STUDIES ON ENZYMES OF

PARAMECIUM CAUDATUM

by

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Dedicated to

Iran Mosadegi and

all courageous mothers

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ABSTRACT

Studies on enzymes in Paramecium caudatum

By screening nine enzymes, α-naphthyl propionate esterase, α-naphthyl butyrate esterase, malate dehydrogenase (NAD-dependent), malate dehydrogenase (NADP-dependent), aspartate amino transferase, superoxide dismutase, phosphogluco isomerase, hexokinase and acid phosphatase in 5 stocks of syngen 1, 6 stocks of syngen 3, 11 stocks of syngen 12 and 2 stocks of syngen 13, all belonging to P. caudatum, it was found that the four syngens could not be distinguished on the basis of enzymatic variation. Indeed the level of intra-syngenic variation was higher than the inter-syngenic variation. For example using a measure of similarity called the coefficient of identity a figure of 73% was recorded between stocks of syngen 3, while in inter-syngenic comparison a figure of 93% was recorded between syngen 3 and syngen 12.

Some enzymes were completely monomorphic, e.g. malate dehydrogenase (both NAD and NADP-dependent). Some were highly polymorphic, e.g. hexokinase and phosphogluco isomerase and others had a moderate degree of polymorphism, e.g. acid phosphatase. Despite the reported general polymorphism in the esterases of other organisms, e.g. Tetrahymena and Paramecium aurelia, α-naphthyl propionate esterase was showed to be completely monomorphic, and α-naphthyl butyrate esterases showed a few intra-syngenic variations.

When 87 wild stocks of P. caudatum were examined for their degree of electrophoretic variation in seven enzymes, in addition to stocks in the four known syngens - 1, 3, 12 and 13, it was revealed that some of the wild stocks could be differentiated from each other and from the stocks of these four syngens. These results indicated that there

were other syngens in nature that could be differentiated utilizing the technique of gel electrophoresis as a sole means of syngens' identification.

Mating type tests were carried out as a further means of identifying syngens. Mating with these unidentified stocks and stocks of syngen 13 were set up to determine if there was a genetic basis to the variations. The occurrence of conjugation was proven by screening two enzymes (phosphogluco isomerase and α-naphthyl propionate esterase) in matings. A conclusion from these two crosses was that these two enzymes were monomers and the variants were under genetic control. Phosphogluco isomerase was screened in the ex-conjugants between two stocks of another syngen - 3, and the enzyme was proposed to be a dimer. Again the variants were under genetic control. This enzyme was potentially useful as a marker in genetic studies.

The effect of bacteria on enzymes of Paramecium was assessed and it was concluded that bacteria in general did not modulate the patterns of enzyme activity in Paramecium. This meant that stocks of Parmecium did not need to be grown on axenic medium, a laborious and time consuming process for studies on enzymes.

The results presented in this study indicated that there was less enzymatic variation and therefore less genetic differentiation between syngens of P. caudatum than in P. aurelia and Tetrahymena. However, more stocks from nature need to be examined in P. caudatum in order to make reliable comparisons between these three protozoa.

ABBREVIATIONS

ATP	:	Adenosine 5'-triphosphate
EDTA	:	Ethylenediaminetetra-acetic acid
MTT	:	[3-(4,5-Dimethylthiozolyl-2)-2,5-diphenyltetrazolium Bromide]
NAD	:	Nicotinamide adenine dinucleotide
NADP	:	Nicotinamide adenine dinucleotide phosphate
PMS	:	Phenazine methosulfate
Tris	:	Trizma Base [Tris(hydroxymethyl)aminomethane]
Tris-HCl	:	Trizma HCl [Tris(hydroxymethyl)aminomethane Hydrochloride]
PVP	:	Polyvinyl Polypyrrolidine

CHAPTER ONE

GENERAL INTRODUCTION

The process of evolution has been studied by various methods. Palaeontological records and physiological studies have in the last few years been supplemented by studies on the evolution of macromolecules. In one approach, the amino acid sequences of single proteins such as insulin (Sanger, 1956), ribonuclease (Anfinsen et al., 1959), hemoglobin (Ingram, 1963), and cytochrome C (Margoliash, 1963), have been investigated in a variety of organisms, e.g., Man, pig, horse, rabbit, chicken and tuna. The conclusion from these studies has been that the basic DNA sequence of the particular gene has been maintained over long periods of time. However, in order to have a better understanding of the evolutionary process several genes representing a number of different proteins should be studied. Although the method of amino acid sequencing is very accurate, it is also very expensive and time consuming. This makes carrying out amino acid sequencing on hundreds of individuals for scores of proteins, impossible. Another experimental approach which has been used to demonstrate protein variation is gel electrophoresis. Electrophoretic techniques were first used by Tiselius (1937; cited by Brewer, 1970) to distinguish multiple fractions of serum proteins migrating through solutions under the influence of an electric current. This technique has been developed since then by (1) improvements in types of supporting media, including the development of the starch gel (Smithies, 1955); (2) the application of histochemical staining methods (Hunter and Market, 1957), which have allowed analysis of electrophoretic variation in enzymatic proteins; and (3) the demonstration that much of the variation was inherited in a simple mendelian fashion. However,

prior to 1963, most studies described variation in terms of single proteins. The initial studies on a large number of proteins was described by Hubby, 1963; Hubby and Throckmorton, 1965; Hubby and Lewontin, 1966; Johnson et al., 1966 and Harris, 1966, using the electrophoretic technique. Proteins are separated by electrophoresis, on the basis of their physiochemical properties. This technique is a sensitive method for detecting minor differences in overtly identical molecules. The most readily detectable differences revealed by this method are amino acid substitutions resulting in charge differences in a protein molecule. This occurs because the substituted amino acid itself carries a different charge or because the charge results in a configurational change in the molecule with consequent alteration of the overall net charge due to covering or uncovering of the ionizable groups. This method has been shown to be capable of distinguishing homozygotes not only from each other, but also from heterozygotes (Hubby and Lewontin, 1966). The different enzymatic forms produced by different alleles at the same locus have been called allozymes by Prakash, Lewontin and Hubby (1969) in order to distinguish them from the more general phenomenon of isozymes. Isozymes are different molecular forms of an enzyme arising from any cause (Market and Møller, 1959), such as binding of a single polypeptide to varying numbers of coenzyme molecules (Jocobson, 1968; Ursprung and Carlin, 1968), or other prosthetic groups (e.g. divalent metal, sialic acid, AMP, etc.); by conjugation or deletion of molecules with reactive groups such as amino, carboxyl, or hydroxyl groups of the amino acid residues of the polypeptide chain.

This technique also has some limitations: (1) it only detects variations in structural loci (i.e., loci coding for proteins) and (2) only a fraction of variations of these loci are detectable. Base

substitutions which do not change the electrophoretic mobilities of protein remain undetected. Shaw (1970) estimated that only 30% of the possible nucleotide substitutions code for amino acids with a different charge. The second limitation can be overcome by exploiting the fact that proteins with different amino acid sequences, but with identical electrophoretic mobilities, may be differentially sensitive to heat denaturation (Bernstein et al., 1973; Singh et al., 1974) or to a change in the conditions of electrophoresis (e.g., varying the pH or buffer). More recently Ramshaw, Coyne and Lewontin (1979) have applied a sequential method of electrophoresis under several conditions involving pH and gel concentrations, to a large sample of human hemoglobins with known amino acid substitutions. This method distinguished 90% of all chemically identical substitutions, when they were at different chain locations, and four out of the five chemically different substitutions at the same site, but which resulted in the same charge. They also reported that amino acid substitutions which are not on the surface of the molecules may show much less effect when they involve charge substitutions, than substitutions involving groups located on the surface. However, they may not be electrophoretically "silent". Substitutions on the surface of the molecule, or those which are partly buried, or having local interactions with chain neighbours are probably the most influential factors in determining the relative mobility of the protein. Ramshaw et al. concluded that all substitutions in these samples of hemoglobins could be distinguished electrophoretically. Specific and unpredictable local interactions may occasionally make two substitutions equivalent electrophoretically, but in general gel electrophoresis is really a powerful tool for detecting protein variations.

Using this technique, widespread enzyme polymorphisms in nature

are now known. The adaptive significance of this variation is unclear (Lewontin, 1974; Ayala, 1976). According to one theory the neutral theory, most allelic substitutions result from random fixation of selectively equivalent alleles, and polymorphism is maintained by neutral and deterious mutations (Kimura and Ohta, 1971; Nei, 1975). The alternative selectionist theory, attempts to demonstrate that there is an adaptive significance for allozymes by relating various parameters of genetic heterozygosity to environmental variations (Bryant, 1974); enzymatic functions, either with regard to substrate heterogeneity (Gillespie and Kojima, 1968; Kojima et al., 1970), or regulation of metabolism (Johnson, 1974).

The use of gel electrophoresis in species identification

The consistent conclusion from electrophoretic studies including many loci is that (1) there is a high degree of genetic similarity between conspecific* populations throughout the range of a species. These populations are nearly identical in allelic content at 85 per cent or more of their loci; and (2) the genetic similarity between different, even very closely related species, is generally much lower and more widely dispersed. Congeneric** species pairs are often completely distinct at one-fifth to four-fifths of their loci (Avise, 1974). The large biochemical differences between species make electrophoretic techniques of great value in describing and identifying members of different species. This technique especially has a great value for distinguishing sibling species which are comprised of morphologically nearly indistinguishable populations, which are reproductively isolated.

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* Belonging to the same species.

** Non-sibling species within a genus.

Sibling species of Drosophila (Hubby and Throckmorton, 1968; Nair et al., 1971; Ayala et al., 1970); Peromyscus (Smith, Selander and Johnson, 1974); Kangaroo rats (Dipodomys) (Johnson and Selander, 1971); and Cotton rats (Sigmodon) (Johnson et al., 1972), show major allelic differences in the range 20 and 50 per cent or more of their loci. Ayala and Powell (1972) found that between 19-32 per cent of loci were diagnostic for any two sibling species of Drosophila. A locus is considered to be diagnostic, if an individual can be assigned to the correct species with a probability of at least 99 per cent.

The technique of gel electrophoresis, has also been applied in both free living and parasitic protozoa, as a means of syngen or varietal identification (Tait, 1970a; Carter and Walliker, 1975; Bagster and Parr, 1973; Godfrey and Kilgour, 1976; Garden et al., 1974; Shirely, 1975).

The term "syngen" was introduced by Sonneborn (1957), for the complex groups of free living protozoa. A syngen is defined by mating groups, which constitute a mendelian population. No mating occurs between syngens. This definition persisted until means of classification were developed without the need for living references. There are 14 syngens recognized in Paramecium aurelia, 5 in P. bursaria, 16 in P. caudatum (Sonneborn, 1957) and 13 in the Tetrahymena pyriformis complex (Elliot, 1970). Many procedures were used for syngen identification (Sonneborn, 1957). These involved geographical distribution, temperature characteristics, i.e. different syngens had different ranges of temperature tolerance, cell size, mating, and serotypes. Of all the mentioned characters, the only possible way of clear identification was through mating type activity. This in itself can be very difficult since different syngens showed different optimal conditions for mating. A further complication is the existence of

asexual strains in some protozoa (e.g. Tetrahymena). This method is also very time consuming and more importantly it is undesirable to base identification upon living material which has to be maintained in laboratories and can lose its mating capability. Such material can also be lost or confused. However, this problem can be overcome by keeping stocks of ciliates for extended periods of time with minimal maintenance by freezing them in liquid nitrogen (Simon, 1971; Simon and Schneller, 1973). To overcome these sorts of problems in syngen identification the gel electrophoresis technique has been used, and it is hoped that by analysing the isozymic patterns of different syngens, it will be possible to differentiate them more conveniently, without the need for carrying out mating experiments.

Tait (1970a) examined 14 syngens of P. aurelia for the six enzymes [Isocitrate dehydrogenase, both soluble (ICDs) and mitochondrial (ICDm); Fumerase (Fum); Hydroxybutyrate dehydrogenase (HBDH); Succinate dehydrogenase (SDH), and Glutamate dehydrogenase (GDH)]. This observation led to the conclusion that if all the five enzymes were examined together (succinate dehydrogenase showed no variation in all syngens), it was possible to distinguish all the enzymes except syngens 1 and 5. Esterases have also been examined in different syngens of P. aurelia (Allen et al., 1971b). Esterases showed both intra and intersyngenic variation, to a greater extent than the enzymes examined by Tait.

It was possible by using gel electrophoresis, to determine the syngen affiliation of a particular stock with the exception of syngens 1 and 5, without resorting to the mating reaction. The status of the P. aurelia was reviewed by Sonneborn (1975) and it was decided to give species status to the various groups of this ciliata previously designated as syngens. The new names replaced the former numerical designation as follows: P. primaurelia for syngen 1, P. biaurelia for

syngen 2, P. triaurelia for syngen 3 and so on. However, to stop any further confusion with earlier reports, the organisms will be referred to here as species 1, 2, ..., 14.

Later, Tait (1978) screened phosphoglucose isomerase (PGI), and showed that a high degree of polymorphism occurred and variants within one species were often indistinguishable from variants in other species. Using this enzyme it was further possible to distinguish syngens 1 and 5. However, it was still necessary to use the mating reaction to separate these two species. This stemmed from the observation that some stocks of species 1 differed as much from each other in their enzyme patterns as they did from the patterns in stocks of species 5. This confuses the differences between stocks of species 1 and 5. Using enzyme variations without access to the mating reaction would have underestimated the number of species. The author concluded from this study that the unpredictability and irregularity of enzyme behaviour in respect to intra and inter-species variation makes the task of species identification in non-mating protozoa very difficult.

In another ciliated protozoan, Tetrahymena pyriformis, the task of syngen identification was more difficult, since (1) there were asexual i.e. non-mating, strains and (2) the mating groups were referred to as syngens (or varieties) even before they had been shown to constitute a single breeding population (Elliott and Hayes, 1955; Gruchy, 1955). Some of these groups have been given latin names (Nanney and McCoy, 1976) but are still of questionable species status. Many attempts have been made to distinguish the syngens of Tetrahymena pyriformis complex such as: geographical distribution, DNA base ratio studies, nucleic acid hybridization, and immunological tests (for review, see Nanney and McCoy, 1976), but again none were proven to be clear cut methods for syngen identification. Esterases and the acid phosphatase

were studied in different syngens of Tetrahymena by Allen and Weremiuk (1971a), and an extensive amount of intra and intersyngenic variation was reported. Borden et al. (1973a) analyzed only the classical amicronucleate strains of this species complex for eight enzymes i.e., β-hydroxybutyrate dehydrogenase, NAD-malate dehydrogenase, NADP-malate dehydrogenase, isocitrate dehydrogenase, glutamate dehydrogenase, tyrosine amino transferase, aldolase and tetrazolium oxidase. They reported that it was possible to assign most of these strains to one of four phenotypic groups called "phenosets". Validity and reproducibility of these results on amicronucleate strains was also reported by Nielsen and Andronis (1975). In the later study of micronucleate mating strains for the eight enzymes mentioned above, Borden et al. (1977) evaluated the enzyme mobility differences among the 12 then recognized syngens. They concluded that closely related syngens had a degree of similarity of 0.67; while distantly related syngens had 0.0 similarity. Nanney and McCoy (1976) also recommended that strains should be considered to belong to a given syngen (or phenoset), if they showed at least 67% similarity in their enzyme mobility for at least these eight enzymes summed together. More recently, Nanney et al. (1980) have re-examined the reliability of isozymes as characters in Tetrahymena taxonomy. The reason for this re-examination was that the syngens within Tetrahymena pyriformis complex were so divergent that it had not been possible to screen enzyme activities in all the syngens under the same conditions in the earlier studies. There had been also many cases where no enzyme activity could be detected in a syngen. In the recent study, Nanney et al. could find optimal conditions for only three out of the eight originally screened enzymes (tetrazolium oxidase, tyrosine amino transferase and isocitrate dehydrogenase) in the 15 groups of Tetrahymenas, i.e. 13 syngens and 2 phenosets. It was

reported that these three enzymes were useful diagnostic traits. The remaining enzymes were either present at very low levels and could not be detected or varied highly in concentration from syngen to syngen. They were not reliable for use as diagnostic characters. Attempts are continuing to find optimal electrophoretic and histochemical conditions for these enzymes (Nanney et al., 1980).

The general conclusion from studies on P. aurelia and T. pyriformis however, supports the use of the electrophoretic technique for detecting taxonomic variations. Electrophoretic mobilities of some isozymes have proven to be the most reliable methods for separating the species in parasitic protozoa, e.g. Trypanosomatids and Plasomdium (Gonvcalves De Lima et al., 1979; Carter, 1973; Kilgour and Godfrey, 1973; Miles et al., 1977).

The work to be reported here is concerned with similar studies in another complex group of free-living ciliates, Paramecium caudatum, which is a complex of 16 breeding groups or syngens with a world wide distribution (Sonneborn, 1957). Attempts were made (Gilman, 1941, 1956b) to distinguish syngens by differences in the optimal temperatures for mating reactivity, the geographical distribution, the sensitivity of stocks to killer strains of P. aurelia and studies of life cycle. It was impossible to identify a syngen by any of these characters except by their mating type characteristics. Cell length ranges from 180 to 280 μ (Vivier, 1974). Each cell contains one micronucleus and one macronucleus. The macronucleus, which is polygenomic, has a somatic function, disintegrates and is replaced during the sexual process, while the micronucleus is diploid and carries the genetic information throughout the sexual process. A new macronucleus develops from the micronucleus after fertilization, but a macronucleus can never produce a micronucleus.

