The photographs were taken at 8 min intervals from ∿ 50 min after attaining the angular velocity, 60,000 rpm, employed in the experiment; phase plate angle was 70°.

(a) Sedimentation velocity of mixtures

Figure (V-7) shows a typical sedimentation velocity pattern obtained with arginase alone (upper pattern) dialyzed free of excess manganese ions, but not exhaustively, with a mixture of the two enzymes (lower pattern) in 0.1M phosphate pH 7.5 at 4°C. The peaks in the lower pattern are clearly resolved, which permitted ready determination of both their sedimentation coefficients and of their areas. In this experiment, the sedimentation coefficient of arginase alone proved to be identical, within experimental error, to that of the slower sedimenting peak in the mixture. This finding is reported in Table (V-3) where it is seen that it was consistently observed in the other reported environments. In each of the reported experiments the concentration given by the slower peak area proved to be slightly greater than that of arginase initially introduced into the mixture, as found by a control experiment similar to that shown by the upper pattern in Figure (V-7). This finding suggests that a Johnston-Ogston effect (frictional interaction) is operating rather than a chemical interaction, a hypothesis substantiated by comparisons made by the last two columns of Table (V-3). It may be concluded that arginase and argininosuccinase do not heterogeneously associate at pH 7.5 over the temperature range 4°C to 20°C where both proteins bear a net negative charge. The experiment conducted at pH 6.0, a pH value between the isoelectric points of arginase and argininosuccinase of pH 5.9 and pH 6.1 respectively, further shows that no chemical interaction is

TABLE V - 3

Sedimentation velocity results on mixtures of argininosuccinase and arginase

ENVIRONMENT	$s_{T,b}$ SLOW PEAK	ARGINASE ALONE	Concentration (g dl^{-1}) of slow peak EXPERIMENTAL	CALCULATED USING EQ.(III-8)†
0.1 M phosphate buffer pH 7.5, 4°C	3.4_6	3.4_6	0.30	0.30_3
0.05 M HCl-tris, 0.05 M NaCl, pH 7.5, 20°C	5.6_2	5.6_2	0.29	0.29_3
0.02 M maleate buffer, pH 6.0, 20°C	5.9_6	5.9_9	0.16	0.17_3

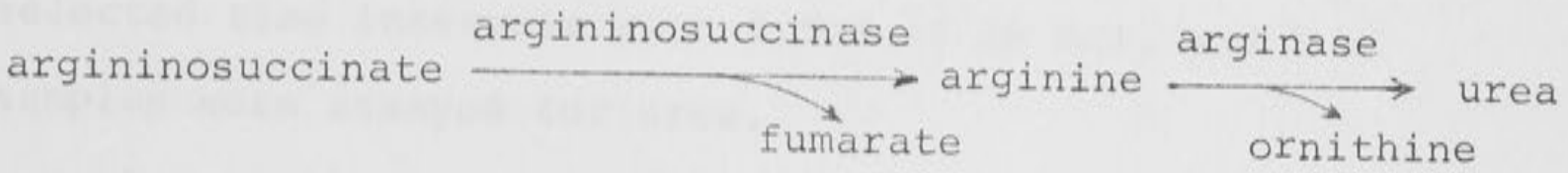
† According to eq.(III-8) the reported calculated value of $c_B^\beta = \bar{c}_B^\alpha\ (v' - \bar{v}_B^\alpha)/v' - v_B^\beta)$ where $\bar{c}_B^\alpha$ was obtained in each case from a control experiment such as illustrated in the top pattern of Fig. (V-7), v' was taken as the measured s of the fast peak in the mixture v_B^β as reported in column 2 of the Table and $\bar{v}_B^\alpha$ was calculated from the *total* concentration of the two proteins in the original mixtures together with k = 0.069 (Chapter III).

induced when the net electrostatic repulsive force is effectively reduced to zero.

Although no chemical interaction between the enzymes is indicated by these physico-chemical studies, it remains to be investigated whether such an interaction is mediated by one or more of the various components of reaction mixtures encountered in a coupled assay.

(b) Results of coupled assays

In these studies the following couple was investigated,



with argininosuccinate as the initial substrate, and by monitoring the rate of production of urea by the diacetyl monoxime method, (detail in Chapter IV). As noted in Chapter III it is desirable with regard to the structural stability of arginase to conduct assays in the presence of 0.01M $MnCl_2$; but in the present studies it was found that this concentration of $MnCl_2$ inhibited argininosuccinase to the extent of $\sim$ 80% rendering meaningful studies on the coupled system impossible. Accordingly the less desirable approach was employed of dialyzing arginase solutions, stored in the presence of excess $MnCl_2$, free of unbound Mn^{++} by the rapid dialysis technique (Englander and Crowe, 1965), immediately prior to the assay. It was shown in control experiments that arginase solutions treated in this way lost only 5% of their total activity in a time period of 8 h, and that the K_m and k_{cat} values found within this time period were identical, within experimental error, with those obtained with the same arginase sample studied in the presence

of excess $MnCl_2$. It is also noted by reference to Figure (III-14a) that the structural integrity of arginase is largely preserved following application of this rapid dialysis procedure. In preparation for the coupled assay a dialyzed solution of arginase was mixed with a solution of argininosuccinase and incubated for 30 min at 25°C, the buffer being 0.05M HCl-tris, 0.05M NaCl pH 7.5. To 2.25ml of the enzyme solution was added 0.25ml of argininosuccinate solution in the same buffer, the reaction being stopped at selected time intervals with 0.5ml of 2M HCl, and 1 ml samples were assayed for urea.

In principle it would be possible to plot the concentration of urea versus t^2 (since two enzymes are involved) and to proceed with an analysis of the kinetic parameters as described in Chapters II and IV. However as shown in the discussion of Chapter IV by utilizing Figure (IV-13) and equation (II-19) it is also legitimate practice to employ a large excess of the second enzyme, which is possible in the present study, and to employ the analysis procedure described by Easterby (1973): both approaches have been shown to be entirely consistent. The Easterby procedure has the advantage as shown in Figure (IV-13) that less reliance is placed on concentration estimations of final product at very early times, where, in certain assays, as in that under discussion, experimental scatter of data values is likely to be the greatest. Moreover it is a suitable method for the qualitative detection of interaction (or lack of it) between the enzymes, in the presence of reaction mixture ligands. It does suffer from the

disadvantage that if an interaction is detected, or product inhibition effects are large (footnote †), the results are not readily interpretable. With the evidence available from the preceding Section, and with the knowledge that the time dependent loss of arginase activity observed in the absence of excess $MnCl_2$ would be relatively minimal in a system containing relatively large concentrations of arginase, it was decided that the Easterby method would be the method of choice for the present study.

---

† The major complicating factor in this respect would be an inhibition or activation of arginase by argininosuccinate, which as the initial substrate is present in relatively high concentrations even at early times. Control experiments showed that such an inhibition does not occur. Similarly no inhibition effects were observed on argininosuccinase by ornithine or urea or on arginase by fumarate. The remaining complications are then the reversible nature of the first reaction and the competitive inhibition of arginase by ornithine (Figures IV - 5 and IV - 6). It has been necessary in the interpretation of the results by the Easterby method, to assume that neither of these complications markedly affects urea production within the relatively short time period of its measurement.

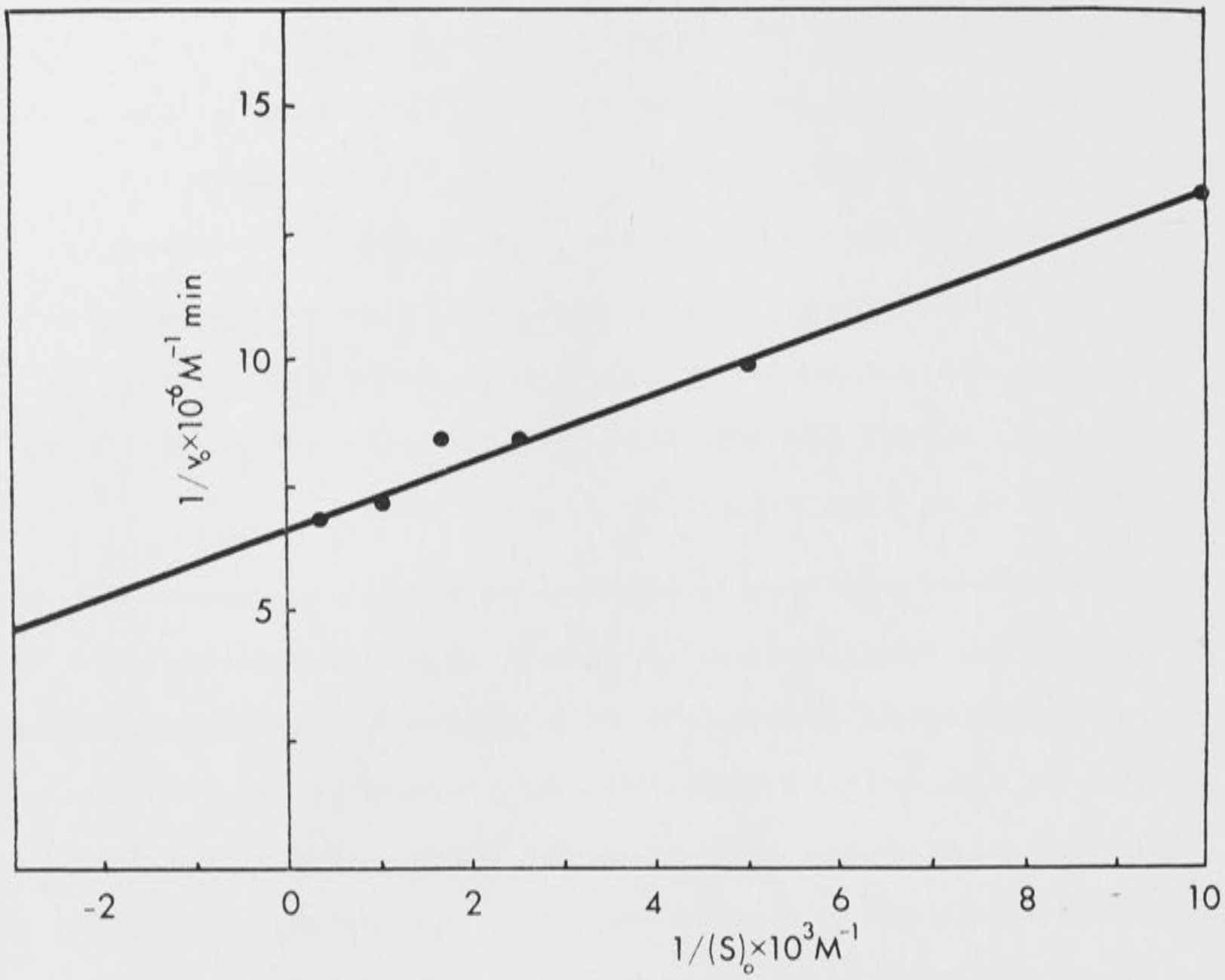


FIGURE V - 8

Double reciprocal plot of data obtained from the coupled reaction catalyzed by argininosuccinase and arginase. The experiment was conducted in 0.05 M HCl-tris, 0.05 M NaCl pH 7.5 at 25°C with initial argininosuccinate concentrations in the range 0.1 to 3mM. The K_m and V_{max} for arginase were 5mM and 2.5×10^{-3} M min^{-1} respectively, and those calculated for argininosuccinase from this plot are given in Section (V-4b).

The initial velocity of depletion of argininosuccinate as defined by equation (II-19) was determined as a function of the initial substrate concentration and plotted in double reciprocal form as is shown in Figure (V-8). The values of K_m and V_{max} for argininosuccinase found from this plot, treated as linear, were $1.0 \pm 0.2 \times 10^{-4}$M and $1.3 \pm 0.2 \times 10^{-5}$M min^{-1} respectively. These values compare favourably with the corresponding values of $1.1 \pm 0.1 \times 10^{-4}$M and $1.2 \pm 0.1 \times 10^{-5}$M min^{-1} found with the same sample of argininosuccinase studied alone, over the concentration range of 0.1 to 3mM, a region in Figure (V-4) where the experimental points may be regarded as approximately linear. Two points emerge from this analysis. First, the results support the contention that arginase is not an effector of argininosuccinase activity, or in other words that no chemical complex formation occurs between the enzymes which alters their catalytic activity. Secondly, the combination of errors involved in interpreting a coupled assay prevents the detailed characterization of argininosuccinase as a non-Michaelis-Menten enzyme (Figure V-6). In this respect, this Section has served to emphasize the limitations of the coupled enzymic assay technique. It is however noted that from the viewpoint of kinetic measurements alone, use of the coupled assay results in demonstrating a lack of interaction is preferable to initial velocity studies in which the inhibitionof argininosuccinase by arginase (or vice versa) is monitored by the rate of production of fumarate (or of urea). Studies of this type indeed showed that inhibition (or activation) did not arise in either case, but only the coupled assay excluded the model where interaction occurs

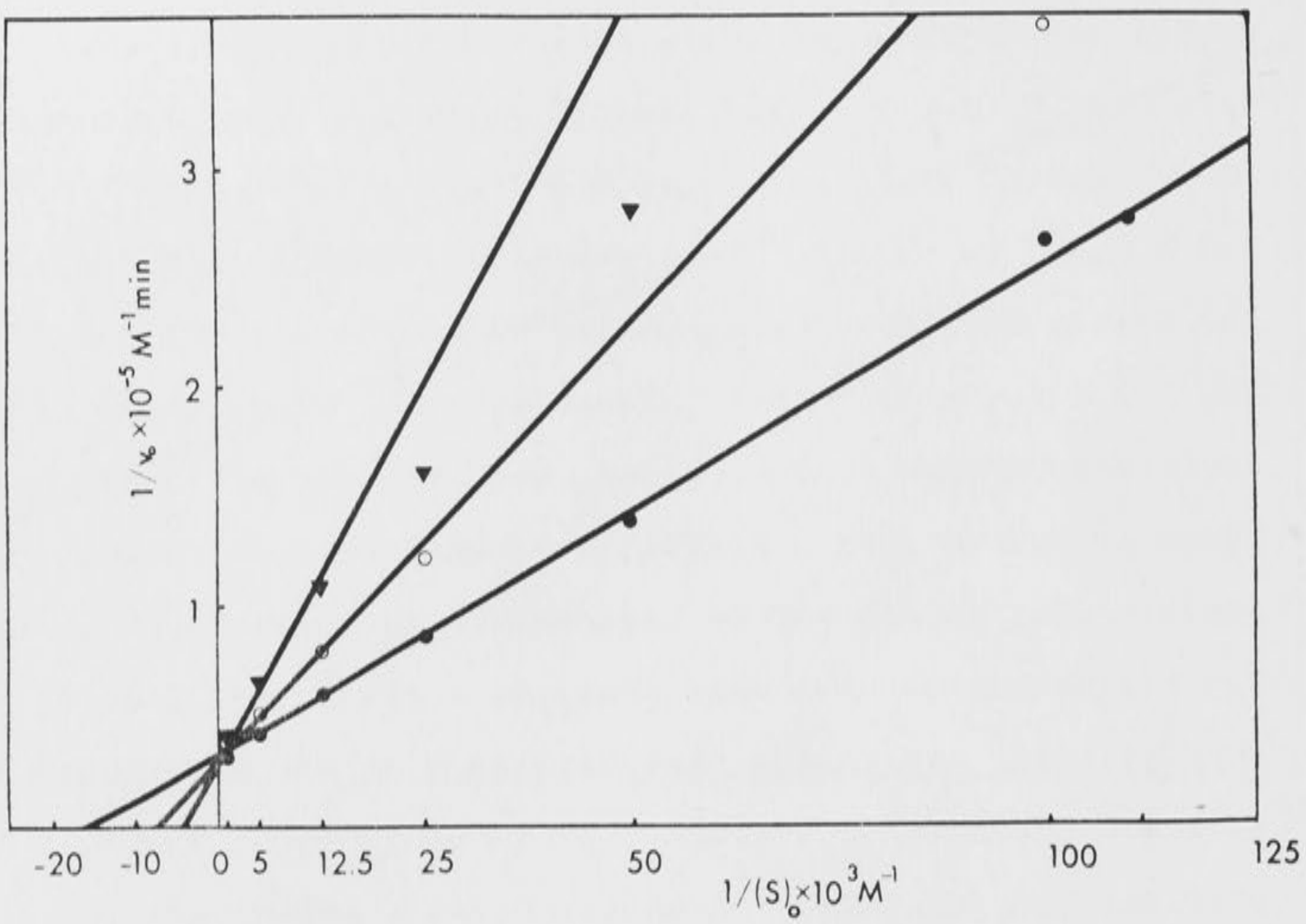


FIGURE V - 9

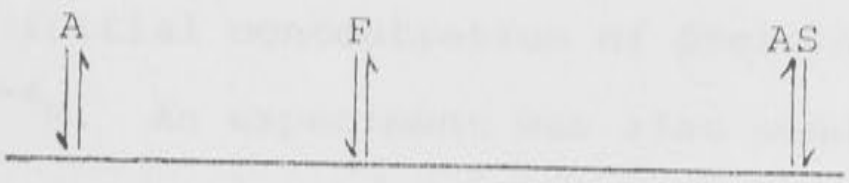
Double reciprocal plot of data from an initial velocity experiment on argininosuccinase with OmM (●), 2mM (O) and 4mM (▼) arginine present in the reaction medium. The assay protocol was as described in the text and the buffer employed was 0.05 M HCl-tris, 0.05 M NaCl pH 7.5 at 25°C.

resulting in a close juxtaposition of the active sites and a consequently elevated rate of final product formation due to a high concentration level of the intermediate compound (arginine) in a local environment.

5. Brief consideration of the reverse reaction catalyzed by argininosuccinase.

Since the reaction, fumarate + arginine $\rightleftharpoons$ argininosuccinate does not involve water and includes only those reactants given in the equation, it may reasonably be classified, in relation to the enzyme argininosuccinase, as a Bi Uni sequential mechanism. Moreover the available evidence suggests that it is an ordered sequential mechanism in which arginine binds to the enzyme prior to the binding of fumarate. Figure (V-9) shows that arginine is an inhibitor in reaction mixtures where argininosuccinate was used as an initial substrate and fumarate was monitored, in initial velocity studies. Since argininosuccinase is likely to be either an allosteric enzyme, or coexists in two isomeric states (Figure V-4), it is somewhat hazardous to treat the data in Figure (V-9) as linear with the indicated competitive inhibition constant 1.6×10^{-3}M. However, the more important point is established by Figure (V-9) that arginine binds to argininosuccinase in the absence of fumarate. It is not a simple matter to perform corresponding kinetic studies to investigate the possible inhibition by fumarate in the initial absence of arginine, since spectrophotometric measurements of fumarate concentrations in experiments of this design would require the subtraction of large blank absorbances. It is noted however, that fumarate in contrast

to arginine, does not prevent the dissociation of argininosuccinase into half-subunits at 4°C in tris or imidazole buffer (Ratner, 1972). It would thus appear that the basic mechanism for the reverse reaction is Bi Uni ordered sequential. Strictly this type of mechanism should be considered in relation to the enzyme which is itself allosteric or an equilibrium mixture of isomeric states; but such an analysis would be extremely difficult and would not seem to have been performed in the literature. In order to proceed therefore, it will be assumed that no cooperative effects (or their equivalent) are operative, which is an assumption, but not an unreasonable one in view of the relatively small deviations from linearity observed in Figure (V-6). The relevant kinetic scheme and its related rate equations becomes (Cleland, 1963a),



(V - 4a)

where A denotes arginine, F fumarate and AS argininosuccinate

$$v = \frac{V_f V_r \left([AS] - \frac{[F][A]}{K_{eq}} \right)}{K_{AS} V_r + V_r [AS] + \frac{K_A V_f [F]}{K_{eq}} + \frac{K_F V_f [A]}{K_{eq}} + \frac{V_f [F][A]}{K_{eq}} + \frac{V_r [AS][F]}{K_F^i}}$$

(V - 4b)

where v is the velocity of the reaction; K_{eq} is defined by equation (V-2b); V_f and K_{AS} are the maximal velocity and Michaelis constant found by employing argininosuccinate as initial substrate; V_r and K_A are the corresponding

parameters for studies in which F is in excess and A is varied as initial substrate; V_r and K_F are the corresponding parameters when A is held in excess; and K_F^i is the non-competitive inhibition constant for F on the reaction initiated with AS.

It is possible to numerically integrate equation (V-4b) when numerical values are assigned to the seven relevant constants. An example is shown by the solid line in Figure (V-10a) where the assigned values of the constants are reported in the caption. This plot refers to the increase of concentration of fumarate with time when argininosuccinate was employed as initial substrate, a situation where clearly at long times the reverse reaction will affect the results. The solid points in Figure (V-10a) are taken from a continuous trace of such an experiment where the initial concentration of argininosuccinate was 3.74×10^{-4}M. An experiment was also conducted in which fumarate and arginine, each of concentration 2×10^{-3}M were used as initial substrates, and the time dependent depletion of fumarate was measured by means of the continuous spectrophotometric trace, points from which are plotted in Figure (V-10b) . The solid line in this Figure was computed by numerical integration of equation (V-4b), utilizing the same values of the constants as reported for Figure (V-10a) except that V_f was adjusted to account for the different concentration of argininosuccinase employed. The consistent fit of the solid lines to the experimental points in both Figures (V-10a and b) lends some support to the view that the kinetic parameters reported are in realistic ranges. It is possible to comment on these values as follows :

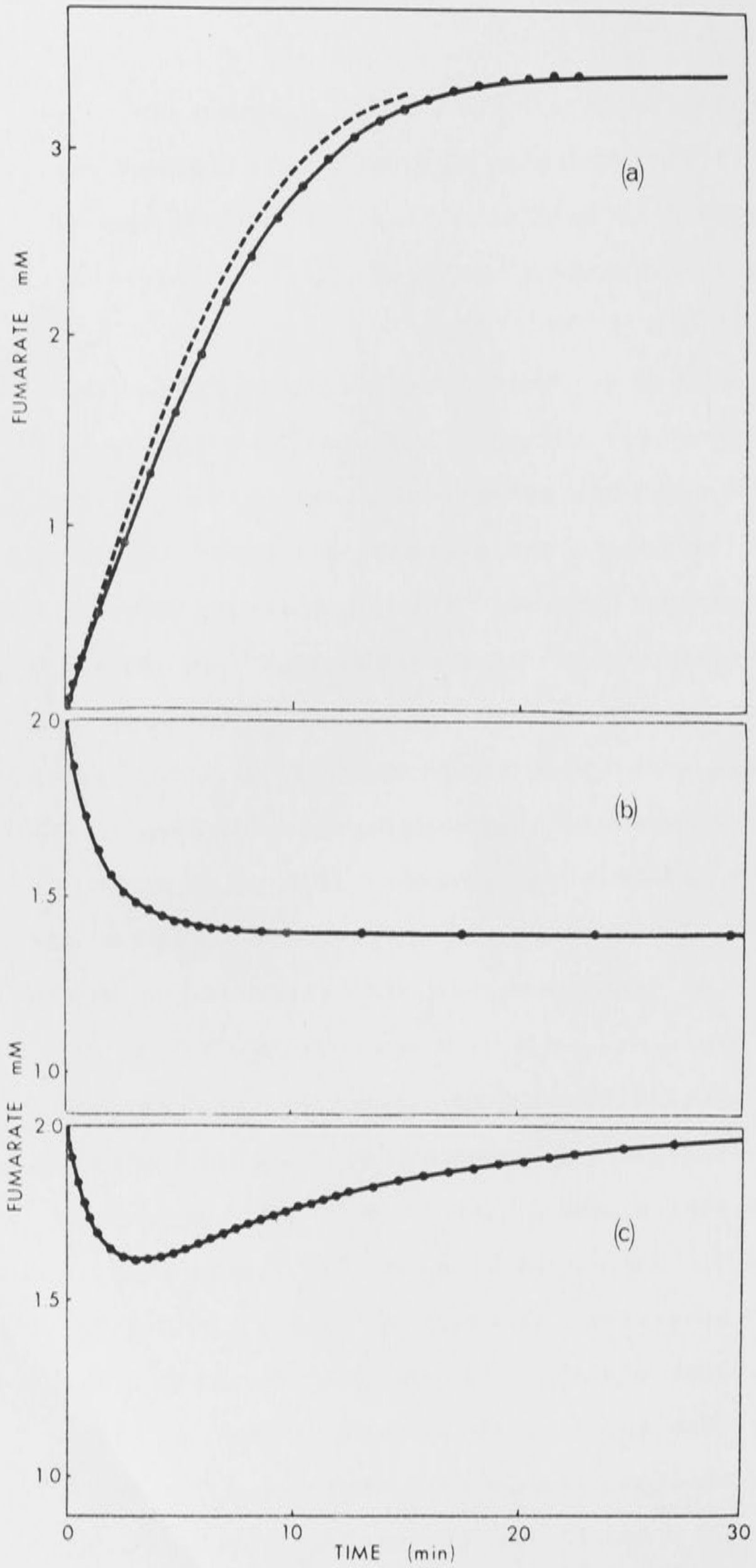

FUMARATE mM
3
2
1
(a)
20
1.5
10
(b)
FUMARATE mM
20
1.5
10
(c)
0
10
20
30
TIME (min)

FIGURE V - 10

Plots of fumarate concentration (•) as a function of time for the argininosuccinase catalyzed conversion of argininosuccinate, in 0.05 M HCl-tris, 0.05 M NaCl pH 7.5 at 25°C.

