3/1974

THE AUSTRALIAN NATIONAL UNIVERSITY

RESEARCH SCHOOL OF BIOLOGICAL SCIENCES

DEPARTMENT OF GENETICS

ANNUAL REPORT 1973

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RESEARCH WORK

The Department of Genetics is mainly interested in studying processes of adaptation and organic evolution by using the combined approaches of genetics and molecular biology. Experimental organisms range from viruses and bacteria to fish, monotremes and mammals and require a wide variety of techniques and apparatus. Genetical methods of mutational dissection, recombination and segregation are used in conjunction with biochemical analysis to study microbial evolution but these methods are difficult to apply to higher diploid organisms. In the latter, special attention is paid to the comparative properties of enzymes of related function, to the population distribution of natural protein variation and to assessments of genetic relatedness by nucleic acid hybridization.

Advances in microbial evolution include the discovery of a complex interaction between genes under natural selection and apparently quite unrelated genes, the demonstration that the majority of newly-arisen mutations have little or no selective disadvantage and a comprehensive model for the origin of mitochondrial mutants in yeast which are deficient in respiration. In the higher organisms, studies of the enzymes of deep-sea fish have shown how they are adaptively modified to withstand high pressures, considerable progress has been made in defining the genetic structure of wild rabbit and marsupial populations and the amino-acid sequences of cytochrome C molecules are being determined to define the evolutionary status of these unique organisms. Further experiments on the transfer of genes from bacteria to the cells of higher organisms have been particularly concerned with the possibility of generating plant cells capable of nitrogen fixation. To this end, attempts are being made to establish benign associations between nitrogen-fixing bacteria and cultured plant cells and to construct in bacteria a self-reproducing genetic system carrying the nitrogen-fixing genes.

1. Evolutionary Adaptation of Enzymes

(a) Catalytic efficiencies of enzymes from ectothermic and endothermic animals (J. Baldwin)

As temperature has a marked effect on the activities and structure of enzymes, the question arises as to how homologous enzymes are adapted to function under the different thermal environments present in different organisms.

Previous studies in this laboratory have been concerned with the effects of temperature on formation of the enzyme-substrate complex and the ability of thermally directed changes in enzyme substrate affinity to stabilize reaction rates over the body temperature range normally experienced by ectothermic animals.

Present investigations deal with the next stage in catalysis: activation of the enzyme-substrate complex. To form the activated enzyme-substrate complex, free energy (ΔG) must be added to the complex, and the magnitude of ΔG can be thought of as the energy

barrier to the reaction. By significantly reducing ΔG values, enzymes enable metabolic reactions to occur at satisfactory rates at biological temperatures. Thus it would seem advantageous if enzymes from cold adapted organisms could reduce the ΔG characteristics of their reactions to a lower level than those of warm adapted species. To test this hypothesis, M_4 lactate dehydrogenase isoenzymes have been purified from a range of animals experiencing different body temperatures and their ΔG values determined. Preliminary results show a positive correlation between ΔG and body temperature, and further, differences in ΔH and ΔS (enthalpy and entropy terms respectively) for the homologous enzymes can be accounted for on the basis of different amounts of weak bond participation in the formation of the activated enzyme-substrate complex.

(b) Regulation of Metabolic Pathways during Thermal Acclimation in fish: (J. Baldwin)

In warm adapted fish, utilization of glucose-6-phosphate (G-6-P) by the pentose shunt is low, probably accounting for less than five per cent of G-6-P metabolized. However following cold acclimation, the percentage participation of the pentose shunt increases as part of an overall reorganization of metabolism. One major role for increased shunt activity is the production of increased amounts of NADPH required for the elevated synthesis of fatty acids in the cold acclimated state. To gain some insight into control mechanisms underlying this change in shunt participation catalytic and regulatory properties of shunt enzymes from warm and cold acclimated rainbow trout are being studied.

(c) Changes in testis specific lactate dehydrogenase isoenzymes during the seasonal spermatogenic cycle of the marsupial *Schoinobates volans* (greater glider) (J. Baldwin, P. Temple-Smith[1] and C. Tideman[1])

The somatic tissues of vertebrates usually contain up to five electrophoretically distinguishable lactate dehydrogenase (LDH) isoenzymes produced by the random association of two subunit types (A and B) to form the tetramers B_4, B_3A, B_2A_2, BA_3 and A_4. An additional LDH isoenzyme (C_4), containing a third subunit type (C) produced at a genetic locus separate from those of the A and B subunits, occurs in sperm and testes of birds and both marsupial and eutherian mammals. Usually only a single C_4 isoenzyme is present, the association of C subunits with A and B subunits to form multiple testis specific isoenzymes being prevented by switching off of A and B subunit production during synthesis of the C subunit.

We have found a similar correlation between the occurrence of testis specific LDH isoenzymes and cyclic spermatogenesis in a marsupial, the greater glider. However, this species differs from most in possessing four testis specific LDH isoenzymes, two of which appear at the onset of spermatogenesis, the other two appearing during regression of the testis at the end of spermatogenesis. An analysis of these testis specific isoenzymes, using kinetic,

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immunochemical and electrophoretic techniques, has shown the subunit compositions of the two initial bands to be C_4 and C_3A, while the regression bands appear to be C-B subunit hybrids. The presence of the C_3A isoenzyme presumably arises from the synthesis of small amounts of the A subunit concurrently with C subunit synthesis, while the appearance of CB hybrids during regression of the testis can be explained in terms of a switching on of B subunit synthesis before production of C subunits has ceased.

(d) Adaptation of enzymes to high pressures: (P.W. Hochachka[2] J. Baldwin and K. Storey[2])

The basic action of pressure on various metabolic processes is described by the relationship:

$$\Delta V = 2.3RT \frac{\log k_{p1} - \log k_{p2}}{p2 - p1}$$

where ΔV is the volume change of activation $\frac{\text{(volume of activated complex)}}{\text{(volume of reactants)}}$ R the gas constant and K the velocity constant at pressure p1 and p2.

