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THE AUSTRALIAN NATIONAL UNIVERSITY

RESEARCH SCHOOL OF BIOLOGICAL SCIENCES

DEPARTMENT OF GENETICS

ANNUAL REPORT 1975

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RESEARCH WORK

During the year the change in the research emphasis of the Department, foreshadowed in last year's Report, was completed. Studies of the cellular genetics of plants, especially by the methods of cytology and tissue culture, has been greatly strengthened by the arrival of Dr Subrahmanyam and Dr Gould to form the Plant Group under Dr Doy's direction. The group has initially been concerned with conditions for maintenance and propagation of a new range of plant stocks, for producing haploid lines and for obtaining new mutants for selection in transgenosis experiments. An interesting new observation, of possible importance to both eukaryotic chromosome structure and the basis of chromosome elimination in hybrids, is that certain bacterial "restriction" endonucleases induce breaks in the chromosomes of some plant species, apparently at the same points in chromatid pairs.

The decision to initiate a new programme of research on lower eukaryotes has now been implemented by the transfer of Dr Clark-Walker from the Department of Developmental Biology, and by the appointment of Dr Gresshoff and Dr Williams as Research Fellows. Dr Clark-Walker continues his work on the molecular basis of _petite_ formation in the yeast _Saccharomyces cerevisiae_, now extended by complementation studies and by the analysis of another yeast, which does not generate _petite_ mutants, in collaboration with Cesira Galeotti, an Italian-Australian Exchange Scholar. Dr Gresshoff's main interest is in the unicellular plant, _Chlamydomonas reinhardi_, in the expectation that it will illuminate problems encountered in the study of higher plants in tissue culture. He is currently isolating, defining and mapping new mutations, has developed a method of protoplast fusion, and is investigating the fate of plasmid and phage DNA genomes taken up by Chlamydomonas protoplasts. Dr Williams' speciality is the cellular slime mould, _Dictyostelium discoideum_ - an amoeba which develops differentiated and organised multicellular structures during parts of its life cycle. In addition to advancing the formal genetic analysis of this organism, he has developed new techniques for isolating diploid strains. An interesting by-product of these studies is the observation that the frequency of double recessive mutations in diploids is only about 10-fold less than that found in haploid strains. The evidence suggests that these homozygous recessive mutations arise by a single mutation followed by mitotic recombination, and not by two independent mutation events.

Other continuing work in the Department includes study of the satellite DNAs in a wallaby and in Drosophila, the control of genetic recombination in _Neurospora crassa_ and of nodulation in plants, nitrogen fixation by bacteria, the role of lysogeny and of natural selection in the evolution of bacteria, the regulation of bacterial cell division and lysogeny, and the formation of hybrid DNA molecules between a bacterial plasmid and a plant virus DNA.

1. Function and evolution of octopine dehydrogenase in molluscs (J. Baldwin)

The enzyme octopine dehydrogenase catalyses the reversible reaction: organine + pyruvate + NADH ⇌ octopine + NAD
It occurs at high concentrations in the muscles of many molluscs and because of the possibility that it may compete with other pathways for pyruvate it is assumed to play an important role in the energy metabolism of these animals. Studies with octopine dehydrogenase purified from the bivalve Pectin have shown that this enzyme is monomeric, an unusual situation among dehydrogenases and one which is of particular interest in relation to the evolution of this class of enzymes. A detailed study is being undertaken of the kinetic, regulatory and structural properties of octopine dehydrogenases isolated from a range of animals to provide insight into both energy metabolsm of mollusca, and the structural, functional and evolutionary relationships of dehydrogenases.

2. Comparative analyses of satellite DNA

A large part of the genome of most higher eukaryotes consists of highly repeated DNA sequences called 'satellite' DNA. This DNA is not transcribed into mRNA. It comprises several families of DNA all containing a simple copy repeated up to a million times. It appears to correlate with the cytologically defined heterochromatin both in quantity and chromosomal location. It biological function is unknown.

(a) Satellite DNA in Macropus rufogriseus (P. Dunsmuir, J. Peacock+)

Two distinct satellite DNAs, amounting to 25% of the total DNA, were isolated from the nuclei of the red-necked wallaby, Macropus rufogriseus. The physical properties of native, single-stranded and reassociated molecules were studied in buoyant-density gradient centrifugation. The homogeneity of each satellite fraction was examined using melting characteristics of native and reassociated DNA, and renaturation kinetics. These data sugest that sequence heterogeneity exists in both fractions. Each satellite fraction was found by in situ hybridization to be localized in heterochromatin of interphase nuclei and in the centromeric regions of metaphase chromosomes. The chromosomal distributions of the two satellite DNAs differentiate the sex chromosomes, which have sequences of only one satellite, from the autosomes which have sequences of both satellites in the centromeric heterochromatin. Giemsa C-banding techniques also showed a differentiation of the centromeric regions of sex chromosomes from those of the autosomes.

The sequence differentiation detected in each of the two satellites may be derived from a basic repeated sequence by random mutation and rearrangement as has been suggested

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for satellites in mouse or alternately, base mismatching in the renatured DNA may reflect the presence of several highly concerved isomeric forms of the repeating sequence of the satellite. Sequence-specific restriction endonucleases have revealed long order periodicity in both satellites. It has been possible to show that there is sequence differentiation between classes of DNA molecules produced by restriction enzyme cleavage of each satellite DNA.

(b) A study of satellite DNA in Drosophila simulans (A.R. Lohe, J. Peacock†)

The type of repeated satellite DNA does not seem to be conserved, even between closely related species such as D.melanogaster and D.simulans, although its highly repeated nature certainly appears to be important to the organism. The aim of the present research is to analyse the satellites of D.simulans to see whether they have particular constraints imposed upon them by evolution. A major part of the year was spent in highly purifying two D.simulans satellites, mainly by hydroxyapatite thermal chromatography. At present a detailed analysis is under way to characterise these satellites by filter and cytological hybridization. Interesting aspects include and labelling of several specific bands and tips of the polytene chromosomes in the cytological hybridization experiments.