In P. caudatum (Hiwatashi, 1959) each syngen consists of two mating types. Each individual produces one mating type substance and conjugation occurs between individuals of complementary mating types not between individuals of the same mating type. Reproduction of paramecium is by binary fission, a common process in many protozoa. It involves mitotic division of the micronucleus.

The main problem of syngen identification in P. caudatum arises from the lack of standard experimental stocks, representing different mating types in each syngen. From 16 originally distinguished syngens, stocks of 4 syngens are now available, the remainder of the standards have been lost from collections. The stocks of these 4 syngens 1, 3, 12 and 13, have all been collected from different parts of Japan by Dr. Hiwatashi and co-workers. The earlier reports (Sonneborn, 1957) also indicated that these 4 syngens were the only syngens collected from Japan and they appeared to be concentrated in warm to moderate regions. Perhaps they are the only syngens in Japan. Syngens 1 and 3 had also been found in the United States. Most of the other syngens seem to be scarce or absent in warm regions (Sonneborn, 1957).

Two other sources of P. caudatum were available, i.e. the Cambridge Culture Collection and the Ann Arbor Culture Collection. The stocks from these collections have not been assigned to any syngen.

has been reported in the literature, in P. caudatum [Lactate dehydrogenase (LDH)] (Agatsuma and Tsukii, 1980). They studied LDH isozymic activity in forty-four stocks belonging to four syngens, i.e. 12 in syngen 1; 17 in syngen 3; 10 in syngen 12 and 5 in syngen 13. With one exception in syngen 3, monomorphism was observed. Their concern in detecting lactate dehydrogenase activity was to investigate the evolution of this enzyme in protozoa in comparison with multicellular organisms. The conclusion from this study

was that LDH, in P. caudatum was a dimer. This enzyme has also been reported to be a dimer in the house fly (Agatsuma and Takeuchi, 1976). In most vertebrates lactate dehydrogenase is a tetramer and under control of two separate gene loci (Ohno et al., 1968). They suggested that these two loci arose by gene duplication from a common ancestor.

Agatsuma and Tsukii did not consider lactate dehydrogenase from the point of view of identification in P. caudatum. Their study nevertheless clearly showed that because of the monomorphism, this enzyme could not be used as a character for distinguishing syngens.

I made several collections during the study to be reported here, to supplement the known syngens of P. caudatum. These collections were made in different parts of England, Scotland and one collection from California. With these samples and also with the samples of the 4 known syngens that were supplied by Dr. Hiwatashi, I set out to study the relationships between these different isolates, using electrophoresis of enzymes and mating reactions. The aims of this study were to assess

(1) the extent of enzyme variation in the known syngens and the unknown isolates and further to determine if any of these enzymes could be used as gene markers;

(2) the usefulness of electrophoresis in syngenic identification in this organism;

(3) the evolutionary relationship of this ciliate with the other two ciliates (Tetrahymena and P. aurelia). It was reported that the differences amongst the syngens of P. aurelia were relatively fewer than those in Tetrahymena pyriformis (Borden et al., 1977). It would be interesting then to determine where P. caudatum stands in comparison with these other two protozoa.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

Media

Stocks of Paramecium caudatum were grown on bacterized medium. The bacterized medium used was modified from that used by Jones (1965). 50 g of dried grass was added to 1 litre of distilled water and autoclaved for 20 minutes at 15 lbs per square inch. This was cooled and filtered through absorbent cotton wool and then through three layers of Whatman No. 1 filter paper. 100 ml of this filtrate was made up to 1 litre with distilled water and dispensed in 1 litre amounts in Thompson bottles of 2 litre capacity. These were then autoclaved at 15 lbs per square inch for 20 minutes. This medium was allowed to cool and then inoculated with Klebsiella pneumoniae and incubated for 2 days at 37°C. At the end of this period of incubation, the pH of medium reached between 6.8 to 7.0 and the medium could be used as required.

Maintenance of paramecia

Stocks of Paramecium were maintained in test-tubes by sub-culturing biweekly in bacterized medium. To ensure that cells were grown in the presence of one species of bacterium (Klebsiella pneumoniae), two measures were taken: (1) before establishing cell lines, different stocks were washed in sterile grass medium and then transferred to bacterized medium (for the details of procedures, see page 118); (2) the feeding process was always carried out in a laminar flow unit. Stocks were kept at 20°C.

The stocks used are listed in Tables I, III, V, VII, IX.

Preparation of extracts

A "clear" test tube culture of P. caudatum was added to one litre of bacterized medium. The cultures were allowed to grow for about a week at 26°C until the cultures became "clear". At this stage most of the bacteria have been eaten by the paramecia. It is very important to use such cultures since bacteria might effect the pattern of enzyme activity in Paramecium (see Chapter Seven). The cleared cultures were filtered through six layers of cheese cloth to remove any large particles in the medium. The filtrate was then centrifuged in an oil testing centrifuge in 100 ml pear-shaped vessels at 600 g for 10 minutes. The paramecia were concentrated at the bottom of the tube. These cells were recentrifuged in a MSE bench centrifuge at 2,000 g in 10 ml conical tubes for about 10 minutes. The pellet of paramecia was taken for the experiments. About 0.5 ml of packed cells were obtained from two litres of culture. The pellet was then stored in glass sample tube at -20°C until the day of use.

The cells were disrupted by twice freeze-thawing and the disrupted cells were harvested by centrifugation at 27,000 g in a Sorvall Super Speed centrifuge for 30 minutes at 4°C. The supernatant was mixed with tracking dye, then it was transferred to capillary tubes and kept at -20°C. These samples were used after these procedures within 3-4 days. The cells were disrupted using an MSE overhead homogenizer or sonicator as an alternative to freeze-thawing. Different methods of sample preparation had no effect on the results obtained. However, because of its simplicity, freeze-thawing was used in the present study to disrupt the cells.

In the screening of enzymes both starch and acrylamide gels were used. The kind of gels used, buffering systems and the specific recipe for staining the gels were very important in detecting the activity of

enzymes. Most of the enzymes to be reported in the following studies would not be detected if any of the mentioned factors were altered.

Starch gels

12% starch* gels were used. For the method of preparation insertion of samples and staining procedures, see Rowe, 1972. Two buffer systems were used.

(A) Tris-boric acid

Gel buffer - 0.03 M boric acid-tris, pH: 7.8.

Electrode buffer - 0.3 M boric acid-tris, pH: 7.8.

(B) Tris-borate-EDTA

Gel buffer - 0.045 M tris, 0.025 M boric acid and 0.002 M EDTA, pH: 8.1.

Electrode buffer - 0.13 M tris, 0.075 M boric acid and 0.006 M EDTA, pH: 8.1.

Enzymes screened by starch gel electrophoresis

Esterases

Buffer system A was used to screen both α-naphthyl propionate esterase and α-naphthyl butyrate esterase. Electrophoresis was carried out for six hours at 300 volts (constant voltage).

(1) Stain for α-naphthyl propionate esterase

1 mM	α-Naphthyl propionate
1 mg/ml	Fast blue RR salt
100 ml	0.1 M Phosphate buffer, pH: 8.0.

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* Starch used was obtained from Connaught Medical Labs., Toronto, Canada.

(2) Stain for α-naphthyl butyrate esterase

1 mM	α-Naphthyl butyrate
1 mg/ml	Fast blue RR salt
100 ml	0.1 M Phosphate buffer, pH: 8.0.

Esterases were also well resolved with buffer (B).

(3) Phosphogluco isomerase

Buffer system B was used. Electrophoresis was carried out for five hours at 250 volts (constant voltage).

Staining method

40 mg	Fructose 6-phosphate
8 mg	NADP
8 mg	MTT
50 unit	Glucose 6-phosphate dehydrogenase
20 mg	$MgCl_2$
50 ml	0.05 M Tris-HCl, pH: 8.0.

After one hour, 1 mg PMS was added.

(4) Malate dehydrogenase

Buffer system B was used. Electrophoresis was carried out for five hours at 250 volts (constant voltage).

Staining method

40 mg	Malic acid
10 mg	MTT
10 mg	NAD
50 ml	0.05 M Tris-HCl, pH: 8.5.

After one hour, 3 mg PMS was added.

(5) Superoxide dismutase

Buffer system B was used. Electrophoresis was carried out for five hours at 250 volts (constant voltage).

Staining method

25 mg MTT

5 mg PMS

50 mg 0.05 M Tris-HCl, pH: 8.5.

(6) Aspartate aminotransferase

Buffer system B was used. Electrophoresis was carried out for five hours at 250 volts (constant voltage). Staining method as reported in Shaw and Prasad, 1970.

Slab acrylamide gels

Davis buffer was used to run the acrylamide gels (Davis, 1964).

Enzymes which were screened on acrylamide gels

(1) Malate dehydrogenase (NADP-dependent) = Malic enzyme

Electrophoresis was carried out for four hours, at 120 volts (30 mA).

Staining method

50 mg Malic acid

20 mg NADP

12 mg MTT

12.5 mg Cl_2Mg

50 ml 0.05 M Tris-HCl, pH: 8.5.

After one hour, 3 mg PMS was added.

(2) Hexokinase

Electrophoresis was carried out for four hours, at 120 volts (30 mA).

Staining method

100 mg D-L-glucose

12.5 mg NADP

12.5 mg $MgCl_2$

10 mg ATP

10 mg MTT

40 units Glucose 6-phosphate dehydrogenase

50 ml 0.05 M Tris-HCl, pH: 7.1.

After one hour, 1 mg PMS was added.

(3) Acid phosphatase

Electrophoresis was carried out for three hours, at 100 volts (25 mA).

Staining method

250 mg PVP

100 mg α-Naphthyl acid phosphatase

75 mg Fast blue BB salt

50 ml 0.125 M acetate buffer.

The gels were soaked in 0.5 M boric acid for two hours before staining.

With the exception of aspartate aminotransferase, all the gels were washed and fixed at 7.5% acetic acid. Asparate amino transferase was washed with water and fixed in glycerol. Gels were stored in polythene bags at 4°C until the day of photographing.

CHAPTER THREE

INTRA AND INTERSYNGENIC VARIATIONS BETWEEN DIFFERENT SYNGENS OF P. CAUDATUM

Stocks and enzymes assayed:

Four different syngens were examined in this study; syngen 1, stock ISn; syngen 3, stocks D119a and KOK; syngen 12, stock Hj1; syngen 13, stocks AK1 and Hj6. These stocks are referred to as the known stocks in the following study. Two stocks of P. caudatum were obtained from the Cambridge Culture Centre - 2C and 2F, one stock was collected from U.E.A.'s pond - stock 42 and one stock was collected from a local pond in Manhattan, California - Cal1. Samples from these stocks were also run on the gels alongside the known stocks, and are referred to as the unknown stocks, i.e., it is not known to which syngens they belong. Table I shows the list of the stocks and their geographical origins.

Table I: Stocks used and their geographical origin.

Stock	Geographical Origin	Syngen	Mating Type
Isn	Iwate, Japan	Syngen 1	I
KOK	Kyoto, Japan	Syngen 3	V
D119a	Kyoto, Japan	Syngen 3	VI
Hj1	Iwate, Japan	Syngen 12	XXIII
AK1	Aomori, Japan	Syngen 13	XXV
Hj6	Iwate, Japan	Syngen 13	XXVI
2C	Devon, England	Unknown	Unknown
2F	Cambridge, England	Unknown	Unknown
42	Norfolk, England	Unknown	Unknown
Cal1	Manhattan, United States	Unknown	Unknown

These ten stocks were assayed for the following enzymes:-

Esterases (EST EC 3.1.1.) (1) α-Naphthyl propionate esterase.
(2) α-Naphthyl butyrate esterase.

(3) Malate dehydrogenase (NAD-dependent) (MDH EC 1.1.1.37).

(4) Malate dehydrogenase (NADP-dependent) (Me EC 1.1.1.40).

(5) Aspartate: 2-oxyglutarate aminotransferase (ASAT EC 2.6.1.1).

(6) Superoxide dismutase (SOD EC 1.15.1.1).

(7) Phosphogluco isomerase (PGI EC 5.3.1.9).

(8) Hexokinase (HK EC 2.7.1.1).

(9) Acid phosphatase (Acph EC 3.1.3.2).

In order to avoid any confusion in this study, when a particular enzyme is described if necessary a brief discussion will follow.

Esterases:

With the exception of acetyl choline esterases and carbonic anhydrases, most carboxylic hydrolyases, commonly known as "esterases", are apparently non-specific. They exhibit overlapping substrate preferences and inhibition characteristics, which can present real difficulties in their classification. In the present study two different substrates, α-naphthyl propionate, and α-naphthyl butyrate were used. Some bands of esterase activity showed more affinity with one of the substrates, while others had the same affinity for both substrates. Since the culture medium was bacterized, and the bacteria may contribute some bands of activity on the gels (Rowe et al., 1971), the bands which were not constantly apparent, have been excluded from this study (also see Chapter Seven).

(1) α-Naphthyl propionate esterase

When gels were stained after electrophoresis for esterases using

α-naphthyl propionate as the substrate, the cathodal part of the gel showed a single band of enzyme activity for each stock (Figure 1). This single band had identical mobility in 8 of the stocks examined. The exceptions were stocks D119a (syngen 3) and Call (unknown syngen). These two stocks had an identical band of mobility which migrated at a slower rate than the identical band in the other stocks.

In the anodal part of the gels, up to three bands of activity appeared in each stock (Figure 1). An identical pattern of enzyme activity was detected in all the known stocks, except stock D119a. Also, the pattern of enzyme activity was identical among the unknown stocks except Call. The latter stock had an identical pattern of enzyme activity as in stock D119a of syngen 3.

As shown in Figure 1, the band of enzyme activity closest to the origin in the known stocks had no corresponding* band in the unknown stocks. The nearest band to the origin migrated faster in the unknown stocks (except Call), than the first band of activity in the known stocks (except D119a). In the two stocks D119a and Call, a darkly stained patch appeared, close to the origin. However, when the samples were diluted before running them on the gels, the patch was separated into two bands whilst a smear still existed between them. One of these bands migrated at a slower rate and the other at a faster rate than the previously described identical band of the known stocks.

The second band of activity from the origin in the known stocks was detectable only in samples that had been kept in the deep-freeze ($-20^{\circ}C$) for more than a few days. Freshly prepared samples, run without being stored, did not show any activity in this region. It was suspected

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* The term "corresponding" was only used to demonstrate the bands with identical mobility regardless of their nature, i.e. whether they represent exactly the same protein or not.

that this band is a sub-band of the first band of activity from the origin.

The unknown stocks 2C, 2F and 42 in most cases developed a smear from their first band toward the anode. However, in some experiments two bands were distinguishable in this region, but because of their inconsistency they were excluded from this study.

The fastest anodal band was identical among the known stocks, including stock D119a and also stock Call (Figure 1).

Other properties of α-Naphthyl propionate esterases:

In all of the stocks, the first anodal band of activity from the origin stained more strongly than the other bands. This band also was more resistant to heat. It was the only band which retained its complete activity after the samples had been heated for 30 minutes at 50°C. Also it was the first band that appeared on the gels soon after the initiation of staining.

(2) α-Naphthyl butyrate esterase

When α-naphthyl butyrate was used as a substrate to detect esterase variations, a single band of activity with identical mobility appeared in the cathodal part of the gels (Figure 2), except in the cases of stocks D119a (syngen 3) and Call (unknown syngen). These two stocks had an identical band of mobility, which migrated at a slower rate than the identical band in the other stocks.

The cathodal band of the α-naphthyl butyrate esterases migrated the same distance from the origin as the cathodal band of α-naphthyl propionate esterases. However, the bands appeared sooner and stained darker when α-naphthyl propionate was used as substrate. It was concluded that these bands represented the same protein, with slightly

more affinity for a α-naphthyl propionate.

In the anodal part of the gel, a single identical band of mobility was detected in all the stocks (Figure 2). The unknown stocks 2C, 2F and 42, had a second band of activity in addition to the former band. This band had a slightly slower mobility. However, it was not always possible to distinguish these two bands especially when their activity was strong (see also Figure 3).

Sometimes, another two faint slower bands of activity were observed in some of the stocks, but because of their inconsistency, they were excluded from this study. It is assumed that these bands represent the same protein as the first and second bands of activity from the origin when α-naphthyl propionate is used as substrate. Since they migrate to the same distance in the gels.

In stocks D119a and Cal1, a strong patch of enzyme activity appeared which migrated only a short distance from the origin (Figure 2).

Comparing Figure 2 to Figure 1, it is clear that only the two stocks D119a and Cal1 have exactly the same pattern of enzyme activity with both substrates. It is assumed that esterases in these two stocks are different from the esterases of the other stocks.

Discussion

As described in the results and as shown in Figures 1, 2 and 3, all the known stocks had identical patterns of esterases activity with regard to the two substrates with the exception of stocks D119a (syngen 3). The unknown stocks also had identical patterns of esterase activity with the exception of Cal1. Interestingly, the four bands of the two stocks D119a and Cal1 had identical mobility. However, there was a greater similarity in esterase pattern of activity between any of the

unknown stocks (except Cal1) and the known stocks (except D119a), than between the known stocks and stock D119a (syngen 3) or, between the unknown stocks and stock Cal1. Two out of five bands were held in common between unknown stocks and the known stocks (excluding stocks D119a and Cal1), one out of five bands were held in common between known stocks and stock D119a, and one out of six bands were held in common between unknown stocks and stock Cal1.