Unlike temperature, which affects all chemical reactions in the same way (by altering the kinetic energy of the reactants), the effect of pressure on reaction rate depends on the associated changes in volume. When the volume of the activated complex exceeds the average volume of the reactants, pressure will decrease reaction rates. At the point where the volumes are equal, there is no change in the reaction rate under pressure. When the volume of the activated complex is less than that of the reactants, pressure accelerates the reaction rate. In this latter case, the reaction can go through a pressure optimum, the pressure at which this optimum occurs often depending on temperature. Thus where two or more enzymes compete for a single metabolite, such differential effects of pressure upon reaction rates can alter the flow of material through critical branch points, and control requirements at such points would be expected to depend critically upon pressure.

Participation in the Alpha Helix Kona expedition in October-November, 1973 provided an opportunity to examine the effects of both temperature and pressure on enzymes from surface, vertically migrating and abyssal animals. The main studies were as follows:

Unusually high activities of citrate synthase in the gill of an abyssal fish.

Citrate synthase of the gill of the deep sea fish, *Antimora rostrata* was isolated and purified to examine its regulatory and catalytic properties at different temperatures and pressures. The enzyme is under feedback inhibitory control by α-ketoglutarate, guanosine triphosphate and adenosine triphosphate, but the last is probably the most important under physiological conditions because of its greater availability. Although none of these regulatory

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properties are altered by high pressure, the maximum velocity of the enzyme shows a pressure optimum at about 3,000 psi and is inhibited about 6,000 psi. The pressure sensitivity of the enzyme is reduced at high temperatures (26°C) but temperature does not affect the apparent activation energy of the reaction. The enzyme occurs in unusually high activity in the gill of this fish (about 6,500 μmoles citrate formed/min/g. fresh weight). This high activity may represent an overall high metabolic capacity of the *Antimora* gill which must sustain normal teleost osmoregulation (the chief energy-consuming work function of the gill) despite the extreme physical conditions of the environment.

Functional properties of the active site of brain acetylcholinesterase in an abyssal fish

The acetylcholinesterases of the brains of an abyssal fish (*Antimora rostrata)* and a surface species, the common mahi mahi, were extracted and compared. Although the enzyme from both species was similarly pressure-inhibited under optimal substrate conditions, the Km for substrate was decreased by pressure in the *Antimora* enzyme but increased by pressure in the enzyme of the surface fish. The basis for this difference was examined by making comparative studies with a series of competitive inhibitors. The active sites of both enzymes were found to be similar except that ligand binding of acetylcholinesterase in the abyssal species is less dependent on hydrophobic interactions than in the surface species. This finding explains the increase in substrate affinity by pressure in *Antimora* but not in mahi mahi, the difference in the inhibitor constants with temperature in the two enzymes, and the consistently higher affinity for the inhibitors tested in the *Antimora* enzyme at low temperature.

The regulatory and catalytic properties of muscle citrate synthase in a fast-swimming pelagic squid

This enzyme is unique among known animal citrate synthases. It is subject to "end-product" inhibition by α-ketoglutarate, the metabolite end-product of the first span of the Krebs cycle, the inhibition being competitive with oxaloacetate. The enzyme is also under "energy charge" control in which adenosine triphosphate or guanosine triphosphate are potent inhibitors while adenosine monophosphate is a potent deinhibitor. These three regulatory effects would be expected to supply this tissue with ample information for the control of the first step of the Krebs cycle. However, reduced nicotine adenine dinucleotide (NADH) is also a strong inhibitor. It is probable that high NADH concentrations *in vivo* would signal anoxia and this regulatory effect would shut off citrate synthase during aerobic-anaerobic transition. This interpretation is supported by the observation that squid muscle cytoplasmic malate dehydrogenase is very sensitive to nicotine adenine dinucleotide inhibition, an effect that would greatly assist in the partitioning of oxaloacetate between citrate synthase and malate dehydrogenase during aerobic-anaerobic transition in accordance with changes in redox potential.

The effects of pressure on all of the above regulatory properties of squid muscle citrate synthase were examined and all

were found to be quite pressure insensitive, as was the maximum velocity of the reaction. Thus the main switching mechanisms of the enzyme are pressure independent which would appear to be advantageous in a highly active predator undergoing substantial pressure changes during daily vertical migrations through the water column.

Further studies are being made of the effects of temperature and pressure on other enzymes of the pelagic squid. Particular attention is being given to enzymes of energy metabolism including muscle malate dehydrogenase and muscle fructose diphosphatase.

2. Ecological Genetics of the Australian Wild Rabbit.

(a) Geographical survey of variation in proteins

(B. J. Richardson, Marjorie A. Coggan[3])

As a first step in detailed studies of this animal, a survey of the geographical variation of the species throughout its mainland range has been started. So far, 1,200 rabbits have been collected from 15 different locations in the eastern States and are being examined by electrophoresis for variation in transferrin, albumin, ceruloplasm, haptoglobin, haemoglobin, iso-citrate dehydrogenase, nucleoside phosphorylase, lactate dehydrogenase, glucose phosphate isomerase, phosphoglycerokinase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, adenylate kinase, adenosine deaminase, indophenol oxidase, phosphoglucomutase and esterases. A further thirty proteins will be examined as time allows. Polymorphisms have already been found in 6-phosphogluconate dehydrogenase, adenosine deaminase, adenylate kinase and an esterase. Insufficient data are available for a geographical analysis to be undertaken at present. Samples will be collected over the next few months from sites throughout the rest of the range of the species, with the aid of CSIRO and the Victorian, South Australian and Western Australian State Departments responsible for rabbit control.

(b) Comparison of the patterns of geographical and temporal change in gene frequency in good and marginal rabbit districts (B. J. Richardson)

Populations on four properties in the Canberra area and six separate populations in the ecologically marginal Snowy Plains area of the Australian Alps are being sampled at regular intervals (1-4 times/year). The populations in each district are from 2-20 miles apart. The effect of weather combined with the different social and genetic structure of these two areas will be studied in situations where gene flow is minimal (due to rabbit proof fences and distance).