(a) The forward reaction. The initial argininosuccinate concentration was 0.37mM, and the solid line is that obtained by fitting the data (•, obtained from a continuous spectrophotometric trace) to equation (V-5b) using the following parameters; $V_f = V_r = 4.4 \times 10^{-4}$M min^{-1}; $K_{AS} = 5 \times 10^{-5}$M; $K_A = K_F = 1 \times 10^{-4}$M; $K_F^{i,AS} = 1 \times 10^{-3}$M. The broken line (---) is that obtained employing $V_f = V_r = 5 \times 10^{-4}$M min^{-1} and $K_{AS} = 8 \times 10^{-5}$M.

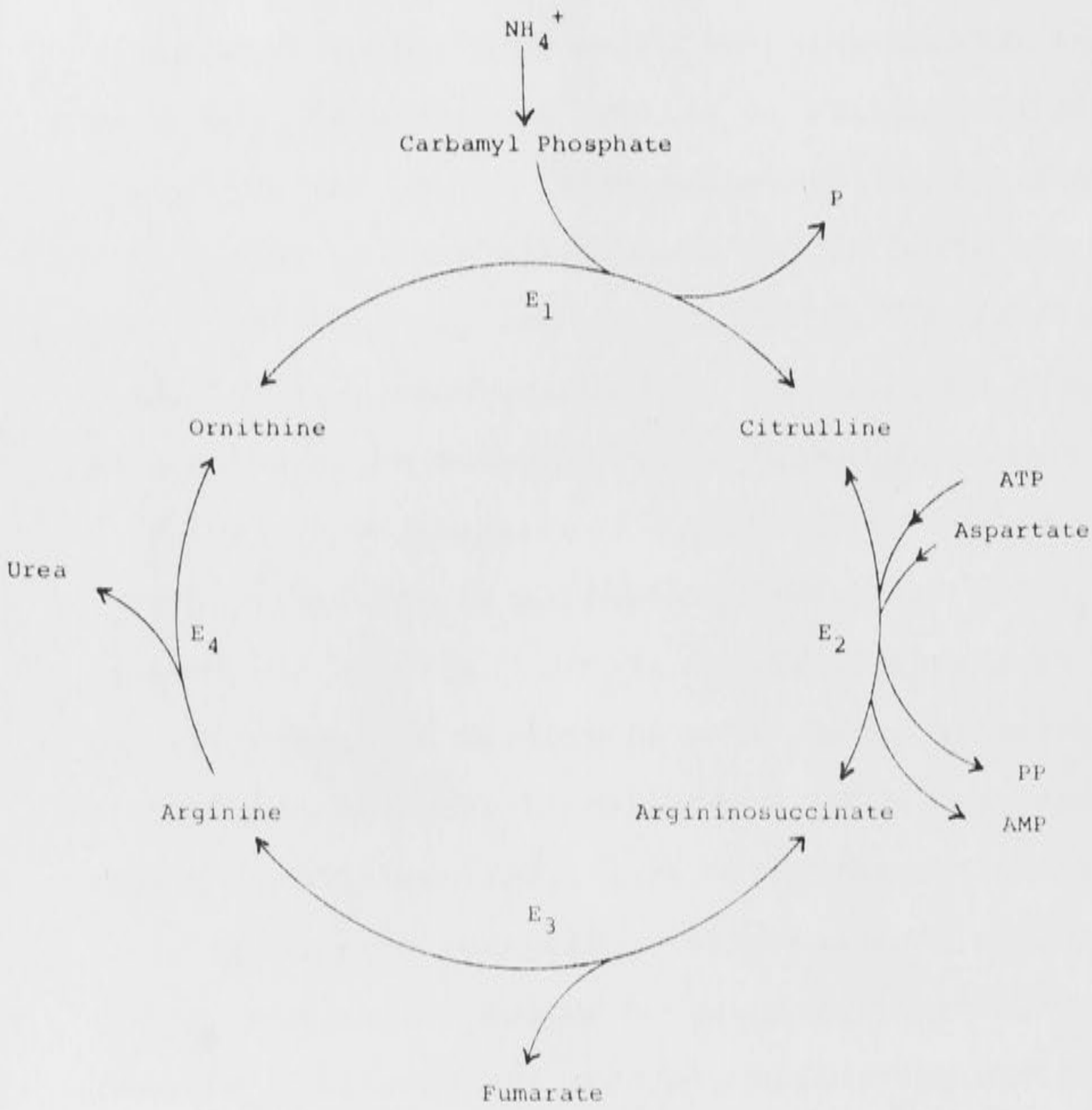
(b) The reverse reaction, with initial fumarate and arginire each of concentration 2mM. The data (•) was again fitted to equation (V-5b), using the same Michaelis and inhibition constants as for curve a, but employing $V_f = V_r = 5 \times 10^{-4}$M min^{-1}.

(c) The forward and reverse reactions. Data (•) from a coupled reaction employing argininosuccinase and arginase with initial arginine and fumarate concentrations of 2mM. These data were fitted (———) by numerical integration of equation (II-28) employing the argininosuccinase parameters employed in curve b, and the additional arginase parameters; $K_m = 5 \times 10^{-3}$M; $K_I = 3 \times 10^{-3}$M; $V_{max} = 1.5 \times 10^{-3}$M min^{-1}.

(1) the value of K_{eq} was that found in this and separate experiments and reported previously; (2) the value of K_{AS} is consistent with that obtainable from Figure (V-4) ignoring the deviations at low and high v_o (and $[S]_o$); (3) as shown by Cleland (1963a) $K_F = K_{eq}K_{AS}V_r/V_fK_A^i$ where K_A^i is the competitive inhibition constant of arginine with argininosuccinate as initial substrate and was given the value 1.6×10^{-3}M (Figure V-9); (4) K_A was set equal to K_F on the basis of the observation made by Ratner, Anslow and Petrack (1953); (5) V_f was set equal to V_r also on the basis of a finding of these authors. It follows that the numerical computations reported could proceed by assigning values to K_A^i (taken arbitrarily as 1×10^{-3}M) and of V_f, the latter being selected on the basis of the best fit to the experimental points. A third experiment was performed in which, to a mixture of argininosuccinase and arginase was added a solution of fumarate and arginine each of final concentration 2mM. The experimental points being shown in Figure (V-10c). The time dependence of fumarate concentration observed is due to the combined effects of the reversible reaction encompassed by equation (V-4b), and the depletion of arginine by arginase, described by equation (II-28a) which includes the competitive inhibition of the enzyme by ornithine (Figures IV-5 and IV-6). It is somewhat gratifying that the solid line computed by numerically integrating these combined equations, and utilizing the basic kinetic parameters previously reported does describe the form of the experimental results which exhibit a clear minimum.

FIGURE V - 11

The ornithine-urea cycle



E_1 = Ornithine transcarbamylase

E_2 = Argininosuccinate synthetase

E_3 = Argininosuccinase

E_4 = Arginase

It is apparent from the complexity of the problem that it would be erroneous to ascribe strong significance to the absolute magnitudes assessed for certain of the kinetic parameters, but one important point emerges quite clearly. It is that the overall rate of the reaction involving, initially, arginine and fumarate is much slower at non-saturating concentrations of these substrates than the rate of the reaction with argininosuccinate of comparable concentration, as initial substrate: this arises as a consequence of the ≈ 300-fold difference in the Michaelis constant rather than in the respective maximal velocities which are approximately equal.

6. The urea cycle.

Since considerable information is now available on the kinetics of the reactions catalyzed by arginase and argininosuccinase, studied separately and in couples, (Chapters III, IV and V), it seems appropriate to comment on their joint contribution to the urea cycle. The cycle in simplified form is summarized in Figure (V-11), where ammonium ions (via carbamyl phosphate), ATP and aspartate are external inputs to the cycle. The rate of change of concentration of these compounds with time is equal to the summed effects of fluxes within the cycle due to the operation of consecutive enzyme catalyzed reactions, and of fluxes from precursors, external to the cycle, which will be assumed to have constant pool concentration, as a consequence of the operation of contiguous pathways not shown. Further comment on this point and on the rates of efflux from the cycle to maintain a constant pool concentration of inorganic phosphate, pyrophosphate, AMP and fumarate will

TABLE V - 4

The symbols used for products and substrates of the urea cycle.

O = ornithine	PP = pyrophosphate
CP = carbamyl phosphate	AMP = adenosine monophosphate
C = citrulline	AS = argininosuccinate
ATP = adenosine triphosphate	F = fumarate
ASP = aspartate	A = arginine
P = inorganic phosphate	U = urea

be made later. For the present attention is directed to fluxes within the urea cycle which must be considered in relation to the mechanisms of the reactions catalyzed by E_1, E_2, E_3 and E_4. The summary of this aspect which follows is necessarily detailed, but it is rewarding in that it leads to the ability to simulate the cycle in kinetic terms (following procedures reviewed by Garfinkel, Garfinkel, Pring, Green and Chance, 1970), and to comment on the effects of certain inborn errors of metabolism in relation to known clinical syndromes.

(a) Individual reaction mechanisms and associated rate constants

Each mechanism of the four urea cycle enzymes will be considered in turn, the arrangement being in increasing order of complexity, and the Sections on arginase and argininosuccinase will serve to summarize much of the kinetic information obtained in this work. Table (V-4) lists the symbolism used for the various substrates and products. It will be shown for each enzyme that it is possible to write an equation describing the time dependent depletion of a substrate on the basis that all enzyme-substrate complexes are in a steady-state: these equations are written in terms of steady-state kinetic parameters which may be evaluated, in principle, by simple experimental procedures not involving flow or relaxation methods. Such equations have the additional advantage that they assume the same form regardless of certain detail in the mechanism of the reaction. For example the equation for a mechanism of the irreversible Michaelis-Menten type is the same regardless of whether a kinetically controlled

interconversion of the enzyme-substrate complex is considered or not, provided the final breakdown step is rate determining. It is possible to utilize such steady-state rate equations in a numerical integration procedure designed to simulate, with the aid of a computer, the operation of the urea cycle. However the validity of such a procedure may immediately be questioned on the grounds that a steady-state of the various enzyme-substrate complexes may not be attained: this is especially worrying because, as will be seen, the *in vivo* concentrations of the various enzymes in the urea cycle are likely to be quite large, as is true for other metabolic pathways (Vegotsky and Frieden, 1958). An alternative procedure for the simulation of the cycle would be to numerically integrate the detailed set of differential equations which describe the rate of change of concentration of all species with time including those of the enzyme-substrate complexes. This procedure offers the immediate advantage that no steady-state assumptions are made; but it suffers from the disadvantages that values must be assigned to each of the individual rate constants and that the computer-based integration would be considerably more time consuming. In the work to be presented both approaches are utilized and compared for the first time.

(i) ARGINASE (E_4). The irreversible production of urea and the competitive inhibition of arginase by ornithine may be represented by,

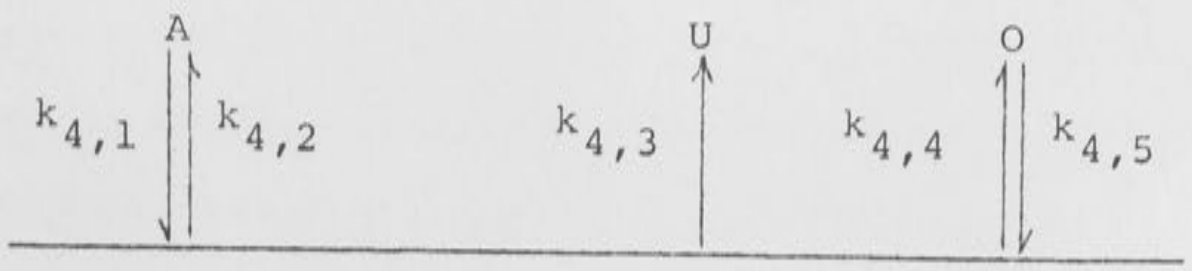


TABLE V - 5

Tabulation of the steady-state kinetic parameters and consistent sets of individual rate constants pertaining to the enzymes of the urea cycle.

ORNITHINE TRANSCARBAMYLASE, E_1

$(k_{cat})_f$	=	1.4×10^{3} sec^{-1}
K_{CP}	=	8.1×10^{-5} M
K_O	=	9.0×10^{-4} M
K_{eq}	=	1×10^{5}
$k_{1,1}$	=	1.7×10^{7} $M^{-1}sec^{-1}$
$k_{1,2}$	=	6.3×10 sec^{-1}
$k_{1,3}$	=	2.1×10^{6} $M^{-1}sec^{-1}$
$*k_{1,4}$	=	1×10^{3} sec^{-1}
$*k_{1,5}$	=	3×10^{3} sec^{-1}
$*k_{1,6}$	=	9×10^{4} $M^{-1}sec^{-1}$
$k_{1,7}$	=	2.6×10^{3} sec^{-1}
$*k_{1,8}$	=	5×10^{5} $M^{-1}sec^{-1}$

ARGININOSUCCINASE, E_3

$(k_{cat})_f$	=	70 sec^{-1} $=(k_{cat})_r$
K_{AS}	=	5×10^{-5} M
K_F	=	1×10^{-4} M
K_A	=	1×10^{-4} M
K_{eq}	=	3.2×10^{-3} M
$k_{3,1}$	=	2.7×10^{6} $M^{-1}sec^{-1}$
$k_{3,2}$	=	7×10 sec^{-1}
$k_{3,3}$	=	7.5×10 sec^{-1}
$k_{3,4}$	=	1.5×10^{6} $M^{-1}sec^{-1}$
$k_{3,5}$	=	1.1×10^{3} sec^{-1}
$k_{3,6}$	=	7×10^{5} $M^{-1}sec^{-1}$

ARGININOSUCCINATE SYNTHETASE, E_2

$(k_{cat})_f$	=	11.2 sec^{-1}
K_C	=	4.6×10^{-5} M
K_{ATP}	=	3.2×10^{-4} M
K_{ASP}	=	3.5×10^{-5} M
K_{eq}	=	8.9
$K_{AS}^{i,C}$	=	3×10^{-4} M
$K_{PP}^{i,C}$	=	1×10^{-4} M
$K_{AMP}^{i,ATP}$	=	3.5×10^{-4} M
$k_{2,1}$	=	2.4×10^{5} M^{-1} sec^{-1}
$k_{2,2}$	=	2.3 sec^{-1}
$k_{2,3}$	=	3.5×10^{5} $M^{-1}sec^{-1}$
$*k_{2,4}$	=	1×10 sec^{-1}
$k_{2,5}$	=	4.8×10^{5} $M^{-1}sec^{-1}$
$*k_{2,6}$	=	1×10 sec^{-1}
$k_{2,7}$	=	2.0×10 sec^{-1}
$k_{2,8}$	=	8.9×10^{5} $M^{-1}sec^{-1}$
$*k_{2,9}$	=	5×10 sec^{-1}
$k_{2,10}$	=	6.4×10^{5} $M^{-1}sec^{-1}$
$*k_{2,11}$	=	5×10 sec^{-1}
$k_{2,12}$	=	1.7×10^{5} $M^{-1}sec^{-1}$

ARGINASE, E_4

$(k_{cat})_f$	=	4.5×10^{3} sec^{-1}
K_A	=	5×10^{-3} M
$K_O^{i,A}$	=	3×10^{-3} M
$*k_{4,1}$	=	1×10^{7} $M^{-1}sec^{-1}$
$k_{4,2}$	=	5.4×10^{4} sec^{-1}
$k_{4,3}$	=	5.3×10^{3} sec^{-1}
$*k_{4,4}$	=	3×10^{4} sec^{-1}
$k_{4,5}$	=	1×10^{7} $M^{-1}sec^{-1}$

* denotes an assumed value

where the first numerical subscript for each rate constant refers to the enzyme E_4. The appropriate steady-state rate equation is

$$v_{E_4} = \frac{d[U]}{dt} = \frac{k_{4,1}k_{4,3}k_{4,4}\,[A][E_4]_o}{k_{4,4}(k_{4,2}+k_{4,3})+k_{4,5}(k_{4,2}+k_{4,3})[O]+k_{4,1}(k_{4,3}+k_{4,4})[A]} \qquad (V-5)$$

where the following identifications can be made,

$(k_{cat})_f = V_f/[E_4]_o = k_{4,3}k_{4,4}/(k_{4,3}+k_{4,4})$;

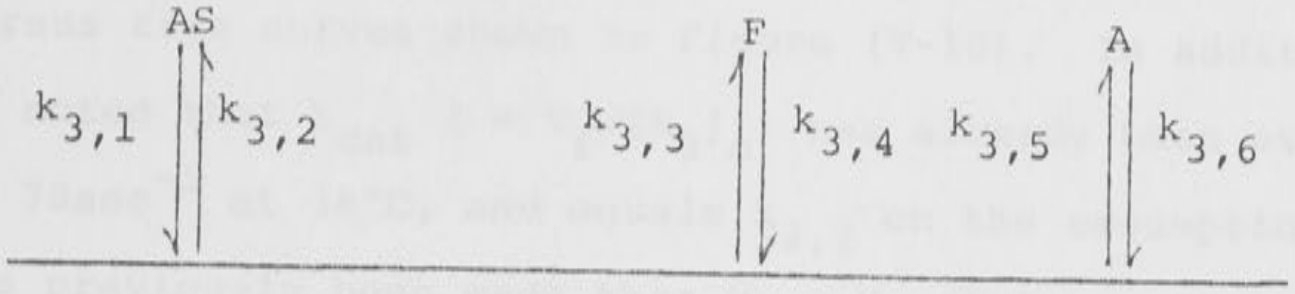
$K_A = k_{4,4}(k_{4,2}+k_{4,3})/k_{4,1}(k_{4,3}+k_{4,4})$; $\quad K_O^{i,A} = k_{4,4}/k_{4,5}$.

The symbol $K_\beta^{i,\alpha}$ denotes an inhibition constant when the compound α is used as the initial substrate (others being held in excess), and β the inhibitor added. The values of the steady-state kinetic parameters found for the arginase catalyzed reaction are summarized in Table (V-5) where the value of $(k_{cat})_f$ was that reported in Chapter IV corrected to 38°C using a Q_{10} of 2.5 : values of Michaelis and inhibition constants are assumed for the present purpose to be temperature independent.

The values of the individual rate constants also tabulated in Table (V-5) were found by assigning, in realistic ranges, values to $k_{4,1}$ and $k_{4,4}$ and utilizing equation (V-5) to evaluate the remainder. It is evident that these values may later be revised in the light of results obtained by pre-steady-state kinetic measurements; but it is emphasized in the present context that they form a consistent set with steady-state kinetic parameters and will therefore permit the required comparison of integration procedures based on the steady-state rate equations, and on rate equations containing the individual rate constants as

explicit parameters.

(ii) ARGININOSUCCINASE (E_3). The mechanism for the reversible reaction involving argininosuccinase will be taken as being described by equation (V-4b) rewritten in the present notation as,



In relation to experimental results where a steady-state of the complexes EAS and EA may be assumed, application of Cramer's Rule leads to,

$$v_{E_3} = \frac{d[F]}{dt} = \frac{\left(k_{3,1}k_{3,3}k_{3,5}[AS] - k_{3,2}k_{3,4}k_{3,6}[F][A]\right)[E_3]_o}{k_{3,5}(k_{3,2}+k_{3,3}) + [AS]k_{3,1}(k_{3,3}+k_{3,5}) + [F]k_{3,2}k_{3,4} + [A]k_{3,6}(k_{3,2}+k_{3,3}) + [F][A]k_{3,4}k_{3,6} + [AS][F]k_{3,1}k_{3,4}} \qquad (V-6a)$$

The coefficients of this expression may thus be rearranged to identify the following steady-state parameters defined in equation (V-4b)

$$(k_{cat})_f = V_f/[E_3]_o = k_{3,3}\,k_{3,5} / (k_{3,3} + k_{3,5})$$

$$(k_{cat})_r = V_r/[E_3]_o = k_{3,2}$$

$$K_{AS} = k_{3,5}(k_{3,3}+k_{3,2}) / k_{3,1}(k_{3,3}+k_{3,5})$$

$$K_F = (k_{3,3}+k_{3,2}) / k_{3,4} \qquad (V-6b)$$

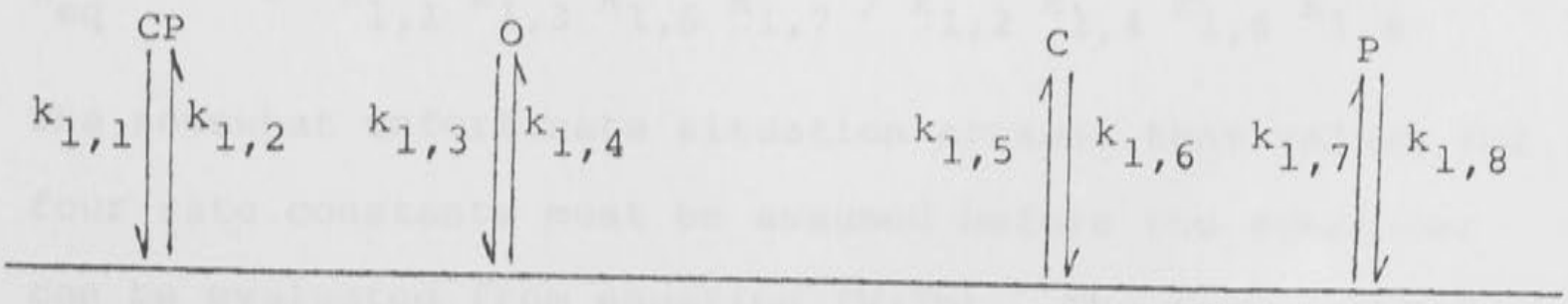
$$K_A = k_{3,2}/ k_{3,6}$$

$$K_{eq} = k_{3,1}k_{3,3}k_{3,5} / k_{3,2}k_{3,4}k_{3,6}$$

$$K_F^{i,AS} = (k_{3,3}+k_{3,5}) / k_{3,4} \qquad (V-6c)$$

The values of the steady-state kinetic parameters stipulated in equation (V-6b) are reported in Table (V-5) and are those summarized in the caption of Figure (V-10): they sufficed to describe both the data from initial velocity studies using argininosuccinate, and the three product versus time curves shown in Figure (V-10). In addition it is noted that k_{cat} ($= V_f/[E_3]_o$) has already been evaluated as $70sec^{-1}$ at 38°C, and equals $k_{3,2}$ on the assumption which has previously been made that $V_f = V_r$ (Ratner, Anslow and Petrack, 1953). With this information it is possible to solve the set of simultaneous equations (V-6b) to obtain the remaining individual rate constants which are also tabulated in Table (V-5). The value of $K_F^{i,AS}$ calculated using equation (V-6c), was 0.8×10^{-3}M which compares well with the value 1×10^{-3}M derived from the simulation shown in Figure (V-10): this lends some support to the contention that the simple Uni Bi mechanism proposed is followed, although it is stressed that the full detail of the argininosuccinase mechanism involving allosteric effects has been neglected for simplicity.