At present, it is not known if the variations between known and unknown stocks represent intra or inter-syngenic differences, since it is not known to which syngens the unknown stocks belong. Chapter Six, deals with the identification of the mating types of these stocks, as a means to identifying their syngenic relationship.

Excluding stock D119a, no intra or inter-syngenic variation was detected in the stocks of four different syngens (1, 3, 12 and 13), which were examined.

(3) Malate dehydrogenase (NAD-dependent)

Malate dehydrogenase (L - Malate: NAD Oxidoreductose EC 1.1.1.37) (MDH) occurs in virtually all eukaryotic cells. It catalyzes the reduction of L-malate to oxaloacetate.

$$\text{L - Malate} + \text{NAD} \rightleftharpoons \text{Oxaloacetate} + \text{reduced NAD}$$

Results

Under the buffer conditions which were used in this study (see Material and Methods, Chapter Two), all forms of the enzyme appeared in the anodal part of the gel. In the region next to the origin, a single band of activity was detectable in all of the stocks, soon after the gel was placed in the staining buffer. This band had identical mobility in all the stocks, but later turned to a smear during the

process of staining (Figure 4).

Another single band of activity appeared in the anode, which migrated faster from the origin than the smear. This band had identical mobility in all the known and unknown stocks with the exception of stocks D119a (syngen 3) and Cal1. These two stocks had an identical band of mobility, which migrated slower than the single identical band of the other stocks (Figure 4).

When the gels were kept for longer periods in the staining buffer, one or two anodal bands of activity were detected in each of the stocks. Although stocks of different syngens showed variations in respect of these banding patterns (i.e. some stocks had a single fast band, some a single slow band and the rest had both bands of mobility), these bands were excluded from this study, because of their inconsistent appearance in different experiments.

(4) Malate dehydrogenase (NADP-dependent) = Malic enzyme

L-Malate: NADP oxidoreductose (decarboxylating) (EC 1.1.1.40) which is more conveniently known as malic enzyme, catalyzes the oxidative decarboxylation of L-malate to pyruvate.

$$\text{L-Malate} + \text{NADP} \rightleftharpoons \text{Pyruvate} + CO_2 + \text{reduced NADP}$$

Results

All the stocks had a single identical sharp band of malic enzyme activity (Figure 5). This band was very unstable and was only detectable in freshly prepared samples. Keeping samples for more than a few days at -20°C completely destroyed the activity of the enzyme.

Other anodal band of activity, which migrated faster from the origin than the single sharp band, were also observed in each of the stocks. This band had identical mobility in all the stocks, with the

exception of stocks D119a and Ca11. These two stocks had an identical band of mobility, which migrated at a slower rate from the origin than the identical bands in the other stocks (Figure 5). The enzyme activity migrating at a faster rate toward the anode represents malate dehydrogenase. (See also page 41.) These bands were fuzzy and gradually disappeared when the gels were kept in distilled water before being fixed with acetic acid (Figure 6). The fuzzy anodal bands were very stable and retained activity in the samples which were kept for a long time (i.e. a few weeks at -20°C).

The slower single sharp band of activity is attributed to malic enzyme since it is NADP-dependent and does not function with NAD. The fuzzy faster bands of activity are attributed to malate dehydrogenase. Malate dehydrogenases react more effectively with NAD, but also retain a low percent of their activity when NADP is used as coenzyme.

(5) Aspartate: 2-oxoglutarate aminotransferase

Aspartate aminotransferase (EC 2.6.1.1) is widely distributed in both the plants and animals (Agren, 1959; Schmidtke and Engel, 1971; Dhanani and Kitto, 1970; Davidson et al., 1970). It catalyzes the reversible reaction:

L-Aspartate + α-Ketoglutarate $\rightleftharpoons$ Oxaloacetate + L-Glutamate

Two well-defined isozymes of glutamate oxaloacetate transaminase have been described in several animal tissues (Fleisher et al., 1960; De Jimeneze et al., 1967). One isozyme exists in the cytoplasm and the other has been shown to exist mainly in the mitochondria.

Results

All of the stocks of P. caudatum, which were investigated, had a single band of aspartate oxaloacetate transaminase. This single band

had identical mobility in all the stocks with the exception of stocks D119a and Cal1 (Figure 7). These two stocks had an identical band of mobility which migrated at a slower rate from the origin than the band in the other stocks. The cathodal band of enzyme activity is very unstable, and it is only detectable in the freshly prepared samples. Heating samples for 30 minutes in 50°C prior to electrophoresis, completely destroys aspartate aminotransferase activity.

A faint smear also appeared in the anodal part of the gels, but because no band was formed, the smear was not considered in this study.

(6) Superoxide dismutase

The enzyme superoxide dismutase was first recognized and described by McCord and Fidvich (1968, 1969). The physiological function of the enzyme is to protect organisms metabolizing oxygen against the potentially deleterious effect of superoxide free radicals (McCord et al., 1971).

This enzyme was known as indolphenoloxidase in human red cells (Brewer, 1967). It was also called deformazan oxidase. In Tetrahymena, the name tetrazolium oxidase (TO) was used for the same enzyme (Borden et al., 1973a).

Superoxide dismutase appears as an achromatic band in the bluish background that develops in the gels, in the presence of light. The enzyme does not require any substrates or coenzymes for its appearance.

Results

This enzyme appeared both in the cathodal and anodal part of the gel (Figure 8).

(I) Cathodal bands: A single identical band of mobility was detected in all the stocks with the exception of stocks D119a and Cal1.

These two stocks had a single band with common mobility which migrated faster than the identical band of the other stocks (Figure 8).

(II) Anodal bands: This was a single band with identical mobility in all the stocks, including D119a and Cal1 (Figure 8).

The cathodal and anodal bands of superoxide dismutase also appeared as a by-product in staining the gels for dehydrogenases at pH: 8.0 or at higher pH's.

Sensitivity of the enzyme

The anodal band appeared to be more stable than the cathodal band. The activity of the cathodal band was destroyed, when the samples were preheated 10 minutes at 50°C before running them on the gel. However, the anodal band of the enzyme remained intact when the samples were preheated for the same period of time and at the same temperature.

(7) Glucose-6-phosphate isomerase

Glucose-6-phosphate isomerase (EC 5.3.1.9) (or more conveniently PGI, i.e. phosphoglucoisomerase), is abundantly present in nature. It catalyzes essential steps in the pathways of glucolytic or oxidative metabolism of carbohydrates.

D-Glucose-6-phosphate $\rightleftharpoons$ O-Fructose-6-phosphate

Results

In P. caudatum, all forms of the enzyme migrated to the anodal part of the gel (Figure 9). The nearest anodal bands to the origin were darkly stained, and appeared consistently in all the experiments. This band had identical mobility in all the known stocks. The unknown stocks, with the exception of stock Cal1, shared an identical band of enzyme mobility, but which migrated at a slower rate than the band in

the known stocks. The unknown stock, Cal1, had an identical band of mobility as the known stocks (Figure 9).

A further anodal band of activity appeared in some of the stocks, but because it appeared inconsistently in different preparations of the same stock, it was excluded from this study.

(8) Hexokinase

Hexokinase (ATP: D-hexose-6-phosphotransferase) (EC 2.7.1.1) has been found in a wide range of organisms from yeast to mammalian tissues. It interacts with the same substrates, although at a lower activity in mammals.

Results

In P. caudatum up to three bands of hexokinase activity were detected in the stocks (Figure 10). The two bands of enzyme activity in stocks D119a and Cal1, which migrated faster toward the anode than the bands in the other stocks, originated from the bacteria and were excluded from this study (see also Chapter Seven).

Both intra and inter-sygenic variation was observed in the stocks. Variation was also observed between the known and unknown stocks.

(I) Intrasyngenic variation:

The two stocks of syngen 13 shared two of their three bands of mobility, numbered as 3 and 4 (Figure 10). The third band of stock AK1 which was slower than the bands 3 and 4, was numbered as 2. The third band of stock Hj6 , that migrated at a faster rate than the bands 3 and 4, was numbered 5.

Of the two stocks of syngen 3, one had no band of activity (stock D119a), the other one (stock KOK) had three bands of activity, numbered 2, 3 and 4. As can be seen, the two stocks of KOK (syngen 3)

and AK1 (syngen 13) had identical patterns of hexokinase activity.

(II) Intersyngenic variation:

Stock ISn of syngen 1 had three bands of activity, numbers 2, 3 and 4. The pattern of enzyme activity in stock Hj1 (syngen 12) was identical to the pattern of activity in stock ISn (syngen 1), as well as to that of stock KOK (syngen 3) and AK1 (syngen 13). If the stocks ISn, KOK, Hj1 and AK1 do represent a syngen pattern of enzyme activity for hexokinase, then there is no intersyngenic variation existing between the four examined syngens of 1, 3, 12 and 13.

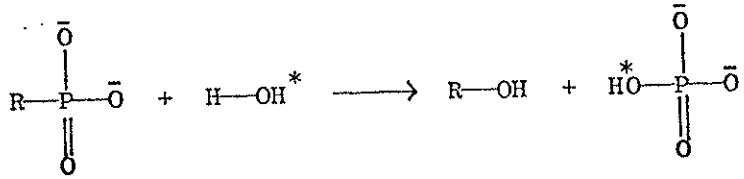
(III) The variation between known and unknown stocks:

The three unknown stocks 2C, 2F and 42 had identical bands of enzyme mobility, numbers 1, 2 and 3. These stocks also shared two of their three bands, numbers 2 and 3, with stock Hj1 (syngen 12), ISn (syngen 1), KOK (syngen 3) and AK1 (syngen 13), (Figure 10).

Stock Cal1 did not show any band of activity.

(9) Acid phosphatase

Acid phosphatase (EC 3.1.3.2) activity is widespread throughout nature. Hydrolysis of a variety of orthophosphate esters as well as transphosphorylation reactions are catalyzed by this enzyme.



Results

Under the electrophoretic conditions used in this study, up to three bands of activity were detected in different stocks of P. caudatum

(Figure 11). Both intra and inter-syngenic variations were observed in the stocks. Variation was also observed between the known and unknown stocks.

(I) Intrasyngenic varition:

The two stocks of syngen 13, AK1 and Hj6, had two identical bands of mobility numbered as 1 and 2 (Figure 11). However, a third band of activity, number 4 was also observed in stock AK1, but stock Hj6 did not have any band corresponding to band number 4 in stock AK1 (Figure 11).

As for the two stocks of syngen 3, one had no band of activity (D119a), and the other (KOK), had two bands of activity, numbers 2 and 4 (Figure 11). A third slower band of activity was also observable in stock KOK, but again this band was inconsistent and was excluded from this study.

(II) Intersyngenic variation:

Stock ISn of syngen 1 had three bands of activity, numbers 2, 3 and 4 (Figure 11). Stock Hj1 of syngen 12 had three bands of activity, numbers 1, 2 and 4, and also a further band or smear which migrated much slower toward the anode. This band has been excluded from the present study, since it might be contributed by bacterial enzyme (for further details, see Chapter Seven).

(III) The variation between known and unknown stocks:

The three unknown stocks 2C, 2F and 42 had two identical bands of enzyme activity, which had also identical mobility to the bands numbered as 1 and 2 in the known stocks of AK1, Hj6 (syngen 13) and Hj1 (syngen 12) (Figure 11). The unknown stock, Cal1, did not show any activity for this enzyme.

Summary of Results and Discussion

(I) By examining two stocks in each of the syngens 3 and 13, and one stock in each of syngen 1 and 12, for intra and inter-syngenic variation, the following results were obtained:

The two stocks of syngen 3 (KOK and D119a) showed variation in eight of the nine examined enzymes. The exception was phosphogluco isomerase. On the other hand, the pattern of enzyme activity was identical in the two stocks D119a and Cal1 (nine out of nine examined enzymes). It is a strong possibility that stock D119a in fact is the same stock as Cal1, which has been mislabelled in our laboratory (for further explanation see page 45). The results in the next Chapter on intrasyngenic variation in stock of syngen 3, reinforces this postulate.

(II) Excluding stock D119a, results for the known stocks can be summarized as follows:

(1) No intra or inter-syngenic variations were observed in seven of the nine examined enzymes (α-naphthyl propionate esterase, α-naphthyl butyrate esterase, malate dehydrogenase, malic enzyme, aspartate aminotransferase, superoxide dismutase and phosphogluco isomerase).

(2) Intra-syngenic variation was observed in the two stocks of syngen 13 for acid phosphatase and hexokinase. The degree of variation was limited. Each of the two enzymes had three bands of enzyme activity. The two stocks of syngen 13, AK1 and Hj6, shared two of their three bands and only differed in one band of enzyme mobility.

(3) Inter-syngenic variation was observed for acid phosphatase and hexokinase. However, the degree of inter-syngenic variation was not greater than the observed intra-syngenic variation for these two enzymes, between the two stocks of syngen 13. In some instances, e.g. with Hj1 (syngen 12) and AK1 (syngen 13), no inter-syngenic variation was observed and these two stocks had identical patterns for the nine enzymes.

(III) The three unknown stocks 2C, 2F and 42 had identical patterns of enzyme activity for all the nine enzymes. It can be suggested that they represent the same syngen. Results of Chapter on mating reactivity reinforces this suggestion.

(IV) Comparing these three unknown stocks to the known stocks it can be said that:

(1) There was no variation as far as four of the nine examined enzymes were concerned, i.e. malate dehydrogenase, malic enzyme, aspartate aminotransferase, superoxide dismutase. These enzymes were also monomorphic in the known stocks.

(2) The three enzymes α-naphthyl propionate esterase, α-naphthyl butyrate esterase and phosphogluco isomerase, which did not show any variation among the known stocks, showed variation in comparing the known stocks to the unknown stocks.

(3) Acid phosphatase and hexokinase which showed intra and inter-syngenic variations, also showed variation between the known and unknown stocks. However, the extent of the variation was not greater than that observed for intra and inter-syngenic variation in these two enzymes.

(V) The unknown stock, Call, had identical patterns of enzyme activity with the known stocks, for phosphogluco isomerase and the fastest anodal band of α-naphthyl butyrate esterase. With the exception of the fastest anodal band of α-naphthyl butyrate esterase, none of the other enzymes had identical patterns of activity, when stock Call was compared to the other unknown stocks 2C, 2F and 42.

Assessment of similarity

To assess the similarity between any of the stocks for the nine examined enzymes, the term "coefficient of identity" has been used. As in Tetrahymena pyriformis (Borden et al., 1973a), the coefficient

of identity was the fraction of enzyme patterns which could not be distinguished between the two compared stocks. This was a simple matching coefficient where the number of positive matches (the number of identical bands) was divided by the total number of comparisons made for any of the two stocks (Table II). The more enzymes which are compared, the more reliable becomes the coefficient of identity.

Stocks		Hj6	AK1	D119a	KOK	ISn	Hj1	2C	2F	42	Cal1
Syngen 13	Hj6	-	93	25	87	87	93	73	73	73	25
	AK1		-	26	93	93	100	80	80	80	26
Syngen 3	D119a			-	26	26	26	18	18	18	100
	KOK				-	93	93	73	73	73	26
Syngen 1 - ISn						-	93	69	69	69	25
Syngen 12 - Hj1							-	80	80	80	26
Unknown	2C							-	100	100	18
	2F								-	100	18
	42									-	18
	Cal1										-

Table II: Value for the coefficient of identity between any of the stocks for nine examined enzymes. The term is expressed as a percentage.

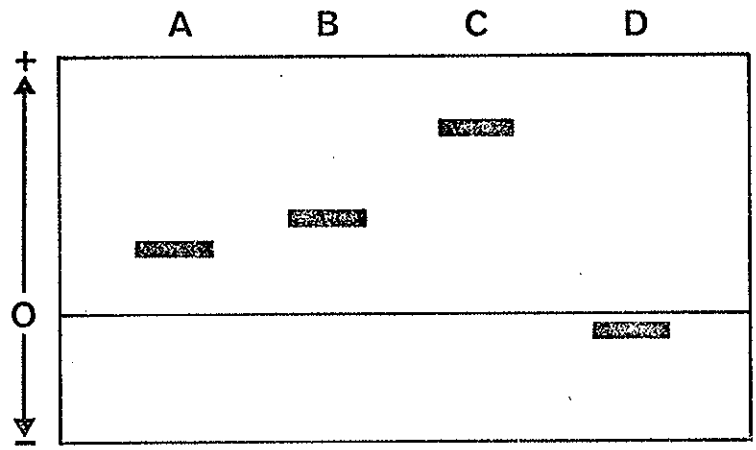
As it is shown in Table II, the figures for coefficient of identity varies from 100, i.e. the absolute identity in the pattern of enzyme activity to 18. The coefficient of identity is higher between any of the two known stocks than between the known and unknown stocks, excluding stocks D119a and Cal1. The least similarity is observed between

the unknown stocks 2C, 2F, 42 and the two stocks Call, D119a.

A surprising comparison shows up between stocks AK1 (syngen 13) and Hj1 (syngen 12), where the coefficient of identity is 100. This indicates a greater similarity between two stocks of two different syngens, than in two stocks of syngen 13. The coefficient of identity between stocks Hj6 and AK1 of syngen 3 is 93.

In general, excluding stock D119a, it is impossible to distinguish any of the four available syngens, by examining nine enzymes in one or two stocks of each of these syngens.

There are some weaknesses involved in this method of measuring phenotypic similarity. For example, if a single band exists in four stocks as it is shown in Figure 12.



The bands in stocks A, B, C and D has zero similarity to one another. However, the band in stock A and B are very similar, but in C and D are very different as judged by their enzyme mobility, and probably greater genetic changes involved with C and D than with A and B.