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Samples from at least twelve different populations in each of these two areas are also being collected for comparative purposes. This will allow a study of the effect of the markedly different ecology of the two areas on the genetic structure of the species. Preliminary results show that the genetic structure of the populations in these two areas is quite different. The allelic frequencies are much the same on each of the properties in the Canberra district while at Snowy Plain there has been apparently random loss of alleles in various populations, presumably due to the occasional catastrophic crashes in numbers found in this area. This may be to the advantage of the species as it allows populations with unusual gene combinations to be tested by the environment.

(c) The microgeographical and temporal distribution of alleles in a marginal and a successful population

(B. J. Richardson, Marjorie A. Coggan[3])

A detailed study of genetic variation in two tagged populations, each covering an area of several hundred acres, has been started. Behavioural and ecological studies have been carried out for many years on both populations by CSIRO Division of Wild Life research scientists, who are actively supporting this study. The first population, at Snowy Plains in Kosciusko National Park is a marginal population which suffers from occasional catastrophic crashes in numbers due to heavy snow falls. The second population, is in Urana, west of Wagga Wagga, in good rabbit country. During the present breeding season, detailed studies are being carried out on the inter-social unit distribution of alleles in each population and also a longitudinal study of the survival of kittens with different genotypes. Kittens are tagged at first capture and attempts are made to recapture all surviving kittens at approximately monthly intervals. Preliminary analysis of some of the data from the Snowy Plain population shows that the gene frequency of an allele of adenosine deaminase can change from 0.5 to 0.9 from one warren complex to the neighbouring one, separated only by social barriers.

If this year's results are satisfactory, perturbation experiments will be started next year by removing animals of particular genotypes before the onset of the breeding season.

Similar programmes will be started next year in association with the South Australian and Western Australian State Departments responsible for rabbit control. The South Australian population is in the arid zone and shows massive cyclical changes in density. The South Australian workers are studying a range of ecological and social factors in this population and these will be related to the genetic dimension of the population. In Western Australia one of the populations is derived from rabbits from a separate release from those found elsewhere in Australia.

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The other population is a normal control population. Again a genetic dimension will be added to a detailed ecological study carried out by the State Department.

(d) Characterization of the three 6-phosphogluconate dehydrogenase isoenzymes found in the wild Australian rabbit (Marjorie A. Coggan,[3] J. Baldwin, B.J. Richardson)

Three types of isoenzyme patterns are found for the dimeric protein 6-phosphogluconate dehydrogenase (6PGD) in the rabbit and their distribution fits that expected for two alleles at one locus. The two homopolymers (from homozygotes) and the heteropolymer (from the heterozygote) were isolated and characterised kinetically. There was no difference in Km (6PG) or Km (NADP) both with and without magnesium. There was no difference in the effect of temperature on Km and the energies of activation of the three proteins were identical. The pH profiles were the same as also were the specific activities. The pattern of thermal inactivation was identical in each case. Whether the alleles are selectively indistinguishable, as implied by these biochemical studies, is unknown.

The two alleles for 6PGD have been found throughout south east Australia, with the exception of the coastal regions including a site near the area of original release. If the alleles are neutral and were both brought into Australia with the original release at Geelong, it is surprising that the rare allele has not been found there. Other alternatives are (i) that the rare allele arose in Australia after the release and has not yet spread throughout the range of the species; (ii) there are selectively detectable differences between the isoenzymes and selection has secondarily removed the rare allele from the coastal populations; or (iii) it is in fact in the coastal populations but by chance has not yet been found.

3. Ecological Genetics of Marsupials

(a) Genetic divergence and phylogenetic relationships in the marsupial family Macropodidae (Kangaroo and Wallabies) (B. J. Richardson)

Previous work has shown that electrophoretically detectable variation in enzymes can be extremely useful in phylogenetic studies of the marsupials. Efforts have been made this year to collect blood and tissue samples from as wide a range of species in this group as possible. Animals from various parts of Australia and from New Guinea have been collected. When the collections are complete the relative migration rates of about fifty different enzymes will be determined. Samples, some of them rather inadequate, have been collected from 35 species of macropodid to date. The phylogenetic analysis will be carried out using the logical system proposed by Hennig.

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(b) Ecological genetics of the Wallaroo (B. J. Richardson)

The wallaroo is a shaggy, heavily-built kangaroo and is found throughout mainland Australia, usually in rough, mountainous or hilly terrain. Earlier studies have shown that the eastern subspecies (*Osphranter robustus robustus*) differs from the inland subspecies (*O.r. erubescens*) in the electrophoretic characteristics of the enzyme glucose-6-phosphate dehydrogenase (G6PD). As the species is found throughout Queensland, with *O.r. erubescens* in the west and *O.r. robustus* in the east, and as this X-linked gene shows haploid expression (due to the method of dosage compensation in marsupials), a series of samples was collected along an east-west line to examine by how much the theoretical sharp change-over point in genotype was affected by migration. This animal is normally sedenary but could travel long distances if necessary.

Samples were collected (from east to west) at Injune, Mitchell, Tambo and Charleville. The Injune and Mitchell animals were all eastern in G6PD type while the Charleville animals, with one exception, were inland in G6PD type. Two samples were shot at Tambo, one 10 miles to the west of the town and the other to the east. The western animals were all inland in G6PD type while the eastern animals, with one exception, were eastern in type. Consequently, even though these animals have a top speed in excess of 25 mph and live for up to 20 years, there was little effective gene flow for this gene between populations separated by as little as 10 miles. Clearly, lumping the animals from the two populations near Tambo together for research or commercial purposes may be misleading; e.g., both populations at Tambo are collected and treated as one by the meat packing company at Tambo.

4. Studies on Sterility

The cytogenetic causes of sterility in male heterogametic organisms have not been extensively studied, and recently some findings in Drosophila seem to be very important to sterility in organisms such as man and mouse. Some of the general rules are being studied in Drosophila and then evaluated in other systems.

(a) Developmental genetics of spermiogenesis in *Drosophila melanogaster* males (G.L.G. Miklos, W.J. Peacock[2], D.J. Goodchild[2])

This is a continuing study of chromosome mechanics during meiosis in Drosophila. A pairing-abnormal spermatid development hypothesis has been developed which relates sex chromosome pairing during meiosis to developmental lesions during spermiogenesis. This hypothesis was jointly conceived during the writing of an extensive review with Dr. J. Peacock, and it seems to be applicable to many

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organisms including man, mouse and even to the B chromosome system in grasshoppers.