(iii) ORNITHINE TRANSCARBAMYLASE (E_1). The available evidence (Cohen and Marshall, 1962; Marshall and Cohen, 1972a, b and c; Ratner, 1973) suggests that the reversible reaction catalyzed by ornithine transcarbamylase is of the Bi Bi ordered sequential type represented by,



The corresponding initial velocity equation, assuming a steady-state of all intermediate complexes, is given by, (with $k_{1,i} = k_i$, i = 1, ..., 8),

$$v_{E_1} = \frac{\left| k_1k_3k_5k_7[CP][O] - k_2k_4k_6k_8[P][C] \right| [E_1]_o}{k_2k_7(k_4+k_5) + k_1k_7(k_4+k_5)[CP] + k_2k_8(k_4+k_5)[P]}$$

$$+ k_3k_5k_7[O] + k_2k_4k_6[C] + k_1k_3(k_5+k_7)[CP][O]$$

$$+ k_6k_8(k_2+k_4)[P][C] + k_1k_4k_6[CP][C] + k_1k_3k_6[CP][O][C]$$

$$+ k_3k_5k_8[O][P] + k_3k_6k_8[O][P][C] \qquad (V - 7a)$$

This equation has been presented by Cleland (1963a) and was verified, in order to check the absence of typographical errors, both by application of Cramer's Rule (involving the expansion of 4 x 4 determinants) and by application of the procedure described by King and Altman (1956). In this case there are only four steady-state kinetic parameters available from the literature on beef liver ornithine transcarbamylase and these are summarized in Table (V-5); the $(k_{cat})_f$ value refers to 38°C (Cohen and Marshall, 1962). It is possible to write expressions in terms of individual rate constants for the available steady-state kinetic parameters (Cleland, 1963a, b and c; Plowman, 1972) as follows,

$$\begin{aligned}
(k_{cat})_f &= k_{1,5}\,k_{1,7} / (k_{1,5} + k_{1,7}) \\
K_{CP} &= k_{1,5}\,k_{1,7} / k_{1,1}(k_{1,5} + k_{1,7}) \\
K_O &= k_{1,7}(k_{1,4} + k_{1,5}) / k_{1,3}(k_{1,5} + k_{1,7}) \\
K_{eq} &= k_{1,1}\,k_{1,3}\,k_{1,5}\,k_{1,7} / k_{1,2}\,k_{1,4}\,k_{1,6}\,k_{1,8}
\end{aligned} \qquad (V - 7b)$$

The somewhat unfortunate situation arises, that values for four rate constants must be assumed before the remainder can be evaluated from equation (V-7b). The four assumed values are indicated in Table (V-5) where also are reported

$$v_{E_2} = \frac{(k_1k_3k_5k_7k_9k_{11}[C][ATP][ASP] - k_2k_4k_6k_8k_{10}k_{12}[PP][AMP][AS])[E_2]_o}{}$$

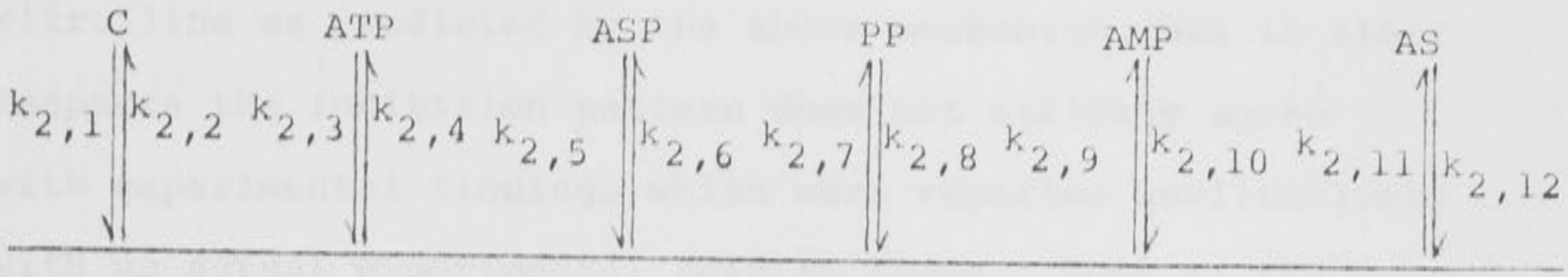
$$
\begin{aligned}
&k_2k_4k_9k_{11}(k_6 + k_7) \\
&+ k_1k_4k_9k_{11}(k_6 + k_7)[C] \\
&+ k_2k_5k_7k_9k_{11}[ASP] \\
&+ k_1k_3k_9k_{11}(k_6 + k_7)[C][ATP] \\
&+ k_1k_5k_7k_9k_{11}[C][ASP] \\
&+ k_3k_5k_7k_9k_{11}[ATP][ASP] \\
&+ k_1k_3k_5(k_7k_9 + k_7k_{11} + k_9k_{11})[C][ATP][ASP] \\
&+ k_2k_4k_6k_8k_{11}[PP] \\
&+ k_2k_4k_9k_{12}(k_6 + k_7)[AS] \\
&+ k_2k_4k_6k_8k_{10}[PP][AMP] \\
&+ k_2k_4k_6k_8k_{12}[PP][AS] \\
&+ k_2k_4k_{10}k_{12}(k_6 + k_7)[AMP][AS] \\
&+ k_8k_{10}k_{12}(k_2k_4 + k_2k_6 + k_4k_6)[PP][AMP][AS]
\end{aligned}
$$

$$
\begin{aligned}
&+ k_1k_4k_6k_8k_{11}[C][PP] \\
&+ k_2k_5k_7k_9k_{12}[ASP][AS] \\
&+ k_1k_3k_6k_8k_{11}[C][ATP][PP] \\
&+ k_1k_4k_6k_8k_{10}[C][PP][AMP] \\
&+ k_3k_5k_9k_{12}[ATP][ASP][AS] \\
&+ k_2k_5k_7k_{10}k_{12}[ASP][AMP][AS] \\
&+ k_1k_3k_5k_8k_{11}[C][ATP][ASP][PP] \\
&+ k_1k_3k_5k_7k_{10}[C][ATP][ASP][AMP] \\
&+ k_1k_3k_6k_8k_{10}[C][ATP][PP][AMP] \\
&+ k_3k_5k_7k_{10}k_{12}[ATP][ASP][AMP][AS] \\
&+ k_3k_6k_8k_{10}k_{12}[ATP][PP][AMP][AS] \\
&+ k_2k_5k_8k_{10}k_{12}[ASP][PP][AMP][AS] \\
&+ k_1k_3k_5k_8k_{10}[C][ATP][ASP][PP][AMP] \\
&+ k_3k_5k_8k_{10}k_{12}[ATP][ASP][PP][AMP][AS]
\end{aligned}
$$

(V - 8a)

the corresponding values of the remaining rate constants consistent with the steady-state kinetic parameters.

(iv) ARGININOSUCCINATE SYNTHETASE (E_2). The mechanism of the reaction catalyzed by this enzyme is not as yet completely defined although, from kinetic and isotope exchange studies (Rochovansky and Ratner, 1967), Ratner (1973) in her review was able to comment "that the three substrates must be bound to the enzyme as a quaternary complex before any product is released". The three substrates mentioned refer to citrulline, ATP and aspartate which add to the enzyme in that order. A possible mechanism is therefore ordered Ter Ter represented by,



The point of uncertainty is the order of release of PP, AMP and AS, but some information can be gained from the inhibition pattern predicted by the rate equation describing this mechanism. The appropriate equation for the steady-state of all intermediate complexes has been derived by Cleland (1963a, b and c) and was checked in this work for possible typographical errors, by the application of the method of King and Altman (1956) ... it is given on the adjacent page, with the simplification that $k_{2,i} = k_i$, $i = 1, \ldots, 12$. From this equation it may be predicted that PP will be an anti-competitive inhibitor for C in the presence of excess ATP and ASP; an anti-competitive inhibitor for ATP in the presence of excess C and ASP; and a mixed inhibitor for ASP in the presence of excess C and

ATP. Rochovansky and Ratner (1967) report that PP is a non-competitive inhibitor in all three sets of experiments under discussion, which in the terminology of certain kineticists, is taken to encompass anti-competitive and mixed inhibition. Indeed inspection of their experimental results indicate that the inhibition pattern suggested for the ordered Ter Ter mechanism, with initial release of PP, is appropriate. For example, double reciprocal plots for experiments in which citrulline was the variable substrate, and PP the inhibitor show parallel straight lines in accordance with an anti-competitive interpretation. It was also observed that AS was a competitive inhibitor for citrulline as predicted by the above mechanism, but in other respects the inhibition pattern does not strictly agree with experimental findings which were reported qualitatively with no actual experimental results shown. Some of the uncertainty may arise from recent observations (Ratner, 1973) that the enzyme may in fact be allosteric in that certain negative cooperativity effects are observed; although from the apparent linearity of double reciprocal plots presented it would appear that these effects are minor. Accordingly, while additional kinetic data are evidently desirable to support the proposed mechanism, it was decided to proceed assuming it to be correct. Values for the steady-state parameters, including $(k_{cat})_f$ which was measured at 37°C, are reported in Table (V-5) and were obtained from the work of Rochovansky and Ratner (1967) on the beef liver enzyme. Expressions for these in terms of individual rate constants stipulated in equation (V-8a) are as follows,

$$(k_{cat})_f = k_{2,7}k_{2,9}k_{2,11}/(k_{2,9}k_{2,11}+k_{2,7}k_{2,9}+k_{2,7}k_{2,11})$$

$$K_C = k_{2,7}k_{2,9}k_{2,11}/k_{2,1}(k_{2,9}k_{2,11}+k_{2,7}k_{2,9}+k_{2,7}k_{2,11})$$

$$K_{ATP} = k_{2,7}k_{2,9}k_{2,11}/k_{2,3}(k_{2,9}k_{2,11}+k_{2,7}k_{2,9}+k_{2,7}k_{2,11})$$

$$K_{ASP} = k_{2,9}k_{2,11}(k_{2,6}+k_{2,7})/k_{2,5}(k_{2,9}k_{2,11}+k_{2,7}k_{2,9}+k_{2,7}k_{2,11})$$

$$K_{eq} = k_{2,1}k_{2,3}k_{2,5}k_{2,7}k_{2,9}k_{2,11}/k_{2,2}k_{2,4}k_{2,6}k_{2,8}k_{2,10}k_{2,12}$$

$$K_{AS}^{i,C} = k_{2,11}/k_{2,12}$$

$$K_{PP}^{i,C} = (k_{2,9}k_{2,11}+k_{2,7}k_{2,9}+k_{2,7}k_{2,11})/k_{2,8}k_{2,11}$$

$$K_{AMP}^{i,ATP} = (k_{2,9}k_{2,11}+k_{2,7}k_{2,9}+k_{2,7}k_{2,11})/k_{2,7}k_{2,10}$$

(V - 8b)

All of these parameters refer to initial velocity studies of the forward reaction where, as we have seen, the order of addition of substrates is known. Their solution in terms of the individual rate constants, reported in Table (V-5) requires the specification of the four rate constants indicated. The values assigned to these four rate constants are not entirely arbitrary as may be seen from the following two considerations. First, a random selection of the four parameters leads in some cases to negative values of the other rate constants, or to unacceptably low values: this imposes a reasonable constraint on the range of values of the set which may be taken. Secondly, expressions for the Michaelis constants of the reverse reaction K_{PP} and K_{AS} may also be formulated from equation (V-8a) and used with the listed individual rate constants to yield the respective calculated values 5×10^{-4}M and 1×10^{-5}M. These values may be compared with those of 1×10^{-4}M and 3×10^{-5}M reported by Rochovansky and Ratner (1967) for beef liver argininosuccinate synthetase: the agreement, at least with respect to the order of magnitude, is reassuring with regard

TABLE V - 6

The intracellular molar concentrations of the enzymes of the urea cycle

ENZYME	LOCATION IN THE CELL	TOTAL ACTIVITY PER kg OF LIVER (μMole min^{-1} of substrate converted)	MAXIMUM SPECIFIC ACTIVITY (units mg^{-1})	TOTAL ENZYME (mg) PER kg of LIVER	ENZYME MOLECULAR WEIGHT	MOLAR CONCENTRATION OF ENZYME
Ornithine trans-carbamylase (E_1)	mitochondria	1.1×10^5	780	1.4×10^2	108,000	2.6×10^{-6}
argininosuccinate synthetase (E_2)	cytoplasm	1.4×10^3	4	3.5×10^2	175,000	4.0×10^{-6}
argininosuccinase (E_3)	cytoplasm	5.0×10^3	23	2.2×10^2	203,000	2.2×10^{-6}
arginase (E_4)	cytoplasm	9.2×10^5	1800	5.1×10^2	114,000	8.9×10^{-6}

to the adoption of the individual rate constants reported, in describing the essential features of the reaction.

(b) Enzyme concentration levels and external fluxes

Table (V-6) summarizes available information pertaining to the concentration levels of the urea cycle enzymes in human liver. The total activities reported in column three are the averages of the values for adults found by Brown and Cohen (1960), Kekomäki, Räihä and Peerheentupa (1967), Morrow, Barness and Efron (1967), Hommes, Degroot, Wilmink and Jonxis (1969), Nuzum and Snodgrass (1971) and Levin (1971), and closely resemble corresponding values reported for the enzymes of beef liver (Cohen and Brown, 1960; Hirsch-Kolb, Heine, Kolb and Greenberg, 1970; Nuzum and Snodgrass, 1971). Column four cites the maximum specific activity of the enzymes reported in Chapters IV and V and those cited by Ratner (1973). These values and those of molecular weight summarized in column five refer to the beef liver enzymes, as do the data in Table (V-5), since insufficient information of this type is available for the human enzymes*. The last column of Table (V-6) provides estimates of the required concentrations of the enzymes, based on the estimate that one kg of liver contains 500g of cytoplasmic water: it is known that one kg of both human and beef liver have a total water content of approximately 700g (Long, 1961). It is quite obvious that the reported enzyme concentration levels, which are some one thousand-fold greater than those employed in enzymic assays, may only be considered as approximate guides for several reasons. These include the following: (1) as reported in column one compartmentalization of at least ornithine transcarbamylase

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* The reader may quite reasonably question the validity of utilizing kinetic and molecular weight parameters, gathered consistently from studies on enzymes extracted from beef liver, for simulations which attempt to comment on human syndromes. It is not possible to justify completely this approach and certainly the need for information on the human enzymes (and the difficulties in obtaining them) need hardly be stressed. It is pertinent however to make two reassuring observations. First, as has been noted above, the maximum activities of each enzyme of the urea cycle per kg of beef liver correspond closely to those in a human liver. Secondly, in the few instances where species comparisons have been made, the steady-state kinetic parameters of urea-cycle enzymes from different sources has been found to approximate each other in magnitude. An example is provided by Table I of Rochovansky and Ratner (1967) where the parameters for argininosuccinate synthetase from beef and pig are compared.

has been suggested (Levin, 1971; Ratner, 1973); (2) the maximum specific activities refer to pH 7.5 and 38°C where as local fluctuations of pH and ionic strength, particularly near membranes (Katchalski, Silman and Goldman, 1971) may well modify the corresponding intracellular values; and (3) the combination of data from human and beef sources is tenuous but has been made since it is desired to correlate the results of computer simulation with known human clinical syndromes. The information summarized in Tables (V-5) and (V-6) completes the set necessary for the consideration of fluxes of compounds within the urea cycle, and thus it remains to consider external fluxes.

It has previously been noted that the rate of change of concentration of X(X = CP, ATP, ASP, P, PP, AMP and F) is due to summed effects which may be represented by,

$$d[X]/dt = (d[X]/dt)_{cycle} + (d[X]/dt)_{external} \qquad (V - 9)$$

The first term denotes the flux within the cycle, which may be represented by rate equations involving the parameters stipulated in Tables (V-5) and (V-6). The second term, in the case of the input compounds will be taken to equal $k_X^*[Y]_{pool}$ where Y is an external precursor of constant pool concentration; for example, for CP, Y = NH_4^+. It is implicit that $(d[X]/dt)_{external}$ is a constant and it will be set equal to the constant steady-state production of urea in the normal liver, which in turn equals the rate of influx of CP, and preserves the 1:1 stoicheiometry of fluxes of ATP and aspartate.

The liver of an "average" 70kg man weighs approximately 1.4 ± 0.2 kg (Davies and Davies, 1961) and

such a man, in nitrogen balance, on a normal protein diet of approximately 80g per day excretes the corresponding nitrogen content (16.5% w/w) in the form of 0.4 moles of urea per day. In terms of the unit of 1kg of liver used in Table (V-6) this means that $d[U]/dt = 6.6 \times 10^{-6}$ M sec^{-1} in 500ml of fluid space.

In the case of the output compounds (X = P, PP, AMP and F), $(d[X]/dt)_{external}$ in equation (V-9) will be taken to equal $-k_X^*[X]$ where [X] is the concentration at time t of the compound generated by the cycle. In these cases it is therefore necessary to assign a value of k_X^* for each X. This may be done on the basis that when d[X]/dt = o, $[X] = [X]_{pool}$ and $(d[X]/dt)_{cycle} = -(d[X]/dt)_{external} = k_X^*[X]_{pool}$. If again $(d[X]/dt)_{external}$ is taken to equal d[U]/dt which is reasonable on stoicheiometric grounds, in the steady-state region, then k_X^* may be directly calculated from values of $[X]_{pool}$. In principle these values may be measured experimentally from liver homogenates, but in practice this is difficult. It is also unwise to infer the concentration of cytoplasmic compounds from known plasma concentrations; for example the plasma concentration of total free phosphate may be estimated to be 1mM (Long, 1961), but this provides an unreliable guide to the intracellular concentration. In order to proceed a value of 1×10^{-4} M will be assumed for each $[X]_{pool}$, corresponding to a k_X^* value of $6.6 \times 10^{-2} sec^{-1}$ for each output compound.

(c) The procedures for the numerical computation

As noted earlier it is desired to compare the results of numerical integration of two different but consistent sets of differential rate equations. Method I is based on the set involving all individual rate constants and which does not assume a steady-state of enzyme substrate complexes: Method II is based on the four steady-state rate equations, (equations V-5, V-6a, V-7a and V-8a).

METHOD I. The salient features of the set of differential rate equations which must be written for the application of this method may be illustrated by writing the subset of equations pertaining to the first enzyme catalyzed reaction

$$d[CP]/dt = k_{1,2}[E_1CP] - k_{1,1}[E_1][CP] + k^*_{CP}\,[NH_4^+]_{pool} \qquad (V - 10a)$$

$$d[O]/dt = k_{1,4}[E_1CPO] - k_{1,3}[E_1CP][O] + k_{4,4}[E_4O] - k_{4,5}[E_4][O] \qquad (V - 10b)$$

$$d[E_1CP]/dt = k_{1,1}[E_1][CP] - k_{1,2}[E_1CP] + k_{1,4}[E_1CPO] - k_{1,3}[E_1CP][O] \qquad (V - 10c)$$

$$d[E_1CPO]/dt = k_{1,3}[E_1CP][O] - k_{1,4}[E_1CPO] - k_{1,5}[E_1CPO] + k_{1,6}[E_1P][C] \qquad (V - 10d)$$

$$d[E_1P]/dt = k_{1,5}[E_1CPO] - k_{1,6}[E_1P][C] - k_{1,7}[E_1P] + k_{1,8}[E_1][P] \qquad (V - 10e)$$

$$d[C]/dt = k_{1,5}[E_1CPO] - k_{1,6}[E_1P][C] - k_{2,1}[E_2][C] + k_{2,2}[E_2C] \qquad (V - 10f)$$

$$d[P]/dt = k_{1,7}[E_1P] - k_{1,8}[E_1][P] - k^*_P[P] \qquad (V - 10g)$$

$$[E_1]_o = [E_1] + [E_1CP] + [E_1CPO] + [E_1P] \qquad (V - 10h)$$

The first equation illustrates equation (V-9) for an input compound, and equation (V-10g) provides illustration for an output compound. Equations (V-10b and f) illustrate a

linkage between consecutive reactions within the cycle, the former equation specifying the closure of the cycle via ornithine. Equations (V-10d and e) pertain to intermediate enzyme complexes which are not *a priori* taken to be in a steady-state. The way in which the enzyme concentrations are employed is illustrated by the final equation (V-10h) which expresses conservation of mass for E_1. There are 30 such differential equations forming the complete set which therefore accounts for the inhibition of arginase by ornithine and all reversible reactions, but for no other type of inhibition or activation due to or separate from enzyme-enzyme interactions.

Before numerical integration of this set of non-linear differential equations may commence it is necessary to specify boundary and initial conditions for t = o. In this regard it is convenient to set the concentrations of all relevant compounds equal to zero at t = o, except for the concentrations of the enzymes, which are given the values reported in Table (V-6), and for ornithine. The intracellular steady-state concentration value of ornithine is in the range 1×10^{-4}M (Cohen and Marshall, 1962) to 3×10^{-4}M (Krebs, Hems and Lund, 1973) and accordingly an initial value of 4.5×10^{-4}M was selected: it will be shown that this results in a steady-state concentration of ornithine in the range cited.

It would of course be possible to assign non-zero concentrations to other compounds as initial conditions, but this would merely alter the time course of attainment of the steady-state on which final comment will be made. The computer programme used for the numerical integration

incorporated the subroutine of Gear (1969) which automatically selects the step size, for what amounts to a variable order predictor-corrector method. The subroutine enables the rapid solution of a set of "stiff" simultaneous differential equations.

METHOD II. This method employed the steady-state rate equations, (equations V-5, V-6a, V-7a and V-8a) and equation (V-9) describing external fluxes to form the following appropriate set,

$$d[C]/dt = + v_{E_1} - v_{E_2} \quad ; \quad d[AS]/dt = + v_{E_2} - v_{E_3}$$

$$d[A]/dt = + v_{E_3} - v_{E_4} \quad ; \quad d[O]/dt = + v_{E_4} - v_{E_1}$$

$$d[U]/dt = + v_{E_4} \quad ; \quad d[CP]/dt = k^*_{CP}[NH_4^+]_{pool} - v_{E_1}$$

$$d[ATP]/dt = k^*_{ATP}[Y]_{pool} - v_{E_2} \; ; \; d[ASP]/dt = k^*_{ASP}[Y]_{pool} - v_{E_2}$$

$$d[PP]/dt = - k^*_{PP}[PP] + v_{E_2} \; ; \; d[AMP]/dt = - k^*_{AMP}[AMP] + v_{E_2}$$

$$d[F]/dt = - k^*_{F}[F] + v_{E_3} \; ; \; d[P]/dt = - k^*_{P}[P] + v_{E_1}$$

(V - 11)

There are twelve equations in this set, in comparison with 30 in the analogous set involving individual rate constants. As a consequence the numerical integration procedure may be performed with a simpler predictor-corrector programme (McCracken and Dorn, 1964) without recourse to a Gear (1969) subroutine, as these equations are "non-stiff". The required boundary and initial conditions were as for Method I. The coefficients in the expressions for v_{E_1}, v_{E_2}, v_{E_3}, and v_{E_4} were determined from the reported steady-state rate equations using the values of the individual rate constants

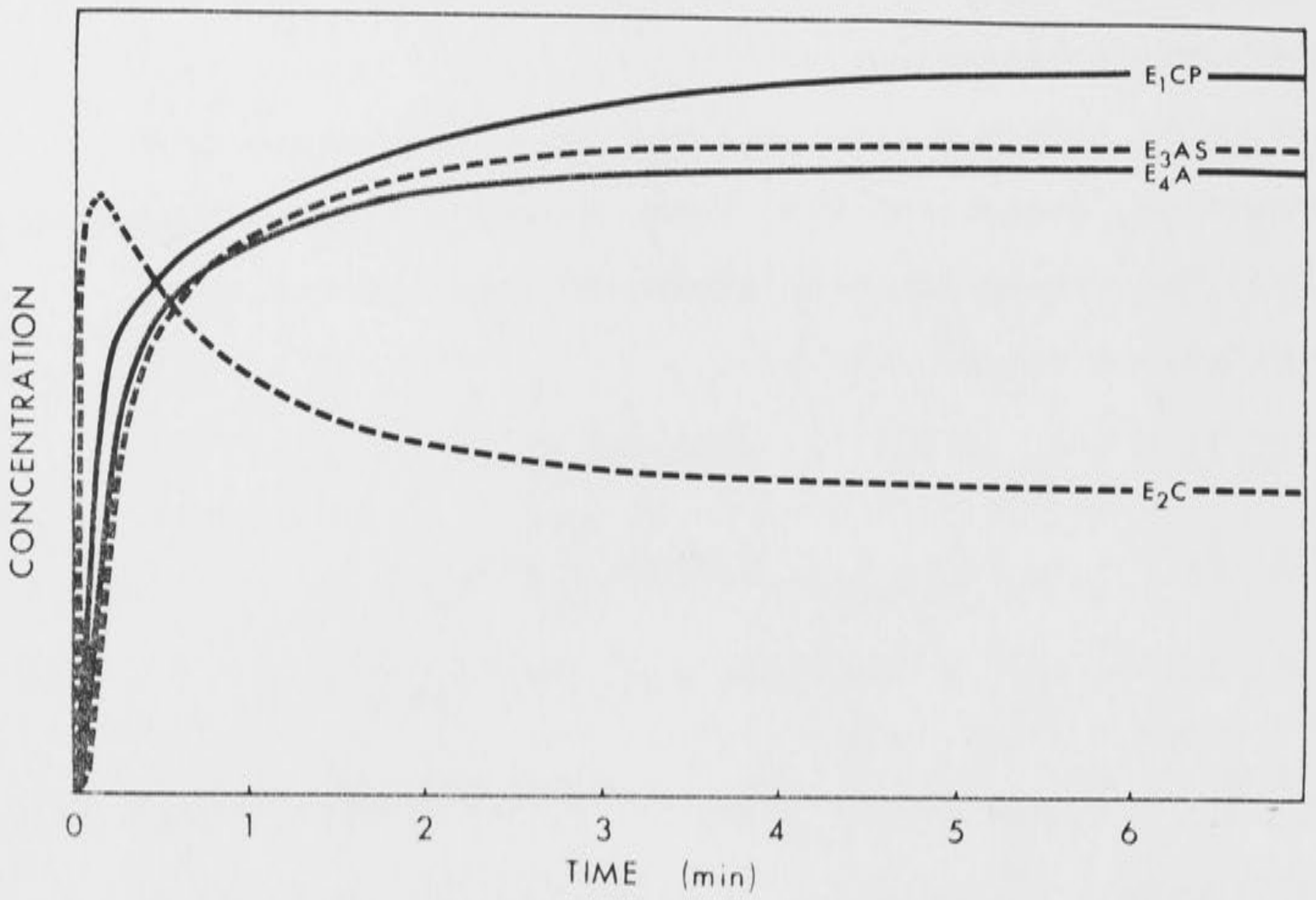


FIGURE V - 12

Plots of the concentration of particular enzyme-substrate complexes during the approach to the steady-state, in a computer simulation of the urea cycle. The enzyme-substrate complexes and their steady-state concentrations at 6 min are; $E_1CP = 1.68 \times 10^{-8}M$; $E_2C = 1.41 \times 10^{-6}M$; $E_3AS = 1.01 \times 10^{-7}M$; $E_4A = 1.22 \times 10^{-9}M$.

given in Table (V-5). It is stressed in this regard that this procedure is entirely equivalent to utilizing the experimentally determinable steady-state kinetic parameters, without knowledge of the individual rate constants.