The other weaknesses in assessing syngenic differences is the lack of examining enough stocks within any of the syngens. Problems

are involved with obtaining stocks within the known syngens. The so-called known stocks have only been obtainable from Dr. Hiwatashi's laboratory in Japan. This collection has been made from different parts of Japan and only four syngens have been isolated, so far, from this part of the world. Recently new collected stocks of syngens 1, 3 and 12 have been sent to our laboratory. Some stocks were dead on their arrival. The rest were grown on bacterized grass medium and some of them had a very poor growth rate and they eventually died. The survivors have been grown and their enzymes screened in order to detect intrasyngenic variation. The results of these experiments will be described in the next Chapter. The aim of utilizing more stocks in each syngen is to have a better assessment of the amount of intra-syngenic variation and in so doing, it would become possible to compare intersyngenic variation with more reliability, i.e. when a single stock is being compared in each syngen, and the intersyngenic variation has been determined. The results cannot be considered reliable since it is not known if a single stock is representative of its syngen.

Figure 1:

α-Naphthyl propionate esterase variations between the known and unknown stocks of P. caudatum.

1 : Hj6
2 : AK1
3 : D119a-Call
4 : KOK
5 : Isn
6 : Hj1
7 : Hj6
8 : 2C-2F-42

Figures 2 and 3:

α-Naphthyl butyrate esterase variations between the known and unknown stocks of P. caudatum.

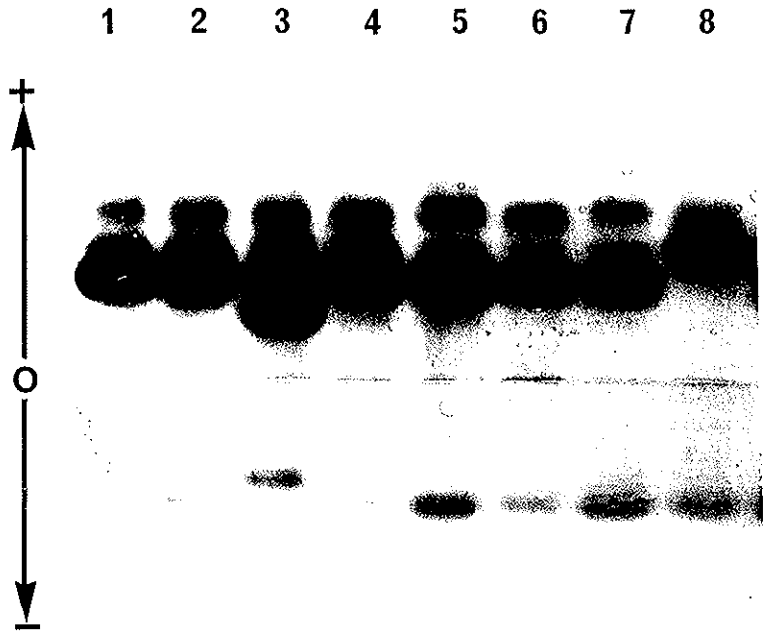
Figure 2

1 : Hj6
2 : AK1
3 : D119a-Call
4 : KOK
5 : Isn
6 : Hj1
7 : Hj6
8 : 2C-2F-42

Figure 3

1 : 2C-2F-42
2 : All the known stocks except D119a

O : Origin


1 2 3 4 5 6 7 8
+
O

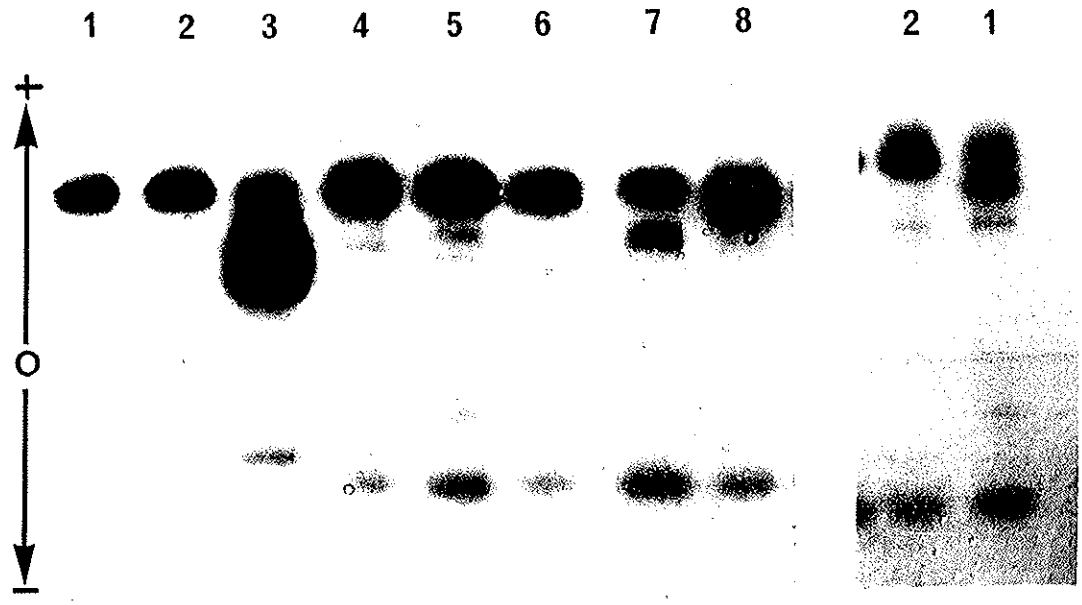

1 2 3 4 5 6 7 8 2 1
+
O

Figure 4:

Malate dehydrogenase variations between the known and unknown stocks of P. caudatum.

1 : Hj6	6 : Hj1	11 : Hj6
2 : AK1	7 : 2C	
3 : D119a	8 : 2F	
4 : KOK	9 : 42	
5 : Isn	10 : Ca11	

O : Origin

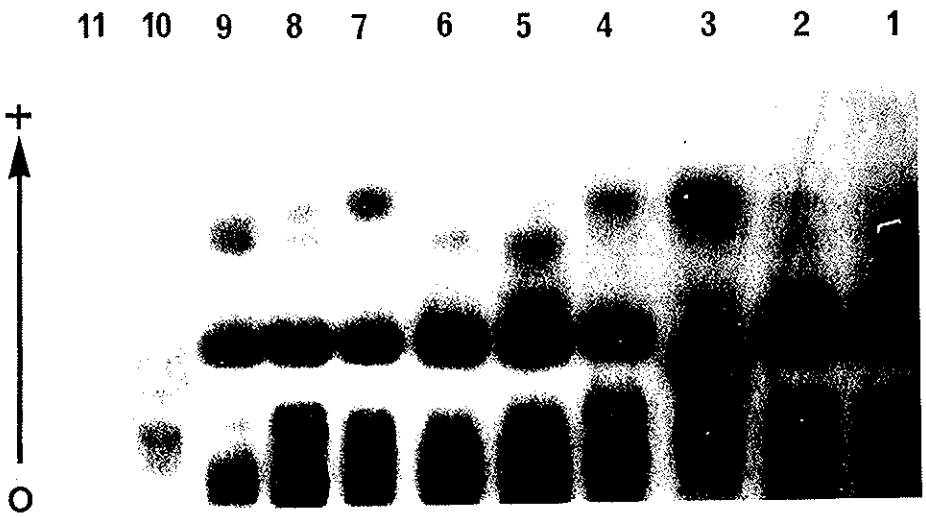

11 10 9 8 7 6 5 4 3 2 1
+
O

Figure 5:

Malic enzyme variations between the known and unknown stocks of P. caudatum. Bands of malate dehydrogenase are also observable.

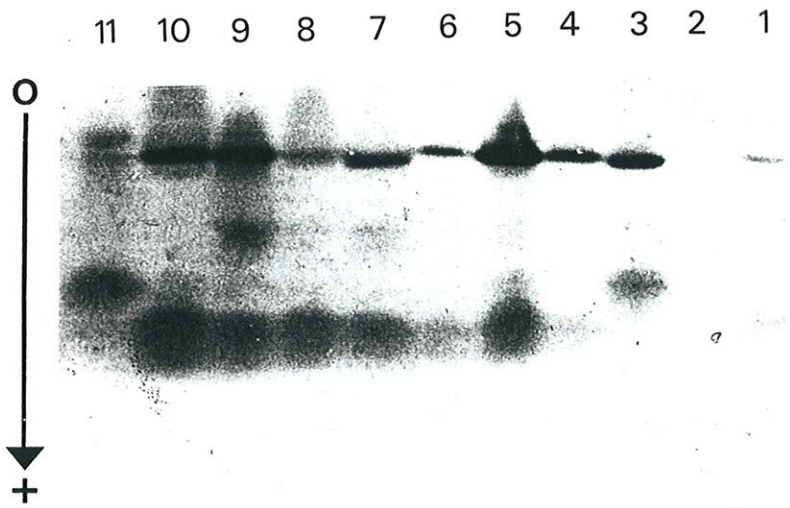
1 :	Hj6	6 :	Hj1	11 :	Cal1
2 :	AK1	7 :	2C		
3 :	D119a	8 :	2F		
4 :	KOK	9 :	42		
5 :	Isn	10 :	Hj6		

Figure 6:

Effect of distilled water on malate dehydrogenase bands.

1 :	Hj6	6 :	Hj1	11 :	Hj6
2 :	AK1	7 :	2C		
3 :	D119a	8 :	2F		
4 :	KOK	9 :	42		
5 :	Isn	10 :	Cal1		

O : Origin


11 10 9 8 7 6 5 4 3 2 1
O
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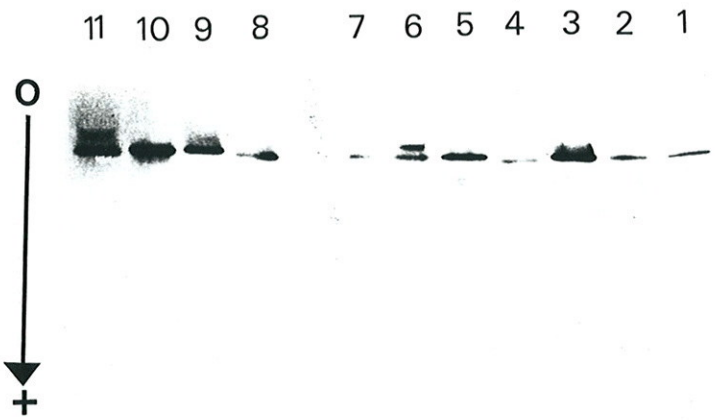

11 10 9 8 7 6 5 4 3 2 1
O
+

Figure 7:

Aspartate aminotransferase variations between the known and unknown stocks of P. caudatum.

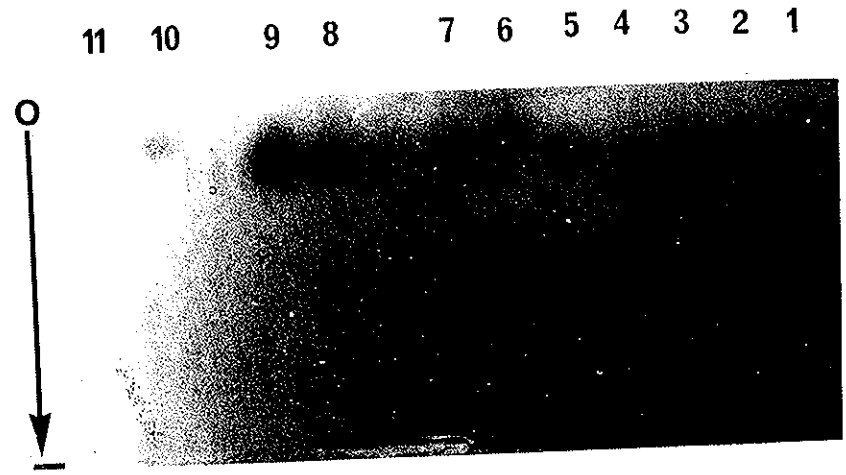
1 : Hj6	6 : D119a	11 : Hj6
2 : AK1	7 : 2C	
3 : Hj1	8 : 2F	
4 : Isn	9 : 42	
5 : KOK	10 : Call	

Figure 8:

Superoxide-dismutase variations between the known and unknown stocks of P. caudatum.

1 : Hj6	6 : Hj1	11 : Hj6
2 : AK1	7 : 2C	
3 : D119a	8 : 2F	
4 : KOK	9 : 42	
5 : Isn	10 : Call	

O : Origin


11 10 9 8 7 6 5 4 3 2 1
O
—

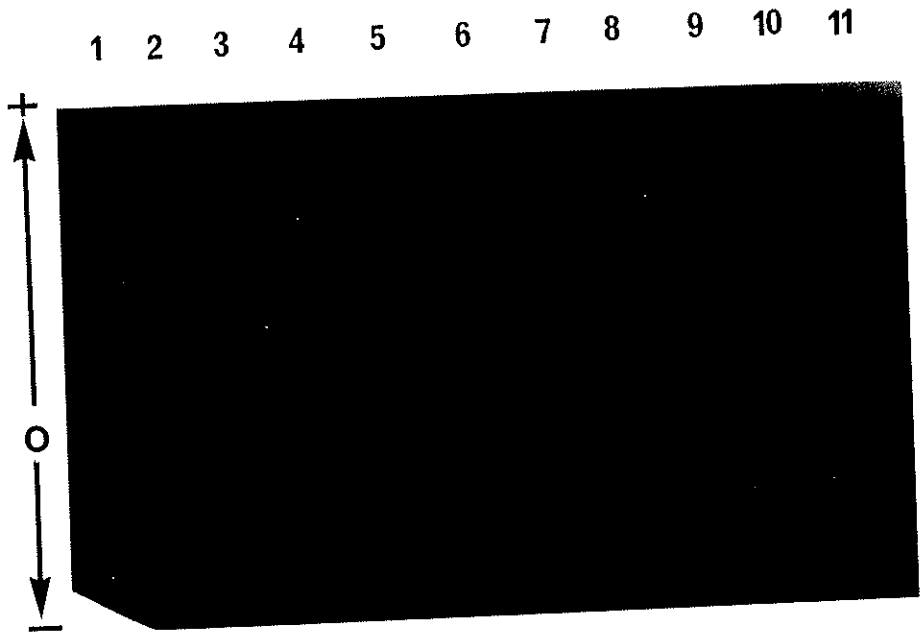

1 2 3 4 5 6 7 8 9 10 11
+
O
—

Figure 9:

Phosphogluco isomerase variations between the known and unknown stocks of P. caudatum.

1 : Call	6 : Isn
2 : Hj6	7 : Hj1
3 : AK1	8 : 2C
4 : D119a	9 : 2F
5 : KOK	10 : 42

O : Origin

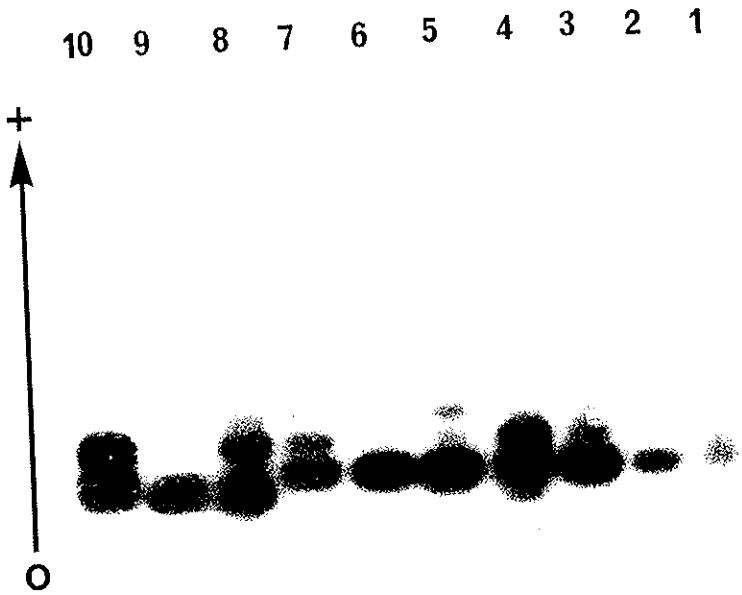

10 9 8 7 6 5 4 3 2 1
+
O

Figure 10:

Hexokinase variations between the known and unknown stocks of P. caudatum.