Plant genetics group (T. Chakrabarti, C.H. Doy, A.R. Gould, H.K. Mahanty, N.C. Subrahmanyam)

This is the first year of operation of the Group and much of the work has been preliminary in terms of two major objectives: (a) transfer of genes between plants and bacteria in either direction, and (b) determination of the degree of uniqueness of genes specifying biosynthetic functions in plants. An interest in cytogenetics and chromosome elimination in inter-specific crosses has been developed due to the previous experience of Dr Gould and Dr Subrahmanyam. Particular attention has been given to the selection and characterisation of plant tissue cultures which may be used for long-term studies. A recurring theme is the use of endonucleases, particularly bacterial site restriction endonucleases, to cleave DNA. The reason for this is that these enzymes produce DNA fragments which can be recombined in vitro to form hybrid DNA molecules not normally found in nature; furthermore the possibility is being considered that restriction endonucleases participate in chromosome elimination following interspecific crosses.

3. Cytogenetic studies in the genus Hordeum

Previous research in Hordeum has been concentrated on finding the genome relationships between species. Although somatic chromosome elimination has been observed in a few interspecific hybrids for over twenty years, the significance

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and potential of this unique phenomenon for the production of haploids was not realised until recently. The main objectives of the present approach are to elucidate the distribution of chromosome elimination among interspecific hybrids, to test the possibility of producing haploids for different species, and to make use of such haploids for meiotic studies which have not been possible before.

(a) Chromosome elimination following interspecific hybridization

It is known that the chromosomes of H.bulbosum ($n = 7$) are selectively eliminated from interspecific cells from the cross H.vulgare X H.bulbosum. Based on this information, various crosses between twenty different species have been made. Seeds collected from the successful crosses have been used for cytological examination. Hybrid embryos from five out of eight crosses showed chromosome elimination. Work on these and other possible combinations is in progress.

(b) Production of haploids and hybrids

The seeds from the interspecific crosses develop for about 10-14 days and tend to abort thereafter which makes it necessary to rely on embryo culture methods to obtain seedlings. The culture methods used are similar to those used for the production of H.vulgare haploids. The seedlings thus obtained have been checked for their ploidy and morphological conformity, and have been classified accordingly as haploids or hybrids. Haploids from three crosses, haploids and hybrids from two crosses, and only hybrids from three combinations have been obtained so far. Work on other combinations is in progress.

4. Plant tissue cultures

Chromosomal variability, cell cycle kinetics, and the morphogenetic potential of the host are being assessed. Such parameters cannot be accurately determined unless very regular transfer regimes are maintained over extended periods.

(a) Characterisation of stocks

Stock suspension cultures of the species Nicotiana sylvestris, Haplopappus gracilis, and Rosa sp. (Paul's Scarlet Rose) have been intensively monitored in various passage lengths and suitable regimes for each species have been set up and rigorously maintained. Rapidly dividing, chromosomal stable cultures of Nicotiana and Rosa are now available and a transfer system in which diploid cells predominate has been developed for Haplopappus. Cultures of Acer pseudoplatanus have been maintained for comparative cell cycle work, and data on the chromosomal variability of callus cultures of Daucus carota, Eucalyptus polybractea, Chenopodium giganteum and Lysopersicon esculentum are also being collected.

(b) Cell cycles and synchrony

Feulgen microdensitometric measurements on Rosa and Nicotiana in culture, suggest that accumulation of plant cells in the G_1 phase during nitrogen-limited growth may provide a general method for the synchronisation of plant tissue cultures. Attempts to duplicate the successful synchronisation of Acer cultures by nitrogen starvation are in progress with various cultured plant cell lines. It is thought that the cell cycle phase of host cells acting as recipients of exogenous DNA or chromosomes, may be critical for the successful integration and replication of such foreign genetic material.

(c) Haploid cell lines in culture

(i) Induction and maintenance

Media recommended by others for the successful induction of callus from haploid plants of Hordeum vulgare, Petunia hybrida and Zea mays were unsatisfactory in this laboratory. A satisfactory medium was developed. Most work has been done with Hordeum vulgare for which the induced callus from haploid plants did not proliferate on subculture. Embryos of the cross H.vulgare X H.bulbosum yield a callus that can be continuously cultured. Due to elimination of H.bulbosum chromosomes the callus is monoploid (for at least six months) for H.vulgare.

(ii) Mutagenesis and selection of mutants

In general there has been a failure to isolate true auxotrophic mutants in plants. A possible reason may be the existence of gene duplication in haploids. Monoploid H.vulgare callus has been used in attempts to induce spontaneous or EMS mutants resistant to 5-methyl tryptophan (also see below).

(d) The study of carrot, soybean, wheat and Arabidopsis tissue cultures

(i) PM6 resistance and site of action (E. Gärtner, P.M. Gresshoff)

Carrot suspensions, carrot callus, and soybean callus were used to provide a higher plant comparison for the algal protoplast system. The same set of endproducts or precusors were found to reverse the herbicide toxicity in plants, callus, bacteria and algae, arguing strongly for the similarity of enzymology or control. Interestingly, reversals are more efficient in plant and bacterial cells indicating more efficient uptake systems (see Section 9d).

(ii) Differentiation and embryogenesis (P.M.Gresshoff)

Carrot and Arabidopsis callus (Columbia wild-type), both derived from protoplast culture, were differentiated to produce complete plants. Plantlet formation of Arabidopsis callus was optimal on media based on the salts of Murashige and Skoog, but containing 2-IP (2-isopentylpurine) at 5 mg/l in the presence of 0.01 mg/l NAA. This procedure also induced plantlets in other Arabidopsis races.

Carrot suspensions (WC2-91) produced embryos by auxin-withdrawal in B5 medium. Subsequent culture of embryoids on Heller-bridges resulted in viable pot grown plants. The development of a fast differentiating system is important in the study of mutational stability throughout differentiation.

5. Applications of Endonucleases

(a) Effect of Endonucleases on Plant Chromosomes

Interspecific hybrids of Hordeum and somatic cell hybrids of mammalian origin are known, in some examples, to result in chromosome degradation. Cytological investigation of such hybrids occasionally reveals chromosome degradation. In an attempt to mimic this cytologically distinguishable phenomenon, the restriction enzymes Hin dII and dIII and Eco RI, and a nonspecific endonuclease-pancreatic DNAase, were supplied to growing root tip cells of Hordeum vulgare and H.bulbosum. Microscopic examinations of the root tips after nuclease treatment revealed that in all cases chromosome cleavage and nuclear degradation had taken place. Treatments with Hin dII and dIII gave more breaks per vulgare chromosome than per bulbosum chromosome. Neither RI nor pancreatic DNAase treatments produced similar differential effects. The number of breaks induced by pancreatic DNAase was however greater than the number of breaks after Eco RI treatments.