(d) Results of the comparative analysis

Only Method I is capable of providing results pertaining to the concentration of the various enzyme-substrate complexes as a function of time, and in a strict sense, of describing instantaneous fluctuations of the system upon the application of a constraint such as the introduction of an inhibitor. Figure (V-12) shows the time dependent production of enzyme-substrate complexes for four arbitrarily selected one to one complexes, involving each of the enzymes. It is seen that a steady-state of these complexes is attained after a period of approximately 6 min, the result being entirely typical of other enzyme complexes in the system (results not shown for reasons of brevity).

This finding has two interesting implications. First, it obviates the concern expressed earlier that such steady-states might not be attained when enzyme concentration levels are relatively high. Secondly, it suggests that the application of Method II would be entirely valid provided results from it, obtained after 6 min, were considered. In fact the findings summarized in Table (V-7) show that this is not only a valid conclusion, but also (and somewhat surprisingly) Method II provides reliable concentration estimates at time periods as short as 10 sec. The situation parallels that discussed in Chapter II when the concentration of an intermediate compound S_2 in a

TABLE V - 7

A comparison of the time dependent production of compounds in the urea cycle as obtained by Methods I and II

Compounds within the cycle

t	O		C		AS		A	
(sec)	I	II	I	II	I	II	I	II
10	3.96×10^{-4}	3.90×10^{-4}	4.81×10^{-5}	5.08×10^{-5}	9.31×10^{-7}	9.51×10^{-7}	3.90×10^{-7}	3.96×10^{-7}
360	2.58×10^{-4}	2.61×10^{-4}	1.84×10^{-4}	1.85×10^{-4}	2.40×10^{-6}	2.43×10^{-6}	8.82×10^{-7}	8.93×10^{-7}
600	2.56×10^{-4}	2.60×10^{-4}	1.86×10^{-4}	1.87×10^{-4}	2.44×10^{-6}	2.44×10^{-6}	9.04×10^{-7}	8.95×10^{-7}

Input compounds

t	CP		ATP		ASP	
(sec)	I	II	I	II	I	II
10	1.67×10^{-7}	1.66×10^{-7}	5.10×10^{-5}	5.11×10^{-5}	5.12×10^{-5}	5.11×10^{-5}
360	1.78×10^{-7}	1.78×10^{-7}	1.86×10^{-4}	1.85×10^{-4}	1.86×10^{-4}	1.85×10^{-4}
600	1.78×10^{-7}	1.78×10^{-7}	1.87×10^{-4}	1.87×10^{-4}	1.87×10^{-4}	1.87×10^{-4}

Output compounds

t	P		PP		AMP		F		U		d[U]/dt	
(sec)	I	II	I	II	I	II	I	II	I	II	I	II
10	4.70×10^{-5}	4.74×10^{-5}	1.08×10^{-5}	1.23×10^{-5}	1.08×10^{-5}	1.23×10^{-5}	9.97×10^{-5}	1.16×10^{-5}	1.2×10^{-5}	1.38×10^{-5}		
360	1×10^{-4}	1×10^{-4}	9.96×10^{-5}	9.96×10^{-5}	9.96×10^{-5}	9.96×10^{-5}	9.97×10^{-5}	9.96×10^{-5}	2.20×10^{-3}	2.18×10^{-3}	6.5×10^{-6}	6.5×10^{-6}
600	1×10^{-4}	1×10^{-4}	1×10^{-4}	1×10^{-4}	1×10^{-4}	1×10^{-4}	1.00×10^{-4}	1×10^{-4}	3.77×10^{-3}	3.77×10^{-3}	6.6×10^{-6}	6.6×10^{-6}

coupled assay, attains in a relatively short time, a sufficiently high concentration to ensure a steady-state of the complex E_2S_2. The second conclusion which may be drawn from Table (V-7) is that both methods show that a steady-state of compounds other than enzyme-substrate complexes (i.e. compounds within the cycle, output compounds and input compounds) have also attained a steady-state concentration after the elapse of approximately 6 min.

It is pertinent to comment briefly on these steady-state concentration levels, which in view of the parameters used to obtain them may be considered to approximate those levels which might be found in normal liver. The following estimates are available from the literature, C = 10^{-5}-10^{-4}M; A = 0 - 10^{-5}M; O = 10^{-4}M, and it can be seen at least in terms of this restricted comparison that the simulation is realistic.

In summary of this Section, it may be concluded that the simpler Method II may be utilized with confidence in simulating various abnormalities which are encountered in the urea cycle. It is noteworthy however that this conclusion could not have been made without the evidence obtained by the application of Method I. The purpose of repeating the simulation of the cycle with certain selected changes of the parameters is two-fold. First, it is hoped that the simulations will provide a rational basis for explaining the concentration levels of certain intermediary compounds which are already known to typify particular inborn errors of metabolism and are used as diagnostic tests. Secondly, it is hoped that the simulations might provide results which extend beyond the explanation of

TABLE V - 8

Computer simulation pertaining to metabolic defects of the urea cycle.

Row	Parameter Varied	Defect	CP	O	C	AS	A	d[U]/dt*
1	$[E_4]_o$ to 3% normal	Hyperarginaemia	1.02	0.88	1.01	1.44	32.8	0.98
2	$[O]_o$ increased 444x	Experiment of Bach et. al (1944)	0.86	770	1.001	1.84	0.62	0.98
3	$[E_1]_o$ to 3% normal	Hyperammonaemia Type II	44.6	1.03	0.96	1.00	1.00	1.00
4	$k_{2,1}$ to 3% normal	Citrullinaemia	3090↑	0.01	2.37	0.89	0.83	0.89
5	$k_{2,1}$ to 20% normal	Citrullinaemia (mild)	1.06	0.72	1.37	0.98	0.97	0.98
6	$[E_3]_o$ to 3% normal	Arginino-succinicaciduria	17000↑	0.004	0.17	170	0.54	0.60
7	$k_{3,1}$ to 3% normal	Arginino-succinicaciduria	1.13	0.54	1.20	33.2	0.97	1.00
8	k^*_{CP} to 20% normal	Hyperammonaemia Type 1	0.18	1.75	0.01	0.18	0.21	0.20
9	$\frac{k^*_{CP}}{[E_1]_o}$ to 20% normal	see text	0.92	1.72	0.78	0.18	0.21	0.10

*Symbols are as for Table (V-4). Concentrations are expressed as ratios to those given in Table (V-7). The ratios for P, PP, AMP, F, ATP and ASP, although available are not cited since in the absence of information on contiguous pathways discussion on the concentration levels of these compounds is hazardous.

known phenomena into the area of prediction.

(e) Inborn errors of metabolism and related phenomena

(i) ARGINASE. The first example of the use of computer simulation in the explanatory area will be the consideration of the effect of lowering the arginase concentration $[E_4]_o$ to 3% of the value previously employed. The result is reported in the first row of Table (V-8) in relation to the concentrations of the key metabolic intermediates found at a time (40 min) when the steady-state of all enzyme-substrate complexes pertained, and indeed had been evident from much earlier times: the concentrations are expressed as ratios of the values found to those reported in Table (V-7). The salient features of these results are, [CP] is marginally elevated, [A] is increased 33-fold and the urea output is virtually unaffected. It has been observed in patients suffering from Hyperarginaemia that indeed the arginine concentration is abnormally high in both blood and cerebro-spinal fluid (CSF), that production of urea is normal and the concentration of the other urea cycle intermediates is normal (Shih and Efron, 1972), as reported in Table (V-8). Patients with this inborn error of metabolism suffer from epilepsy, spastic diplegia and mental retardation (Shih and Efron, 1972). It is generally believed that these conditions are the direct result of elevated ammonia levels (hyperammonaemia) and this could be tentatively associated with the observed slight increase of [CP], an effect which could be increased further on protein ingestion. The observation that the patients lived for relatively long periods before diagnosis, e.g. 5 yr, (Terheggen, Schwenk, Lowenthal, Van Sande and

these compounds is hazardous.

Colombo, 1969), is in accord with the hypothesis that the hyperammonaemia is not chronically severe. It could also be noted that the same metabolic effects could arise by increasing the K_m of arginase rather than decreasing its concentration. Thus the absolute steady-state concentration of arginine even in this pathological condition (3×10^{-5}M) is well below the normal K_m of arginase, implying that its rate of depletion is linear with respect to its concentration, the slope of the linear region being directly proportional to V_4/K_A.

The result is also pertinent to experimental studies which were conducted in an attempt to negate the conclusion formulated by Krebs that urea biosynthesis is effected solely by the ornithine-urea cycle. A typical paper in this controversy was that presented by Bach, Crook and Williamson (1944). These authors added 0.1M ornithine to liver slices and measured the rate of urea production. They used the finding that urea production was essentially unaffected as support for their negation; but it is clear that competitive inhibition of arginase by this concentration of ornithine (effectively increasing K_m 30-fold) does not in itself markedly alter d[U]/dt. Indeed the second row of Table (V-8) which pertains to a simulation with increased $[O]_o$ equal to 0.2M entirely supports this finding, and reflects the effect of increased ornithine concentration on steps both catalyzed by arginase and ornithine transcarbamylase, and hence the whole cycle.

Comment is now possible on a case of lysine intolerance reported by Colombo, Bürgi, Richterich and Rossi (1967) in which the patient suffered from marked hyperammonaemia on

the ingestion of lysine. The workers recalled that lysine is a competitive inhibitor for arginase and made the not unreasonable suggestion that this was the basis for the symptoms of the intolerance. It appears however, in view of the above discussion, that the explanation is untenable unless the patient synthesized a genetic variant of arginase which was inhibited much more strongly by lysine than normal.

(ii) ORNITHINE TRANSCARBAMYLASE. The third row of Table (V-8) refers to a simulation in which $[E_1]_o$ was decreased to 3% of its normal value: this reduced level is that indicated from activity measurements on biopsy and necropsy specimens of liver from sufferers of Hyperammonaemia Type II (Shih and Efron, 1972). All tabulated concentration levels are similar to those normally found except for a marked 45-fold increase of [CP]. The result is strikingly in accord with clinical-biochemical findings. First, sufferers of Hyperammonaemia Type II die at an early age (3 weeks) indicating that the hyperammonaemia corresponding to high [CP] is severe. Secondly the patients excrete reasonably large amounts of orotic acid, uridine and uracil as a consequence of the overactivity of the pyrimidine biosynthetic pathway, but normal amounts of urea. Thirdly, an abnormally high level of glutamine is found, possibly as a result of the amination of glutamic acid by glutamine synthetase in the brain. Fourthly, normal levels of ornithine are found in the plasma, which has puzzled some authors (Levin, 1971; Shih and Efron, 1972), but is predicted by the simulation. It is for this reason that the complaint has been termed

Hyperammonaemia Type II which is sensible terminology because ornithine does not accumulate even though the enzyme catalyzing its removal is deficient.

(iii) ARGININOSUCCINATE SYNTHETASE. The fourth row in Table (V-8) was computed by reducing the value of $k_{2,1}$ from $2.4 \times 10^{5} M^{-1} sec^{-1}$ reported in Table (V-5) to $8 \times 10^{3} M^{-1} sec^{-1}$. This has the effect of increasing K_C in accordance with the observation that the Michaelis constant of the enzyme obtained from fibroblast cultures of a sufferer of Citrullinaemia was increased 25-fold (Tedesco and Mellman, 1967). A salient observation from these calculations is that the citrulline concentration is elevated as is found in patients suffering from this lesion, although in practice the level in the blood is very high due to poor renal clearance (high tubular reabsorption) of this amino acid (Levin, 1971). The urea output is reduced in both theoretical and practical observations (Morrow, Burness and Efron, 1967) and the tabulated high level of [CP] is again consistent with the knowledge that Citrullinaemia is associated with severe hyperammonaemia which manifests itself in these cases by epilepsy, mental retardation and early death. It is apparent in this particular simulation that [CP] fails to attain a steady-state in the time taken for the simulations. It is also noted however that many of the intermediate metabolites are already in a steady-state. These effects arise because of the severely rate limiting nature of this enzyme especially when defective. A simulation with $k_{2,1}$ reduced to 20% of its 'normal' value yields the data in row 5 of Table V-8.

It is interesting to note that [C] is elevated 1.4 times above normal, which is approximately half the elevation seen in the simulation of a more severe case of Citrullinaemia (row 4). This occurs despite a six-fold increase in $k_{2,1}$ in comparison with the severe case.

(iv) ARGININOSUCCINASE. In the case of arginase it was noted, that appropriate alteration of either its maximal velocity or of its Michaelis constant or both could give rise to the biochemical signs that characterize Hyperarginaemia. This however is not a safe generalization in relation to other enzymes in the urea cycle. The point was explored in relation to argininosuccinase with simulations in which, first, $[E_3]_o$ was lowered to 3% of its previous value (row 6 of Table V-8) and, secondly, K_{AS} was increased 33-fold by reducing $k_{3,1}$ (Table (V-8) row 7). In this connection it is of interest that Miller and McLean (1967) partially purified argininosuccinase from the liver of a patient who had suffered from Argininosuccinicaciduria, and found that the specific activity was 3% of normal, but it is by no means certain that this was a maximal specific activity. Comparison of the results in rows 6 and 7 shows that the simulation is capable of predicting quite different behaviour with respect to all intermediates and particularly with respect to [CP] which in the case of variation of $[E_3]_o$ accumulates markedly with time and does not approach a steady-state. Consider first the consequences which would follow if in a patient $[E_3]_o$ was indeed extremely low. It would be expected that the excess CP would result in stimulation of the pyrimidine biosynthetic pathway, and that products such as orotic acid would appear in the urine.

Secondly, extreme hyperammonaemia and early death would be likely. Thirdly, such a hypothetical patient would exhibit low levels of citrulline, high levels of argininosuccinate and reduced production of urea. Observation in real cases show that none of these predictions are fulfilled except for high argininosuccinate. In fact the observations are entirely consistent with resultsreported in row 7 simulated with increased K_{AS}. In particular [C] as well as [AS] are high (up to 2.5g of AS being excreted per day in the urine, Shih and Efron, 1972), urea production is normal and hyperammonaemia is not extreme. Thus Argininosuccinicaciduria, the first metabolic disorder of the urea cycle to be described (Allan, Cusworth, Dent and Wilson, 1958) has been diagnosed in several teenage patients (Shih and Efron, 1972).

This discussion provides an example of the power of coupling computer simulated results with clinical-biochemical observation in predicting the likely detailed cause of reduced enzymic activity. In the case of Arginino-succinicaciduria it appears to be the result of an increased Michaelis constant, and the hypothesis finds support in enzyme induction studies performed on rats (Schimke, 1973). The possibility is of course not excluded, that a patient may be encountered with a low argininosuccinase concentration level. In this case the computer simulations may aid diagnosis in that they suggest analysis of the urine for orotic acid *and* argininosuccinate: both levels would be high in contrast to the simulation encountered with Hyperammonaemia Type II where only the former would be elevated.

(v) CARBAMYL PHOSPHATE SYNTHETASE. Row 8 of Table (V-8) was obtained by reducing k^*_{CP} 5-fold in order to mimic a defect in mitochondrial - bound NH_3-dependent carbamyl phosphate synthetase and to comment briefly on the lesion termed Hyperammonaemia Type I. The clinically observed decrease in the rate of urea synthesis is illustrated by the results which also suggest that examination of the ornithine level may also aid diagnosis, a suggestion hitherto unexplored. It is of interest that a case has been reported (Levin, 1971; Shih and Efron, 1972) where hyperammonaemia was associated with hyperornithinaemia. An explanation of the relation was given in terms of inhibition of arginase by ornithine which we have seen earlier to be untenable in view of the results in row 2 of Table (V-8). The more likely explanation is that the patient suffered from Hyperammonaemia Type I and this stresses the relevance of the present prediction that ornithine levels should be explored in relation to this lesion. The nature of the present calculations which have not explicitly considered carbamyl phosphate synthetase prohibits further detailed comment except that it is noted, with a decreased CP input, that the NH_3 level must increase with the clinically observed result that patients suffer from hyperammonaemia and related effects.

It would of course be possible to simulate examples where combinations of various disorders were considered, by altering kinetic parameters of two or more enzymes. One example will suffice to illustrate this type of approach, and to emphasize that it may, in some instances be difficult, from the pattern of intermediate metabolite concentrations,

to identify uniquely the disorder. The final row of Table (V-8) was obtained by reducing k^*_{CP} and $[E_1]_o$ to 20% of their normal values. It is clear from the results presented therein that the metabolite pattern is qualitatively similar to that in the preceding row except that [CP] and [C] are somewhat higher. In both cases however the patient would suffer from hyperammonaemia and decreased urea output. It is of interest to speculate that reduced k^*_{CP} and $[E_1]_o$ might reasonably arise as one consequence of CCl_4 poisoning since both carbamyl phosphate synthetase and ornithine transcarbamylase are known to be membrane bound.

7. Discussion

A major finding in this Chapter is that numerical integration methods find considerable use in the understanding of kinetically controlled fluxes within the sequence of reactions which comprise the urea cycle. It is clear that the simulations have proceeded on the basis of several assumptions, and since these have been specified in detail in the text it suffices to comment at this point on one which has particular relevance in the present context. This concerns the neglect in the simulations of control effects which may operate at the molecular level as a consequence of allosteric or self-interaction properties of particular enzymes or as a result of heterogeneous associations between different enzymes (Chapter IV). Two specific examples of this type of complication are as follows: (1) Figure (V-4) shows that the mechanism of the forward reaction catalyzed by argininosuccinase is not strictly Michaelis-Menten as assumed and (2) while there is

definite evidence that argininosuccinase and arginase do not interact, recent studies on arginase and ornithine transcarbamylase obtained from yeast (Messenguy, Penninckx and Wiame, 1971) indicate that the possibility of heterogeneous association reactions between mammalian enzymes should be explored. It would be possible to consider such effects in writing the basic set of differential rate equations in terms of individual rate constants but the task of evaluating these constants in (for example) the case of a multi-site allosteric enzyme catalyzing a reversible reaction seems prohibitively difficult at this time. It is therefore particularly relevant that the work in the present Chapter has shown, at least for the urea cycle, that the use of steady-state rate equations provides a satisfactory alternative in obtaining solutions in terms of intermediary metabolite patterns. The potential exists therefore for the use of phenomenological rate equations such as equation (V-4) which contain constants available from relatively simple *in vitro* kinetic studies. It was considered however that the use of equation (V-3) would be premature until more information is available on the reverse reaction and on the control potential of ornithine transcarbamylase and argininsuccinate synthetase.

In conclusion it may be fairly said that while the present numerical simulations pertaining to the urea cycle are in some senses preliminary the results are definitely encouraging in that the gross metabolite concentration patterns observed clinically in patients suffering from particular inborn errors of metabolism have been reasonably reproduced. In addition it has been possible to comment on

the relative interplay between enzyme concentration levels, and altered Michaelis constants in dictating overall fluxes. The search for ways of describing the dynamic operation of metabolic pathways will no doubt continue in the future along the lines of both generalization (e.g. Kacser and Burns, 1973; Reich and Sel'kov, 1974) and by the utilization of detailed rate equations formulated for particular enzymes. It is hoped that the present work which was aimed at a coalescence of *in vitro* kinetic results, including those obtained with coupled systems, and numerical integration methods, has contributed to this broad, complicated and fascinating problem.

CHAPTER VI

MATERIALS AND METHODS

TABLE VI - 1

Summary of argininosuccinase purification

STEP	VOLUME	MASS OF PROTEIN	SPECIFIC ACTIVITY RUmg^{-1} AVE.	SPECIFIC ACTIVITY RUmg^{-1} MAX.	TOTAL ACTIVITY (RU)	YIELD (% OF ORIGINAL U
1. EXTRACTION a. Homogenate of 3kg of calf liver	9ℓ	3kg liver	-	-	-	-
1. EXTRACTION b. Supernatant	5.7ℓ	-	-	-	325,000[a]	100
2. FIRST $(NH_4)_2SO_4$ FRACTIONATION	-	-	-	-	-	-
3. SECOND $(NH_4)_2SO_4$ FRACTIONATION	-	-	-	-	-	-
4. ISOELECTRIC PRECIPITATION AND HEAT	25ml	-	-	-	290,000	90
5. CM52 CHROMATOGRAPHY	170ml	1500mg	180	250	260,000	80
6. DE52 CHROMATOGRAPHY	315ml	210mg	560	1000	120,000	37
7. CRYSTALLIZATION	20ml	65mg	1200	1400	78,000	24

[a] This value is likely to be artifically low because of the presence of fumarase in the liver supernatant at this stage of the preparation.

1. Materials.

(a) Proteins

Commercial samples of arginase (bovine lyophilized powder 22 U mg^{-1} lot no. 80C-0910, Sigma) and urease (jack bean highly purified type VII powder, 110,000 Sumner units g^{-1}. batch no. 101C-5040, Sigma) were obtained and used without further purification. The commercial arginase sample, brown in colour, was only employed in early studies to establish assay procedures : all physico-chemical characterizations of the enzyme reported in Chapters III, IV and V were conducted with highly purified arginase prepared as described in detail in Chapter III.