Light and electron microscope studies on spermiogenesis are being performed on an attached XY stock in which this compound chromosome folds on itself during meiosis.

(b) Electron microscope studies of Drosophila sterility

(G. L. G. Miklos, R. W. Whitty[4])

One of the genotypes under study in relation to the hypothesis is the XYY male. The XYY male in Drosophila contrasts markedly in its cytogenetic behaviour to man and mouse. In humans, the extra Y chromosome is usually eliminated prior to meiosis so that primary spermatocytes are XY; however the cytological picture is a very variable one. XYY mice on the other hand are invariably sterile, with X-Y or Y-Y bivalents, XYY trivalents, or all sex chromosomes as univalents at meiosis. In Drosophila, the three sex chromosomes form a trivalent and sperm breakdown is visible in the electron microscope. XYY's are characterised by a wide spectrum of fertilities, varying from complete sterility to almost normalcy.

We are examining the same XYY male genetically as well as in the electron microscope. This project is being maintained almost solely by the electron microscope unit, which provides a very large proportion of the facilities and workload. In fact, without the enthusiasm and ready availability of the unit, this project could not be carried out owing to time pressures on other projects.

5. Developmental and Biochemical Genetics of Plants

(a) Haploid cell lines in culture (P.M. Gresshoff, C.H. Doy)

Experiments have continued with the haploid cultures of *Lycopersicon esculentum* and *Arabidopsis thaliana* that have been maintained for 2½-3 years. The only way to keep cell lines at present is to sub-culture. Many changes have been observed, mostly unfavourable, as would be expected with the accumulation of genetical and epigenetical changes. It is usually possible to revitalise cultures by adjusting the chemical and physical environment. All cultures which green on differentiation medium have been found by electron-microscopy to contain many mitochondria and chloroplasts. The finding of chloroplasts is particularly interesting when it is recalled that the cultures derive from the male gametic cell line. *L. esculentum* cultures have lost the ability to differentiate into plantlets whereas the *A. thaliana* cultures still form masses of haploid plantlets.

A haploid cell line of *Vitis vinifera* had died after a number of sub-cultures.

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4 Member of the Electron Microscope Unit

Many unfavourable changes to cultures seemed to follow the move to the new building and may be related to the various breakdown in services leading to sudden changes in the physical environment such as temperature and light.

(b) Thiamine auxotrophs of *Arabidopsis thaliana* in culture (C. H. Doy)

Diploid cultures of thiamine auxotrophs obtained from overseas have not behaved in culture as would have been expected. It is possible that the cultures were harmed during transit to Australia. Various methods have been tested for nutritional and cross-feeding experiments in culture.

(c) Spheroplast - protoplasts from *Arabidopsis thaliana* in culture (P. M. Gresshoff, C.H. Doy)

It has proved relatively simple to make protoplasts from callus cultures of *A. thaliana* using a single enzyme preparation obtained from Dr. Gamborg. Fusions of protoplasts have been readily observed without using the special methods of Cocking and co-workers. Our studies would support the view of Gamborg and co-workers that healthy protoplasts can be fused in normal nutrient medium. In some experiments the difficulty has been to prevent fusion rather than to encourage it.

(d) *Lycopersicon esculentum* ANU-H27-1 transgenosed for *Escherichia coli* gene Z (C.H. Doy)

Cultures of *L. esculentum* ANU-H27-1 transgenosed for *E. coli* gene Z (see Doy, Gresshoff and Rolfe, 1972 report) were established on lactose-supplemented differentiation medium in the light. Cultures that had not been exposed to phage-mediated gene Z transgenosis did not establish themselves under these conditions. Sub-cultures from cell-lines that had previously been shown to contain high levels of *E. coli* β-galactosidase were observed to gradually blacken and degenerate as did all cultures left in the dark. Micro-organisms could not be recovered from such cultures. Some of these losses can be attributed to difficulties associated with the change of building and to laboratory accidents. Other cultures in which the *E. coli* β-galactosidase was, at most, five times the basal level of analogous enzyme activity characteristic of the plant cells, continued to grow in the light, some greening and some not. In no examples were plantlets produced, although electron-microscopy revealed many chloroplasts. This failure to form plantlets is paralled by changes in non-transgenosed plant material. It became progressively more difficult to be certain that cultures contained *E. coli* gene Z specified β-galactosidase. A complete survey more than a year after transfer of cultures to light and medium containing lactose and growth factors promoting differentiation, shows no trace of gene Z specified enzyme, although the characteristic low levels of plant enzyme can be found. The diagnostic test is that *E. coli* gene Z specified β-galactosidase is protected against heat denaturation by its specific

antiserum but plant enzyme is not protected. It should be especially noted that these cultures grow at various rates on lactose, a property that was not characteristic of the plant cell line before transgenosis, although a few controls did grow.

Cultures maintained for up to one year on lactose-differentiation medium in the light (and 1½ years after transgenosis) grew faster when sub-cultured to glucose or sucrose medium, although greening was often better on lactose medium.

It is suggested that plant cells transgenosed for *E. coli* gene Z specified β-galactosidase are gradually eliminated from cultures maintained on lactose in the light. Survival of the cultures on lactose medium is then presumably due to genetical or epigenetical changes which have made available the lactose, although no change in the normal level of plant enzyme has been observed; or, alternatively or in addition, because of photosynthesis. The loss of all cultures kept in the dark (or light) and known to have contained high levels of *E. coli* β-galactosidase suggests that plant cells cannot sustain a high level of transgenosis of foreign genes. It should be emphasised that transgenosis of normal phage genes is likely to occur (other workers have shown this) as well as gene Z. This is undesirable, since it must divert metabolic and other resources. Evidence for the undesirable effects of exposure to phage has been provided by observing sub-cultures of *L. esculentum* ANU-H27-1 maintained on glucose - non-differentiation medium in the dark after exposure to neutral phage (phage without bacterial genes of survival value). At first, growth is like controls, but sequential sub-cultures grow ever more slowly and eventually die.