(b) Model systems for analysis of endonucleitic chromosome cleavage (with P.M. Gresshoff)

The cleavage of barley chromosomes by restriction and other endonucleases has important implications for chromosome structure and may allow the isolation of discrete chromosome fragments. However, a more tractable system than barley root tips is needed if the phenomenon of chromosome cleavage is to be fully characterised. Two members of the Compositae, Haplopappus gracilis and Crepis capillaris have low chromosome nombers (n=2 and n=3 respectively) and large chromosomes. Rapidly dividing tissue cultures of H.gracilis are being extensively analysed in terms of nuclear cytology, and whole plants are being grown up from seed. Much effort has been put into the development of suitable cytological and protoplast isolation techniques which should now facilitate the on-going analysis of the effects of exogenously supplied nucleases on plant chromosomes. Work with C.capillaris will commence once seed is obtained.

(c) Isolation and characterisation of plant endonucleases (with K.M. Ip)

To test the possible existence of site specific endonucleases which may be involved in the selective elimination of H.bulbosum chromosomes from Triticum aestivum X H.bulbosum hybrids, wheat seedling nuclease has been isolated. This nuclease has been tested using root tip cells of H.vulgare,

H.bulbosum and T.aestivum. Preliminary results suggest that the wheat enzyme is ineffective against wheat chromosomes but cleaves the chromosomes of other species.

(d) Effects of exogenously supplied endonucleases on meiotic recombination

It has been suggested that if endogenous repair systems can maintain viability after short controlled treatments of seeds or embryos with suitable site-specific nucleases, it should be possible to generate a wide spectrum of cytogenetic stocks. Also, non-homologous exchange between chromosomes fragmented by nuclease treatment might facilitate the transfer of chromosome fragments from one species to another. Only limited success was achieved in experiments designed to test whether barley embryos can germinate after treatment with restriction endonucleases. It was therefore decided to investigate the consequences of restriction enzyme treatment with another meiotic system that can be genetically analysed.

Preliminary studies with the ascomycete fungus Sordaria fimicola demonstrate that recombination can be drastically affected by exogenously supplied endonucleases. Treatment with either pancreatic DNAase or the restriction enzymes Hin dII and dIII before crossing a black-spored strain with a tan-spored strain, results in a high frequency of non-reciprocal recombinants including those of the 5:3 and 7:1 types.

6. Isolation of plant DNA for gene transfer experiments

A number of methods of isolating DNA have been applied to material from plant tissue cultures. A relatively new method which depends on the molecular sieving of high molecular weight DNA has proven rapid, reliable and capable of yielding DNA of high purity.

7. Isolation of 6-methyl tryptophan-resistant mutants from tissue cultures

It is intended to use 5-methyl tryptophan-resistant mutants, of the class defective in feedback inhibition at anthranilate synthase, in experiments designed to test for transfer, expression and inheritance of genes reciprocally between plants and the bacterium Escherichia coli. Briefly, the presumptive transfer will first be detected on the basis of transfer of prototrophy to appropriate deletion mutants and for the acquisition of a 5-methyl tryptophan-resistant phenotype. The preferred source of resistance is plant DNA from a monoploid (hence the work with H.vulgare monoploid cultures already discussed) but, until these become available, emphasis has been placed on 5-methyl tryptophan-resistant mutants from well characterised polyploid cultures of tobacco and carrot. Presumptive 5-methyl tryptophan-resistant mutants have been obtained from N.sylvestris and carrot cultures and are presently being purified before characterisation. A fluorometric enzymic method has been developed to test for the required class of mutants.

8. Pollen as a vehicle for gene transfer

It is postulated that the time of development of the germination tube of pollen may be a useful stage for the introduction of foreign DNA. Consequently conditions for the germination of Hordeum pollen has been investigated. This is also of general interest since, for graminaceous plants, pollen of only four species have been successfully germinated in vitro and no information is available for barley. A method has been developed for the germination and pollen tube development of dihaploid pollen (72%) and monohaploid pollen (58%). Chemical factors involved are sucrose, Ca^{++} ions and EDTA.

9. Cell biology of Chlamydomonas reinhardi

A research programme initiated during 1974 in Germany was continued to explore the use of the unicellular, green alga Chlamydomonas reinhardi as a model system to study genetical, developmental and biochemical problems presently encountered in the study of higher plant cells in tissue culture, and their related uses in somatic cell genetics, mutagenosis and gene transfer (transgenosis).

The work centred mainly around a cell-wall-less mutant of C.reinhardi (CW15), which allowed many experimental approaches presently utilized in plant protoplast biology. Research was subdivided as follows:

(a) General Culture Conditions (P.M. Gresshoff, E.Gärtner)

Protoplast strain CW15 was previously grown on undefined media. The development of a defined medium for suspension and plate culture was a major prerequisite for further work, especially in the field of mutagenesis, mutant selection and gene transfer.

A simple medium comprising simple salts, trace elements and acetate as carbon source was found to provide high (90-100%) plating efficiencies and doubling times in liquid culture of 6-9 hours. Mannitol (0.1M) was added as an optimal osmotic protectant, as it resulted in higher plating efficiencies and lower DNAase activity in the extra-cellular environment.

(b) Electron Microscopy of CW15 (P.M. Gresshoff, H.K. Mahanty, K.C. Hopkinson)

The cell-wall-less state of CW15 was confirmed by electronmicroscopy. Protoplasts also showed the absence of flagella, which is thought not to be due to loss of synthetic ability but, rather, to mechanical shearing, since basal bodies and small stumps were still observable. A supportive function of the cell-wall in the maintenance of flagellar integrity is postulated.

(c) Mutagenesis and Mutant Isolation of CW15 (P.M. Gresshoff)

Owing to the limited spectrum of genetic markers in C.reinhardi and higher plants in general, investigations on the efficiency of mutagens (ultraviolet radiation, EMS

and/or MNNG) in the induction of resistances to antibiotics and nucleotide/aminoacid analogues were continued. A large number of such mutants were isolated and are being presently characterised and genetically mapped. These will provide useful genetic markers for all experiments concerned with somatic cell genetics and gene transfer.