Argininosuccinase was prepared from young beef livers by the procedure devised by Ratner (1970) which is summarized in Table (VI-1). The first four steps including the initial extraction in 0.02 M phosphate pH 7.0 proceeded as previously described, the first minor modification of the Ratner procedure occurring in Step 5. In this Step 25ml of protein solution, previously dialyzed for 7 h to remove excess ammonium sulphate, was loaded onto a CM-cellulose (Whatman CM52 microgranular preswollen) column (5.6 x 35 cm), pre-equilibrated at room temperature with 0.005 M phosphate buffer pH 6.15. The column was eluted at a flow rate of 100ml h^{-1} with the same phosphate buffer, the eluant being monitored continuously at 280nm. The 15ml fractions collected were also assayed for enzymic activity after the addition of 1.5ml of 1M phosphate buffer pH 7.5 to each fraction. The first 150ml of emerging protein solution proved to be associated with the highest relative enzymic activity and was cooled to 4°C prior to the

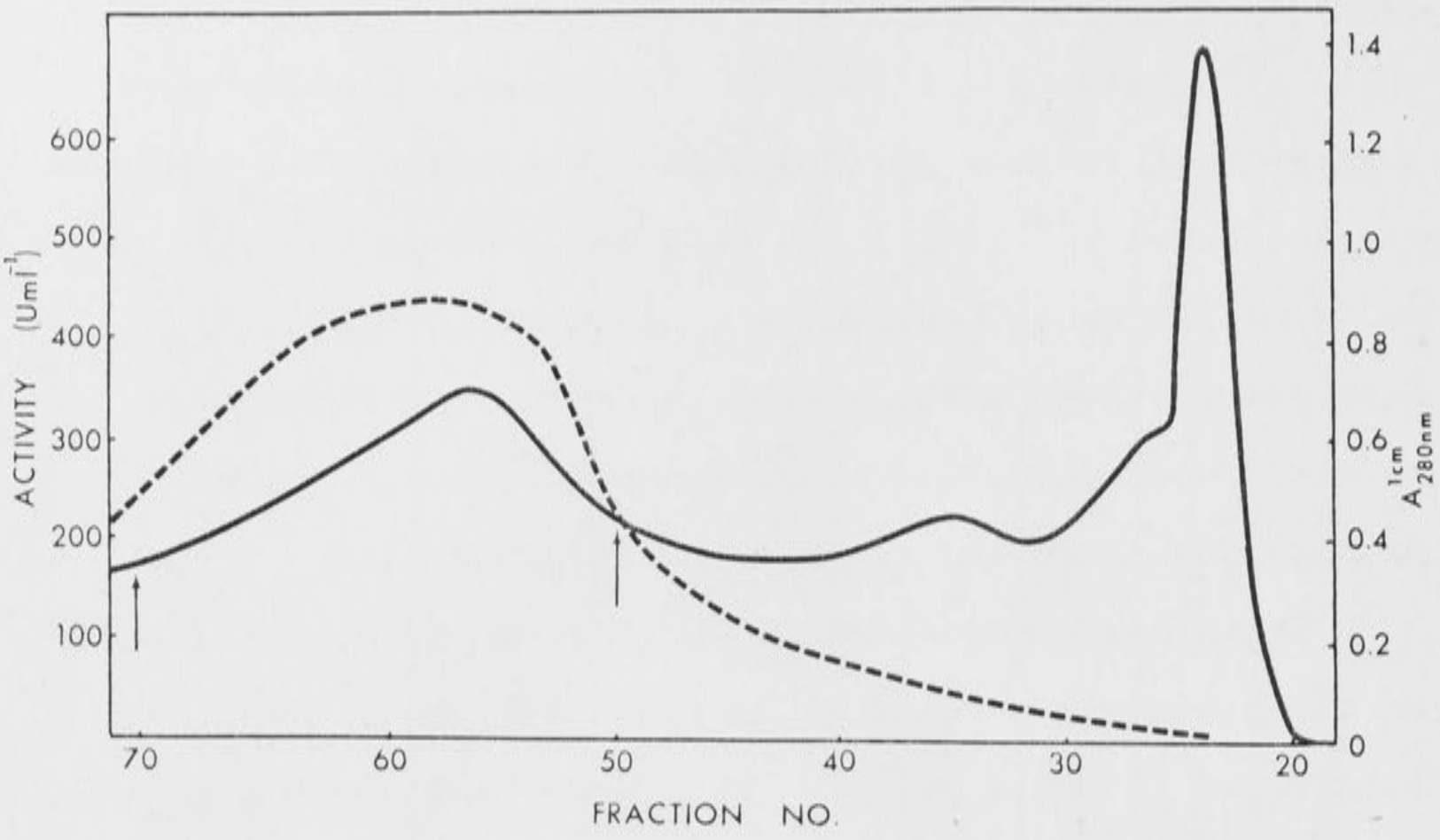


FIGURE VI - 1

Elution profile of beef liver argininosuccinase from a column of Whatman DE52 (4 x 29cm) at room temperature. The eluting buffer was 0.04 M phosphate pH 5.5 and the flow rate was 250ml h^{-1}. The solid line refers to absorbance at 280nm, and the dotted line to argininosuccinase enzymic activity. 15ml fractions were collected.

addition of 38g of $(NH_4)_2SO_4$ which precipitated the enzyme. The pellet obtained by centrifugation was resuspended in 10ml of 0.1 M phosphate buffer pH 7.5. In Step 6, after dialysis, this solution was applied to a DEAE-cellulose (Whatman DE52 microgranular, preswollen) column (4 x 29 cm) pre-equilibrated at room temperature with 0.01 M phosphate buffer pH 6.6. Elution proceeded at a constant flow rate of 250ml h^{-1} in three stages : (1) 530ml of the same buffer was applied under pressure to give colourless fractions high in protein content but possessing no argininosuccinase activity; (2) 640ml of 0.01 M phosphate buffer pH 6.1 was applied under pressure, and even though no protein was eluted at this stage resolution of the original dark brown band into two zones was observed on the column; and (3) the column was finally eluted with 0.04 M phosphate buffer pH 5.5 to give the elution profile shown in Figure (VI-1). The individual fractions were pooled to give a final volume of 285ml to which 30ml of 1M phosphate buffer pH 7.5 was added, prior to the final $(NH_4)_2SO_4$ fractionation conducted at 4°C by the addition of 25g, per 100ml, of the salt. The resulting solid was redissolved in 0.1 M phosphate buffer pH 7.5 to give a final protein concentration of greater than 10mg ml^{-1} suitable for the application of the crystallization procedure employing $(NH_4)_2SO_4$ described by Ratner (1970).

The crystals obtained are shown in Figure (V-1) and when dissolved exhibited a specific activity of 1,400 RU mg^{-1}, the concentration being estimated spectrophotometrically by employing an $E^{1cm,1mg\ ml^{-1}}_{280nm}$ of 1.298 (Lusty and Ratner,

1972). The protein was stored in crystalline form or in concentrated solution (10mg ml^{-1}) at -12°C and was stable for several months.

(b) Substrates and inhibitors

The following compounds were obtained commercially in their highest grade of purity (AR); L-arginine (free base, Calbiochem) urea (Mallinkrodt); fumaric acid (British Drug Houses Ltd.); manganese chloride.$2H_2O$ (Mallinkrodt); and L-ornithine (free base, Calbiochem).

Argininosuccinate was prepared by incubating a solution of 0.125 M arginine and fumarate in 0.1 M phosphate buffer pH 7.5 with 80,000 RU of argininosuccinase at 30°C for 3.5 h. For this purpose argininosuccinase was prepared from pig kidney (Ratner, 1957), a source essentially free of arginase (and of fumarase after the heat step in the isolation procedure). Under the conditions cited an equilibrium position is attained in which ∿ 90% of the substrates are converted to argininosuccinate (Chapter V). The final product was extracted from the mixture by ether extraction and precipitation with $BaCl_2$ (Ratner, 1957). Its purity was confirmed by thin layer chromatography on silica gel in butanol, pyridine, water (1:1:1) (Smith, 1958). An R_f value of 18 was obtained, by staining with ninhydrin, a value greater than that obtained (13) with a control experiment conducted with arginine alone. It was also found that the argininosuccinate preparation gave negative responses to the Sakaguchi reaction (eliminating the possibility of an arginine contaminant) and on exposure to I_2 vapour (eliminating the possibility of substantial fumaric acid content). It is perhaps noteworthy that a

yield of 40g of argininosuccinate was obtained, in four days, of value $1,600. The barium salt of the substrate, which was stable if stored as a dry powder at -12°C, was converted, prior to use, to the potassium salt used in all assay experiments reported, by addition of 0.5 M K_2SO_4 (1 ml per 100mg of argininosuccinate) and removal of the precipitated barium sulphate by Millipore filtration.

(c) Buffers

Aqueous buffers with compositions reported in the text were prepared from glass distilled water and analytical grade reagents supplied by British Drug Houses Ltd., Hopkins and Williams Ltd., Ajax Chemicals Ltd., and Sigma. The pH values of the buffers were measured using a Radiometer pH meter (Model 26) equipped with glass and calomel electrodes, the measurements being made either at the cited temperatures or at 20°C with corrections made employing d(pH)/dT coefficients (Dawson, Elliott, Elliott and Jones, 1969). The method used in the text for describing a buffer is to specify first the compound whose final molarity is cited, followed by the compound used to adjust the pH value. In the case of phosphate buffers the cited molarity refers to potassium dihydrogen phosphate, pH adjustment being effected with KOH.

2. Methods.

(a) General laboratory methods

Weighings were made with Mettler H1OT balances(weighings in the milligram range were made with a Mettler micro-balance), and solutions were adjusted to their final volumes in volumetric flasks. In general protein concentrations were estimated spectrophotometrically employing a Zeiss PMQII or Carey 14 spectrophotometer and extinction coefficients cited in the text. In relation to ultracentrifuge studies protein concentrations (in the required refractometric units) were obtained using a Brice Phoenix differential refractometer. All protein solutions were passed through a washed 0.4μ Millipore filter immediately prior to spectrophotometric and refractometric examinations.

Dialyses were performed (generally at 4°C) with Visking 8/32 (for analytical work) and 23/32 (for preparative work) cellophane tubing which had been immersed for 2h in boiling 50% ethanol (one change), boiled in two changes of 10mM $NaHCO_3$, 1mM EDTA then rinsed and stored in distilled water at 4°C (McPhie, 1971). The minimum time of conventional dialysis was 24h with two changes of buffer at 4h and 8h, and in most cases there was at least 100 volumes of buffer to 1 volume of protein solution. In instances specifically cited in the text where exhaustive dialysis was undesirable with regard to content of manganese ions, a rapid dialysis apparatus (Englander and Crowe, 1965) was employed.

All glassware was routinely washed using 7X detergent, thoroughly rinsed with many changes of distilled water, rinsed with 3% acetic acid, and finally with several changes

of glass distilled water.

(b) Electrophoresis

(i) Analytical. Polyacrylamide gel electrophoresis was performed according to the method of Davis (1964) on a Buchler apparatus. 7.5% acrylamide gels were produced in 0.5 x 7.5cm tubes and no spacer gel was employed. The gels were prepared without potassium ferricyanide and were photopolymerized. The electrode buffer was 0.006 M tris-0.047 M glycine (pH 8.3), and 1 ml of 0.001% bromophenol blue was added to the buffer in the cathode compartment to act as a migration marker. The protein solution in 20% glycerol or concentrated sucrose solution was loaded onto the top of the gels, under buffer, by use of 5, 10, or 20 µl "Microcaps" disposable micropipettes. A constant current of 5mA per tube was employed and the jacketed anode chamber was cooled with tap water. After the run, the gels were removed from their tubes and placed in 0.02% amido black 10B (George T. Gurr Ltd.) in 7.5% v/v acetic acid for 15 min after which time they were destained by soaking in two changes of 7.5% acetic acid for 20h.

Electrophoresis on cellulose acetate was conducted at 25°C using a Beckman Model R-100 Microzone electrophoresis system, and electrode buffer (Beckman) of pH 8.5. The applied potential was 250V, and staining was effected with 0.02% amido black 10B in 7.5% v/v acetic acid.

(ii) Isoelectric focusing. The procedure was that of Vesterberg and Svensson (1966). A 110ml column thermostated at 4°C was employed with a sucrose density gradient ranging from 0 to 25% w/v sucrose from the top to the base of the column. The sucrose gradient was produced by the use of

24 test-tubes into which were delivered, from burettes, graded amounts of 50% w/v sucrose (containing 0.3% ampholyte pH 5-7, Ampholine L.K.B. Produkter) and water (containing 1.0% ampholyte pH 5-7), (Vesterberg and Svensson, 1966). Protein, amounting to no more than 20mg was added to tube number 13, the volume replacing that of the light solution and the gradient was formed by pouring the solution into the top of the column, the whole process taking 30 min. The voltage was increased in a step-wise manner from ∿ 300V to 600V over 8h care being taken to ensure that the total power consumption was less than 1W. After 70h the power was switched off and fractions were collected from the base of the column. Each fraction was assayed for enzymic activity, its pH was measured and its absorbance at 280nm was recorded.

(c) Enzymic assays

The detailed conduct of enzymic assays has been described in Chapters IV and V and it therefore suffices to make brief comment on the chemical means employed to monitor the concentrations of the following compounds.

(i) Urea. Urea was estimated by the diacetyl monoxime method (Ceriotti and Spandrio, 1963; Sakai and Murachi, 1969) which utilizes two reagent solutions : Solution I, 0.25g of diacetyl monoxime (Sigma) per 100ml of 3.0% v/v glacial acetic acid in water and Solution II, 0.8g of antipyrine (Sigma) per 100ml of 80% v/v sulphuric acid in water. The composition of these solutions was selected to ensure that the concentrations of reagents in the final assay medium was optimal for the development of the coloured urea-chromophore on heating (Ceriotti and Spandrio, 1963). The only

difficulty encountered was the hydrolysis of diacetyl monoxime in Solution I which resulted in high blank absorbances after several days. The difficulty was simply overcome by preparing fresh solutions of Solution I prior to each assay.

(ii) Arginine. Full details of the spectrophotometric method employed (Ward and Srere, 1967) have been given previously.

(iii) Ammonium ions. The Conway micro-diffusion method proved to be the most reliable and accurate procedure for the extraction of ammonia from reaction mixtures (Conway, 1939, 1947). The success of this procedure was reliant on completely covering the inner compartment with a film of the solution containing boric acid. This was achieved by adding excess solution initially rather than by employing the wetting agent BRIJ 35 which proved to react with Nessler's reagent. The Nessler's solution (alkaline potassium mercuri iodate) was prepared by a standard method described by Hillebrand and Lundell (1953).

(iv) Fumarate. Fumarate was estimated spectrophotometrically at 240nm following the method devised by Racker (1950) using the molar extinction coefficient of 2.44×10^3 (Alberty, Massey, Frieden and Fuhlbrigge, 1954).

(d) Enzymic activity units

(i) Arginase. One unit of arginase activity is defined as that amount of enzyme that will hydrolyze 1 μmole of arginine in 1 min. For the isolation procedure assays the temperature was 25°C and an initial arginine concentration of 1.6mM was employed with the pH adjusted to pH 9.5 with HCl. Under these conditions the arginase is ∿ 60% saturated

and the reaction proceeds at a rate which equals that at pH 7.5 in 0.05 M HCl-tris, 0.02 M NaCl, 0.01 M $MnCl_2$ at 25°C with extrapolation to saturating arginine.

(ii) Argininosuccinase. One RU (Ratner Unit) is defined as the amount of enzyme that will convert 1 μmole of argininosuccinate per hour at 38°C in 0.1 M phosphate buffer pH 7.5 with an initial argininosuccinate concentration of 1mM (correction with the factor 1.1 was made for the undersaturation). When corrections for temperature were employed the value taken for Q_{10} was 2.25 (Ratner, 1970).

(iii) Urease. One Sumner Unit is defined as that amount of enzyme that liberates 1mg of ammonia nitrogen from a 3% w/v solution of urea in 0.1 M phosphate at 20°C in 5 min. It equals 14.28 International Units (Reithel, 1971).

(e) Frontal analysis experiments

All quantitative chromatography experiments were performed with jacketed glass columns supplied by Chromatronix Inc. Temperature control was achieved by circulating water through the jacket from a thermostated bath. Sephadex beads from Pharmacia Fine Chemicals were equilibrated with buffer following the procedure recommended by the manufacturer. Immediately prior to packing the column the suspension was degassed by rapid swirling under vacuum. Packing was achieved with the aid of an Isco Metering Pump which ensured a steady flow of buffer.

The protein solution and the eluting buffer were introduced in order onto the column by means of the displacement apparatus shown schematically in Figure (VI-2). The five way tap also shown schematically in the Figure was designed to facilitate the exacting procedure of

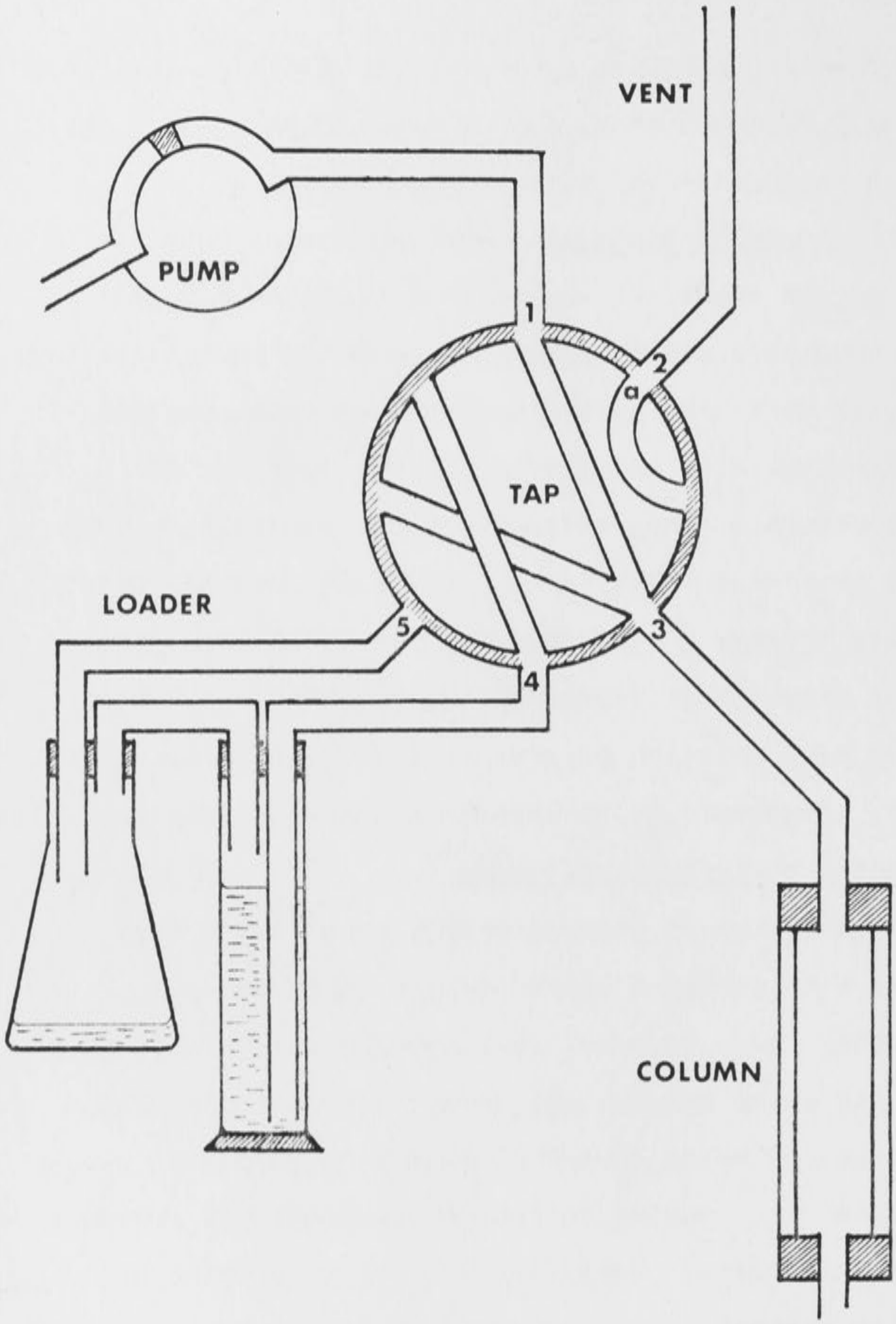


FIGURE VI - 2

Schematic representation of the displacement apparatus utilized to introduce protein and eluting buffer onto a Sephadex column in frontal gel chromatography experiments.

quantitatively delivering protein solution onto the column without introducing air bubbles. Consequently the tap was designed with small internal volumes and was constructed to be operated in sequence so that all internal tubes were vented free of air before the buffer and solution were allowed to proceed along these channels. The tap was operated in the following sequence: (1) with tube *a* in juxtaposition with position 1 buffer was allowed to fill the small loop and overflow into the vent; (2) tube *a* was brought into juxtaposition with position 2, as shown in Figure (VI-2), allowing buffer to be pumped onto the column; (3) juxtaposition 3*a* caused the introduction of buffer into the loader and hence portion into the vent; (4) juxtaposition 4*a* introduced protein onto the column, of weight determined from the tared cylinder; (5) juxtaposition 5*a* enabled protein solution in the vent to run onto the column; and (6) juxtaposition 6*a* reintroduced buffer as the eluant.

As soon as loading had begun small aliquots of eluant were collected by means of an ISCO (Golden Retriever) fraction collector in pre-tared tubes. The weight of each aliquot was determined and divided by a density term to give the corresponding volume. Each tube was assayed for enzymic activity as described in the text. Void volumes were obtained by eluting a 0.1% solution of Blue Dextran 2000 ($\bar{M}$=2 x 10^6, Sigma) and monitoring the eluant at 620nm.

(f) Partial specific volume

Density measurements for use in equation (III-20) were made using an Anton Paar DMA 02C Digital Precision Densitymeter thermostated to within 0.01°C of 20°C by means of water circulation from a thermostat. The oscillator tube

was flushed with distilled water and dried after rinsing with AR ethanol. The solution of interest was introduced into the tube, without bubbles, and the tube sealed with teflon plugs. The filled tube is subjected to electronic oscillation and adopts a frequency given by,

$$f = \frac{1}{2\Pi}\sqrt{\frac{c}{m}} \qquad \text{(VI - 1)}$$

where c is the elasticity constant of the oscillator (glass plus fluid) and m its total mass (equal to $M_o + \rho V$) where M_o is the mass of the tube, V its effective volume and ρ the density of the solution. It follows that,

$$\rho = (T^2 c/4\Pi^2 V) - (M_o/V) = A(T^2 - B) \qquad \text{(VI - 2)}$$

Thus the difference between two densities ρ_1 and ρ_2 of two different fluids is given by,

$$(\rho_1 - \rho_2) = A(T_1^2 - T_2^2) \qquad \text{(VI - 3)}$$

where T_1 and T_2 are the periods for a finite number of oscillations (10^4 employed herein). Values of T were normally measured approximately 30 times and averaged to minimize slight variation due to thermal fluctuations. The instrument constant A was determined to be 2.695275×10^{12} using air and water as reference fluids and values of their densities obtained from standard tables.

(g) Ultracentrifuge methods

All ultracentrifuge experiments were conducted in a Spinco Model E analytical ultracentrifuge fitted with an electronic speed control. Temperature was controlled to within $\pm$ 0.1°C by the RTIC unit.

(i) Sedimentation velocity. In general an An-D rotor was employed but certain high speed experiments were performed with the titanium An-H rotor. Single sector 12mm cells (aluminium filled, or Kel F centre pieces) were used with quartz windows, comparative experiments being performed with two cells one of which was equipped with a 1° wedge window. Schlieren optics were used and photographic records obtained on Kodak metallographic or A40 plates.

The schlieren patterns (plots of refractive index gradient versus radial distance) were measured using a two dimensional comparator (Gaertner Scientific Corporation). The method used for correcting horizontal measurements to corresponding radial distances in the cell may be illustrated with reference to the position of the air-liquid meniscus. The comparator distance $X_R - X_m$ (cm) was measured as the distance on the plate between the meniscus and reference line, the latter being generated by edges in either the counterpoise or cell rotor. Division of $(X_R - X_m)$ by the horizontal magnification factor (determined using a cell window transparent scale) yields the actual radial distance in the cell. Since the distance from the axis of rotation to the edge generating the reference line is known (5.70 cm), the distance from the meniscus to the axis of rotation is made available by direct addition. Invariance of x_m, thus obtained, with time was used as a test for the absence of cell leakage. In an entirely similar manner the position of the maximum ordinate of an observed schlieren peak x_p was determined and used in equation (III-2b) to calculate the sedimentation coefficient, $s_{T,b}$, referring to the temperature

and buffer environment. Where appropriate $s_{T,b}$ was converted to a value at 20°C in water utilizing the relation (Svedberg and Pedersen, 1940),

$$s_{20,w} = s_{T,b} \frac{\eta_{T,w}}{\eta_{20,w}} \eta_{rel} \frac{(1 - \bar{v}\rho)_{20,w}}{(1 - \bar{v}\rho)_{T,b}} \qquad (VI - 4)$$

where $\eta_{T,b}$ and $\eta_{20,w}$ are the viscosities of water at temperatures T and 20°C, respectively; η_{rel} is the relative viscosity of the buffer; $(1 - \bar{v}\rho)$ is the bouyancy term in the indicated environments.

In boundary analysis experiments and in the determination of weight-average sedimentation coefficients corresponding values of x (corrected as above) and of dn/dx (the height of the schlieren peak above the base line) were obtained across the entire schlieren pattern again using the two dimensional comparator. These values were employed directly in equations (III-2a) and (III-11) which include summations leading to the area under the peak (trapezoidal integration).

(ii) Sedimentation equilibrium. Aluminium An-D and An-J rotors were used for sedimentation equilibrium studies. The latter being of particular value for runs performed at speeds below 10,000 rpm. Double sector 12mm carbon-filled epon centre pieces and sapphire windows were used in all experiments. The Rayleigh interference optical system with an off-set limiting aperature was employed, leading to photographic records (on Kodak IIGA plates) of plots of interference fringes. One fringe corresponds to 0.025g dl^{-1} of protein with a specific refractive increment of 4.6×10^{-5}.