These results are consistent with transgenosis as defined in publications but show that there is no evidence of permanent beneficial genetical inheritance or phenotypic expression. A reasonable conclusion is that as the result of using normal specialised transducing phage unequivocal evidence of gene Z transgenosis was found up to 6-9 months after initiation of experiments but not thereafter. This is not unexpected, and it is suggested that development of the method depends on modifications to vector phage to prevent expression of non-required genes or the use of plasmids and other DNA similarly modified or ideally, carrying only the genes for which transgenosis is required. The ideal to aim for is permanent inheritance of an appropriate gene dosage whether nuclear or cytoplasmic with controlled expression of transgenosis maintained in balance with the natural plant cell metabolism.

(e) Novel associations between nitrogen fixing bacteria and plant cells in culture (C. H. Doy)

Work has begun on attempts to set up novel associations between naturally autonomous nitrogen fixing bacteria (such as *Klebsiella*) and plant cells. Preliminary experiments suggest that

cultures of haploid *Arabidopsis thaliana* can partly control the growth of wild-type *Klebsiella* and that further control can be achieved by the use of a bacterial auxotroph.

6. Bacterial Gene Transfer

(a) Expression of bacterial genes in human cells

(C. R. Merril[2], B.G. Rolfe[5])

Cultured human cells from a patient with a congenital lack of an enzyme necessary for the utilization of the sugar galactose can have their enzyme deficiency largely restored by infection with a bacteriophage (transducing phage) carrying bacterial genes for galactose metabolism. Bacterial gene expression occurs only when the infected cells are in mid-logarithmic phase. The properties of the restored enzyme activity are those of a hybrid enzyme containing both human and bacterial components. A selection system has been developed whereby certain human cell lines can be rescued by the infection of the galactose transducing phage.

(b) Development of a suppression system in human cells

(P. D. Cooper[6], B.G. Rolfe[5], H. Naora[7])

As part of a programme examining the introduction of bacterial genes into cells of higher organisms, the bacterial genes used to correct (suppress) certain nonsense mutations in bacteria have been introduced into human cells. Cell cultures carrying this added genetic information have been used for the plating of stocks of mutagenized polio virus. Viruses which can only grow on those cell lines which carry the added bacterial genes have been isolated and are being characterised in detail.

(c) Introduction of bacterial plasmids into plant cells

(B. G. Rolfe[5], J. Campbell[2], J. Schell[2])

This problem is being pursued as follows:

(i) Plasmid carrying the genes for nitrogen fixation

Work has begun on constructing a plasmid (a heritable genetic element) which carried all the genes necessary for the formation of an anaerobic environment, ATP biosynthesis and ability to fix nitrogen. When complete, the final plasmid DNA will be introduced into plant cells by selecting for the maintenance of the ability to fix atmospheric nitrogen.

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5 Visiting Research Worker

7 Member of the Molecular Biology Unit

6 Member of the Department of Microbiology

(ii) Drug resistance plasmids into *Agrobacterium tumefaciens*

The bacterial strain *A. tumefaciens* C58, which is pathogenic and causes the formation of tumors (crown gall) in a variety of plants, normally contains a large plasmid. Upon the loss of this plasmid the strain becomes non-pathogenic. Different drug resistant plasmids have been transferred into this cured strain to see if the tumor-inducing principle can be restored. Attempts to make a hybrid plasmid between drug resistant plasmids and the plasmid in *A. tumefaciens* are under way so that it can be more readily transferred into other strains.

7. Control of Genetic Recombination in *Neurospora crassa*
(D. G. Catcheside)

Work on the recombination genes which control the frequency of genetic recombination locally has continued. Recombination in several sectors of the region between *arg-3* and *ad-3* in linkage group I is controlled by *rec-2*$^{+}$ and *rec-3*$^{+}$. However, the section between *his-2* and *his-3* is apparently controlled by a gene not yet identified.

One *histidine-3* mutant (TM429) is apparently due to an interchange with one break within the *his-3* locus. This results in the removal of the distal part of the *his-3* locus together with distal parts of linkage group I to another chromosome. The original strain of TM429 has the gene *cog*$^{+}$ which, in the absence of *rec-2*$^{+}$ (= *rec-w*$^{+}$), normally results in six times as much allelic recombination in the *his-3* locus as when only *cog* is present. In crosses of TM429 to other *his-3* mutants which carry *cog*, there is no difference due to a substitution of *rec-2* for *rec-2*$^{+}$ if the site of the mutation is proximal to TM429, but an increase of about 10 to 15 times occurs if the mutation is distal to TM429. In crosses of TM429 to other *his-3* mutants which carry *cog*$^{+}$, the differential effect of *rec-2* and *rec-2*$^{+}$ occurs irrespective of position in the locus. The results are explicable if initiation of recombination occurs preferentially in *cog*$^{+}$ when it is present and if the initial nick in a DNA chain can migrate some distance before establishing a cross strand exchange with the homologue. Events of this kind occurring in the TM429 interchange chromosome cannot penetrate to the proximal part of the *his-3* locus.

The apparent third allele at the *rec-3* locus has been shown to be truly an allele of *rec-3* and *rec-3*$^{+}$ with a high degree of probability. Fairly exhaustive tests have failed to disclose any undoubted recombinant between *rec-3*^{L}, derived from the Lindegren A strain, and either *rec-3* or *rec-3*$^{+}$. Recombinants were sought by selecting in the *acridine-3 arginine-3* interval, about 2.5 centimorgans long, in crosses designed so that the action of the *rec-3* alleles could be measured at both the *amination-1* and the *histidine-2* loci. While the results show some variation within classes, due partly to minor genetic differences and partly to uncontrolled experimental errors, they

are quite clear. In terms of the frequencies of prototrophs formed per hundred thousand ascospores the effects are as follows:

	rec-3^{+}	*rec-3*^{L}	*rec-3*
am-1	1	8	25
His-2	7	7.5	42

There is an effect *rec-3*^{L} on recombination at the *his-2* locus. It reduces recombination when compared with *rec-3*, but if *rec-3* were not known it would be judged that *rec-3*$^{+}$ and *rec-3*^{L} had no differential effect at the *his-2* locus. The results make more likely the theory that products of the *rec* genes associate with recognition sites in or near the target loci and act to exclude an event which would initiate recombination. It is less likely that the products are regulators of enzymes which prescribe specific endonucleases.