An auxotrophic mutant selection system, based on the fact that sodium-arsenate kills growing cells in preference to non-growing ones, was refined. Reconstruction experiments using wild-type C.reinhardi and an arginine-requiring auxotroph in minimal medium containing 5 to 25 mM arsenate have shown considerable enrichment for the mutant over wild-type. As yet no new class of auxotroph (other than arginine, acetate or vitamin requiring) has been isolated. This selective screen may be of particular significance for the plant cell tissue culture system.

(d) Interaction of CW15 with a plant herbicide
(E. Gärtner, P.M. Gresshoff)

The post-emergence herbicide phosphono-methyl-glycine (PM6) kills CW15 at relatively high levels (10mM) as compared to whole plants (0.1-2mM) and cultured plant cells (1-3mM). This killing effect was partially reversable by the addition of phenylalanine, tyrosine and tryptophan together ((0-1mM), or by the former two in combination. Shikimate, the aromatic end products alone, other amino acids and a large variety of metal ions failed to show this reversal. This level of reversal was qualitatively the same as observed in E.coli HfrH, Rhizobium japonicum, carrot plants, carrot callus and suspension cultures, soybean callus culture, and duckweed, as the same precursors allowed reversal but to a more efficient level than found in CW15.

It is thought that these data indicate the general impermiability of Chlamydomones membranes to exogenously supplied amino acids, sugars and nucleotides. Comparative sutdies have failed to show any difference between the susceptibilities of CW15 and itswalled parent, thus inferring that the cellwall offers no hindrance to small molecules.

The site of PM6 action is thought to be in the phenylalanine-tyrosine branch, most likely in chorismate mutase or the control of these enzymes. Biochemical studies have been initiated, but have failed to provide conclusive results as yet (in cooperation with Mr. Kam Ming Ip).

(e) Fusion of genetically marked CW15 protoplasts
(P.M. Gresshoff)

Protoplast fusion induced by PEG (polyethylene-glycol) or calcium nitrate at high pH was observed. Using genetic resistance markers it was possible to isolate fusion products by complementation. These strains carrying both resistance markers are isolated at frequencies well above spontaneous mutation frequencies. The method provides a somatic cell system for the study of mitotic recombination

and complementation studies, both of which are very desirable for studies of plant tissue cultures. Studies with CW15 protoplasts have already begun to indicate new avenues of research.

(f) Gene transfer and presumptive expression (P.M. Gresshoff, D. Hess+)

Bacterial genes, carried on an E.coli B - derived kanamycin-resistance factor (R-factor) were transformed to, and seemingly expressed in, protoplasts of CW15-9. The addition of mannitol (0.1M) to the preculture medium was found to decrease intercellular DNase activity, most likely by preventing high degrees of cellular bursting. Kanamycin-resistant clones arose, after treatment with R-factor DNA, at frequencies three orders of magnitude higher than spontaneous mutation frequencies in controls treated with other DNA (not coding for kanamycin-resistance) or buffer.

The acquired phenotype, however, was unstable and was lost over 40 generations of culture on selective media. The data are taken to support strongly the present belief that prokaryotic genes are transferable and expressed in eukaryotic cells, but that this transfer lacks stability. An elucidation of the reasons for instability is in progress.

(g) Phage uptake and interaction with CW15 (P.M. Gresshoff, B.G. Rolfe)

Bacteriophages (λ, T4 or ϕ80) were mixed with CW15 cells and phage uptake was monitored. Significant levels of plaque forming units of phage were reisolated from the cellular pellet after successive washing and SDSlysis. The cellular location of the phage is yet unknown and tight binding to the membrane is as yet not eliminated. Possible transfer and subsequent expression of transducing phage carrying functions needed for the growth of CW15 or CW15-derived mutants has been initiated.

10. The interaction of polychlorinated biphenyls (PCB) with algae

(a) PCB and Chlamydomones reinhardi wildtype and protoplast (P.M. Gresshoff, E. Gärtner, H.K. Mahanty)

Cells grown in suspensions to which variable amounts of PCB (Aroclor 1242 - Monsanto Chem.Co.) were added, were initially strongly affected by PCB (as measured by cell number and viability), but then acquired resistance, since growth in the presence of PCB became as efficient as that of non-PCB grown controls. This "resistance" did not persist upon subculture in new PCB, but an identical cycle of sensitivity-resistance occurred. Pre-incubation of PCB and media without cells, followed by inoculation of cells at fixed time intervals, revealed that PCB disappeared from the aqueous phase within 2-3 days of shaking in the light. Chemical analysis of the site of PCB deposition (most likely the glass surfaces) has been initiated.

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Protoplasts (CW15) showed an identical sensitivity-resistance pattern, although the effective PCB level was considerably lower.

(b) PCB and Chlorella species (H.K. Mahanty, P.M. Gresshoff)

Two species of Chlorella (211/8K and 211/8B, Camb.Culture Collection) were found to be PCB resistant and sensitive respectively. The resistance of 211/8K was genetic and not of the C.reinhardi type (see (a) above). 211/8K was resistant to 1000-fold higher levels of PCB as compared with 211/8B which never acquires resistance like C.reinhardi. Electron-microscopic studies have as yet failed to provide a working morphological explanation for the observed difference. 211/8B was found to contain sporopollenin, while 211/8K did not have this cellwall component.

To clarify the resistance phenomenon of 211/8K a mixed culture of 211/8B and 211/8K in 5ppm PCB was set up. Survival of 211/8B was not affected, thus argueing that 211/8K resistance was not due to a withdrawal of PCB from the medium either by biological amplification or enzymatic breakdown.

The precise molecular mechanisms for the observed resistance of 211/8K and C.reinhardi is still open to conjecture.

11. Differentiation in Dictyostelium discoideum

The cellular slime mould Dictyostelium discoideum is a simple eukaryote which occupies the border between unicellular and multicellular organisms. Growing amoebae are solitary, but when starved they aggregate together to form a slug in which three cell types differentiate, ultimately producing a fruiting body. The overall aim of the research program is to attempt to discover the nature of cell differentiation in this organism, initially using a genetic approach. Since genetic analysis in D.discoideum is relatively new, at this stage considerable effort is being directed towards developing genetic techniques, constructing a linkage map, and examining the chromosomes.