Each sedimentation equilibrium experiment was preceded

by an experiment designed to determine the initial concentration of the protein solution in refractometric units. For this purpose 0.14ml of dialyzed protein solution (Casassa and Eisenberg, 1963) was introduced into 1 sector of a 12mm double sector synthetic boundary cell (capillary type): the other sector was filled with 0.44ml of dialysate. Acceleration of the rotor to 10,000 rpm resulted in the formation of the synthetic boundary, whereupon a photograph was taken immediately and utilized to determine the fringe fraction. The rotor was decelerated to 6,000 rpm and maintained at this angular velocity to permit resolution of the fringes by diffusion before a second photograph was taken to obtain a count of the total number of fringes. A cell deviation plot used for correcting the fringe fraction was obtained by mixing the contents of the cell after the run and reaccelerating to 6,000 rpm to obtain a baseline photograph.

In the actual sedimentation equilibrium experiments 0.01ml of inert fluoro-carbon FC43 was introduced into both sectors of a double sector cell (without capillary), followed by dialyzed protein solution in one sector and dialysate in the other, of volume required to obtain column lengths of 3mm. The rotor was initially accelerated to an angular velocity of 1.4ω where ω is the final velocity appropriate to the sedimentation equilibrium equation: this overspeeding technique suggested by Hexner, Radford and Beams (1961) and Howlett and Nichol (1972a) was designed to shorten the time required to reach the final equilibrium position. The time of overspeeding was obtained from Figure 4 of Howlett and Nichol (1972a) and

that of ω (to obtain a fringe density at the cell base of approximately 100 fringes per cm) was calculated from the relation provided by Richards, Teller and Schachman (1968). Both calculations require assignment of an estimate of the diffusion coefficient. In practice the criterion that equilibrium had been achieved at the values of ω cited, was that the interferogram was time-invariant between two photographs taken near the end of the run, at an interval of 1h. The final equilibrium distribution was measured according to the method described in detail by Richards, Teller and Schachman (1968), corrections being made for cell deviations, and led to a plot of $\log_{10}$ fringe displacement versus x^2 (Figure III-10a). Apparent weight-average molecular weights were calculated on the basis of equation (III-14) using a computer programme written by Baghurst (1972) based on a flow-chart of Richards, Teller and Schachman (1968).

In specific instances cited in Chapters III and V the sedimentation equilibrium experiments were of the meniscus depletion type originally described by Yphantis (1964). The protocol for these experiments was as described above except that no overspeeding was employed (Teller, Horbett, Richards and Schachman, 1969) and higher values of ω were used. In these experiments it is not required to obtain values of the initial loading concentration since measurement of the final equilibrium distribution provides a direct measure of the concentration (in terms of fringe displacement) as a function of radial distance.

(h) Computations

(i) Digital computing. In the interests of brevity fine detail of the various computer programmes employed in this work will not be given. The numerical methods utilized, although relatively complex in some instances, were standard and were obtained from texts, original papers or from computer libraries. The programmes obtained from these sources were appropriately modified to suit the requirements of any given problem. The computer simulation of the urea cycle in terms of its individual unitary reactions (Method I of Chapter V), and other numerical solutions of "stiff" differential equation systems presented in the text, were performed with a programme developed by Professor D.T. Elmore of Queen's University, Belfast and Dr D.V. Roberts. The programme coded in FORTRAN and requiring ∿ 35K storage, accepts input data in a format of chemical equations, with rate constants and concentration terms, which it automatically converts to the appropriate flux equations, and the numerical integration is achieved by the use of the subroutine DIFSUB (Gear, 1969). (These two features are also shared by the programmes CHEK and CHEKMAT developed by Curtis and Chance, 1972.) The computer simulation of the urea cycle employing the steady-state differential equations, and the simulation of coupled enzymic assays were performed with a second order predictor-corrector programme which incorporated the automatic selection of step-size (McCracken and Dorn, 1964). The programmes used for the urea cycle simulation, coded in FORTRAN, were executed on a Univac 1108 computer. The predictor-corrector programme used routinely for coupled assay simulations was

coded in FOCAL and executed on the smaller PDP - 8/1 computer. Because of the relatively slow operation and moderate storage requirement of the predictor-corrector programme, a 4^{th} order Runge-Kutta programme was written for the numerical integration of simultaneous differential equations of the "non-stiff" type, and was particularly useful in preliminary modelling studies.

Many other calculations were performed routinely with the PDP - 8/1 computer. These included the weighted least squares fitting of data, the calculation of sedimentation coefficients, the processing of sedimentation equilibrium data and boundary analysis. Because of limited storage capacity of the PDP - 8/1 computer, which normally operated on 7-user mode, some problems were processed using the single-user mode ($\sim$ 12K storage).

(ii) Analogue computing. As described in Chapter IV the full time course of the coupled arginase-urease reaction was first simulated using an analogue computer. The choice was made because of the rapidity with which solutions to differential equations are obtained. The general purpose analogue computer was an E.A.I. PACE TR-48 with reference voltage of 100V and with a standard complement of summers and integrators. The instrument also contained three, quarter-square multipliers each consisting of four x^2 diode function generators. Figure (VI-3) is an illustration of the circuit employed for the simulation of the operation of the coupled reaction. The circuit is seen to be roughly symmetrical around a central horizontal line and the upper section of the circuit is that representing arginase. It has a feed back loop to simulate the product inhibition of

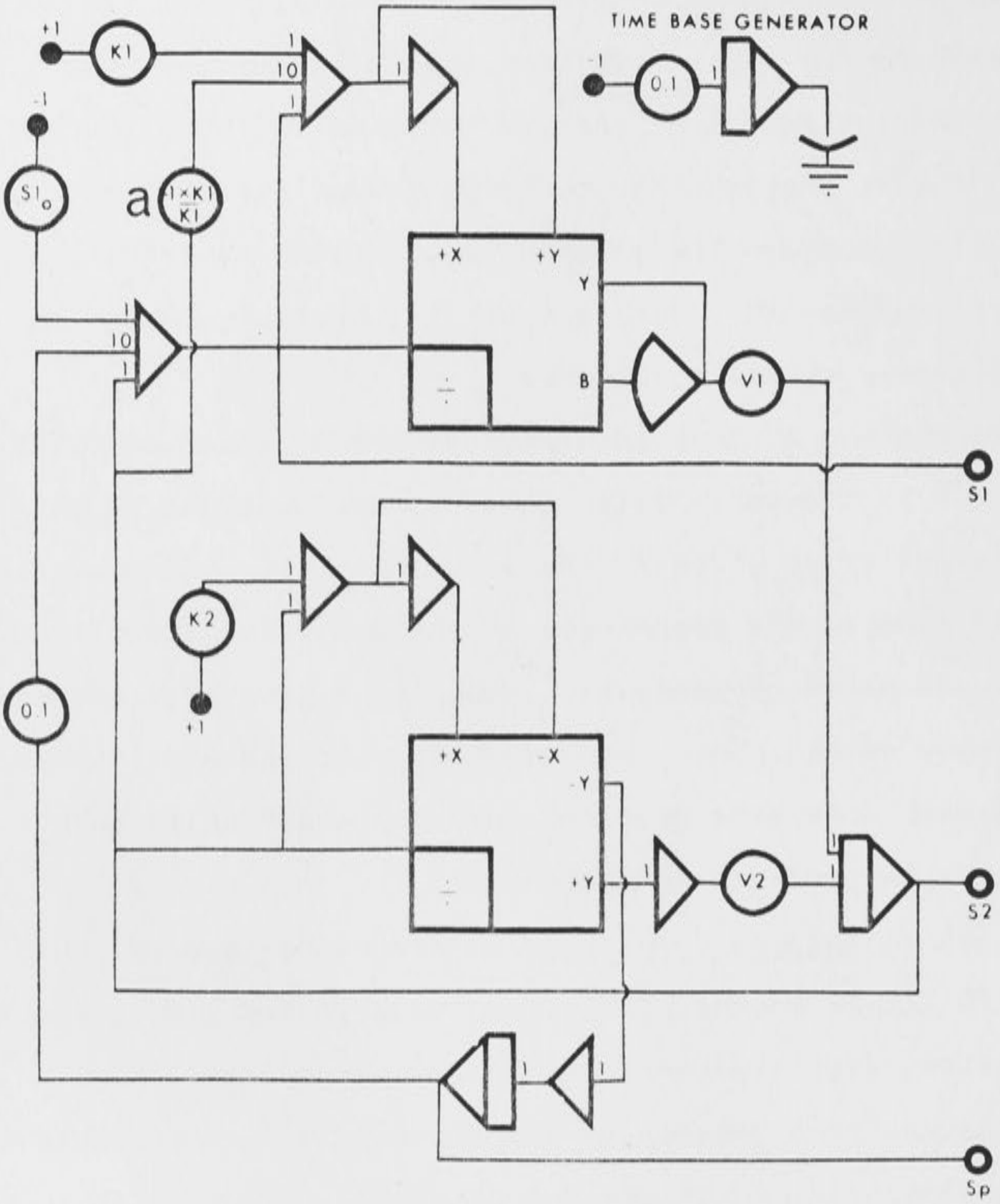


FIGURE VI - 3

Circuit diagram representing the consecutive reactions catalyzed by arginase and urease used in the analogue computation of the concentration of reactants versus time (Figure IV - 11). The potentiometers are labelled to specify those which correspond to the magnitudes of maximal velocities and Michaelis constants : potentiometer a was varied to simulate different values of the inhibition constant.

the reaction by ornithine, and the potentiometer a was set to give the ratio K_m/K_I. The values of K_m and V_{max} for arginase and urease were set on the various potentiometers, to values obtained from the experimental initial velocity studies of these enzymes (Chapter IV). The output was plotted on transparent graph paper overlying the experimental data points, and the fitting was achieved by adjustment of the appropriate potentiometers followed by resimulation.

ABSTRACT

The work commences with consideration of a linear sequence of two reactions, each catalyzed by an enzyme of the Michaelis-Menten type. The steady-state differential equations are integrated using an approach employing Maclaurin polynomials to yield a solution, in open form, which expresses the concentrations of each reactant and of final product as explicit functions of time. The formulation suggests that experimentally available results of the increase of final product concentration with time be plotted with t^2 as the abscissa, since the slope of the limiting tangent to this curve, drawn as t approaches zero, is then given by a defined analytical function from which the steady-state kinetic parameters relating to the first enzyme catalyzed reaction may readily be evaluated. The analysis procedure is extended by consideration of the following: (1) more than two enzymes in a linear sequence; (2) mechanisms involving single substrates other than the simple Michaelis-Menten mechanism (especially those pertaining to control phenomena); (3) reactions involving enzymes acting on two substrates (Appendix I); and (4) sequences of reactions forming a cycle (Appendix I). The general finding emerges that in all cases a plot of final product concentration versus time raised to the power of the number of reactions in the sequence, be constructed to permit evaluation of the slope of the limiting tangent : this slope is given by an analytical function which specifies the contribution to final product formation of the steady-state kinetic parameters of each of the enzymes involved.

The above concepts embodied in Chapter II were explored in relation to consecutive reactions catalyzed by arginase and urease. As a prelude to this study the physico-chemical properties of beef liver arginase were determined and are reported in Chapter III. In addition to confirming previously established values of basic parameters such as molecular weight (114,000) and of establishing others such as the apparent specific volume (0.734 $g^{-1}ml$), the findings in Chapter III also show that arginase does not dissociate on dilution to concentration levels generally employed in enzymic assays, and that it does not heterogeneously associate with sulphite modified urease in environments used for kinetic analysis. Accordingly, the arginase-urease couple was judged suitable as a system to explore the application of the theory presented in Chapter II. A report on the kinetic studies is given in Chapter IV where it is concluded that the suggested analysis procedure is indeed pertinent to results obtained in a coupled assay. While the results also confirm the suggestion that the urease and arginase do not heterogeneously associate, the opportunity is taken at the end of Chapter IV to formulate theory pertaining to the effect of enzyme-enzyme interaction on results obtained in coupled assays. It is shown, for example, that such an interaction between two enzymes which exhibit Michaelis-Menten kinetics when studied alone, can result in behaviour paralleling that found when an enzyme of the Michaelis-Menten type is coupled with one of the control type. The finding is discussed in relation to possible biological implications and to experimental detection of such macromolecular interactions by simple

kinetic measurements.

Chapter V commences with a characterization of the molecular and kinetic properties of beef liver argininosuccinase. The physico-chemical findings include support for the suggestion that the enzyme, of molecular weight 203,000, comprises four ellipsoidal subunits arranged with tetrahedral D_2 symmetry : dissociation of the 203,000 unit does not occur in the environment and dilution range used for the conduct of subsequent kinetic experiments. The kinetic findings indicate that the Eadie-Hofstee plot obtained with argininosuccinate as initial substrate is not linear, as suggested previously, but is interpretable in terms of models which involve either cooperativity (both positive and negative) between the active sites, or isomerization of the enzyme with preferential binding of substrate to one isomeric state which possesses two non-equivalent sites. The reverse reaction with arginine and fumarate as initial substrates was less extensively investigated but application of numerical integration methods permitted estimation of the relevant steady-state kinetic parameters. Both physico-chemical (sedimentation velocity) and kinetic (coupled assay) findings show that arginase and argininosuccinase do not form a complex in the environments studied.

Chapter V concludes with a description of fluxes associated with the urea cycle and utilizes parameters found for arginase and argininosuccinase together with literature values for ornithine transcarbamylase and argininosuccinate synthetase in a numerical integration of flux equations. The integration is considered in relation

to both steady-state differential rate equations and to a more extensive set which does not assume a steady-state of enzyme-substrate complexes. It is concluded that both approaches lead to the same intermediate metabolite pattern, defined as the concentration of relevant species, found as early as 10 sec from the time of commencement of the operation of the cycle. Specified kinetic parameters are modified in an attempt to simulate certain inborn errors of metabolism, the resulting intermediate metabolite patterns being correlated with observed clinical-biochemical findings. Although it is stressed throughout this Section that several clearly specified assumptions must necessarily be made, it is felt that the results demonstrate the potentiality in both explanatory and predictive areas.

A basic theme of the work is the expression of the concentrations of species in consecutive reactions as a function of time, either by applying numerical integration methods or by utilizing Maclaurin polynomial expansions of the relevant differential equations to obtain open solutions. The theme is continued in Appendix II where it is shown that application of Laplace-Carson transforms may lead in certain circumstances to closed solutions describing both pre-steady- and steady-states.

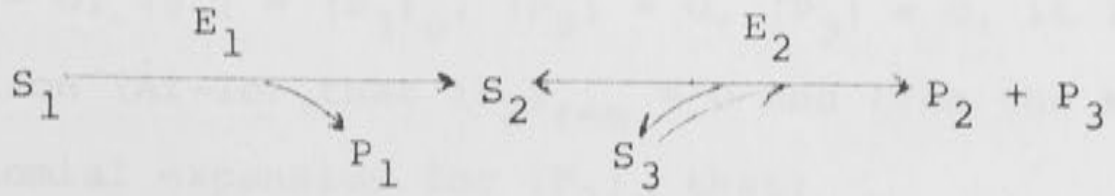
APPENDIX I

EXTENSION OF THE MACLAURIN POLYNOMIAL APPROACH TO SYSTEMS INVOLVING ENZYMES ACTING ON TWO SUBSTRATES

The use of Maclaurin polynomials to give an open solution of the concentration of final product as a function of time has proved useful in suggesting procedures for the interpretation of coupled assays. In Chapter II the following systems were shown to be amenable to such treatment; (1) irreversible consecutive reactions where each enzyme acted on a single substrate : enzymes of both Michaelis-Menten and control types were considered; (2) two consecutive irreversible Michaelis-Menten mechanisms with inhibition (competitive) of the first enzyme by its product S_2; and (3) two consecutive reversible Michaelis-Menten reactions. The purpose of this Appendix is to explore the possibility that the Maclaurin polynomial approach may prove useful in interpreting steady-state kinetic results on coupled systems of more complicated nature. It is of course not possible to be encyclopaedic with regard to all reaction mechanisms (and combinations of them) that may be encountered in practice; but it is hoped that the following additional examples will suffice to indicate the generality of the approach. The examples to be presented differ from those given earlier in that one enzyme of the sequence acts on two substrates in a given direction and thereby introduces the biochemical reality that most enzyme catalyzed reactions cannot be considered to involve single substrates.

1. Coupling of a Michaelis-Menten mechanism to an ordered sequential Bi Bi mechanism.

The scheme may be represented by,



It could be taken to represent a coupled assay involving the urea cycle enzymes, arginase (E_1) and ornithine transcarbamylase (E_2) with arginine (S_1) as initial substrate forming, irreversibly, urea (P_1) and ornithine (S_2), which together with carbamylphosphate (S_3) binds to E_2 to form a ternary complex which breaks down to form citrulline (P_2) and inorganic phosphate (P_3). The appropriate steady-state rate equations are,

$$\frac{d[S_1]}{dt} = - \frac{V_1 [S_1]}{[S_1] + K_1(1 + [S_2]/K_{S_2}^{I,S_1})} \qquad \text{(AI - 1a)}$$

$$\frac{d[S_2]}{dt} = \frac{V_1 [S_1]}{[S_1] + K_1(1 + [S_2]/K_{S_2}^{I,S_1})} - \frac{d[P_2]}{dt} \qquad \text{(AI - 1b)}$$

$$\frac{d[P_2]}{dt} = \frac{d[P_3]}{dt} = \frac{\left(\alpha_1 [S_2][S_3] - \alpha_2 [P_2][P_3]\right)[E_2]_o}{\psi} \qquad \text{(AI - 1c)}$$

$$\psi = \alpha_3 + \alpha_4[S_3] + \alpha_5[P_3] + \alpha_6[S_2] + \alpha_7[P_2] + \alpha_8[S_2][S_3] + \alpha_9[P_2][P_3] + \alpha_{10}[S_2][P_2] + \alpha_{11}[S_2][S_3][P_2] + \alpha_{12}[S_2][P_3] + \alpha_{13}[S_2][P_2][P_3] \qquad \text{(AI - 1d)}$$

where (AI - 1a) is expressed in terms of the maximal velocity V_1, Michaelis constant K_1 and inhibition constant for ornithine (S_2) binding to E_1; equation (AI-1c and d) is identical with equation (V-7a) with the constant coefficients $\alpha_1, \cdots, \alpha_{13}$ so identified in terms of individual rate constants.

With the initial conditions that at $t = 0$, $[S_1] = [S_1]_o$, $[S_2] = 0$, $[S_3] = [S_3]_o$, $[P_2] = 0$, $[P_3] = 0$, it follows from equation (AI-1c) that $[P_2]_{t=o} = 0$ and from the Maclaurin polynomial expansion for $[P_2]_t$ that;

$$\lim_{t\to o} \frac{[P_2]_t}{t^2} = \frac{1}{2}[P_2]''_{t=o} = m \qquad \text{(AI - 2)}$$

Differentiation of equation (AI-1c) with respect to time yields

$$[P_2]'' = [E_2]_o[\psi\{\alpha_1[S_2][S_3]' + \alpha_1[S_3][S_2]' - \alpha_2[P_2][P_3]' - \alpha_2[P_3][P_2]'\} - (\alpha_1[S_2][S_3] - \alpha_2[P_2][P_3])\psi']/\psi^2 \qquad \text{(AI - 3a)}$$

and thus,

$$[P_2]''_{t=o} = \alpha_1[S_3]_o[E_2]_o[S_2]'_{t=o} / \psi_{t=o} \qquad \text{(AI - 3b)}$$

From (AI-1b), $[S_2]'_{t=o} = V_1[S_1]_o/(K_1+[S_1]_o)$ and from equation (AI-1d), $\psi_{t=o} = \alpha_3 + \alpha_4[S_3]_o$ and thus equation (AI-2) may be written,

$$m = \frac{\alpha_1 [E_2]_o}{((\alpha_3/[S_3]_o) + \alpha_4)} \quad \frac{V_1 [S_1]_o}{2(K_1 + [S_1]_o)} \qquad \text{(AI - 4a)}$$

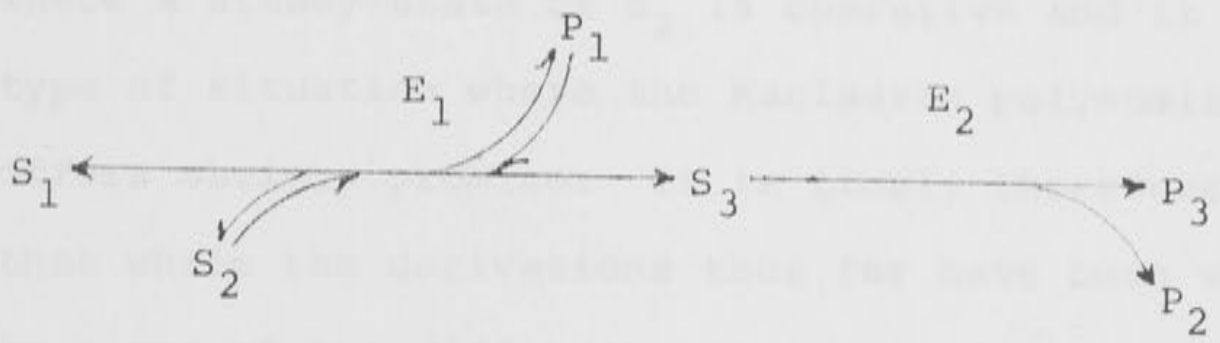
If S_3 were initially in excess so that $\alpha_3/[S_3]_o$ is negligible, equation (AI-4) becomes with the identities from equation (V-7a)

$$m = \frac{k_3k_5}{k_4+k_5} [E_2]_o \frac{V_1[S_1]_o}{2(K_1+[S_1]_o)} = \frac{(V_2)_f}{(K_{S_2})} \frac{V_1[S_1]_o}{2(K_1+[S_1]_o)} \qquad \text{(AI - 4b)}$$

where $(V_2)_f = k_5k_7[E_2]_o/(k_5+k_7)$ is the maximal velocity of the second reaction in the forward direction and $K_{S_2} = k_7(k_4+k_5)/k_3(k_5+k_7)$ is the Michaelis constant referring to S_2.

The important point emerges from equation (AI-4b) that evaluation of the initial slope of a plot of $[P_2]_t$ *vs.* t^2 as a function of $[S_1]_o$ with $[S_3]_o$ large permits

evaluation of the steady-state kinetic parameters of the first enzyme in the sequence if those of the second enzyme ($(V_2)_f$ and K_{S_2}) are known from separate studies. These findings are consistent extensions to those formulated in Chapter II. It may also be shown by entirely analogous reasoning that if the situation were reversed in that an ordered sequential Bi Bi reaction were followed by a Michaelis-Menten reaction according to,



that

$$m = \lim_{t \to o} \frac{[P_3]_t}{t^2} = \frac{V_2\, \alpha_1 [E_1]_o [S_1]_o [S_2]_o}{2K_2\, (\alpha_3 + \alpha_4 [S_1]_o + \alpha_6 [S_2]_o + \alpha_8 [S_1]_o [S_2]_o)} \qquad \text{(AI - 5)}$$

where the conventional form of the ordered two substrate initial velocity equation has been modified by the scaling factor $(V_2/2K_2)$, reflecting the effect of the second enzyme. Equation (AI-5) with the α_3 term omitted also pertains to a system in which the mechanism of the first reaction is ping pong Bi Bi since equation (V-7a) applies with terms involving α_3, α_{11} and α_{13} omitted (Cleland, 1963a).

2. A single enzyme possessing independent active sites catalyzing different but consecutive reactions

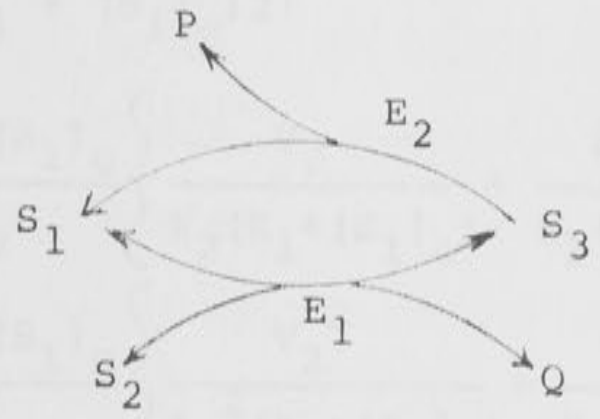
In certain instances, such as with chorismate mutase-prephenate dehydrogenase, a situation is encountered where a single protein molecule possesses two distinct active

sites. The first may bind a substrate S_1 converting it to S_2 ($S_1 \xrightarrow{E_1} S_2$), which then binds together with an additional substrate S_3, at the second active site with the result that final product P is formed ($S_2 + S_3 \xrightarrow{E_1} P$). In such situations it is not possible by changing the enzyme concentration to affect the maximal velocity of the second reaction without affecting that of the first. It follows that it may not be possible to attain conditions where a steady-state of S_2 is operative and it is in this type of situation where the Maclaurin polynomial solution offers obvious promise. It is timely therefore to note that while the derivations thus far have been visualized in terms of two <u>different</u> enzymes acting consecutively (for simplicity of presentation), they apply directly to the system under discussion where two independent active sites are situated on the same enzyme. For example, if the $S_1 \rightarrow S_2$ reaction is of the Michaelis-Menten type and the $S_2 + S_3 \rightarrow P$ is ordered sequential, then eq (AI-4b) describes the functional relationship between the experimentally determinable m and $[S_1]_o$. If then additional studies are performed with S_2 and S_3 as initial substrates, the indicated steady-state kinetic parameters may be evaluated.