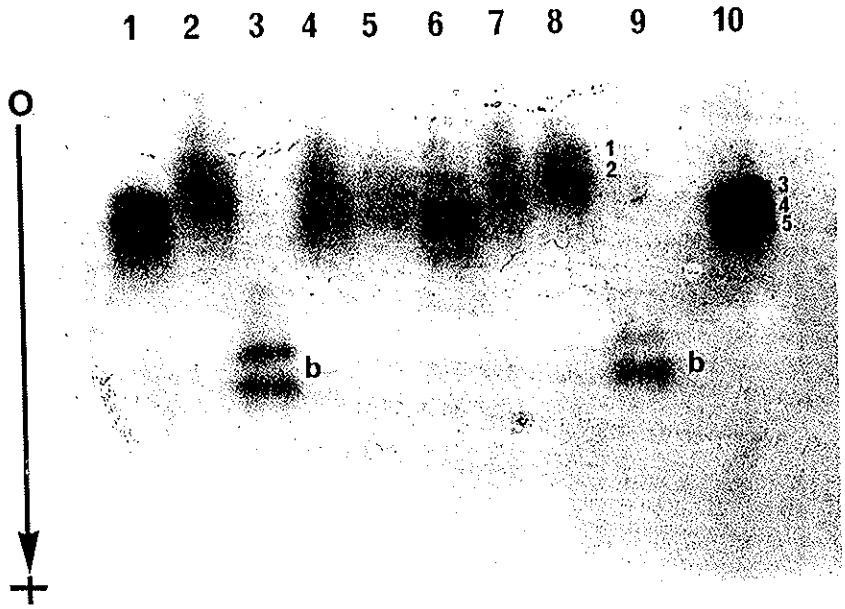
1 :	Hj6	6 :	Hj1
2 :	AK1	7 :	2C
3 :	D119a	8 :	2F
4 :	KOK	9 :	Cal1
5 :	Isn	10 :	Hj6

Figure 11:

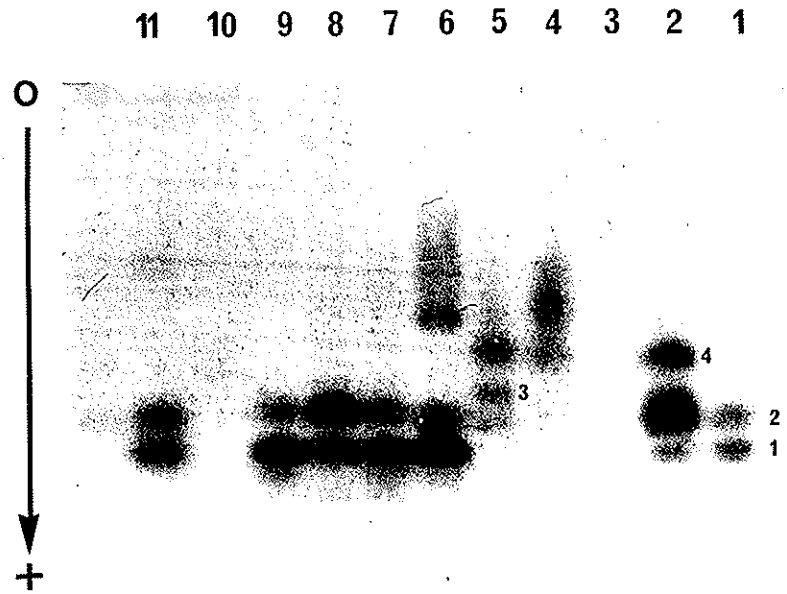
Acid phosphatase variations between the known and unknown stocks of P. caudatum.

1 :	Hj6	6 :	Hj1	11 :	Hj6
2 :	AK1	7 :	2C		
3 :	D119a	8 :	2F		
4 :	KOK	9 :	42		
5 :	Isn	10 :	Cal1		

O : Origin



b: bacerial enzyme



CHAPTER FOUR

SECTION I

INTRASYNGENIC VARIATION BETWEEN STOCKS OF SYNGEN 3 IN PARAMECIUM CAUDATUM

The stocks of syngen 3, which are available, are listed in Table III, with their geographical site of origin and their mating type.

Stock	Geographical Origin	Mating Type
KOK	Kyoto, Japan	V
KT2	Kyoto, Japan	V
YT1	Yamagata, Japan	V
KY	Kyoto, Japan	VI
YT4	Yamagata, Japan	VI
YT3	Yamagata, Japan	VI
D119a	Kyoto*, Japan	VI

Table III: Stocks of syngen 3 used and their geographical origin.

* Stock D119a is derived from crosses of natural stocks collected in Kyoto.

For the methods of preparation of stocks and enzyme essay, see Materials and Methods (Chapter Two). Enzymes used in this study are as follows:

Esterases
- (1) α-Naphthyl propionate esterase.
- (2) α-Naphthyl butyrate esterase.

(3) Malate dehydrogenase (MDH).

(4) Malic enzyme (Me).

(5) Aspartate : 2-oxoglutarate aminotransferase (ASAT).

(6) Superoxide-dismutase (SOD).

(7) Phosphogluco isomerase (PGI).

(8) Hexokinase (HK).

(9) Acid phosphatase (AcPh).

When interpreting the electrophoretic banding patterns, the following nomenclature is used. An abbreviation is chosen to designate each protein, e.g. EST. In the case of multiple forms of the same enzyme, a number follows the abbreviates, e.g. EST_1 signifying the band with the least anodal mobility, i.e. the closest band to the origin. The next band is numbered 2 and so on. This form of numbering the bands is necessary as the more anodal bands of the examined enzymes are not usually very clear and not consistent in their appearance. If the enzyme migrated to the cathode, the same principles are followed only the numbers are represented in the negative form. The band with the least cathodal migration is designated -1, e.g. EST_{-1}. The most common form of a band arbitrarily designated 100, e.g. PGI_1^{100}. This figure represents the unit distance the isozyme migrates. Other forms of the same enzyme are then assigned a numerical value representing their mobility relative to this unit distance, e.g. PGI_1^{75} and PGI_1^{120}. When two bands migrated very closely no numerical value is attributed, e.g. EST_3.

This simple nomenclature is very useful for the purposes of intra and inter-syngenic comparisons. It may also indicate a genetic basis for determination of the proteins.

Esterases

(1) α-Naphthyl propionate esterase

When gels were stained using α-naphthyl propionate as substrate, up to 4 bands of enzyme activity were detected in each of the stocks (Figure 13). One band occurred in the cathodal part and the others in the anodal part of the gel. The single cathodal band had identical mobility in all the stocks, with the exception of stock D119a. This stock had a single band with slower mobility. As shown in Figure 13, some of the stocks, e.g. KOK and YT1, also had a faint slower band which migrated the same distance from the origin as the single cathodal band of stock D119a. However, this band was not consistent and was excluded from the study.

In the anodal part of the gel, the band closest to the origin was the most darkly stained. This band had identical mobility in all the available stocks of syngen 3 with the exception of stock D119a, which had a slow band.

The second anodal band from the origin only appeared in samples which had been kept for a few days in the deep freeze. The exception was the second anodal band of stock D119a, which was observed in all the samples.

The third anodal band from the origin had identical mobility in all the stocks with the exception of the two stocks, YT4 and YT3. The identical band in these two stocks migrated at a slightly slower rate than the common band in the other stocks.

(2) α-Naphthyl butyrate esterase

When α-naphthyl butyrate was used to detect esterases, a single identical band of mobility appeared in the cathodal part of the gel in all the stocks, with the exception of stock D119a. This stock had a single band which migrated at a slower rate than the identical band

in the other stocks. The pattern of enzyme activity in the cathodal part of the gel was identical with both substrates used in the present study. However, the intensity of the cathodal band was slightly greater when α-naphthyl propionate was used as substrate. It was assumed that the cathodal bands represented the same protein, with slightly more affinity for α-naphthyl propionate.

In the anodal part of the gel, the first and second bands nearest to the origin were very faint, with the exception of stock D119a (Figure 14). These bands corresponded to the first and second anodal bands from the origin when α-naphthyl propionate was used. It was assumed that these bands represented the same protein, which had a greater affinity for α-naphthyl propionate. The first and second anodal bands of activity in stock D119a appeared with the same intensity with both substrates. As discussed in Chapter Three the nature of these proteins in stock D119a differed from that of the first and second anodal bands of enzyme activity in the other stocks.

Further toward the anode, a darkly stained single band of activity appeared in each of the stocks (Figure 14). This band had an identical mobility in all the stocks with the exception of stocks YT3 and YT4. These two stocks had identical bands of mobility which migrated at a slower rate than the band in the other stocks. This pattern was exactly identical to the pattern of enzyme activity when α-naphthyl propionate was used. It was concluded that these bands represented the same protein interacting with both substrates.

Categorization of the esterases according to their substrate activities.

As has been described above, three zones of activity can be differentiated in each gel stained for esterases (Figures 13 and 14).

1) The cathodal zone:

The bands in this zone had a slightly greater intensity for α-naphthyl propionate. The faster band which was the most common cathodal band was designated as EST_{-1}^{100} and the slower band in stock D119a was designated as EST_{-1}^{75}.

2) The zone nearest to the anode:

With the exception of stock D119a, whose bands of activity had the same intensity for both substrates, the bands of activity of the other stocks in this zone were predominately detectable only with α-naphthyl propionate. Occasionally faint bands appeared in this zone, when α-naphthyl butyrate was used, but these bands were not regularly detectable.

The closest band of activity to the origin was designated as EST_1^{100} in stock D119a and EST_2^{100} in the other stocks of syngen 3.

3) The most anodal zone:

The bands in this zone had the same intensity for both the substrates. Slight differences in mobility were observed between the single bands of different stocks in this zone, but they were designated as EST_3.

(3) Malate dehydrogenase (MDH)

A smear appeared from the origin up to 1 cm to the anode. Next to this smear, a single anodal band of activity was detected in all the stocks. This band had identical mobility in all the stocks with the exception of stock D119a (Figure 15). The single band in the stock D119a had a slower rate of migration compared with the common band of the other stocks. The common band was designated as MDH_1^{100} and the slower band as MDH_1^{82}.

A further band of activity was also observed in some of the stocks.

This band migrated at a faster rate toward the anode, but because of its inconsistency in different experiments, this band was excluded from the present study.

(4) Malic enzyme (Me)

This enzyme appeared as a single sharp band of activity, on polyacrylamide gels (Figure 16). The single band had an identical mobility in all the stocks of syngen 3 and was designated as Me_1^{100}.

A faster set of fuzzy bands also appeared in the anode (Figure 16). All the stocks had a single identical band with the exception of stock D119a, which showed a slower band of activity. When gels were run under the same conditions as used for malic enzyme, but stained for malate dehydrogenase (MDH), a set of fast anodal bands identical to the fuzzy fast band which has been already described, were detected. However, the slow, sharp single band of activity disappeared from the gel (Figure 17). It has been concluded that the single sharp band represents malic enzyme activity and the fast fuzzy band represents malate dehydrogenase activity.

(5) Aspartate aminotransferase (ASAT)

A single band of activity appeared in the cathodal part of the gel (Figure 18). This single band had an identical mobility in all the stocks of syngen 3 with the exception of stock D119a. The single band in stock D119a migrated at a slower rate than the single band in the other stocks. The common form of the band was designated as $ASAT_{-1}^{100}$ and the band in stock D119a as $ASAT_{-1}^{85}$.

The cathodal band of enzyme activity was very unstable and only detected in freshly prepared samples.

In the anodal part of the gel, a faint smear was detected in each of the stocks, and was therefore excluded from this study.

(6) Superoxide dismutase (SOD)

In the anodal part of the gel, a single band with identical mobility was detected in each of the stocks. This band was designated as SOD_1^{100} (Figure 19).

In the cathodal part of the gel, a single band appeared in each of the stocks. This band had identical mobility in all the stocks with the exception of stock D119a (Figure 19). The latter stock had a single band which migrated faster from the origin than the single identical band in the other stocks. The common band of enzyme activity was designated as SOD_{-1}^{100} and SOD_{-1}^{175} for that in stock D119a.

(7) Phosphogluco-isomerase (PGI)

One or two bands of activity were observed in each stock (Figure 20), after the gels were stained for phosphogluco isomerase. The first band of activity nearest to the origin was the more stable and active band. It also appeared in all the stocks and in different electrophoretic runs. The second and faster band of activity appeared as a faint band, which was not observable in all the stocks and also was not reproducible in different runs. This band was not considered in the present study.

The first band of activity had identical mobility in stocks KOK, YT1, KY, YT4. This band was designated as PGI_1^{100}. Stock YT3 had a slower band of mobility designated as PGI_1^{75} and stock KT2 had a faster band than the common band which was designated as PGI_1^{120} (Figure 20).

(8) Hexokinase (HK)

The three bands of hexokinase activity were not clear and a smear existed between the bands (Figure 21). This enzyme was chosen however, for study because of the extent of the intersyngenic variation which

had been observed (see Chapter Three). In syngen 3 of P. caudatum, up to three bands of hexokinase activity were detected. The two fast bands of enzyme activity in stock D119a, were bacterial enzyme and for this reason were excluded from this study (see also Chapter Seven).

The most common band of hexokinase activity in stocks of syngen 3 was designated as HK_1^{100} (Figure 22). The mobility of other bands were calculated relative to the HK_1^{100} band. This method of designating the bands was chosen to make their identification easier in comparing the pattern of hexokinase activity in different stocks. Otherwise, it is not known if the three bands in each stock are the product of the action of three alleles at a single locus or different loci are involved in contributing these bands.

The two stocks YT3 and YT4, in addition to the common band HK_1^{100}, had two other slower bands of mobility (HK_1^{88} and HK_1^{76}). Stocks KT2, YT1 and KY had two faster bands of mobility in addition to the common band (HK_1^{112} and HK_1^{124}). Stock KOK had one slower (HK_1^{88}) and one faster (HK_1^{112}) band of mobility in addition to the common band (HK_1^{100}) (Figure 22).

(9) Acid phosphatase (AcPh)

Under the electrophoretic conditions used in this study, up to three bands of acid phosphatase activity were detected in different stocks of syngen 3 (Figure 23). These three bands had identical mobility in all the stocks. However, the third band of mobility from the origin was not detected in stock KOK. The second band of activity from the origin was usually more intense than the other two bands. This band was designated as $AcPh_1^{100}$, the slower band as $AcPh_1^{75}$, and the faster band as $AcPh_1^{125}$ (Figure 23).

Here again, this method of designating bands was chosen because

it made the comparisons of pattern of acid phosphatase activity easier, in different stocks. However, it is not known if the three bands are the product of the actions of three alleles at a single locus or different loci are involved in contributing these bands.

No bands of acid phosphatase activity were observed in stock D119a. It is possible that a "null" or "silent" allele exists in this stock.

Assessment of similarity among examined stocks of syngen 3

To assess the similarity, the term "coefficient of identity" has been used. The definition of this term and the way it has been calculated was described earlier (see Chapter Three). Table IV shows the calculated value for the coefficient of identity between any of the two examined stocks of syngen 3.

Stocks	KOK	KT2	YT1	KY	YT4	YT3	D119a
KOK	-	80	86	86	80	73	26
KT2		-	93	93	73	73	18
YT1			-	100	80	73	25
KY				-	80	73	25
YT4					-	93	18
YT3						-	12
D119a							-

Table IV: Value for the coefficient of identity between stocks of syngen 3 for nine examined enzymes.

Two major conclusions can be extracted from Table IV:

1) The degree of similarity between stock D119a and the other

stocks is very low. Considering this stock is derived from crosses of natural stocks collected in Kyoto, it would be expected to show a similar pattern of enzyme activity and therefore a greater coefficient of identity, to the stocks collected from the same geographical site (KOK, KT2 and KY). However, the calculated coefficient of identity between stock D119a and the other stocks from the same geographical site was as low as the value for the coefficient of identity between stock D119a and the stocks from different geographical sites. Because of the unexpected exceptional low coefficient of identity between stock D119a and other stocks of syngen 3, and its absolute identity to stock Cal1, it is concluded that the stock labelled as D119a does not represent syngen 3. Instead, this stock represents one of the stocks which have been collected from California (also see Chapter Three). For this reason, the stock which has been mislabelled as D119a was discarded.

2) The second conclusion is that there is high percentage of similarity between any two stocks. The figure for coefficients of identity varies from 100, e.g. absolute identity between stocks YT1 and KY to 73. The latter figure is observed between all but one comparison of stock YT3 with the other stocks. This relatively low similarity indicates that the stock YT3 is genetically more isolated from the other stocks except YT4. The coefficient of identity is 93 between YT3 and YT4. Stock YT3 was also the only stock of syngen 3 which showed variation in Lactate dehydrogenase (Agatsuma and Tsukii, 1980).

Categorization of enzymes

The enzymes can be categorized in terms of their monomorphism and in terms of their degree of polymorphism.

1) The following proteins are monomorphic: EST_2; EST_{-1}; MDH_1;

Me_1; SOD_1; SOD_{-1} and $ASAT_{-1}$.

2) The polymorphic enzymes according to their degree of polymorphism are as follows:

a) Acid phosphatase ($AcPh_1$): only one of the six stocks showed variations for this enzyme. The variant stock (KOK) lacks a single band which appeared in the other stocks. However, stock KOK shared the two other bands with the rest of the stocks. The lack of a single band in stock KOK might be due to the low concentration of this protein rather than inactivity of an allele responsible for determining this protein in the latter stock.

b) Esterase 3 (EST_3): this was the only esterase which showed polymorphism. The single band in two of the stocks (YT3, YT4) had a minor difference in mobility from the single band in the other stocks.

c) Hexokinase (HK_1): three different patterns of activity were observed among the six stocks of syngen 3. At least one and at most two out of three bands of activity were held in common between stocks showing different patterns.

d) Phosphogluco isomerase (PGI_1): this enzyme demonstrated the greatest amount of polymorphism amongst the stocks of syngen 3. Two of the six stocks were variants. There was no common band of activity between the two variants or between the variants and the other four stocks. As a result, three different phenotypic forms of phosphogluco isomerase patterns were observed in stocks of syngen 3.

Figure 13:

Intra-syngenic variations between stocks of syngen 3, for α-naphthyl propionate esterase.