(a) The size and proportion of fruiting bodies of D.discoideum (F.O. Stenhouse, K.L. Williams)

Early work in D.discoideum suggests that under standard conditions the proportion of spore to stalk cells in the fruiting body is relatively constant. The size of fruiting bodies can be altered by aggregating amoebae at different densities, and there are a few reports of mutants which form large or small fruiting bodies. The initial aim of this project is to isolate and characterise two classes of mutants: those with altered proportions of spore and stalk cells (pattern mutants) and those with normal proportions but with fruiting bodies whose size is altered (scale mutants). The basal disc cells will also be studied. This program requires the development of rapid techniques for counting the numbers of cells in a fruiting body and determining the numbers of each type of cell.

Fortunately the three cell types are physically separated in the slug. The anterior amoebae in the slug ultimately form stalk cells, the central and posterior amoebae form spore cells, and the amoebae at the very end form the basal disc cells. Using vital staining to identify each cell type, techniques are being developed to dissect each of the three cell types from the slug and count them.

(b) The chromosomes of D.discoideum (G.E.Robson, K.L. Williams)

In earlier studies a simple method, using Giemsa stain, was developed for examining the chromosomes of D.discoideum. A major problem was the low mitotic index of exponentially growing amoebae of D.discoideum (0-2%) and resistance to anti-mitotic agents, e.g. colchicine, vinblastine. It has been reported that aggregating amoebae of D.discoideum become synchronised. This suggested a possible way of increasing the mitotic index. Amoebae were aggregated and subsequently returned to growth medium. For about 6 hours the mitotic index is zero, then there is a sharp rise to about 6% followed by possibly two more peaks and troughs in the mitotic index, suggesting some synchrony for more than one cell division. The mitotic index of 6% is sufficiently high for studies aimed at karyotyping the seven haploid chromosomes of D.discoideum. Several Giemsa banding techniques have been tried including C banding techniques (e.g. $Ba(OH)_2$ followed by hot 2x SSC) and G banding techniques (e.g. hot 2x SSC, trypsin, Ca^{++}, Mg^{++}-free salt solutions). A karyotype based on G banding should be available shortly.

(c) Construction of a linkage map of D.discoideum (K.L. Williams, P.C. Newell+)

For ease of genetic analysis using the parasexual cycle it is important to have recessive selective markers on each arm of each linkage group. At present it is not possible to grow D.discoideum on a defined medium, so auxotrophic mutants have not been isolated. Recessive resistance mutations of several types have been isolated and these are being assigned to linkage groups.

(i) Metal ion resistance: Cd^{++} resistance. One locus cadA has been located on linkage group I. Co^{++} resistance. Final crosses are in progress to assign the cobA locus to linkage group V, VI, or VII. Several other cobalt resistant mutants are being examined.

(ii) Alcohol resistance: previous work located methanol resistance mutations at the acrA locus on linkage group II. Mutants resistant to cyclohexanol have been isolated, and the cyhA locus has been assigned to linkage group I.

(iii) Ethidium bromide resistance: Chromosomal mutations resistant to ethidium bromide have been isolated and one locus has been located on linkage group IV. A mutation which enhances resistance to ethidium bromide and acriflavin has been tentatively located on linkage group I.

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(d) New techniques for isolating diploid strains of D.discoideum (K.L. Williams)

Genetic analysis in D.discoideum is based on the parasexual cycle. One aspect of this cycle involves the formation of diploid strains resulting from the fusion of two haploid amoebae. Diploid formation is a rare event in D. discoideum (10^{-5}). Currently diploids are isolated by co-aggregating haploid amoebae of two strains each containing different recessive growth temperature-sensitive mutations, and isolating diploids at the restrictive temperature. The haploid strains die at the restrictive temperature. Future genetic analysis of differentiation in D.discoideum will involve the use of conditional (temperature-sensitive) developmental mutants. Such analysis is most cumbersome when the strains already carry growth temperature sensitive mutations. Hence ways of isolating diploid strains without using temperature-sensitive mutations have been sought. One possibility is the isolation of dominant resistance mutants. Two independently derived dominant mutants resistant to cobaltous chloride have been isolated. Diploids have been obtained between a growth temperature-sensitive cobalt resistant haploid strain and a wild type (grows at restrictive temperature) cobalt-sensitive strain, by selection at the restrictive temperature in the presence of cobaltous chloride.

(e) Mutation frequency at recessive loci in haploid and diploid strains of D.discoideum (K.L. Williams)

For studies on the control of differentiation, developmental mutations that are dominant to wild type are likely to be very important. Most developmental mutations in D. discoideum are recessive to wild type; for example only about 1% of aggregateless mutations are dominant. A possible way of enriching for dominant developmental mutations is to look for mutants in diploid rather than haploid strains. It has been assumed that isolating mutants at recessive loci in diploids would require 2 independent mutagenic events, thus the frequency of observing such mutants in diploids would be extremely low (e.g. 10^{-6} haploid, 2×10^{-12} diploid). Preliminary studies with a diploid strain suggested that several aggregateless mutants isolated were due to homozygous recessive mutations rather than dominant mutations. This suggested that mutants at recessive loci occurred at a very high frequency, close to that observed in haploids. Similar observations have been made in a variety of tissue culture systems. To investigate this puzzling result, the mutation frequency at a specific recessive locus, acrA, was determined in haploid and diploid strains of D.discoideum. The frequency of mutants at acrA in diploid strains was only about 10-fold less than that found in haploid strains. Studies using strains with flanking markers suggest that homozygous recessive mutations arise in diploid strains by a single mutation followed by mitotic recombination, rather than by two independent mutagenic events.

12. The mechanism of petite formation in the yeast - Saccharomyces cerevisiae.

This problem is under investigation from several directions.

(a) Complementation studies (G.D. Clark-Walker, G.L.G. Miklos)

It has been established that spontaneously arising respiratory deficient (petite) mutants of yeast can reform respiratory competent cells when suitable crosses are established. This process is termed complementation. Analysis of the patterns obtained from a matrix leads to the conclusion that complementation involving the rebuilding of a complete mitochondrial genome does not depend on recombination between reciprocally deleted circular molecules. This means that some mitochondrial genomes formed by complementation may contain transpositions, duplications or inversions of DNA sequences. Indeed such gene rearrangements may be the cause of the instability of some complementation diploids.