3. <u>Cyclic schemes</u>

The discussion of the urea cycle in Chapter V proceeded solely on the basis of numerical integration of the appropriate sets of differential rate equations. The question arises whether analytical solutions in the form of

polynomials in t (open solutions) may be used as an alternate (and potentially more general) way of expressing the operation of a cycle. Consider a very simple scheme in which two substrates S_1 and S_2 bind in an ordered sequence to E_1 producing, irreversibly, products Q and S_3 : the compound S_3 is transformed to S_1 by an irreversible Michaelis-Menten mechanism catalyzed by enzyme E_2 with the release of product P,



It is helpful to visualize the *in vitro* situation where to a mixture of E_1, E_2 and S_1, S_2 in large excess is added at t = o and the concentration of P with time, monitored. The rate of disappearance of S_1 due to the reaction catalyzed by E_1 is given by equation (V-7a) with $k_6 = 0$ and $k_8 = 0$ and becomes on division by $[S_2]$ of large magnitude $V_1[S_1]/(K_1+[S_1])$ where $V_1 = k_5k_7[E_1]_o/(k_5+k_7)$ and $K_1 = k_5k_7/k_1(k_5+k_7)$. Thus, it is possible to formulate the set,

$$\frac{d[S_1]}{dt} = -\frac{V_1[S_1]}{K_1+[S_1]} + \frac{V_2[S_3]}{K_2+[S_3]} \qquad \text{(AI - 6a)}$$

$$\frac{d[S_3]}{dt} = \frac{V_1[S_1]}{K_1+[S_1]} - \frac{V_2[S_3]}{K_2+[S_3]} \qquad \text{(AI - 6b)}$$

$$\frac{d[P]}{dt} = \frac{V_2[S_3]}{K_2+[S_3]} \qquad \text{(AI - 6c)}$$

There is close analogy between equations (AI-6) and (II-2) except that equation (AI-6a) contains an additional term reflecting the operation of the cycle : indeed the particular simple cycle was selected to permit the analogy.

Application of the Maclaurin polynomial by successive differentiation of equation (AI-6) yields,

$$[P]_t = \frac{V_2V_1[S_1]_o t^2}{K_2(K_1 + [S_1]_o)2!}$$

$$- \frac{V_2V_1[S_1]_o}{K_2}\left\{\frac{V_2}{K_2(K_1+[S_1]_o)} + \frac{2V_1[S_1]_o}{K_2(K_1+[S_1]_o)^2} + \frac{V_1K_1}{(K_1+[S_1]_o)^3}\right\}\frac{t^3}{3!}$$

$$+ \frac{V_2V_1[S_1]_o}{K_2}\left\{\frac{V_2^2}{K_2^2(K_1+[S_1]_o)} + \frac{V_2V_1[S_1]_o}{K_2^2(K_1+[S_1]_o)^2}\right.$$

$$\left. + \frac{V_2\,V_1\,K_1}{K_2(K_1+[S_1]_o)^3} + \frac{3V_1^2K_1[S_1]_o}{K_2(K_1+[S_1]_o)^4}\right. \qquad \text{(AI - 7)}$$

$$\left. + \frac{V_1K_1[V_1K_1K_2+V_2K_1^2+2[S_1]_o(V_2K_1-V_1K_2)+V_2[S_1]_o^2]}{(K_1+[S_1]_o)^5}\right\}\frac{t^4}{4!}$$

Comparison of equation (AI-7) with equation (II-14) reveals two salient points. First, the first term of each is identical and thus analysis of the data from the cyclic reaction may proceed as for the linear sequence of reactions by determining m as the initial slope of a plot of $[P]_t$ versus t^2. (This conclusion has been verified with cycles involving more than two enzymes, where, for example, with a cycle involving three enzymes a plot of $[P]_t$ versus t^3 is appropriate). Secondly, the second terms of equation (AI-7) and (II-14) are also identical, which may at first suggest that the Maclaurin polynomial has failed to reflect

the operation of the cycle : however, the respective third terms differ showing that the cycle is reflected in this and higher order terms. It would indeed be possible to generate higher order terms and thus approach an analytical (open) solution describing the time-increase of output-product from the cycle. It must be stressed however that this generation of terms becomes increasingly laborious with increasing degreeof t^* and with increasing numbers of compounds in the cycle. Evidently the numerical integration procedures illustrated in Chapter V offer greater promise in handling complex cycles with external inputs, the Maclaurin polynomial approach retaining its usefulness in analyzing *in vitro* initial velocity studies of sequences of reactions in linear or cyclic array.

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*FOOTNOTE:

In this connection it is noted that equation (II-14) with the t^4 term retained, and using parameters appropriate to Figure (II-3), describes $[S_p]_t$ to within 8% of the value obtained by numerical integration when 15% of the final product has been formed. Evidently to describe the full time course of product formation given in Figure (II-3) the alternating power series would have to be generated to powers of t much greater than four. This particular example therefore serves to emphasise the laborious nature of the problem.

APPENDIX II

CONSIDERATION OF COUPLED ENZYME SYSTEMS IN THE TRANSIENT REGION OF THE REACTION

Chapters II and IV and Appendix I were concerned with the analysis of kinetic results obtained with coupled enzyme systems in terms of steady-state kinetic parameters appropriate to each enzyme in the sequence. It is well established that parameters such as Michaelis constants are in fact functions of individual rate constants, the types of functions encountered being illustrated in Chapter V. With certain postulated mechanisms, describing reversible reactions, the values of the individual rate constants may be obtained from the steady-state kinetic parameters (e.g. arginino-succinase in Table V-5), but with other postulated mechanisms, such as the irreversible Michaelis-Menten mechanism appropriate to arginase, only joint analyses of steady-state and pre-steady-state results yield the individual values. In some cases, it may be necessary to couple the enzymic reaction of interest to that of another enzyme catalyzed reaction in order to record the concentration of final product as a function of time in the pre-steady-state period of the joint reactions. The purpose of this Appendix therefore is to present theoretical equations which may aid in the analysis of pre-steady-state kinetic data obtained with a coupled enzyme system in terms of the relevant individual rate constants.

Consider the reaction scheme defined in equation (II-1) for which it is a simple matter to write the set of equations relating $d[i]/dt$, ($i = E_1, S_1, E_1S_1, S_2, E_2S_2, S_p$), to the individual rate constants and concentrations of specified compounds. For example,

$$d[E_1S_1]/dt = k_1[E_1][S_1] - (k_{-1}+k_2)[E_1S_1] \qquad \text{(AII - 1)}$$

The solution of this set of simultaneous differential equations may proceed in the simple and conventional manner of algebraically solving simultaneous equations, provided the Laplace-Carson transform is taken of each side of each equation. The definition of a function so transformed is,

$$L\{f(t)\} = \psi \int_0^\infty e^{-\psi t} f(t)\, dt \qquad \text{(AII - 2)}$$

and the salient properties of Laplace-Carson transformations are as follows; (1) the transform of a sum of functions is the sum of the individual transforms; (2) the transform of a constant multiplied by the function is equal to the constant multiplied by the transform (the linearity properties); (3) the transform of a constant is the constant; and (4) the transform of a first derivative is given by, $L\{f'(t)\} = -\psi f(o) + \psi L\{f(t)\}$, which may be readily verified by application of integration by parts to equation (AII-2). The difficulty arises in transforming equation (AII-1), that a product of two variables $[E_1][S_1]$ is encountered for which there is no transform. The problem may be overcome by assuming that $[S_1] \approx [S_1]_o$ = constant, a reasonable assumption in the early time domain, provided that also the amount of S_1 bound to E_1 is relatively small. With this assumption the transform of equation (AII-1) may be written, using the above specified properties, as,

$$\psi[e_1 s_1] = k_1[e_1][S_1]_o - (k_{-1}+k_2)[e_1 s_1] \qquad \text{(AII - 3)}$$

where the transformed time dependent species are represented by lower case letters (e.g. $L\{[E_1S_1]_t\} = [e_1 s_1]$). Similar transformed equations may be written for each of the members of the original set of differential equations provided it is

assumed that $[E_2] \approx [E_2]_o$ = constant; a reasonable assumption if relatively high concentrations of the second enzyme are employed.

The next step of the procedure is to solve (using Cramer's Rule) for $[s_p]$, the set of non-homogeneous transformed rate equations. This leads directly to the expression,

$$[s_p] = \frac{k_1k_2k_3k_4[E_1]_o[E_2]_o[S_1]_o}{\psi(\psi+\lambda_1)(\psi+\lambda_2)(\psi+\lambda_3)} \qquad \text{(AII - 4)}$$

where, $\lambda_1 = k_1[S_1]_o + k_{-1}+k_2$

$$\lambda_2 = \frac{A + \sqrt{A^2 - 4B}}{2}, \qquad \lambda_3 = \frac{A - \sqrt{A^2 - 4B}}{2}$$

and $A = k_3[E_2]_o+k_{-3}+k_4, \quad B = k_3k_4[E_2]_o$

The remaining problem is to find the inverse of equation (AII-4), since $L^{-1}\{[s_p]\} = [S_p]_t$, and the desired expression for the concentration of final product as a function of time will be obtained. The inverse of the right hand side of equation (AII-4) is conveniently obtained from tables (Rodiguin and Rodiguina, 1964; Capellos and Bielski, 1972) leading to the final result,

$$[S_p]_t = \frac{k_1k_2k_3k_4[E_1]_o[E_2]_o[S_1]_o\, t}{\lambda_1\,\lambda_2\,\lambda_3} - P_1(1 - e^{-\lambda_1 t}) - P_2(1 - e^{-\lambda_2 t}) - P_3(1 - e^{-\lambda_3 t})$$

where, $P_i = \dfrac{k_1k_2k_3k_4[E_1]_o[E_2]_o[S_1]_o}{\lambda_i^2 \prod_{\substack{j=1 \\ j\neq i}}^{3} (\lambda_j - \lambda_i)}$ $\qquad$ (AII - 5)

$(i=1,2,3)$

It is now noted that since $[S_p]$ may be readily monitored it is possible to obtain pre-steady-state and steady-state kinetic data utilizing E_2 and S_2 alone, and thereby obtain the individual values for the constants k_3, k_{-3} and k_4 (Gutfreund, 1955; Kasserra and Laidler, 1970). With this information, equation (AII-5) is seen to describe an experimentally available result in terms of the three remaining unknowns, k_1, k_{-1} and k_2, which may be evaluated as follows. At relatively long times,

$\sum_{i=1}^{3} P_i(1 - e^{-\lambda_i t})$ in equation (AII-5) approaches zero, and

$[S_p]_t$ becomes a linear function of t of slope v_o*. This quantity which is the coefficient of t in equation (AII-5) may readily be identified by use of the definitions of λ_i as,

$$v_o = k_2[E_1]_o[S_1]_o / ([S_1]_o + K_{E_1}) \qquad \text{(AII - 6a)}$$

$$K_{E_1} = (k_{-1} + k_2) / k_1 \qquad \text{(AII - 6b)}$$

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*FOOTNOTE:

It is noted that it is only when $[E_2]_o >> [E_1]_o$ that a linear region is observable in a plot of $[S_p]_t$ versus t. Even in these cases, as has been discussed in Chapter II, description in linear format, of data obtained at early times and when a steady-state of enzyme-substrate complexes has been achieved, requires a plot of $[S_p]_t$ versus t^2.

It follows that the repetition of the coupled kinetic experiment at different values of $[S_1]_o$ permits the evaluation of k_2 and K_{E_1}. Substitution of $k_1 = (k_{-1}+k_2)/K_{E_1}$ into equation (AII-5) now leads to an equation in the single unknown k_{-1} which may be solved for any values of the point ($[S_p]$, t) in the early time domain : since k_{-1} is an implicit parameter in this expression its value can only be obtained by numerical approximation, a simple task with the aid of a computer. It follows that the six individual rate constants originally envisaged have been evaluated. It could also be noted that an alternative procedure involving the determination of the transient time has been outlined for the evaluation of the final rate constant, (Kuchel and Roberts, 1974).

Inspection of the above mathematical analysis procedure shows that the crucial step in its application is the conversion of the differential rate equations to a form of first degree. Thus the products $[E_1][S_1]$, and $[E_2][S_2]$ are encountered, and it is required to make each of these terms a product of a constant and a variable : the above formulation utilized $[S_1]$ = constant and $[E_2]$ = constant. Clearly three other combinations could have been examined, but the two involving $[S_2]$ = constant are physically unrealistic in relation to the transient period of a coupled enzymic reaction. It remains therefore to comment on the combination $[E_1] = [E_1]_o$ = constant and $[E_2] = [E_2]_o$ = constant which is not only capable of analysis in terms of Laplace-Carson transforms, but it is also experimentally attainable by utilizing high concentrations of both enzymes. In this case solution for $[s_p]$, of the

set of simultaneous transformed rate equations gives, on inversion,

$$[S_p]_t = [S_1]_o \left\{ 1 - \sum_{i=1}^{4} \left(e^{-\lambda_i t} \prod_{\substack{j=1 \\ j \neq i}}^{4} \frac{\lambda_j}{(\lambda_j - \lambda_i)} \right) \right\} \qquad \text{(AII - 7)}$$

where $\lambda_1 = (A + \sqrt{A^2 - 4B})/2$, $\lambda_2 = (A - \sqrt{A^2 - 4B})/2$

$A = k_1[E_1]_o + k_{-1} + k_2$, $B = k_1 k_2 [E_1]_o$

$\lambda_3 = (C + \sqrt{C^2 - 4D})/2$, $\lambda_4 = (C - \sqrt{C^2 - 4D})/2$

$C = k_3[E_2]_o + k_{-3} + k_4$, $D = k_3 k_4 [E_2]_o$

Under these conditions there is no steady-state, $[S_p]_t$ rises from zero in a complicated exponential fashion to a final concentration $[S_1]_o$. As a consequence the evaluation of k_1, k_{-1} and k_2 must proceed in a less direct fashion than described above even when values of k_3, k_{-3} and k_4 have been obtained from separate studies on E_2 alone. A suggested procedure is based on the observation that in general $\lambda_1 >> \lambda_2$ and $\lambda_2 \approx k_2[E_1]_o/([E_1]_o + K_{E_1})$. A plot of $1/\lambda_2$ versus $1/[E_1]_o$ would lead to values of k_2 and K_{E_1} and hence with the use of equation (AII-7) a complete solution. λ_2 corresponding to a particular $[E_1]_o$ must be obtained by fitting equation (AII-7) to the experimental results by a reiterative procedure.

It may be concluded from the above illustrations that the application of Laplace-Carson transforms provides a powerful means of obtaining mathematical descriptions of the time dependent formation of final product in a coupled assay, which apply to both pre-steady- and steady-states.

Indeed it is a relatively simple matter to extend the analysis to mechanisms more complicated than those proposed. Kuchel and Roberts (1974) for example have treated systems where each enzyme forms two enzyme-substrate complexes before final product is released. It will suffice, in illustrating the breadth of the approach to give a final example which relates to the Section in Chapter IV concerning the possiblity of heterogeneous association between the two enzymes involved in a coupled assay. This work has not been published and is presented here to stress that both steady-state kinetic data (Chapter IV) and pre-steady-state information may be used to explore the possibility of such an interaction. The scheme considered is again equation (II-1) with the additional reaction

$$E_1 + E_2 \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} E_1E_2$$

being considered. Several possibilities arise but for brevity, and consistency with Chapter IV, it will be taken that the catalytic activity of E_2 is unchanged in the complex-state while only uncomplexed E_1 possesses catalytic activity. The basic set of rate equations is written as before, account being taken of terms involving k_5 and k_{-5}, and their Laplace-Carson transforms written with the assumption that $[S_1]$ = constant and $[E_2]$ = constant. Solution of these equations, and subsequent inversion gives,

$$[S_p]_t = \frac{V_{E_1}[S_1]_o\, t}{K_{E_1}\left(1 + \frac{[E_2]_o k_5}{k_{-5}}\right) + [S_1]_o}$$

$$+ \sum_{i=1}^{4} \left(\prod_{j=1}^{4} k_j \right) [E_1]_o [E_2]_o [S_1]_o \left\{ \frac{(\lambda_i - k_{-5})(1 - e^{-\lambda_i t})}{\lambda_i^2 \prod_{\substack{j=1 \\ j \neq i}}^{4} (\lambda_j - \lambda_i)} \right\} \qquad \text{(AII - 8)}$$

It is seen from equation (AII-8) that at relatively long times when the second term approaches zero a plot of $[S_p]$ versus t approaches linearity, but with a slope smaller than v_o due to the $(1 + [E_2]_o k_5/k_{-5})$ term of K_{E_1} in the denominator of the first term. In other words, in this time domain, E_2 by its heterogeneous association with E_1 is effectively acting as a competitive inhibitor, with the inhibition constant k_{-5}/k_5 being identified as the equilibrium constant governing the E_1E_2 dissociation. Equation (AII-8) therefore may provide a basis for experimentally detecting associations between enzymes when both may be studied separately as well as in combination, and a potential means of evaluating the individual rate constants (although the fitting procedure will become more difficult as the number of parameters increases).

A final general observation is relevant. One consistent theme of this work has been the attempt to write $[S_p]_t$ as an explicit function of time. It is clear that application of the Laplace-Carson transform method achieves this, in the form of closed solutions, such as equations (AII-5), (AII-7) and (AII-8). This type of solution is clearly

superior to solutions obtained either by numerical integration (Chapter V), or in open form by use of the Maclaurin polynomial (Chapters II and IV). However, it is stressed that the closed solutions can only be written when certain assumptions are made concerning the constancy of quantities which strictly should be treated as variables. These assumptions will be reasonable in certain situations encountered both *in vivo* and *in vitro* but quite unreasonable in other circumstances. It follows that each type of solution will in all probability find its particular use in solving a broad spectrum of problems encountered in the study of diverse sequences of enzyme catalyzed reactions.

BIBLIOGRAPHY

Adair, G.S. (1925) *J. Biol. Chem. 63*, 529.

Adams, E.T., and Fujita, H. (1963) in *"Ultracentrifugal Analysis in Theory and Experiment"*, *Williams, J.W., Ed., Academic Press, New York*, p. 119.

Alberty, R.A., Massey, V., Frieden, C., and Fuhlbrigge, A.R. (1954) *J. Am. Chem. Soc. 76*, 2485.

Allan, J.D., Cusworth, D.C., Dent, C.E., and Wilson, V.K. (1958) *Lancet 1*, 182.

Andrews, P. (1966) *Br. Med. Bull. 22*, 109.

Archibald, W.J. (1942) *Ann. N.Y. Acad. Sci. 43*, 211.

Bach, S.J. (1946) *Nature 158*, 376.

Bach, S.J., Crook, E.M., and Williamson, S. (1944) *Biochem. J. 38*, 325.

Bach, S.J., and Killip, J.D. (1958) *Biochim. Biophys. Acta 29*, 273.

Bach, S.J., and Killip, J.D. (1961) *Biochim. Biophys. Acta 47*, 336.

Baghurst, P.A. (1972) *Ph.D. Thesis. Australian National University.*

Baghurst, P.A., Nichol, L.W., and Sawyer, W.H. (1972) *J. Biol. Chem. 247*, 3199.

Baldwin, E. (1936) *Biol. Rev. 11*, 247.

Baldwin, R.L. (1957) *Biochem. J. 65*, 503.

Barwell, C.J., and Hess, B. (1970) *Z. Physiol. Chem. 351*, 1531.

Bates, D.J., and Frieden, C. (1973) *J. Biol. Chem. 248*, 7878.

Bergmeyer, H-U. (1953) *Biochem. Z. 324*, 408.

Bier, M. (1962) in *"Methods in Enzymology"*, *Colowick, S.P., and Kaplan, N.O., Eds., Vol. V, Academic Press, New York*, p. 33.

Blakeley, R.L., Webb, E.C., and Zerner, B. (1969) *Biochemistry 8*, 1984.

Bloomfield, V., Dalton, W.O., and Van Holde, K.E. (1967) *Biopolymers 5*, 135.

Bray, R.C., and Ratner, S. (1971) *Arch. Biochem. Biophys. 146*, 531.

Briggs, G.E., and Haldane, J.B.S. (1925) *Biochem. J. 19*, 338.

Brown, G.W., and Cohen, P.P. (1960) *Biochem. J. 75*, 82.

Cann, J.R. (1970) *"Interacting Macromolecules"*, *Academic Press, New York.*

Capellos, C., and Bielski, B.H.J. (1972) *"Kinetic systems"*, *Wiley-Interscience, New York.*

Carlisky, N.J., Sadnik, I.L., and Menendez, J.L. (1972) *Comp. Biochem. Physiol. 42B*, 81.

Casassa, E.F., and Eisenberg, H. (1964) *Adv. Protein Chem. 19*, 287.

Ceriotti, G., and Spandrio, L. (1963) *Clin. Chem. Acta 8*, 295.

Changeux, J.-P., and Rubin, M.M. (1968) *Biochemistry 7*, 533.

Cleland, W.W. (1963a) *Biochim. Biophys. Acta 67*, 104.

Cleland, W.W. (1963b) *Biochim. Biophys. Acta 67*, 173.

Cleland, W.W. (1963c) *Biochim. Biophys. Acta 67*, 188.

Clementi, A. (1914) *Atti Accad. Lincei 23*, 612.

Clementi, A. (1919) *Atti Accad. Lincei 27*, 299.

Cohen, P.P., and Brown, G.W. (1960) in *"Comparative Biochemistry"*, *Florkin, M. and Mason, H.S., Eds., Vol. II, Academic Press, New York*, p. 161.

Cohen, P.P., and Marshall, M. (1962) in *"The Enzymes", 2nd ed., Boyer, P.D., Lardy, H., and Myrbäck, K., Eds., Vol. 6, Academic Press, New York*, p. 327.

Cohen, R., and Mire, M. (1971a) *Eur. J. Biochem. 23*, 267.

Cohen, R., and Mire, M. (1971b) *Eur. J. Biochem. 23*, 276.

Cohn, E.J., and Edsall, J.T. (1943) *"Proteins, Amino Acids and Peptides", Reinhold, New York.*

Colombo, J.P., Bürgi, W., Richterich, R., and Rossi, E. (1967) *Metab. Clin. Exp. 16*, 910.

Colombo, J.P., Terheggen, H.G., Lowenthal, A., Van Sande, M., and Rogers, S. (1973) in *"Inborn Errors of Metabolism", Hommes, F.A., and Van Den Berg, C.J., Eds., Academic Press, New York*, p. 239.

Conway, E.J. (1939) *"Micro-Diffusion Analysis and Volumetric Error", Crosby Lockwood and Son Ltd., London.*

Conway, E.J. (1947) *"Microdiffusion Analysis and Volumetric Error", Revised ed., Crosby Lockwood and Son Ltd., London.*

Cook, R.A., and Koshland, D.E. (1970) *Biochemistry 9*, 3337.

Cox, D.J. (1969) *Arch. Biochem. Biophys. 129*, 106.

Crank, J. (1970) *"The Mathematics of Diffusion", Clarendon Press, Oxford.*

Creeth, J.M., and Nichol, L.W. (1960) *Biochem. J. 77*, 230.

Curtis, A.R., and Chance, E.M. (1972) in *"Analysis and Simulation of Biochemical Systems", FEBS VIIITH Meeting Proceedings, Hemker, H.C., and Hess, B., Eds., Vol. 25, North Holland Publishers, Amsterdam*, p. 39.

Dalziel, K. (1968) *FEBS Letts. 1*, 346.

Davies, D.V., and Davies, F. (1961) *"Gray's Anatomy"* *33rd ed.*, *Longmans, Green and Co. Ltd., London*, p. 1481.

Davis, B.J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404.

Davtyan, M.A., and Bunyatyan, G.K. (1970) *Biokhimiya 35*, 341. (Eng. trnsl.)

Dawson, R.M.C., Elliott, D.C., Elliott, W.H., and Jones, K.M. (1969) *"Data for Biochemical Research"*, *2nd ed.*, *Clarendon Press, Oxford*, p. 475.

Dixon, M., and Webb, E.C. (1958) *"Enzymes"*, *Academic Press, New York*, p. 118.