Work is going forward on the mapping of two *amination-1* mutants which result in alterations of adjacent amino acids in the polypeptide out of which glutamic dehydrogenase is constructed. Also evidence has been found of a genetic difference in the stocks in respect of a factor expressed as strong or weak in allelic recombination at the *am-1* locus.

(S.T. Chang[5])

No evidence for any *recomination* genes affecting the *pan-2*, *his-5* and *his-7* loci has been found. None of the known genes at the *rec-1*, *rec-2* and *rec-3* loci have any effect and no other genes of differential effect have been found in a search of a considerable number of wild strains.

In the course of this work, a specific suppressor of one, out of four, alleles at the *pan-2* locus was found. It is not linked to *pan-2* and has no effect on a number of known nonsense and missense mutants at other loci.

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8. Control and Organisation of Aromatic biosynthesis in *Neurospora crassa*

(a) DAHP synthase (K.M. Ip, C.H. Doy, Dorothy M. Halsall[2])

The allosteric isoenzyme DAHP synthase (Trp) appears to exist in many forms as shown by the analytical ultracentrifuge and acrylamide gels. Some of these have been purified to homogeneity, including at least one active form of the enzyme. Kinetic experiments show that at high enzyme protein concentrations a more active polymer is formed. Catalysis proceeds *via* a ping-pong mechanism, although this may not hold at low substrate concentrations. Inhibition by L-tryptophan is competitive with respect to one substrate (phosphoenolpyruvate, PEP) and non-competitive with respect to the other (erythrose-4-phosphate, E4P). Since PEP is the first substrate to bind and E4P the second, the overall velocity of the reaction is controlled by the effort of tryptophan on the binding of PEP, the initial ligand of the reaction. Whereas PEP is essential for stabilising active DAHP synthase (tyrosine), a sulphydryl reagent is required for maintaining the stability of active DAHP synthase (tryptophan).

It may now be possible to proceed to compare the amino acid composition and peptide maps of the tyrosine and tryptophan DAHP synthetases, with a view to an assessment of their evolutionary relatedness.

(b) Purification and stability of the multienzyme complex encoded in the *arom* gene cluster of *Neurospora crassa*

(J. W. Jacobsen[2], B.A. Hart[2], C.H. Doy, N.H. Giles[2])

A method has been developed for the purification in high yield of the *arom* multi-enzyme complex from *Neurospora crassa*. The product of purification is an aggregate of approximately 230,000 molecular weight having five enzyme activities which function in the common pathway in aromatic amino acid biosynthesis. Conditions promoting dissociation of the enzyme complex have been determined. Alkaline pH values, storage in low ionic-strength buffers, and high temperature all promote dissociation. The aggregate fragments resulting from dissociation are quite labile and have not been thoroughly characterised. However, in all but one of the fragments observed 5-dehydroshikimate reductase (shikimate: $NADP^+$ oxidoreductase, EC 1.1.1.25) and 5-dehydroquinate dehydratase (5-dehydroquinate hydro-lyase, EC 4.2.1.10)

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activities are associated. These two activities have been found alone or in association with one of the three additional activities. A presumptive dimer of 3-enolpyruvylshikimic acid 5-phosphate synthetase has also been observed. To explain the variety of fragments observed, the minimum number of polypeptides present is postulated to be four. Thus, the possibility that the 115,000 molecular weight subunits of the complex are a single multifunctional polypeptide has been excluded.

9. Studies on Membrane Function (B. G. Rolfe[5], J. Campbell[2] E. H. Creaser)

This is a continuing study of the relationship between structure and function of cell membranes by investigating the influence particular proteins have on membrane associated functions. In particular, a set of bacterial membrane mutants which are no longer killed by protein antibiotics (colicins) have been studied.

(a) Mutants in which the efficiency of integration of several phages into the bacterial chromosome has been altered. The increased integration of the bacteriophage λ in one of these strains results from an over production of the phage repressor which in turn results from an anomalous RNA polymerase activity within this mutant. A large molecular weight component is absent from membrane fractions of this strain but reappears in revertants of the original mutant strain.

(b) Mutants with defective electron transport properties and oxidative phosphorylation properties have enabled an investigation to be made of the mechanisms of phage and colicin induced lysis in bacteria. Both lysing agents require a normal oxidative phosphorylation while reduced levels of intracellular cyclic AMP can inhibit phage directed lysis. These studies have been used to investigate the biological clock of phage λ

10. Natural Selection and the Evolution of Nutritional Requirements (D. Dykhuizen)

Natural selection is being studied as a phenomenon or force which can be experimentally dissected using selection for auxotrophy as a model system. The particular experimental system used up to the present time is selection for tryptophan mutants of *Escherichia coli* against wild-type in a glucose-limited chemostat with excess tryptophan.

Previous work has shown that there are three phases during an experiment. Initially there is a lag period in which the *trp*$^+$, and *trp*$^-$ strains seem to be selectively neutral. Then there is a period of selection when the selection rate for a

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particular experiment is constant and can be experimentally determined. During the final phase the ratio of the genotypes tends to fluctuate in an unpredictable manner.

The selection rate per generation depends upon the dilution rate of the chemostat and which gene is mutated, but presumably not upon the amount of tryptophan between 10δ mg/ml and 50δ mg/ml, the initial glucose concentration between 0.3 and 0.5 mg/ml, the size of the chemostat or the specific gene mutation.

This year, strains were reconstructed using a different background, but the same mutations as before. The previous results were obtained, except it seems that for the new background there is a little greater selection for any given mutation at a given dilution rate.