(b) Instability of complementation diploids (K. Oakley, G.D. Clark-Walker)

Some respiratory competent diploid yeasts formed by complementation between two haploid petites give rise to a larger than normal number of respiratory deficient mutants. It has been established by sporulation and ascus dissection that the instability is cytoplasmically transmitted. Furthermore when matrices are established with petites derived from haploid segregants, complementation is again observed. Some of the resulting diploid respiratory competent colonies are now stable with regard to petite production. This led to the working hypothesis that instability may be due to mitochondrial DNA containing transpositions, inversions or duplications and that the yeast mitochondrial genome can be formed in a number of different ways.

(c) Mitochondrial DNA in other petite positive yeasts (R.M. O'Connor, C.R. McArthur, G.D. Clark-Walker)

The petite mutation is not confined to the genus Sacchromyces but occurs in other yeasts. Comparative studies were therefore undertaken to test the generalization that the petite mutation arises in these yeasts as a result of peculiar structural properties of their mitochondrial DNAs. Mitochondrial DNA has been isolated and characterized from the yeast Torulopsis glabrata and found to be only a quarter the size (6 μm) of that from S.cerevisiae. Spontaneously arising petite mutants have been included in this study and have been shown to contain much smaller circular DNA molecules, similar to the situation in S.cerevisiae. The frequency of occurence of these mutants in T.glabrata is 1000-fold less than in S.cerevisiae, suggesting that the S.cerevisiae mitochondrial genome may result from two dimerizations of a basic 6 μm molecule. Such an enlarged genome may have many more sites of homology facilitating a higher rate of deletion by internal recombination and excision.

13. Mitochondrial mutants in Kluyveromyces lactis (.C.Galeotti, G.D. Clark-Walker)

Kluyveromyces lactis is a petite-negative yeast which does not form cytoplasmically inherited respiratory deficient mutants. The interesting question therefore arises as to why this yeast does not form petites. It has been decided to tackle this problem both by examining the structure of the mitochondrial DNA and by studying the postulated presence of viability genes on the mitochondrial genome for which mitochondrial mutants are being sought by selecting for resistance to erythromycin.

14. Circular DNA in leukemic patients (G.D. Clark-Walker, F. Firkin+)

Circular mitochondrial DNA from leukemic patients is known to contain abnormal forms such as tandem dimers, catenated dimers and higher oligomers. However drugs used in leukemia therapy also stimulate the formation of abnormal circular DNA forms in experimental animals. The circular DNA of leukemic patients is therefore being re-examined before drug therapy regimes are introduced. All six patients examined to date also show abnormal circular DNA forms. In addition differences have been observed between patients, one having many circular dimers while the others contain increased frequencies of catenated molecules.

15. Control of genetic recombination in Neurospora crassa (D.G. Catcheside)

Further study of the control of recombination between the loci of arginine-1 and serine-3, to which mating type is close, has disclosed that various wild strains carry factors which reduce recombination in this region. Several different factors are concerned, but it is not possible at present to determine how many, nor how they interact, nor on what particular segments they act.

It has been shown that conversion at some loci (e.g. me-2) shows an apparent reversal in polarity. It is known that alleles which manifest this phenomenon were derived from different wild strains and it is possible that a gene outside the locus itself causes the effect, as cog^{+} does at the his-3 locus. Efforts have been made to find such genes near to the me-2 and am-1 loci, but have been unsuccessful so far due to difficulties in preparing the requisite stocks. A more fruitful approach might be to derive more mutants from the wild strains thought to carry genes affecting polarity of conversion.

A by-product of work on the am-1 locus was a measurement of recombination (0.5×10^{-6} prototrophs) between two mutant sites four base pairs apart. This compares with about 5×10^{-4} for the most widely separated mutant sites in a gene that is 1356 nucleotide pairs long. Recombination between the closest sites is about a third of that expected if proportional to physical length.

\+ not a member of this University

16. Virus control of host cell metabolism and membrane functions

A microbial model system is being developed to examine how cells co-ordinate their intra-cellular physiological processes and their responses to environmental stimuli. The long term aim of this approach is to develop a "genetics of macroregulation" - the overall co-ordinated control of the biosynthesis and activity of cellular macromolecules.

(a) Lysogenic conversion of E.coli by phage lambda (B.G. Rolfe, J.H. Campbell*)

E.coli carrying the bacteriophage lambda intercalated in its chromosome normally expresses two phage genes, cI and rex. These genes modify the bacterial hosts' macro-regulation of cell division. The rex protein is closely related to host genes tol B (colicin-tolerance) and lon (cell elongation control) which maintain the orderly sequence of steps in cell division. Phage lambda uses this host macroregulatory system to regulate its own viral activities. The rex function appears to insulate this mechanism from environmental and genetic factors which otherwise would interfere with the normal life cycle of phage lambda. Our present findings show that: (i) the lambda rex function partially suppresses the tol B phenotype; (ii) the tol B cells are defective in cell division control; (iii) lambda rex activity is closely related physiologically to colicin K which disrupts the control of cell division and the energized state of the membrane; (iv) the lambda rex gene aids in lysogeny under certain conditions, particularly during anaerobic growth; (v) the rex gene aids in the normal timing of host-cell lysis of lambda infected bacteria.

(b) Control of host-cell lysis by phage lambda (B.G. Rolfe, J.H. Campbell*)

The timing of host-cell lysis by bacteriophage lambda is controlled by two separable systems. The S gene product acts as a positive effector of lysis while another protein, the lysis regulator. is an inhibitor of lysis. If the continuous function of the lysis regulator is interrupted in phage-infected cells, immediate lysis ensues. This lysis requires metabolic energy but not s gene activity. In contrast, S protein activity is stimulated by agents which uncouple and which block oxidative phosphorylation. The lysis regulator is thermolabile and several lines of evidence suggest that it involves the lambda rex gene product.

(c) Competitive advantage of the lambda lysogenic state (D. Dykhuizen, B.G. Rolfe)

Studies, using chemostats containing varying concentrations of glucose and different ratios of cell innocula, have shown that lysogens of lambda phages defective in their rex function are the preferentially selected cell population. However, under high concentrations of glucose, the normal wild-type lambda lysogen does not have the selective advantage of the lambda rex defective lysogen.