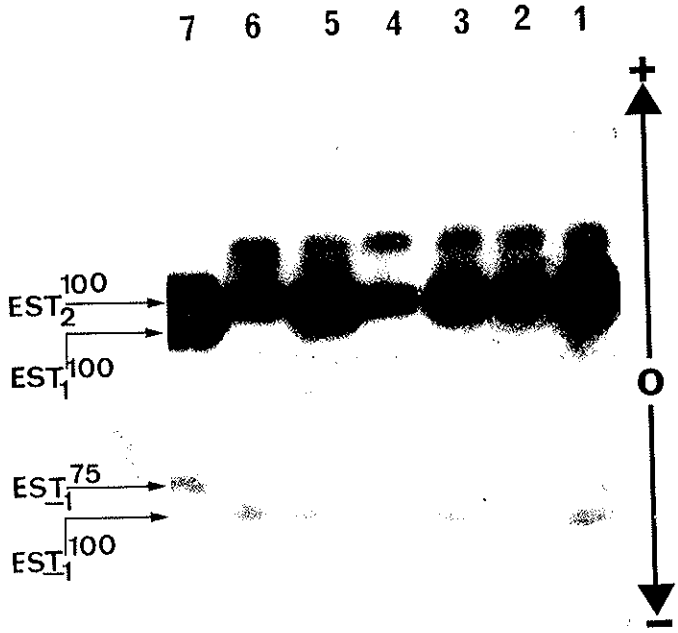
1 : KOK	4 : KY	7 : D119a
2 : KT2	5 : YT4	
3 : YT1	6 : YT3	

Figure 14:

Intra-syngenic variations between stocks of syngen 3, for α-naphthyl butyrate esterase.

1 : KOK	4 : KY	7 : D119a
2 : KT2	5 : YT4	
3 : YT1	6 : YT3	

0 : Origin


7 6 5 4 3 2 1
+
EST2 100
EST1 100
O
EST1 75
EST1 100
-

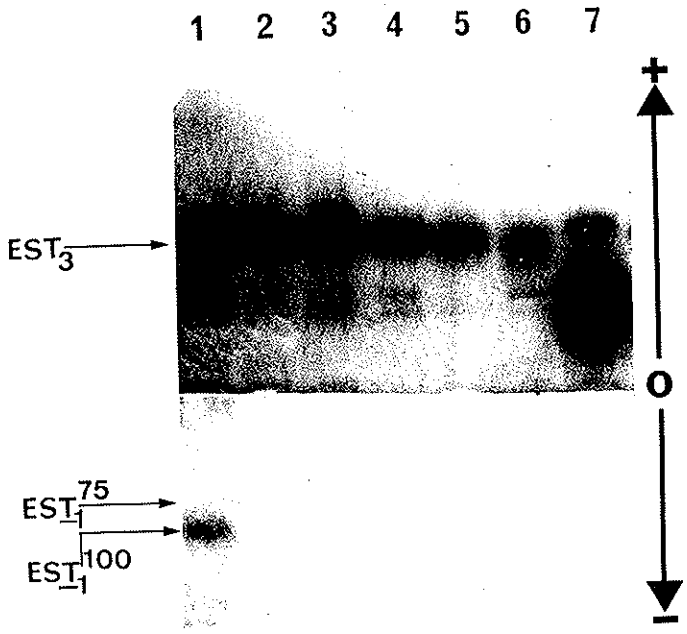

1 2 3 4 5 6 7
+
EST3
O
EST1 75
EST1 100
-

Figure 15:

Intra-syngenic variations between stocks of syngen 3 for malate dehydrogenase.

Figure 16:

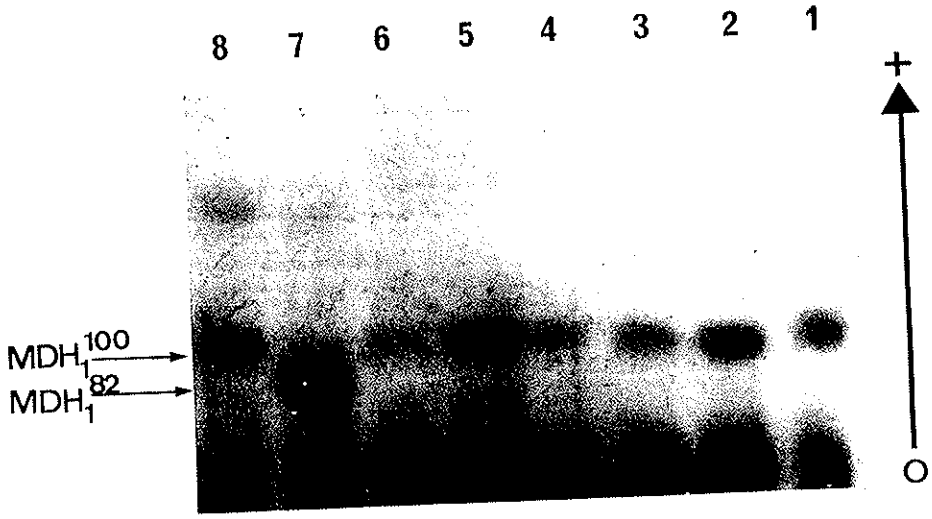
Intra-syngenic variations between stocks of syngen 3 for malic enzyme. Bands of malate dehydrogenase are also observable.

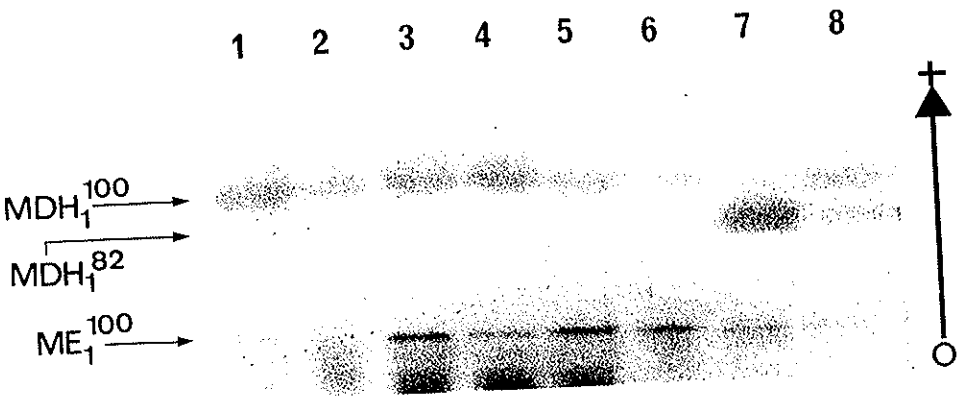
Figure 17:

Intra-syngenic variations between stocks of syngen 3 for malate dehydrogenase. (The electrophoretic conditions are the same as Figure 16, only NAD was used instead of NADP.)

1 : KOK	4 : KY	7 : D119a
2 : KT2	5 : YT4	8 : 2C → unknown
3 : YT1	6 : YT3	

0 : Origin


8 7 6 5 4 3 2 1
+
O
MDH1 100
MDH1 82


1 2 3 4 5 6 7 8
+
O
MDH1 100
MDH1 82
ME1 100

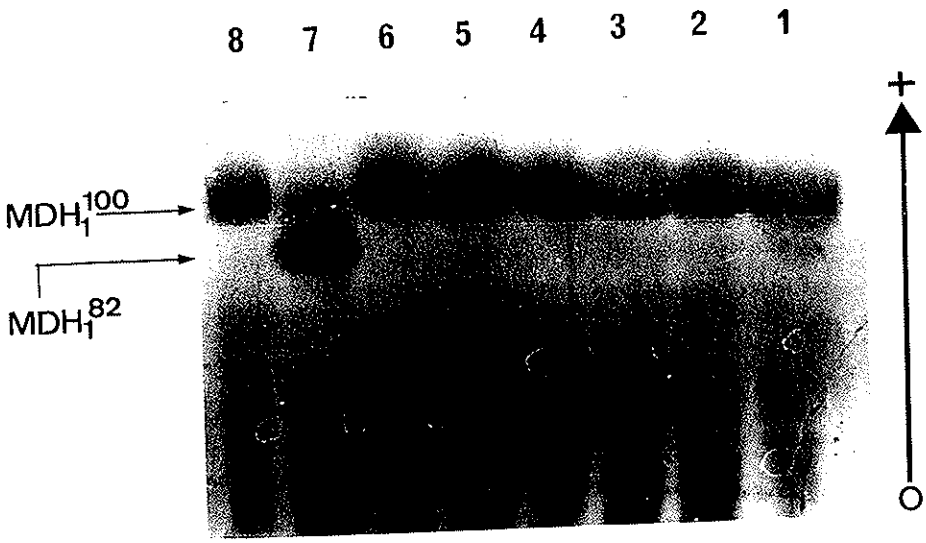

8 7 6 5 4 3 2 1
+
O
MDH1 100
MDH1 82

Figure 18:

Intra-syngenic variations between stocks of syngen 3 for aspartate aminotransferase.

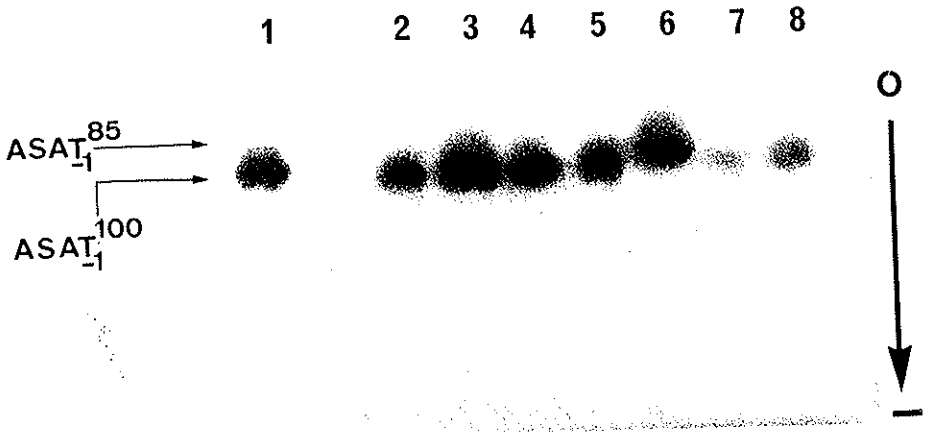
1 : KOK	4 : KY	7 : YT3
2 : KT2	5 : YT4	8 : 2C → unknown
3 : YT1	6 : D119a	

Figure 19:

Intra-syngenic variations between stocks of syngen 3 for superoxide dismutase.

1 : KOK	5 : YT4	8 : 2C → unknown
2 : KT2	6 : YT3	
3 : YT1	7 : D119a	

O : Origin


1
2
3
4
5
6
7
8
O
ASAT$_{-1}^{85}$
ASAT$_{-1}^{100}$
–

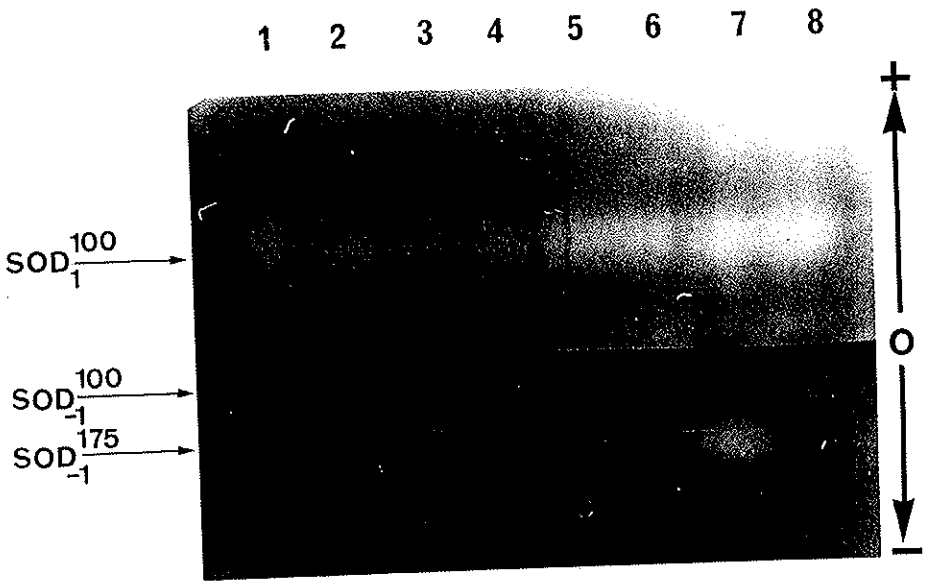

1
2
3
4
5
6
7
8
+
O
–
SOD$_{1}^{100}$
SOD$_{-1}^{100}$
SOD$_{-1}^{175}$

Figure 20:

Intra-syngenic variations between stocks of syngen 3 for phospho-gluco isomerase.

1 : KOK	4 : KY	7 : D119a
2 : KT2	5 : YT4	
3 : YT1	6 : YT3	

0 : Origin

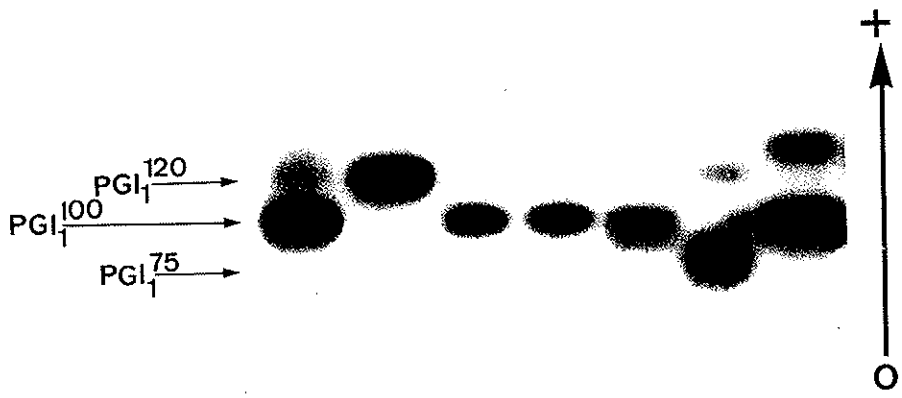

1
2
3
4
5
6
7
+
O
PGI1 120
PGI1 100
PGI1 75

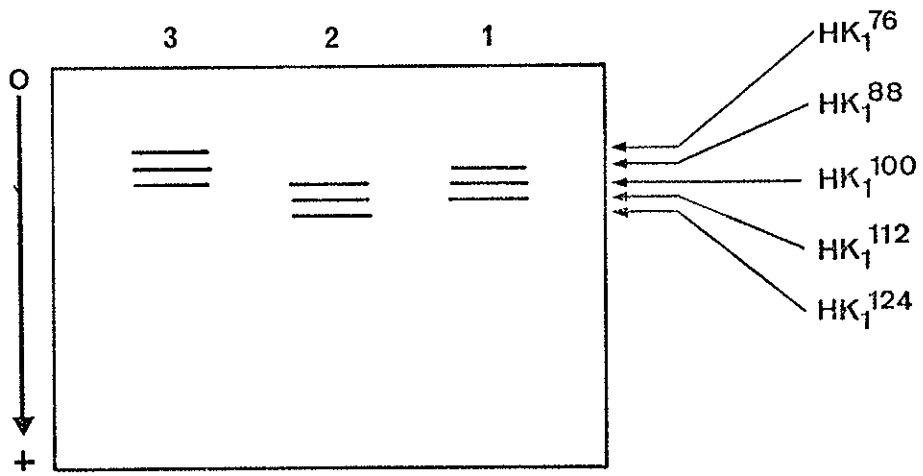
Figure 21:

Intra-syngenic variations between stocks of syngen 3 for hexokinase.

1 : KOK	4 : KY	7 : D119a
2 : KT2	5 : YT4	
3 : YT1	6 : YT3	

Figure 22:

The three different patterns of hexokinase activity, in the examined stocks of syngen 3.

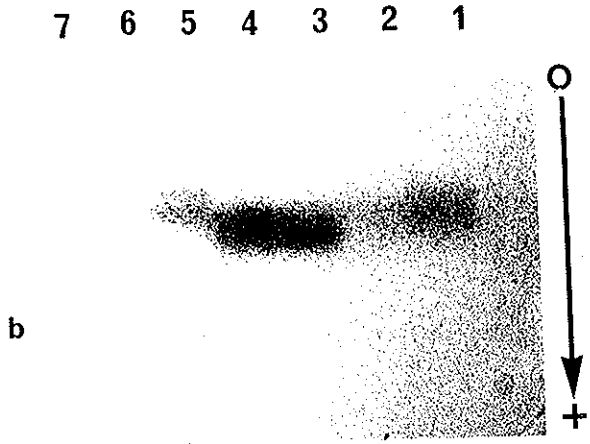


1 : KOK

2 : KT2, KT1, KY

3 : YT4, YT3

O : Origin



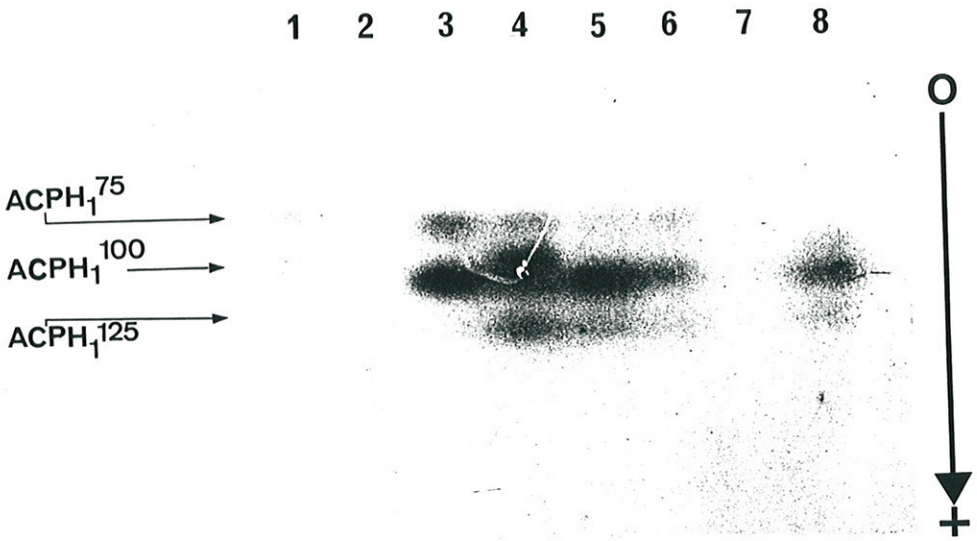
b : bacterial enzyme

Figure 23:

Intra-syngenic variations between stocks of syngen 3 for acid phosphatase.