Also it appears that the number of generations for the initial lag is dependent upon the size of the chemostat. In the small chemostat there is a significant increase in the number of generations of this lag phase at a tryptophan concentration of 10 mg/ml. Otherwise the number of generations of the lag phase seems to be constant over different dilution rates, different mutations in different genes and different tryptophan levels.

A most surprising preliminary result found this year is that when the background of both the mutant and wild-type is made tryptophanase negative, the initial lag period disappears. Tryptophanase is a catabolic enzyme which splits tryptophan into indole and serine. The selection rate for the *trp E*$^-$ versus wild-type for a given dilution rate seems to be the same in the tryptophanase minus and the tryptophanase plus backgrounds.

11. Studies on Circular DNA

(a) Circular DNA in yeast (G. D. Clark-Walker[8], G.L.G. Miklos)

Rapid progress has been made in studies on a class of covalently closed circular DNA molecules in *Saccharomyces cerevisiae*. We have found that this yeast contains 62 molecules per cell of a class of covalently closed circular DNA termed omicron (oDNA) of buoyant density 1.701 g/cm^3 with a major size class of 1.9 µm. The localization of oDNA has been investigated in a respiratory deficient mutant $2e\rho^{-1}$ containing altered mitochondrial DNA (ρDNA) in the form of covalently closed circles. There are 81 ρDNA molecules per cell and they range in size from 0.05 µm to 1.6 µm and have a buoyant density of 1.676 g/cm^3. When this mini-circular ρDNA is used as a

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marker of cytoplasmic contamination, we find that purified nuclei do not contain oDNA. We conclude from the cytoplasmic location of oDNA that it does not represent a gene amplification product nor is it related to "informosomal" DNA. Further molecular studies are being pursued in regard to oDNA.

A study is being made of the mechanism of the petite mutation in yeast, whereby respiratory competent cells "mutate" with high frequency (~1%) to respiratory deficiency. These processes and certain aspects of mitochondrial genetics are being examined by a consideration of the mechanics and behaviour of covalently closed circular mitochondrial DNA molecules.

(b) Structure and function of plasmid DNA in *Escherichia coli* (J. Langridge, G.L.C. Miklos)

Some *E. coli* mutants produce up to seven times the quantity of β-galactosidase that is made by constitutive mutants. Preliminary equilibrium density centrifugation and electron microscope studies are being made to determine if the mechanism of this high level of enzyme production occurs by excision of a portion of the *E. coli* genome, which then cyclises, undergoes covalent closure, and acts as a template for transcription.

No covalently closed circular molecules smaller than F' factors have been found and the study has been complicated by the finding of cryptic plasmids in different strains. Reisolation of the original mutants and further centrifugation and hybridization studies are planned.

12. Genetic Approaches to Enzyme Structure (J. Langridge)

(a) Quaternary structure of β-galactosidase

An outline of the architecture of β-galactosidase in terms of the positions of substrate binding sites, the catalytic site and the folding pattern of the protein chain has already been obtained by the selection, characterization and mapping of appropriate mutants. The latest work, using similar techniques, resulted in the localization of the regions of the protein which make the contacts between inactive subunits to give the catalytically active tetrameric molecule. A model of the enzyme's structure has been proposed in which the substrate-binding sites of the enzyme are thought to be provided by the junctures between subunits of the tetramer.

(b) The mutation spectra of representative genes

It has lately been argued that many mutations are not deleterious but neutral to natural selection. This proposal has been tested by determining the mutation spectra, the frequencies of mutants with different levels of activity, of the gene for β-galactosidase and the gene for galactoside permease in *Escherichia coli*. Of the 733 amino-acid substitutions calculated to have occurred during the course of the experiments, only 11 reduced β-galactosidase activity below 50 percent. These and other data indicate that metabolic enzymes are highly buffered against the effects of mutation and that most mutations affecting such proteins are nearly neutral to natural selection under customary conditions. The general conclusion was that resistance of genes to mutation is greatest in those specifying soluble enzymes, less in those whose products must fit into a structural framework and least in those involved in protein-nucleic acid interactions.

(c) Patterns in the translation of a gene messenger

In groups of genes which are expressed via a single messenger RNA molecule, many mutations affect the translation of distal RNA regions. Studies of suppressed enzymes of the gene for β-galactosidase indicate that gradients of translation exist within the gene as well as in adjacent genes. These results have been interpreted in terms of a secondary structure to the messenger RNA.

(d) The genetic structure of the gene for galactoside permease

In order to test certain models for the organization of the galactoside permease gene, about 70 mutants of it were isolated, characterized and mapped. The data indicate that this gene consists of only one cistron coding for a single protein molecule responsible for the facilitated diffusion of substrates into the cell. A second function of the gene, that of active transport, additionally requires the products of other genes outside the lactose operon.

(e) Bacterial degradation of benzene compounds

In studies of the biological breakdown of the benzene components of anti-life compounds, certain bacteria have been isolated from the local sewage works. They have been examined for their catabolic abilities and attempts are now being made to increase the range of compounds utilized by chemical mutagenesis.

13. Protein Evolution

(a) Evolutionary studies on histidinol dehydrogenases (E. H. Creaser)

Histidinol dehydrogenase, the terminal enzyme in histidine biosynthesis is being used as a model protein to study aspects of evolutionary adaptation. In particular, the manner in which organisms adapt to sudden increases in the medium of the substrate for an essential metabolic enzyme is being studied. In *Arthrobacter histidinolovorans* and *Pseudomonas aeruginosa* such adaptation to L-histidinol is being examined. This study which is nearing completion, has shown that the two organisms have a completely different method of adapting to use histidinol. In Arthrobacter excess histidinol in the medium promotes the synthesis of an inducible enzyme with high activity towards histidinol. It has been possible to separate and partly purify both this induced enzyme and the normal biosynthetic enzyme. The two enzymes differ electrophoretically and have slightly different molecular weights. However, they have similar kinetic properties and it is possible that the induced enzyme is the result of gene duplication plus the acquisition of a control mechanism. Amino acid analysis and peptide mapping is being used to confirm this proposition.