* former member

17. Nitrogen fixation and control of nodulation in leguminous plants.

The aim of this project is to increase our understanding of the regulation of nitrogen fixation in both the free-living nitrogen-fixing bacteria and in the symbiotic bacteria which fix atmospheric nitrogen within root nodules of certain plants. We also want to determine the principles involved in the successful establishment of root nodules. With this information, our long term aim is to try to make a nitrogen-fixing wheat plant.

(a) Studies on nitrogen fixation in free-living bacteria (M.L. Warren-Wilson, B.G. Rolfe)

We have previously reported that Rhizobium trifolii and certain strains of E.coli K12 can grow on nitrogen-free media, in an atmosphere devoid of ammonia, provided the environment is aerobic. However, we have had varying success in repeating the positive acetylene reduction results although growth on NFM media is consistently found. We have been able to exclude contamination as an explanation in those situations where we have successfully repeated the phenomenon. Present investigations suggest that there are crucial physiological conditions associated with positive acetylene reduction in both bacterial species.

(b) Control of nodulation in soybean plants (P.M. Gresshoff, B.G. Rolfe)

Two isogenic soybean varieties, differing only in their ability to nodulate (a Mendelian character) in rich soil were callused to provide a further tool in the investigation of the processes underlying the control of nodulation. Significant growth differences were observed when callus from nod$^+$ and nod$^-$ (nodulating and non-nodulating) plant material was grown on synthetic media containing nitrate as the sole nitrogen source. Enzymatic studies on callus as well as whole plants reinforce the biological observation that nod$^-$ plant material has the inability to utilize NO_3/NO_2 as a source of nitrogen. A model explaining these observations and their relevance to nodulation is currently being tested.

18. Natural selection in the evolution of bacteria.

(a) Ecological and genetic effects of competition between a generalist and specialist in the experimental model of E.coli in a chemostat (D. Dykhuizen, M. Davies)

The experimental model and preliminary data were described in last years annual report under the title "Selective advantage and niche dimension". The work is now finished and being prepared for publication. The results may be summarized as follows: (i) Coexistence is possible between a specialist and less efficient generalist when some food which only the generalist can use is present. In these experiments the food unique to the generalist is lactose. (ii) The equilibrium numbers of the generalist during coexistence depend upon the relative efficiencies of the specialist and the generalist and upon the percentage of the food (lactose).

(iii) There is an upper limit and a lower limit on the percentage of lactose permitting coexistence. (iv) The positions of the upper and lower limits shift as the relative efficiencies of the specialist and generalist shift. (v) A genetic change was seen in the generalist which increased its fitness on lactose, its unique resource, but decreased its fitness on the resource shared with the specialist. (vi) In one case, three genotypes coexisted on two resources, contradicting the competitive exclusion principle.

(b) The selective sequential response hypothesis
(D. Dykhuizen)

The sequential response hypothesis, postulates that if a population is placed in a new environment, the genetic changes which are most important to fitness are selected first and the others are left to later. It is assumed that the environment is constant and that the necessary genetic variability is present in the population. By analogy with tuning an engine, this hypothesis proposes that the course adjustments in fitness are done first and then, in sequence, finer and finer adjustments are made. A different hypothesis often used in population genetics models postulates that if a population is placed in a new environment, there will be concurrent selection for all gene variants which can improve fitness. The genetic load argument uses this hypothesis.

Certain predictions can be made from the sequential response hypothesis which are different from those from the alternative concurrent response hypothesis: (i) Certain allelic differences will appear neutral for a considerable period before selection starts. Experiments show that, in a glucose-limited chemostat with excess tryptophan, there is a 55 generation lag, during which the alleles are selectively neutral, before selection begins for a trp mutation (trp^-) over wild-type (trp^+). (ii) By changing the genetic background, the importance of various genetic differences may be changed. A mutation (tna) yielding a non-functional tryptophanase, an enzyme which breaks down excess tryptophan, disturbs amino-acid pool sizes and protein synthesis. When the background of the strains is made tna^-, the importance of the trp^+ - trp^- genetic difference increases and the lag disappears. (iii) By changing the environment, the sequence can be changed., Experimentally, when lactose is used instead of glucose in trp^+ - trp^- selection, against a tna^- background, a 25 generation lag is introduced. Thus in each case, the data support the new sequential response hypothesis.

19. Control of cell division and lysogeny in bacteria
(E. Elizur, S.M. Luther-Davies, W. Hayes)

Studies of thymine-requiring bacterial mutants, which elongate without cell division and die in the absence of thymine (an essential constituent of DNA), but are suppressed by recA mutations, led to the isolation of secondary mutations of B.subtilis and E.coli which are thymineless-death resistant (tdr) and divide normally under thymine deprivation (see previous report). A primary interest in a temperature-

sensitive E.coli mutation (tif) which, at 40°, results in thermal induction of λ phage and filament formation in a non-lysogenic strain, maps very close to the recA gene and is also suppressed by a recA mutation, suggested that the tdr mutations might be located in this same small region. However, technical difficulties led to postponment of this mapping project and to concentration on the tif phenomenon.

A striking feature of the tif mutation is that its expression is greatly enhanced by the presence of adenine, and it has been proposed that the tif^+ gene may act in the regulation of repair of DNA damage. The present research shows that the primary effect of the tif mutation is on translation of messenger RNA and involves the ribosomes. This was initially suggested by the new observation that in the tif mutant, streptomycin tolerance is greatly increased by prolonged incubation with adenine, a phenomenon not observed with the tif^+ control. When cultures with increased streptomycin tolerance are kept on streptomycin, the tif phenotype, namely temperature sensitivity, is suppressed. Introduction of an F-prime factor bearing the tif^+ allele abolishes this streptomycin-tolerance and all other expressions of the tif mutation, showing that the mutation is recessive.

Translation by ribosomes derived from tif^+ and tif^- cells grown under different conditions, was assayed with the following results. (i) Using either phage MS2 mRNA or poly-U as messenger, ribosomes from tif^- cells, grown at 37° in the presence of adenine, require an optimal Mg^{++} concentration 4 to 6 mM higher than similar cultures grown without adenine. (ii) Ribosomes from tif^- cells grown at 37° translate phage MS2 mRNA and poly-U messenger with only 25% and 50% the efficiency, respectively, of those derived from cells grown at 30°. (iii) In the poly-U system the expression of the tif mutation drastically reduces the misincorporation of isoleucine instead of phenylalanine into the derivative polypeptide, implying that the ribosomes are more restrictive in translation.