1 : KOK	4 : KY	7 : D119a
2 : KT2	5 : YT4	8 : 2C → unknown
3 : YT1	6 : YT3	

O : Origin


1 2 3 4 5 6 7 8
O
ACPH$_1^{75}$
ACPH$_1^{100}$
ACPH$_1^{125}$
+

SECTION II

INTRASYNGENIC VARIATION BETWEEN STOCKS OF SYNGEN 12 IN PARAMECIUM CAUDATUM

The stocks of syngen 12 are listed in Table V with their geographical site of origin and their mating type.

Stock	Geographical Origin	Mating Type
TK 104	Tochigi, Japan	XXIV
TK 306	Tochigi, Japan	XXIV
TK 305	Tochigi, Japan	XXIII
TK 301	Tochigi, Japan	XXIII
Wa 33	Miyagi, Japan	XXIII
Wa 24	Miyagi, Japan	XXIII
Wa 16	Miyagi, Japan	XXIV
Nn 4	Niigate, Japan	XXIII
Nn 2a	Niigate, Japan	XXIV
Nn 1a	Niigate, Japan	XXIV
Hj 1	Iwata, Japan	XXIII

Table V

In order to make reliable comparisons between syngens, stock KOK (syngen 3) was run as standard, alongside the eleven stocks of syngen 12. For the methods of preparation of stocks and enzyme assay, see Materials and Methods (Chapter Two). The enzymes examined in this study are as follows:

Esterases
- (1) α-Naphthyl propionate esterase.
- (2) α-Naphthyl butyrate esterase.

(3) Malate dehydrogenase (MDH).

(4) Malic enzyme (Me).

(5) Aspartate: 2-oxoglutarate aminotransferase (ASAT).

(6) Superoxide dismutase (SOD).

(7) Phosphogluco isomerase (PGI).

(8) Hexokinase (HK).

(9) Acid phosphatase (AcPh).

Esterases

(1) α-Naphthyl propionate esterase

When α-naphthyl propionate was used to detect the pattern of esterase activity, up to 4 bands of activity were detected in stocks of syngen 12. One of these bands appeared in the cathodal part of the gels (Figure 24). This band had identical mobility in all the eleven stocks of syngen 12. This band also had identical mobility with the cathodal band of stock KOK (syngen 3), which was run alongside the other stocks as standard. This band was designated as EST_{-1}^{100}.

A second slower cathodal band was also detectable in some of the stocks (e.g. stock Nn2a in Figure 24). However, it did not appear consistently in different experiments and therefore the band was not considered in this study.

In the anodal part of the gels three bands of activity were detected (Figure 24). The nearest band to the origin was also the most darkly stained and had identical mobility in all eleven stocks of syngen 12. This band also appeared in stock KOK (syngen 3) and was designated as EST_{2}^{100}.

The second band of activity from the origin only appeared in the

samples which were kept in the deep-freeze for a few days and was excluded from this study.

The third band of enzyme activity which migrated furthest toward the anode was identical in all the stocks except stocks Nn 2a, Nn 4 and Wa 24 where it migrated at a slightly slower rate (Figure 24). The difference in the mobility of these two bands was particularly noticable when two stocks, one with fast and the other with slower form of the enzyme, were run side by side on the same gel (e.g. Nn 1a and Nn 2a in Figure 24). Because of the minor differences in the mobility of the furthest anodal band, it was designated generally as EST_3.

(2) α-Naphthyl butyrate esterase

A single cathodal band of enzyme activity with identical mobility was detected in all stocks, when the gels were stained with α-naphthyl butyrate as the substrate (Figure 25). This band migrated the same distance from the origin as the cathodal band when the other substrate α-naphthyl propionate was used, although the intensity of the cathodal band was greater with the latter substrate. It was assumed that these bands represented the same protein.

In the anodal part of the gel a single band of activity was observed (Figure 25). This band had identical mobility in eight of the stocks of syngen 12 as well as stock KOK of syngen 3. The remaining three stocks (Nn 2a, Nn 4 and Wa 24) shared an identical band which migrated slightly slower than the identical band of the other stocks. These bands corresponded to the bands designated as EST_3, when α-naphthyl propionate was used as substrate.

(3) Malate dehydrogenase (MDH)

This enzyme appeared as a single anodal band of activity. This band had identical mobility in all the stocks of syngen 12 (Figure 26). It should be noted that the single identical band of enzyme activity in syngen 12 had identical mobility with the band in stock KOK (syngen 3). This band was designated as MDH_1^{100}. Stock TK 306 had, in addition to the common band, a band of faster mobility. The latter band was excluded from this study, since it could have originated from bacteria (see Chapter Seven).

A further anodal band of activity also appeared in some of the stocks, but because of its inconsistency in different experiments, this band was excluded from the present study.

As shown in Figure 26, a smear existed from the origin for 1 cm in the direction of the anode. The smear became very faint or disappeared when the samples were heated for 30 minutes in 50°C before electrophoresis. This smear probably was contributed by a locus different from the one which is responsible for contributing the common band. Heating the samples for the same period of time at the same temperature did not have any effect either on the single identical band or on the faster band of stock TK 306.

(4) Malic enzyme (Me); (5) Aspartate aminotransferase (ASAT);

(6) Superoxide dismutase (SOD)

These three enzymes were monomorphic in the stocks of syngen 12. Their pattern of activity was identical to the pattern in stocks of syngen 3 for these three enzymes (see Figures 16, 18, 19).

(7) Phosphogluco isomerase (PGI)

A single identical band of mobility was observed in all eleven

stocks of syngen 12. Two stocks, Nn 2a and Nn 1a, had a slower identical band of mobility in addition to the common band (Figure 27).

The common band of activity, which had also the same mobility with the band in stock KOK (syngen 3), was designated as PGI_1^{100} and the slower band of mobility as PGI_1^{86}. The slower band of mobility in stocks Nn 2a and Nn 1a had identical mobility with the band in stock 2C (unknown) (Figure 27).

A further anodal band of activity was observed in some of the stocks, but because of its inconsistency, it was excluded from this study.

(8) Hexokinase (HK)

Three bands of the enzyme activity were detected in each of the stocks (Figure 28). The most common band observed among the stocks was designated as HK_1^{100}. This band corresponded to the most common band in syngen 3. Stocks TK 306, TK 305, Wa 33 and Hj 1 had two slower bands (HK_1^{76}, HK_1^{88}) and the rest of the eleven stocks had two faster bands (HK_1^{112}, HK_1^{124}) in addition to the common band of activity (Figure 29). Band HK_1^{124} is very faint and is not clearly distinguishable on the accompanying photograph.

(9) Acid phosphatase (AcPh)

Under the electrophoretic conditions used in this study, three bands of enzymic activity with identical mobility were detected in all eleven stocks of syngen 12 (Figure 30). The relative intensity of the bands varied from one stock to the other. The second band of activity from the origin was the most stable and darkly stained among the three bands. This band was designated as $AcPh_1^{100}$, the first band of activity from the origin as $AcPh_1^{75}$, and the fastest band of activity as $AcPh_1^{125}$.

The first and second bands of activity from the origin have identical mobility with stock KOK (syngen 3).

In addition to the three described bands, a smear with a slower mobility also appeared in stock Hj 1 (syngen 12) (Figure 30). The smear was always present in this stock, in different experiments. However, because of the possibility that the smear is representing bacterial enzyme it was excluded from this study (for further details see Chapter Seven).

Assessment of similarity among examined stocks of syngen 12

Table VI shows the calculated value for coefficient of identity between any of two examined stocks of syngen 12.

Stocks	TK 104	306	305	301	Wa 33	24	16	Nn 4	2a	1a	Hj 1
TK 104	-	86	86	100	86	93	100	93	87	93	86
TK 306		-	100	86	100	80	86	80	75	80	100
TK 305			-	86	100	80	86	80	75	81	100
TK 301				-	86	93	100	93	86	93	86
Wa 33					-	75	86	80	75	86	100
Wa 24						-	86	93	93	86	80
Wa 16							-	93	86	93	86
Nn 4								-	93	86	86
Nn 2a									-	93	75
Nn 1a										-	81
Hj 1											-

The coefficient of identity varies from 75 to 100. The four cases, where the figure 75 is observed for the coefficient of identity involve

stock Nn 2a. It can be suggested that this stock is genetically more isolated from the other stocks of syngen 12. On the other hand, a coefficient of identity as high as 93 is also observed in some pair-wise comparisons (e.g. Wa 24/Nn 2a). There is also no correlation between the degree of similarity and the geographical origin of the stocks. For example, the figure 100 is observed between two stocks of the same geographical origin such as TK 104 and TK 301 as well as two stocks of different geographical origin such as TK 104 and Wa 16 or Wa 24 and Nn 4.

Categorization of enzymes

The enzymes can be categorized in terms of being monomorphic and in terms of their degree of polymorphism.

1) The following enzymes were monomorphic in the eleven stocks of syngen 12: EST_{-1}; EST_2; MDH_1; Me_1; $ASAT_1$; SOD_1; SOD_{-1}; $AcPh_1$.

2) The polymorphic enzymes according to their degree of polymorphism were as follows:

a) Phosphogluco isomerase (PGI_1); two of the stocks were variants, although they possessed the common band of the other stocks, as well as an extra band. The two variants had identical pattern .

b) Esterase 3 (EST_3); three of the eleven stocks had variant bands. The variant stocks (Nn 2a, Nn 4 and Wa 24) had identical patterns amongst themselves.

c) Hexokinase (HK_1); there were two patterns of enzyme activity in the eleven stocks. The two types shared only one of their three bands.

Figure 24:

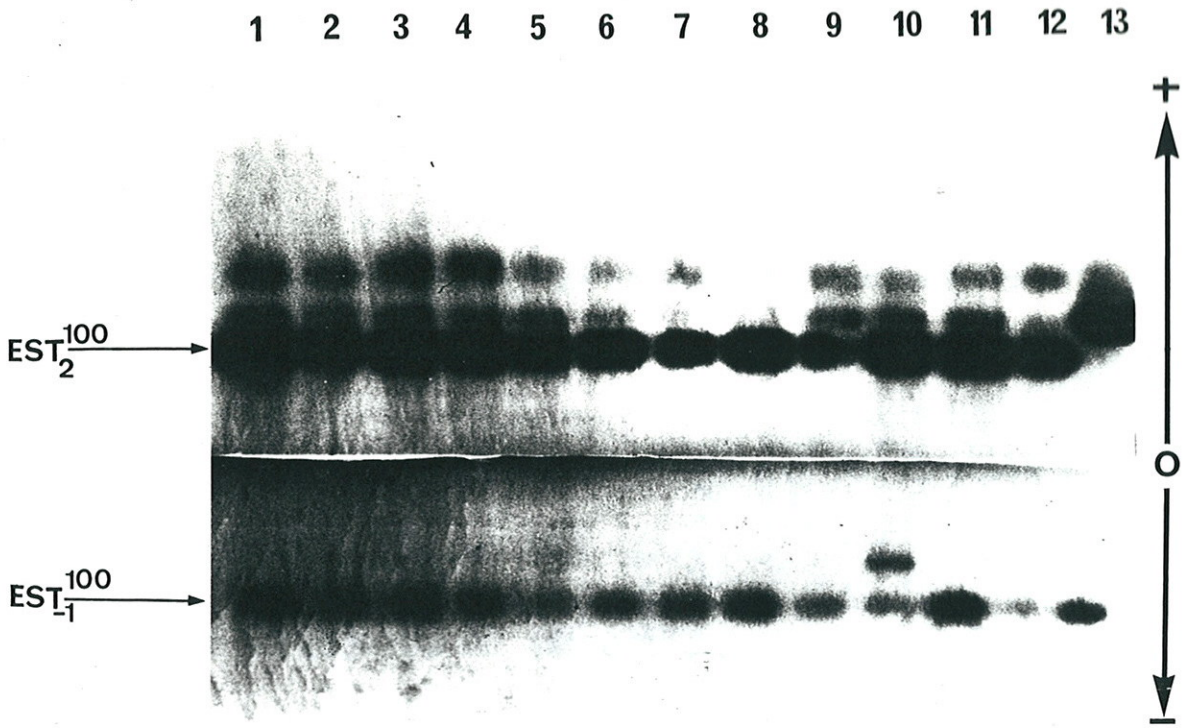
Intra-syngenic variations between stocks of syngen 12 for α-naphthyl propionate esterase.

Figure 25:

Intra-syngenic variations between stocks of syngen 12 for α-naphthyl butyrate esterase.

1 :	KOK	syngen 3	7 :	Wa24	syngen 12
2 :	TK104	syngen 12	8 :	Wa16	" "
3 :	TK306	" "	9 :	Nn4	" "
4 :	TK305	" "	10 :	Nn2a	" "
5 :	TK301	" "	11 :	Nn1a	" "
6 :	Wa33	" "	12 :	Hj1	" "
			13 :	2C	unknown

0 : Origin


1 2 3 4 5 6 7 8 9 10 11 12 13
+
O
−
EST$_2^{100}$
EST$_{-1}^{100}$

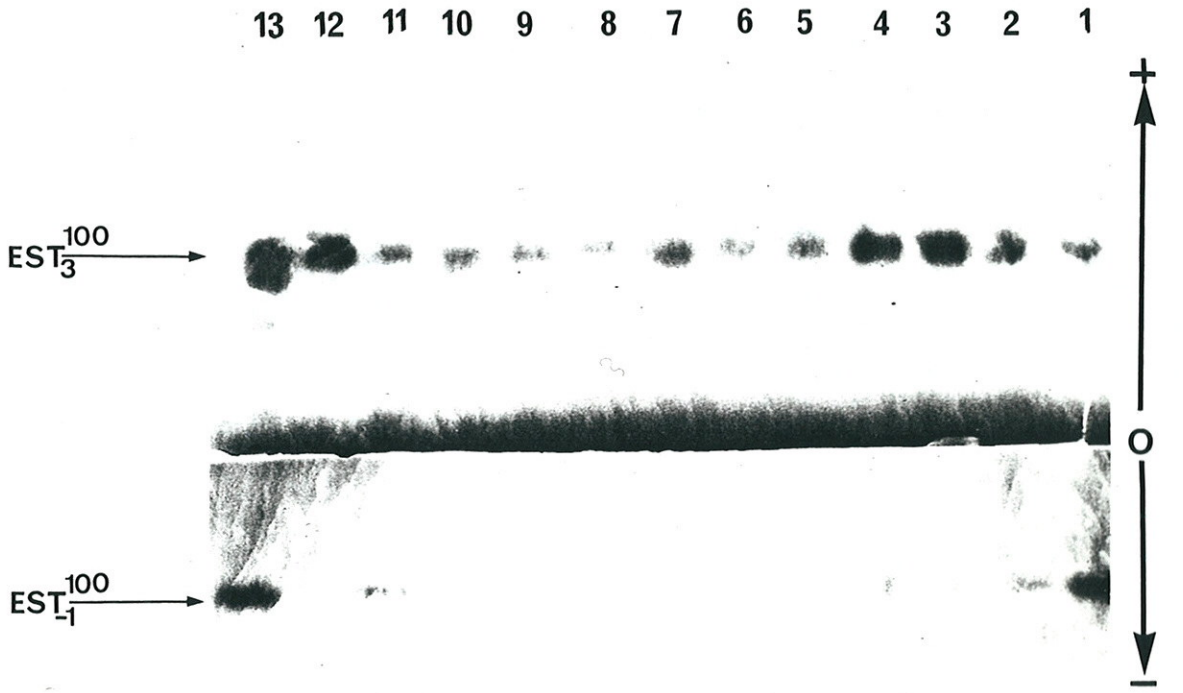

13 12 11 10 9 8 7 6 5 4 3 2 1
+
O
−
EST$_3^{100}$
EST$_{-1}^{100}$

Figure 26:

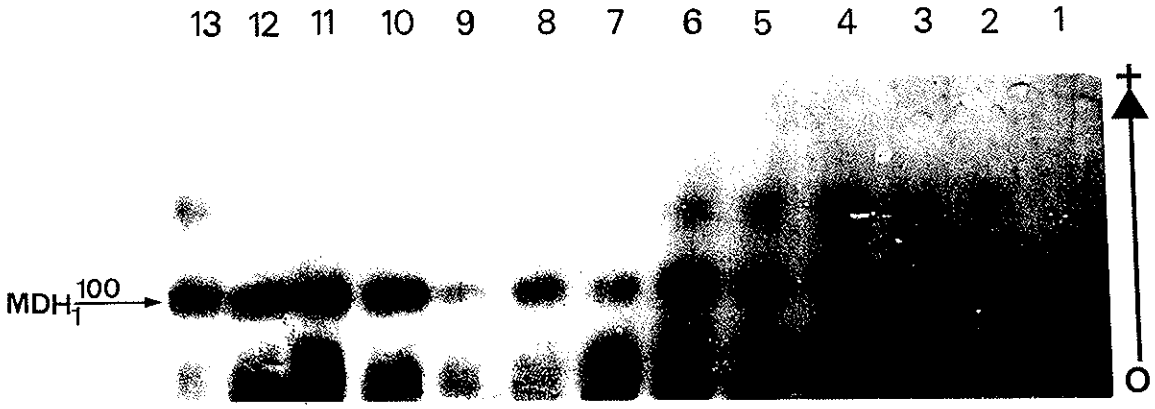
Intra-syngenic variations between stocks of syngen 12 for malate dehydrogenase.