Eadie, G.S. (1942) *J. Biol. Chem. 146*, 85.

Easterby, J.S. (1973) *Biochim. Biophys. Acta 293*, 552.

Edlbacher, S. (1917) *Z. Physiol. Chem. 100*, 111.

Edmond, E., Farquhar, S., Dunstone, J.R., and Ogston, A.G. (1968) *Biochem. J. 108*, 755.

Einstein, A. (1905) *Ann. Physik 17*, 549.

Englander, S.W., and Crowe, D. (1965) *Analyt. Biochem. 12*, 579.

Fahien, L.A., and Smith, S.E. (1974) *J. Biol. Chem. 249*, 2696.

Farron, F. (1973) *Analyt. Biochem. 53*, 264.

Faxén, H. (1929) *Arkiv. Mat. Astron. Fysik 21B*, (3).

Fearon, W.R. (1939) *Biochem. J. 33*, 902.

Folley, S.J., and Greenbaum, A.L. (1946) *Biochem. J. 40*, 46.

Foster, R.J., and Niemann, C. (1953) *Proc. Nat. Acad. Sci. U.S.A. 39*, 999.

Frieden, C. (1967) *J. Biol. Chem. 242*, 4045.

Frieden, C. (1971) *Ann. Rev. Biochem. 40*, 653.

Frieden, C., and Colman, R.F. (1967) *J. Biol. Chem. 242*, 1705.

Fuchs, B. (1921) *Z. Physiol. Chem. 114*, 101.

Fujita, H. (1956) *J. Chem. Phys. 24*, 1084.

Fujita, H. (1959) *J. Phys. Chem. 63*, 1092.

Fujita, H. (1962) *"The Mathematical Theory of Sedimentation Analysis", Academic Press, New York.*

Garfinkel, D., Garfinkel, L., Pring, M., Green, S.B., and Chance, B. (1970) *Ann. Rev. Biochem. 39*, 473.

Garfinkel, D., and Hess, B. (1964) *J. Biol. Chem. 239*, 971.

Gear, C.W. (1969) *Inf. Process. 68*, 187.

Gilbert, G.A. (1955) *Disc. Far. Soc. 20*, 68.

Gilbert, G.A. (1959) *Proc. R. Soc. A250*, 377.

Gilbert, G.A. (1966) *Nature 210*, 299.

Gilbert, L.M., and Gilbert, G.A. (1973) in *"Methods in Enzymology", Hirs, C.H.W., and Timasheff, S.N., Eds., Vol. XXVII, part D, Enzyme Structure. Academic Press, New York*, p. 273.

Gilbert, G.A., and Jenkins, R.C.L. (1959) *Proc. R. Soc. A253*, 420.

Gilbert, G.A., and Kellet, G.L. (1971) *J. Biol. Chem. 246*, 6079.

Glass, R.D., and Knox, W.E. (1973) *J. Biol. Chem. 248*, 5785.

Goldberg, R.J. (1953) *J. Phys. Chem. 57*, 194.

Gosting, L.J. (1956) *Adv. Protein Chem. XI*, 429.

Grassmann, W., Hormann, H., and Janowsky, O. (1958) *Z. Physiol. Chem. 312*, 273.

Greenberg, D.M. (1951) in *"The Enzymes" 1st ed., Sumner, J.B., and Myrbäck, K. Eds., Vol. 1, Academic Press, New York*, p. 893.

Greenberg, D.M. (1960) in *"The Enzymes", 2nd ed., Boyer, P.D., Lardy, H., and Myrbäck, K., Eds., Vol. 4, Academic Press, New York*, p. 257.

Greenberg, D.M., Bagot, A.E., and Roholt, O.A. (1956) *Arch. Biochem. Biophys. 62*, 446.

Gutfreund, H. (1955) *Disc. Far. Soc. 20*, 167.

Hamilton, P.D. (1965) *Nature 205*, 284.

Harell, D., and Sokolovsky, M. (1972) *Eur. J. Biochem. 25*, 102.

Havir, E.A., Tamir, H., Ratner, S., and Warner, C. (1965) *J. Biol. Chem. 240*, 3079.

Hearon, J.Z. (1949a) *Bull. Math. Biophys. 11*, 29.

Hearon, J.Z. (1949b) *Bull. Math. Biophys. 11*, 83.

Hearon, J.Z. (1950a) *Bull. Math. Biophys. 12*, 57.

Hearon, J.Z. (1950b) *Bull. Math. Biophys. 12*, 85.

Hearon, J.Z. (1953) *Bull. Math. Biophys. 15*, 121.

Heinrich, R., and Rapoport, T.A. (1974a) *Eur. J. Biochem. 42*, 89.

Heinrich, R., and Rapoport, T.A. (1974b) *Eur. J. Biochem. 42*, 97.

Hellerman, L., and Perkins, M.E. (1935) *J. Biol. Chem. 112*, 175.

Hess, B., and Boiteux, A. (1972) in *"Protein-Protein Interactions", 23. Colloquium der Gesellschaft für Biologische Chemie, 13.-15. April 1972 in Mosbach/Baden. Jaenicke, R., and Helmreich, E., Eds., Springer Verlag, Berlin*, p. 271.

Hess, B., and Wurster, B. (1970) *FEBS Letts. 9*, 73.

Hexner, P.E., Radford, L.E., and Beams, J.W. (1961) *Proc. Nat. Acad. Sci. U.S.A. 47*, 1848.

Hillebrand, W.F., and Lundell, G.E.F. (1953) *"Applied Inorganic Analysis", Wiley, New York*, p. 790.

Hirsch-Kolb, H., and Greenberg, D.M. (1968) *J. Biol. Chem. 243*, 6123.

Hirsch-Kolb, H., Kolb, H.J., and Greenberg, D.M. (1971) *J. Biol. Chem. 246*, 395.

Hirsch-Kolb, H., Heine, J.P., Kolb, H.J., and Greenberg, D.M. (1970) *Comp. Biochem. Physiol. 37*, 345.

Hofstee, B.H.J. (1952) *Science 116*, 329.

Hommes, F.A. (1962) *Arch. Biochem. Biophys. 96*, 28.

Hommes, F.A., Degroot, C.J., Wilmink, C.W., and Jonxis, J.H.P. (1969) *Arch. Dis. Childhood 44*, 688.

Howlett, G.J., and Nichol, L.W. (1972a) *J. Phys. Chem. 76*, 2740.

Howlett, G.J., and Nichol, L.W. (1972b) *J. Biol. Chem. 247*, 5681.

Hunter, A., and Dauphinee, J.A. (1924) *Proc. R. Soc.*B97 , 209.

Hunter, A., and Dauphinee, J.A. (1929) *J. Biol. Chem. 85*, 627.

Hunter, A., and Downs, C.E. (1944) *J. Biol. Chem. 155*, 173.

Hunter, A., and Pettigrew, J.B. (1936) *Enzymologia 1*, 341.

Illingworth, J.A., and Tipton, K.F. (1969) *Biochem. J. 115*, 511.

Jeffrey, P.D., and Pont, M.J. (1969) *Biochemistry 8*, 4597.

Johnson, P. (1946) *Trans. Far. Soc. 42*, 28.

Johnston, J.P., and Ogston, A.G. (1946) *Trans. Far. Soc. 42*, 789.

Kacser, H., and Burns, J.A. (1973) in *"Rate Control of Biological Processes", Symposia of the Society for Experimental Biology, No. XXVII, Cambridge University Press, Cambridge*, p. 65.

Kalb, A.J., and Levitzki, A. (1968) *Biochem. J. 109*, 669.

Kasserra, H.P., and Laidler, K.J. (1970) *Can. J. Chem. 48*, 1793.

Katchalski, E., Silman, I., and Goldman, R. (1971) *Adv. Enzymol. 34*, 445.

Kauzmann, W. (1959) *Adv. Protein Chem. XIV*, 1.

Kekomäki, M., Räihä, N.C.R., and Peerheentupa, J. (1967) *Acta Paediat. Scand. 56*, 631.

King, E.L., and Altman, C. (1956) *J. Phys. Chem. 60*, 1375.

Kirschner, K. (1968) *Curr. Topics Microbiol. Immunol. 44*, 123.

Kirschner, K., and Wiskocil, R. (1972) in *"Protein-Protein Interactions", 23. Colloquium der Gesellschaft für Biologische Chemie, 13.-15. April 1972 in Mosbach/Baden. Jaenicke, R., and Helmreich, E., Eds., Springer Verlag, Berlin*, p. 245.

Kistiakowsky, G.B., Mangelsdorf, P.C., Rosenberg, A.J., and Shaw, W.H.R. (1952) *J. Am. Chem. Soc. 74*, 5015.

Kistiakowsky, G.B., and Rosenberg, A.J. (1952) *J. Am. Chem. Soc. 74*, 5020.

Klotz, I.M. (1946) *Arch. Biochem. Biophys. 9*, 109.

Klotz, I.M. (1953) in *"The Proteins", 1st ed., Neurath, H., and Bailey, K., Eds., Vol. IB, Academic Press, New York*, p. 727.

Koch, G.L.E., Shaw, D.C., and Gibson, F. (1971) *Biochim. Biophys. Acta 229*, 795.

Koch, G.L.E., Shaw, D.C., and Gibson, F. (1972) *Biochim. Biophys. Acta 258*, 719.

Koshland, D.E., and Neet, K.E. (1968) *Ann. Rev. Biochem. 37*, 359.

Koshland, D.E., Némethy, G., and Filmer, D. (1966) *Biochemistry 5*, 365.

Kossel, A., and Dakin, H.D. (1904a) *Z. Physiol. Chem. 41*, 321.

Kossel, A., and Dakin, H.D. (1904b) *Z. Physiol. Chem. 42*, 181.

Krebs, H.A., Hems, R., and Lund, P. (1973) *Adv. Enz. Regulation 11*, 361.

Krebs, H.A., and Henseleit, K. (1932) *Z. Physiol. Chem. 210*, 33.

Kuchel, P.W., and Roberts, D.V. (1974) *Biochim. Biophys. Acta 364*, 181.

Kupke, D.W. (1960) *Adv. Protein Chem. 15*, 57.

Laidler, K.J. (1955) *Can. J. Chem. 33*, 1614.

Laidler, K.J., and Bunting, P.S. (1973) *"The Chemical Kinetics of Enzyme Action", 2nd ed., Clarendon Press, Oxford*, p. 73.

Lamm, O. (1929) *Arkiv. Mat. Astron. Fysik 21B*, (2).

Laurent, T.C., and Killander, J. (1964) *J. Chromatog. 14*, 317.

Levin, B. (1971) *Adv. Clin. Chem. 14*, 65.

London, W.P. (1966) *J. Biol. Chem. 241*, 3008.

Long, C.L. (1961) *"Biochemists' Handbook", Spon Ltd., London*, p. 679.

Longsworth, L.G. (1959) in *"Electrophoresis, Theory, Methods and Applications", M. Bier, Ed., Academic Press, New York*, p. 91.

Lusty, C.J., and Ratner, S. (1972) *J. Biol. Chem. 247*, 7010.

Lynen, F. (1964) in *"New Perspectives in Biology", Sela, M., Ed., B.B.A. Library Vol. 4., Elsevier, Amsterdam*, p. 132.

Lynn, K.R. (1967) *Biochim. Biophys. Acta 146*, 205.

McClure, W.R. (1969) *Biochemistry 8*, 2782.

McCracken, D.D., and Dorn, W.S. (1964) *"Numerical Methods and Fortran Programming", Wiley, New York*, p. 330.

Maclaurin, C. (1742) *"Fluxions"*.

McKenzie, H.A., and Murphy, W.H. (1970) in *"Milk Proteins, Chemistry and Molecular Biology", McKenzie, H.A., Ed., Vol. 1, Academic Press, New York*, p. 157.

McPhie, P. (1971) in *"Methods in Enzymology", Jakoby, W.B., Ed., Vol. XXII, Academic Press, New York*, p. 23.

Mamiya, G., and Gorin, G. (1965) *Biochim. Biophys. Acta 105*, 382.

Marshall, M., and Cohen, P.P. (1972a) *J. Biol. Chem. 247*, 1641.

Marshall, M., and Cohen, P.P. (1972b) *J. Biol. Chem. 247*, 1654.

Marshall, M., and Cohen, P.P. (1972c) *J. Biol. Chem. 247*, 1669.

Masters, C.J., Sheedy, R.J., Winzor, D.J.,and Nichol, L.W. (1969) *Biochem. J. 112*, 806.

Mepham, T.B., and Linzell, J.L. (1966) *Biochem. J. 101*, 76.

Mepham, T.B., and Linzell, J.L. (1967) *Nature 214*, 507.

Messenguy, F., Penninckx, M., and Wiame, J.-M. (1971) *Eur. J. Biochem. 22*, 277.

Michaelis, L., and Menten, M.L. (1913) *Biochem. Z. 49*, 333.

Miller, A.L., and McLean, P. (1967) *Clin. Sci. 32*, 385.

Mohamed, M.S. (1949) *Acta. Chem. Scand. 3*, 988.

Mohamed, M.S., and Greenberg, D.M. (1945) *Arch. Biochem. Biophys. 8*, 349.

Monod, J., Wyman, J.,and Changeux, J.-P. (1965) *J. Mol. Biol. 12*, 88.

Morrow, G., Barness, L.A., and Efron, M.L. (1967) *Pediatrics 40*, 565.

Newmeyer, D. (1957) *J. Gen. Microbiol. 16*, 449.

Nichol, L.W., Bethune, J.L., Kegeles, G., and Hess, E.L. (1964) in *"The Proteins"*, *Neurath, H., Ed., Vol. II, Academic Press, New York*, p. 305.

Nichol, L.W., and Creeth, J.M. (1963) *Biochim. Biophys. Acta 71*, 509.

Nichol, L.W., Jackson, W.J.H., and Winzor, D.J. (1967) *Biochemistry 6*, 2449.

Nichol, L.W., Jackson, W.J.H., and Winzor, D.J. (1972) *Biochemistry 11*, 585.

Nichol, L.W., Janado, M., and Winzor, D.J. (1973) *Biochem. J. 133*, 15.

Nichol, L.W., and Ogston, A.G. (1965a) *Proc. R. Soc. B163*, 343.

Nichol, L.W., and Ogston, A.G. (1965b) *J. Phys. Chem. 69*, 4365.

Nichol, L.W., Ogston, A.G., and Preston, B.N. (1967) *Biochem. J. 102*, 407.

Nichol, L.W., Ogston, A.G., and Rescigno, A. (1970) *Proc. R. Soc. A317*, 153.

Nichol, L.W., Ogston, A.G., and Winzor, D.J. (1967) *J. Phys. Chem. 71*, 726.

Nichol, L.W., and Roy, A.B. (1965) *Biochemistry 4*, 386.

Nichol, L.W., Smith, G.D., and Ogston, A.G. (1969) *Biochim. Biophys. Acta 184*, 1.

Nichol, L.W., Smith, G.D., and Winzor, D.J. (1969) *Nature 222*, 174.

Nichol, L.W., and Winzor, D.J. (1972) *"Migration of Interacting Systems", Clarendon Press, Oxford.*

Nuzum, C.T., and Snodgrass, P.J. (1971) *Science 172*, 1042.

Oncley, J.L. (1941) *Ann. N.Y. Acad. Sci. 41*, 121.

Perrin, F. (1936) *J. Phys. Radium 7*, 1.

Plowman, K.M. (1972) *"Enzyme Kinetics", McGraw-Hill, New York.*

Porembska, Z., Jachimowicz, J., and Gasiorowska, I. (1971) *Bull. Acad. Pol. Sci. 19*, 27.

Racker, E. (1950) *Biochim. Biophys. Acta 4*, 211.

Rapoport, T.A., Heinrich, R., Jacobasch, G., and Rapoport, S. (1974) *Eur. J. Biochem. 42*, 107.

Ratner, S. (1957) in *"Methods in Enzymology", Colowick, S.P., and Kaplan, N.O., Eds., Vol. III, Academic Press, New York*, p, 643.

Ratner, S. (1970) in *"Methods in Enzymology", Tabor, H., and Tabor, C.W., Eds., Vol. XVIIA, Academic Press, New York*, p. 304.

Ratner, S. (1972) in *"The Enzymes", 3rd ed., Boyer, P.D., Ed., Vol. VII, Academic Press, New York*, p. 167.

Ratner, S. (1973) *Adv. Enzymol. 39*, 1.

Ratner, S., Anslow, W.P., and Petrack, B. (1953) *J. Biol. Chem. 204*, 115.

Ratner, S., and Kunkemueller, M. (1966) *Biochemistry 5*, 1821.

Ratner, S., and Petrack, B. (1953) *J. Biol. Chem. 200*, 175.

Ratner, S., Petrack, B., and Rochovansky, O. (1953) *J. Biol. Chem. 204*, 95.

Reddy, S.R.R., and Campbell, J.W. (1970) *Comp. Biochem. Physiol. 32*, 499.

Reich, J.G., and Sel'kov, E.E. (1974) *FEBS Letts. 40 Suppl.*, S119.

Reithel, F.J. (1971) in *"The Enzymes", 3rd ed., Boyer, P.D., Ed., Vol. IV, Academic Press, New York*, p. 1.

Ricard, J., Mouttet, C., and Nari, J. (1974) *Eur. J. Biochem. 41*, 479.

Richards, M.M., and Hellerman, L. (1940) *J. Biol. Chem. 134*, 237.

Richards, E.G., Teller, D.C., and Schachman, H.K. (1968) *Biochemistry 7*, 1054.

Richet, C. (1894) *Compt. Rend. Soc. Biol. 46*, 525.

Richet, C. (1897) *Compt. Rend. Soc. Biol. 49*, 743.

Roche, J., Thoai, N., and Verrier, J.-M. (1953) *Compt. Rend. 236*, 143.

Rochovansky, O., and Ratner, S. (1967) *J. Biol. Chem. 242*, 3839.

Rodiguin, N.M., and Rodiguina, E.N. (1964) in *"Consecutive Chemical Reactions : Mathematical Analysis and Development", Sneider, R.F., Ed., Engl. ed ., Van Nostrand, Princeton.*

Rogers, S. (1959) *Nature 183*, 1815.

Rogers, S. (1964) in *"Control of Cell Division and the Induction of Cancer", Nat. Cancer Inst. Monograph, 14*, p. 107.

Rosenberg, H., Ennor, A.H., and Morrison, J.F. (1956) *Biochem. J. 63*, 153.

Sakai, T., and Murachi, T. (1969) *Physiol. Chem. Phys. 1*, 317.

Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660.

Schachman, H.K.(1959) *"Ultracentrifugation in Biochemistry", Academic Press, New York.*

Schimke, R.T. (1964) *J. Biol. Chem. 239*, 3808.

Schimke, R.T. (1973) *Adv. Enzymol. 37*, 135.

Schulze, I.T., Lusty, C.J., and Ratner, S. (1970) *J. Biol. Chem. 245*, 4534.

Schwert, G.W. (1949) *J. Biol. Chem. 179*, 655.

Shih, V.E., and Efron, M.L. (1972) in *"The Metabolic Basis of Inherited Disease"*, *3rd ed., Stanbury, J.B., Wyngaarden, J.B., and Fredrickson, D.S., Eds., McGraw-Hill, New York*, p. 370.

Smith, I. (1958) *"Chromatographic Techniques : Clinical and Biochemical Applications"*, *Heinemann, London*, p. 152.

Sober, H.A. (1968) *"Handbook of Biochemistry"*, *The Chemical Rubber Co., Cleveland, Ohio*. p. B55.

Sophianopoulos, A.J. (1969) *J. Biol. Chem. 244*, 3188.

Stacey, K.A. (1956) *"Light-Scattering in Physical Chemistry"*, *Butterworths, London*.

Stadtman, E.R. (1970) in *"The Enzymes"*, *3rd ed., Boyer, P.D., Ed., Vol. I, Academic Press, New York*, p. 397.

Steiner, R.F. (1952) *Arch. Biochem. Biophys. 39*, 333.

Stokes, G.G. (1856) *Trans. Cambridge Phil. Soc. 9, Part II*, 8.

Svedberg, T. (1925) *Kolloid - 2. 36, Erg. - Bd.*, 53.

Svedberg, T., and Pedersen, K.O. (1940) *"The Ultracentrifuge,"* *Clarendon Press, Oxford*.

Sweeney, J.R., and Fisher, J.R. (1968) *Biochemistry 7*, 561.

Tedesco, T.A., and Mellman, W.J. (1967) *Proc. Nat. Acad. Sci. U.S.A. 57*, 829.

Teipel, J., and Koshland, D.E. (1969) *Biochemistry 8*, 4656.

Teller, D.C., Horbett, T.A., Richards, E.G., and Schachman, H.K. (1969) *Ann. N.Y. Acad. Sci. 164*, 66.

Terheggen, H.G., Schwenk, A., Lowenthal, A., Van Sande, M., and Colombo, J.P. (1969) *Lancet 2*, 748.

Thompson, C.B. (1948) *Experientia, 4*, 432.

Tiselius, A. (1930) *Nova Acta R. Soc. Scient. Upsal. (4) 7, No. 4*, 1.

Tschudy, D.P., and Bonkowsky, H.L. (1972) *Fedn. Proc. Fedn. Am. Socs. Exp. Biol. 31*, 147.

Van Holde, K.E. (1960) *J. Phys. Chem. 64*, 1582.

Van Holde, K.E. (1967) *"Sedimentation Equilibrium", Fractions No. 1 Bechman Instruments, Inc., Palo Alto, Calif.*

Van Slyke, D.D., and Archibald, R.M. (1942) *Fedn. Proc. Fedn. Am. Socs. Exp. Biol. 1*, 139.

Van Slyke, D.D. and Archibald, R.M. (1946) *J. Biol. Chem. 165*, 293.

Vassent, G. (1974) *J. Theoret. Biol. 44*, 241.

Vegotsky, A., and Frieden, E. (1958) *Enzymologia 19*, 143.

Vielle-Breitburd, F., and Orth, G. (1972) *J. Biol. Chem. 247*, 1227.

Vesterberg, O., and Svensson, H. (1966) *Acta Chem. Scand. 20*, 820.

Waley, S.G. (1964) *Biochem. J. 91*, 514.

Walker, J.B. (1953) *J. Biol. Chem. 204*, 139.

Wall, M.C., and Laidler, K.J. (1953) *Arch. Biochem. Biophys. 43*, 299.

Walter, C. (1966) *J. Theoret. Biol. 11*, 181.

Walter, C., and Morales, M.F. (1964) *J. Biol. Chem. 239*, 1277.

Ward, R.L., and Srere, P.A. (1967) *Anal. Biochem. 18*, 102.

Wiel, L., and Russell, M.A. (1934) *J. Biol. Chem. 106*, 505.

Winzor, D.J. (1966) *Biochem. J. 101*, 30C.

Winzor, D.J., Loke, J.P., and Nichol, L.W. (1967) *J. Phys. Chem. 71*, 4492.

Winzor, D.J., and Nichol, L.W. (1965) *Biochim. Biophys. Acta 104*, 1.

Winzor, D.J., and Scheraga, H.A. (1963) *Biochemistry 2*, 1263.

Wong, J.T-F. (1965) *J. Am. Chem. Soc. 87*, 1788.

Wurster, B., and Hess, B. (1970) *Z. Physiol. Chem. 351*, 1537.

Wyman, J. (1964) *Adv. Protein Chem. 19*, 223.

Yphantis, D.A. (1964) *Biochemistry 3*, 297.

LIST OF PUBLICATIONS

Sections of the following publications were used in this work:

1. "Steady State Kinetics of Consecutive Enzyme Catalysed Reactions Involving Single Substrates : Procedures for the Interpretation of Coupled Assays".
   Kuchel, P.W., Jeffrey, P.D., and Nichol, L.W. (1974) *J. Theoret. Biol.* (in press).
2. "Interpretation of Coupled Enzymic Assays. Studies on Consecutive Reactions Catalysed by Arginase and Urease".
   Kuchel, P.W., Jeffrey, P.D., and Nichol, L.W. (1974) *Proc. Aust. Biochem. Soc.*, *7*, 18.
3. "The Detection and Consequences of Association of Two Enzymes Involved in Catalysing Consecutive Reactions".
   Nichol, L.W., Kuchel, P.W., and Jeffrey, P.D. (1974) *Biophys. Chem.* (in press).
4. "Active Site Directed Effectors of Allosteric Enzymes".
   Smith, G.D., Roberts, D.V., and Kuchel, P.W. (1974) *Biochim. Biophys. Acta* (in press).
5. "The Behaviour of Coupled Enzyme Systems in the Transient and Steady-State Regions of the Reaction".
   Kuchel, P.W., and Roberts, D.V. (1974) *Biochim. Biophys. Acta* *364*, 181.