These findings suggest that when the tif mutation is expressed at 37° in the presence of adenine, prophage induction and inhibition of cell division in non-lysogens could occur by from a decrease in the number or activity of key regulatory molecules below a required threshold as a result of inefficient protein synthesis.

20. Genetics of thermophily (J.A. Lindsay)

Research on the molecular basis of thermophily was continued during the past year. Initial studies had shown that the property of thermophily is transferable, by DNA-mediated transformation, from B.caldolyticus to B.subtilis and had suggested linkage of the high temperature (HT) genes with the streptomycin locus, thus implying control of thermophily at the ribosomal or t-RNA level (see previous report). Further transformation experiments have since been performed using other B.subtilis recipients characterised by various markers such as temperature sensitivity and derepression of the histidine pathway, and other donors such as one of the

original B.subtilis thermophilic transformants and a rifampicin-resistant strain of B.caldolyticus. The results show (i) that B.subtilis can be transformed to thermophily at a higher frequency by the thermophilic transformant than by B.caldolyticus itself; (ii) that thermosensitive B.subtilis mutants can be transformed to thermophily; (iii) that transformation to thermophily appears to depend on more than one gene, since the transformants show considerable heterogeneity in growth rates; (iv) that the genes determining thermophily appear to be more closely linked to rifampicin resistance than to streptomycin resistance, although proper linkage studies have not yet been performed; and (v) that regulation of the histidine pathway is unaffected by transformation to thermophily. (See also the Annual Report of the Protein Biochemistry Unit.)

21. Preparation of a molecular vector for plant cells (J. Langridge)

A hybrid molecule is being prepared which is expected to multiply in both plant and bacterial cells. Its purpose is to provide a means of introducing genes into plant cells by by-passing the sexual process. The DNA sequence for replication in plants is being obtained from Cauliflower Mosaic virus, which contains double-stranded DNA with a molecular weight of 4.5×10^6 daltons. The replication sequence for bacterial cells is provided by the plasmid, RP1, of molecular weight 40×10^6, from Pseudomonas aeruginosa. The preparation of the hybrid molecule involves the use of restriction enzymes to generate self-annealing ends and of ligases to join them together covalently.

Methods based on the sieving properties of controlled pore glass have been developed for the isolation of cauliflower mosaic virus, which has been obtained in high yield from mustard plants. Antiserum to the viral coat protein has been made by Dr Dudman and a fluorescent conjugate with the purified antibodies has been prepared. The fluorescent complex will be used to identify the presence of the viral genome in plant cells. The circular DNA of cauliflower mosaic virus is cut in one place by a restriction endonuclease from Bacillus amyloliquifaciens.

The bacterial plasmid, RP1, has been transferred from Pseudomonas to Escherichia coli, where it is more readily isolated. The plasmid has been extracted and purified from the bacterial cells and tested for reaction with a range of endonucleases. The plasmid carries a gene for resistance to the "genetic misreader", kanamycin, which also inhibits plant cell division. This gene is to serve as a marker for the identification of the plasmid part of the hybrid molecule in both plant and bacterial cells.

22. DAHP synthase from Neurospora crassa (K.M. Ip, C.H. Doy)

The work reported in earlier years has continued and is now concluded. It has been shown that proteins that co-purify with DAHP synthase (Trp), the product of the aro 8 locus, are not degraded forms of this isoenzyme. The interesting possibility remains that they are naturally associated with the aro 8 gene product.

OTHER ACTIVITIES

Professor Hayes delivered the first lecture, on "Prokaryotic Genetics", of a series of lectures on Genetics organised by the JCSMR. He also gave a CSIRO/ANU Genetics Seminar on "Some aspects of the history of molecular biology" and, with Professor Catcheside, participated in a small formal symposium on "The future of genetics" to mark the opening of the new Department of Genetics building at Monash University. He chaired a symposium on "Genetic Engineering" organised by ANZAAS at the University of Melbourne, and gave courses of lectures on bacterial genetics to 3rd year Biochemistry students in the Department of Biochemistry, SGS, ANU, and in the James Cook University at Townsville.

Professor Catcheside completed a book entitled "Genetics of Recombination" for publication by Edward Arnold (Publishers) Ltd., London.

Dr Doy was on study leave for two months attending the 2nd Gordon Conference on Plant Cell and Tissue Culture, and visiting various research centres in the U.S.A. and Europe.

Dr Clark-Walker gave eleven lectures on molecular genetics to 3rd year Biochemistry students in SGS, ANU. He, Mr O'Connor and Ms Warren-Wilson read papers at the annual meeting of the Australian Biochemical Society.

Dr Rolfe spent four months on study leave, mainly working with Dr J.H. Campbell*, School of Medicine, University of California, Los Angeles. He also gave an invited paper at the 5th International Conference on "Biology of Temperate Bacteriophage" in Virginia, U.S.A., attended the Gordon Conference on Plant Cell and Tissue Culture, gave papers/seminars at the Bacteriophage Meetings, Cold Spring Harbor, N.Y. and at the Ontario Cancer Institute, Toronto, and had discussions with staff members of the A.R.C. Nitrogen-fixation Unit, University of Sussex.

While en route to ANU, Dr Gresshoff gave lectures at the A.R.C. Nitrogen-fixation Unit, University of Sussex, while Dr Williams lectured at Harvard and Brown Universities in the U.S.A.

Ms Dunsmuir and Mr Lohe read papers to the annual meeting of the Genetics Society of Australia.

Following publication of his novel research on the basis of thermophily in bacteria, Mr Lindsay was invited to read a paper at the International Conference on "Proteins from Thermophilic Microorganisms" at Zurich. He was also the recipient of a Visiting Fellowship in the Department of Molecular Biology and Biophysics at the University of Zurich to enable him to act as co-editor of a book on the conference proceedings. He was further invited to discuss his work at the University of Cambridge, England.

Early in the year Dr Baldwin left to take up the appointment of Lecturer in the Department of Zoology, Monash University.

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