A different adaptation occurs in Pseudomonas where a mutation occurs to change the control system of the normal biosynthetic enzyme so that it is produced in greatly enhanced amounts. The increased enzyme appears to be identical to the wild-type and the mutant does not map in the region of the structural gene for histidinol dehydrogenase.

The main problem in studying histidinol dehydrogenases has been the very low content of enzyme in the cells. For example, in Neurospora there is approximately five mg of enzyme per kilogram of mycelium and the amount recovered after purification is less than this. Therefore, organisms should be used in which the enzyme content can be increased by derepression or induction. However, in many of the organisms of interest the amount of available enzyme recoverable remains very low. Recently the use of affinity chromatography to increase yields has been investigated by Creaser, Bentley and Lindsay. The use of histamine appears promising, in that it acts as a competitive inhibitor for all histidinol dehydrogenases tested and we are presently attaching it to Sepharose via a six carbon spacer unit. Preliminary results are encouraging.

(b) Proteins from thermophilic bacteria (J. A. Lindsay, E. H. Creaser)

Extreme thermophiles are particularly interesting bacteria because they grow at temperatures which inactivate and occasionally coagulate most mesophilic enzymes and also cause the *in vitro* melting of hydrogen bonds which maintain the secondary and tertiary structures of nucleic acids. Despite several searches there have been no reports of thermally protective components in the thermophilic bacteria. From studies of the enzyme, histidinol dehydrogenase, from a range of temperature-adapted *Bacillus* species, it appears that thermostability is imparted by the amino acid sequence of the enzyme. The active site of the enzyme remains unaltered, and sequences outside the active site have no influence on catalytic activity, but only on thermostability. Transformation experiments between high and low temperature adapted *Bacillus* species are also underway, in an attempt to find a genetic component of thermostability.

(c) Cytochrome C as an evolutionary marker (E. H. Creaser, J. Baldwin, K. W. Bentley)

Studies on cytochrome c have provided very interesting results when corrections are made between changes in the primary sequence of this protein and the evolutionary relationships of the organisms containing it. To date all the work has been carried out on plants and animals from the Northern Hemisphere and it is desirable to extend the evolutionary tree of cytochrome c to distinctive organisms from Australia. Dr. M. McDowall, who is an expert in purification and sequencing of cytochrome c, spent some months in the Department helping to prepare and characterise cytochrome c from echidna and platypus. These proteins are currently being sequenced and it is proposed to sequence cytochrome c from other Australian species during the coming year.

14. Protein Biochemistry (K. W. Bentley, E. H. Creaser)

Investigations continue on the use of cobalt chelates in peptide degradation and synthesis. Peptides and proteins with a free NH_2 terminal group react with the *cis*-β-hydroxyaquo (triethylenetetramine)cobalt(III) ion at pH 8.0 and 45°C. Raising the pH to 10.5 cleaves the N-terminal bidentate amino acid cobalt complex which can be identified directly or converted to the free amino acid. The procedure takes approximately one hour with no acid hydrolysis step, and thus no destruction of labile amino acids. The method has been applied to the identification of the N-terminal amino acid of twenty small peptides and ten proteins, and to the quantitative N-terminal amino acid determination of insulin, glucagon and a range of small peptides. Cycling the process gives sequential hydrolysis from the N-terminus and eventually total hydrolysis.

Complete quantitative hydrolysis of insulin A and a hexa-peptide containing tryptophan and methionine has been observed. Unambiguous sequencing of peptides up to six residues has been achieved.

Methods for the synthesis and modification of biologically important peptides has, with few exceptions, little economic usefulness for preparations containing more than 20 aminoacyl residues. This is largely due to incomplete reactions forming prematurely truncated sequences and failure sequences. Elimination of these problems by using activated aminoacyl derivatives is being investigated.

Bidentate cobalt(III) chelation of compounds of the form $NH_2CHR-C-Y$ permits selective modification to the R group. This provides a facile method of preparing reagents useful in peptide synthesis and degradation.

The condensation of preformed amino acids into peptide linkages in a prebiological environment requires that certain stereochemical criteria be observed. This has been investigated for aspartic and glutamic acids and is at present being extended to include lysine, histidine, arginine, glutamine and asparagine.

OTHER ACTIVITIES

Professor W. Hayes, at present head of a Medical Research Council Unit and Professor in the Department of Molecular Biology of Edinburgh University, has been appointed Head of the Department of Genetics from 1 January, 1974.

Professor Catcheside was appointed President of the Botany Section of ANZAAS and gave an address on "Chemicals, radiation and heredity". Dr. Doy chaired and participated in an ANZAAS intersection symposium on transfer of genetical information. Dr. Rolfe also participated in this symposium.

Dr. Doy served as Registrar of the annual scientific meeting of the Australian Society for Microbiology and was elected Chairman of the society in the A.C.T.

Dr. Doy was appointed Chairman of the genetics section of the Gordon Research Conference on Plant Cell and Tissue Culture held in New Hampshire and was awarded a travel grant from the Australian-American Research Foundation.

Whilst overseas Dr. Doy also attended the 1973 Brookhaven Symposium and lectured at Edinburgh University, Nottingham University, The John Innes Institute, the Brookhaven National Laboratories, Yale University, Harvard University, the Connecticut Agricultural Station, Oak Ridge National Laboratory and the University of Georgia.

Dr. Doy continued as an Honorary Associate of the Department of Microbiology, University of Sydney and delivered lectures in various Australian universities and CSIRO Divisions.

Mr. Gresshoff has been awarded an Alexander von Humbolt Scholarship and left in December to work at the Hohenheim University in West Germany.

Dr. Chooi, who has been on leave from the Department since May 1972, has now resigned to continue her work at the University of Washington in Seattle.

A symposium in Barcelona on the Origin of Life and the International Congress of Biochemistry in Stockholm was attended by Dr. Creaser, the International Congress of Genetics in California was attended by Professor Catcheside, the Brookhaven Symposium was attended by Dr. Doy and the Gordon Research Conference in New Hampshire was attended by Dr. Doy and Mr. Gresshoff.

A course of lectures and practical work in Microbial Genetics was given by Dr. Rolfe to students in the School of General Studies. Dr. Doy gave special lectures to students of the Botany Department.

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