Figure 27:

Intra-syngenic variations between stocks of syngen 12 for phospho-gluco isomerase.

1 :	KOK	syngen 3	7 :	Wa24	syngen 12
2 :	TK104	syngen 12	8 :	Wa16	" "
3 :	TK306	" "	9 :	Nn4	" "
4 :	TK305	" "	10 :	Nn2a	" "
5 :	TK301	" "	11 :	Nn1a	" "
6 :	Wa33	" "	12 :	Hj1	" "
			13 :	2C	unknown

O : Origin


13 12 11 10 9 8 7 6 5 4 3 2 1
MDH1 100
+
O

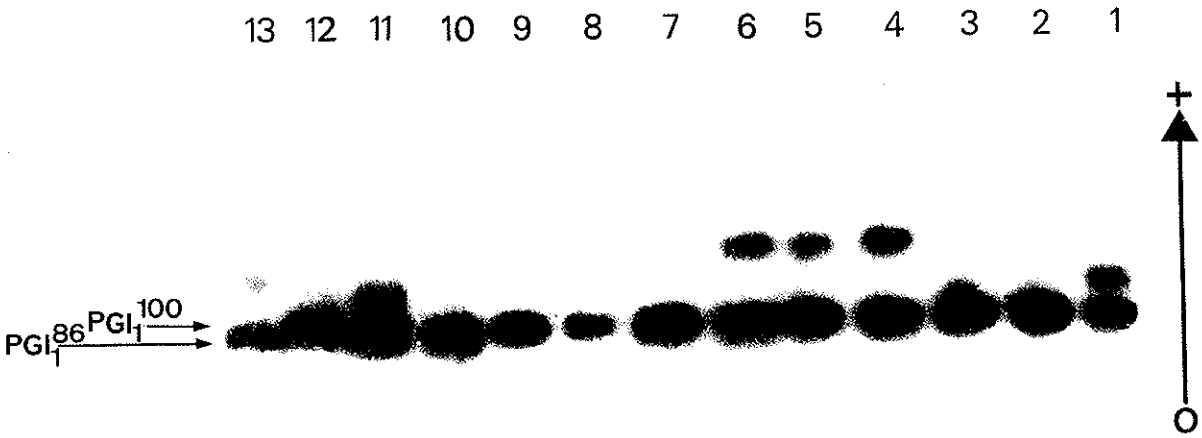

13 12 11 10 9 8 7 6 5 4 3 2 1
PGI 86 PGI1 100
+
O

Figure 28:

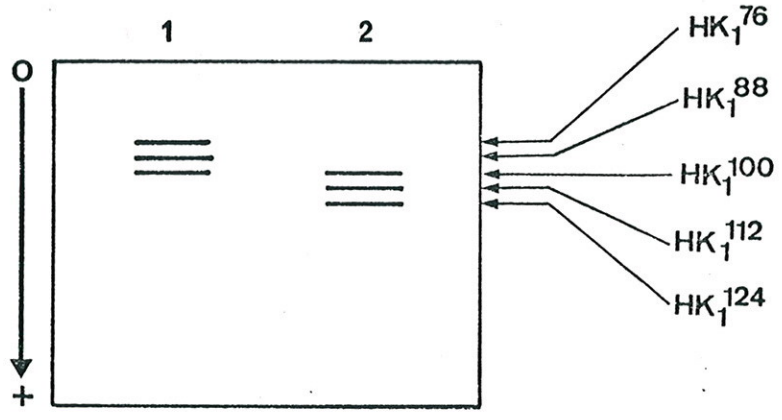
Intra-syngenic variations between stocks of syngen 12 for hexokinase.

1 :	KOK	syngen 3	7 :	Wa24	syngen 12
2 :	TK104	syngen 12	8 :	Wa16	" "
3 :	TK306	" "	9 :	Nn4	" "
4 :	TK305	" "	10 :	Nn2a	" "
5 :	TK301	" "	11 :	Nn1a	" "
6 :	Wa33	" "	12 :	Hj1	" "
			13 :	2C	unknown

O : Origin

Figure 29:

The two different patterns of hexokinase activity, in the examined stocks of syngen 12.



1 : TK306, TK305, Wa33, Hj1

2 : TK104, TK301, Wa24, Wa16,
Nn4, Nn2a, Nn1a

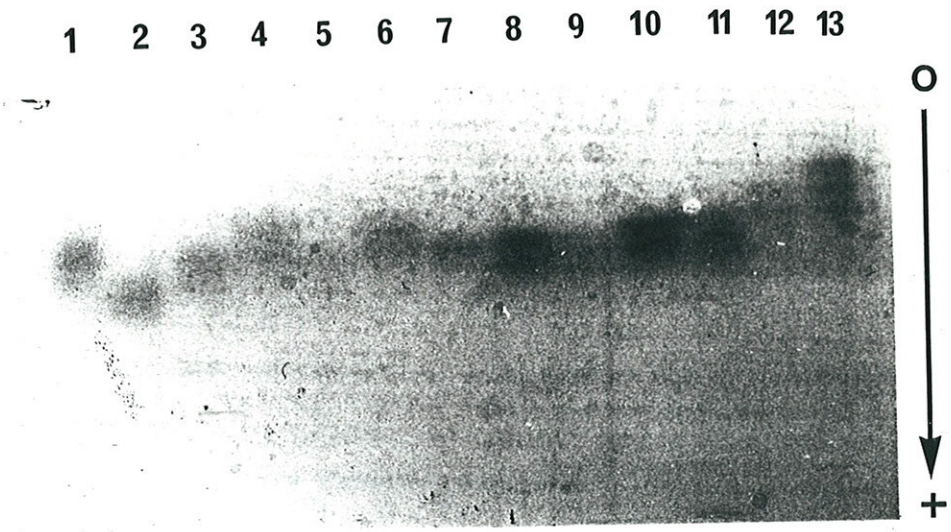
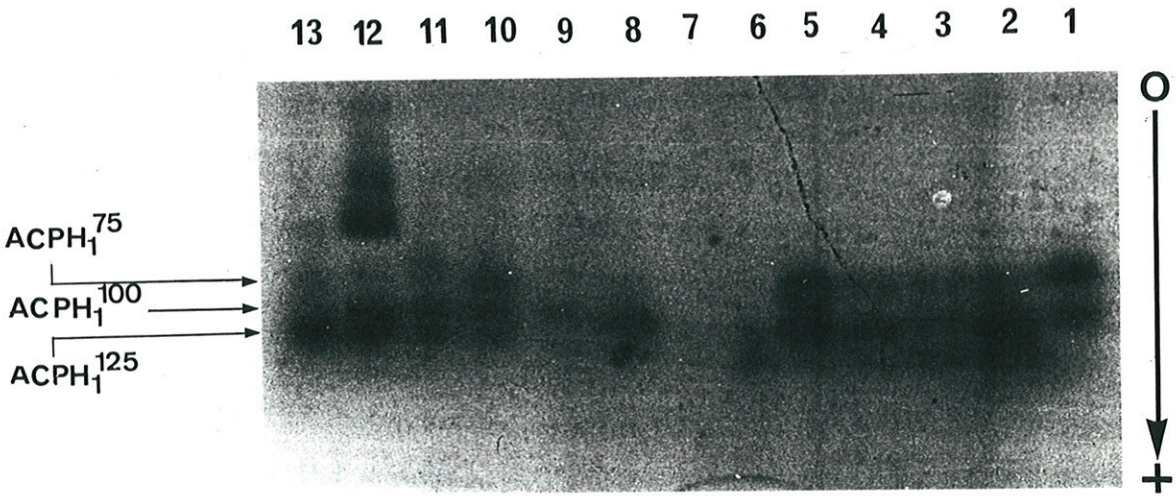

1 2 3 4 5 6 7 8 9 10 11 12 13
O
+

Figure 30:

Intra-syngenic variations between stocks of syngen 12 for acid phosphatase.

1 :	KOK	syngen 3	7 :	Wa24	syngen 12
2 :	TK104	syngen 12	8 :	Wa16	" "
3 :	TK306	" "	9 :	Nn4	" "
4 :	TK305	" "	10 :	Nn2a	" "
5 :	TK301	" "	11 :	Nn1a	" "
6 :	Wa33	" "	12 :	Hj1	" "
			13 :	2C	unknown

O : Origin


13 12 11 10 9 8 7 6 5 4 3 2 1
O
+
ACPH1 75
ACPH1 100
ACPH1 125

SECTION III

INTRASYNGENIC VARIATION BETWEEN STOCKS OF SYNGEN 1 IN PARAMECIUM CAUDATUM

The stocks of syngen 1 are listed in Table VII, with their geographical site of origin and their mating types.

Stock	Geographical Origin	Mating Type
MS 25	Miyagi, Japan	I
ISn	Iwate, Japan	I
NS 2	Nagano, Japan	I
Ih 4	Iwate, Japan	II
Ih 2	Iwate, Japan	I

Table VII

In order to make reliable comparisons between syngens, stock KOK (syngen 3) was run alongside the five stocks of syngen 1. For the method of preparation of samples and enzyme assay, see Materials and Methods (Chapter Two). Enzymes examined in this study were as follows:

Esterases (EST)
- (1) α-Naphthyl propionate esterase.
- (2) α-Naphthyl butyrate esterase.

(3) Malate dehydrogenase (MDH).

(4) Malic enzyme (Me).

(5) Aspartate : 2-oxoglutarate aminotransferase (ASAT).

(6) Superoxide dismutase (SOD).

(7) Phosphogluco isomerase (PGI).

(8) Hexokinase (HK).

(9) Acid phosphatase (AcPh).

Esterases

(1) α-Naphthyl propionate esterase

A single band of the enzyme was detected in the cathodal part of the gel when α-naphthyl propionate was used as substrate (Figure 31). This band also had the same mobility as the cathodal band of stock KOK (syngen 3). This band was designated as EST_{-1}^{100}.

In the anodal part of the gel, three to four bands of the enzyme activity appeared in each of the stocks. The nearest band to the origin was the darkest staining and the most stable of all the bands. This band had the same mobility in all the five stocks of syngen 1 and the nearest band to the origin in stock KOK (syngen 3) (Figure 31). This band was designated as EST_{2}^{100}. The second anodal band only appeared in the samples which were kept in deep-freeze for more than a few days and was excluded from this study.

The furthest anodal band had identical mobility in all five stocks. This band also corresponded with the third anodal band of stock KOK (syngen 3). However, one of the stocks (NS 2), had a faster band of mobility, in addition to the common band (Figure 31). The furthest anodal band was designated as EST_{3}.

(2) α-Naphthyl butyrate esterase

A single band was observed in the cathodal part of the gel (Figure 32). This band corresponded to the single cathodal band when α-naphthyl propionate was used as substrate. It was assumed that the cathodal band of activity represented the same protein which interacted with both

substrates. However, the intensity of interaction was slightly greater when α-naphthyl propionate was used.

In the anodal part of the gel, a single band of activity was detected. This band had identical mobility in all the stocks. Stock NS 2 had, in addition to this band, a second band which migrated slightly faster (Figure 32). These bands corresponded to the bands designated as EST_3, when α-naphthyl propionate was used as substrate.

(3) Malate dehydrogenase (MDH); (4) Malic enzyme (Me); (5) Aspartate aminotransferase (ASAT); and (6) Superoxide dismutase (SOD)

These four enzymes were monomorphic in the examined stocks of syngen 1. Their pattern of activity was identical to the described pattern in stocks of syngen 3 (see Figures 15, 16, 18, 19).

(7) Phosphogluco isomerase (PGI)

The first anodal band of activity from the origin had an identical mobility in all the stocks, with the exception of stock Ih 2. The single band of the latter stock migrated at a slower rate than the common band (Figure 33). The common band of activity, which also had identical mobility to the band in stock KOK (syngen 3) was designated as PGI_1^{100} and the slower band in stock Ih 2 as PGI_1^{86}.

A second band of enzyme activity also appeared in some of the stocks. Variation was observed between these bands, but because of their inconsistency in appearance, they were excluded from this study.

(8) Hexokinase (HK)

Three bands of activity were detected in each of five stocks of syngen 1 (Figure 34). The most common band of activity in stocks of syngen 1, corresponded to the band designated as HK_1^{112} in stock KOK

(syngen 3). In order to avoid any further confusion, when comparing stocks of different syngens, the most common band in syngen 1 was designated as HK_1^{112} instead of HK_1^{100}. Three stocks, Ms 2S, ISn and Ih 2, in addition to the band HK_1^{112}, had two slower bands of enzyme mobility (HK_1^{100} and HK_1^{88}). Stock NS 2 in addition to the bands HK_1^{100} and HK_1^{112}, had a third faster band HK_1^{124}. The remaining stock Ih 4 had the bands HK_1^{112}, HK_1^{124} and HK_1^{136} (Figure 35).

(9) Acid Phosphatase (AcPh)

All five stocks of syngen 1 had three bands of enzyme activity. These three bands had identical mobilities in the five stocks. The band which was designated as $AcPh_1^{100}$, in stock KOK (syngen 3), appeared to be very faint in stocks of syngen 1. This faint band was therefore designated as $AcPh_1^{100}$ in the stocks of syngen 1. The slower band next to the $AcPh_1^{100}$ was designated as $AcPh_1^{89}$. This band had no corresponding band in stock KOK (syngen 3). The slowest band of mobility was designated as $AcPh_1^{75}$, and had a corresponding band in stock KOK (Figure 36).

Assessment of similarity between stocks of syngen 1

Table VIII shows the calculated value for the coefficient of identity between any of the two stocks of syngen 1.

Stocks	Ms 2S	ISn	NS-2	Ih-4	Ih-2
Ms 2S	-	100	87	87	93
ISn		-	87	87	93
NS-2			-	87	81
Ih-4				-	81
Ih-2					-

The coefficient of identity varies between 81 and 100, no particular close or distant relationship can be discerned between stocks of syngen 1 from Table VIII.

Categorization of enzymes

The reported enzymes can be categorized as to their monomorphism and in terms of their degree of polymorphism.

1) The following enzymes were monomorphic in the five stocks of syngen 1: EST_2; EST_{-1}; MDH_1; Me_1; $ASAT_{-1}$; SOD_1; SOD_{-1}; $AcPh_1$.

2) The polymorphic enzymes according to their degree of polymorphism were as follows:-

a) Esterase 3 (EST_3); only one of the five stocks showed variation for this enzyme. The variant (NS2) had an additional band of activity.

b) Phosphogluco isomerase (PGI_1); one of the five stocks was variant. This stock (Ih 2) had a slower band, instead of the common band in the other stocks.

c) Hexokinase (HK_1); three different patterns of enzyme activity detected amongst the five stocks. However, each stock had at least one common band of mobility with the other stocks.

Figure 31:

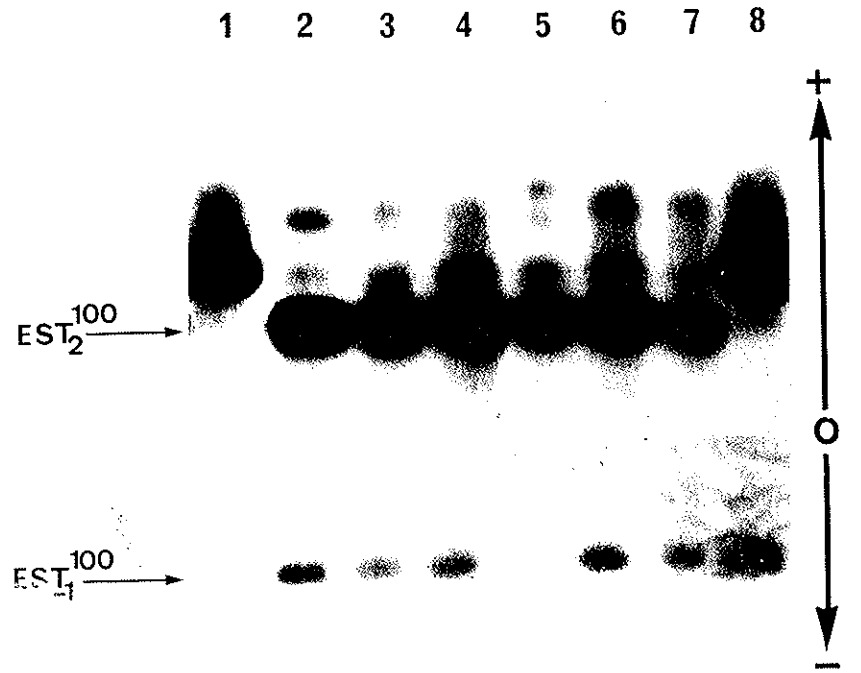
Intra-syngenic variations between stocks of syngen 1 for α-naphthyl propionate esterase.

Figure 32:

Intra-syngenic variations between stocks of syngen 1 for α-naphthyl butyrate esterase.

1 :	2C	unknown	5 :	Ns2	syngen 1
2 :	KOK	syngen 3	6 :	Ih4	" "
3 :	Ms25	syngen 1	7 :	Ih2	" "
4 :	Isn	" "	8 :	2C	unknown

O : Origin


1 2 3 4 5 6 7 8
+
EST2100
O
EST1100
−

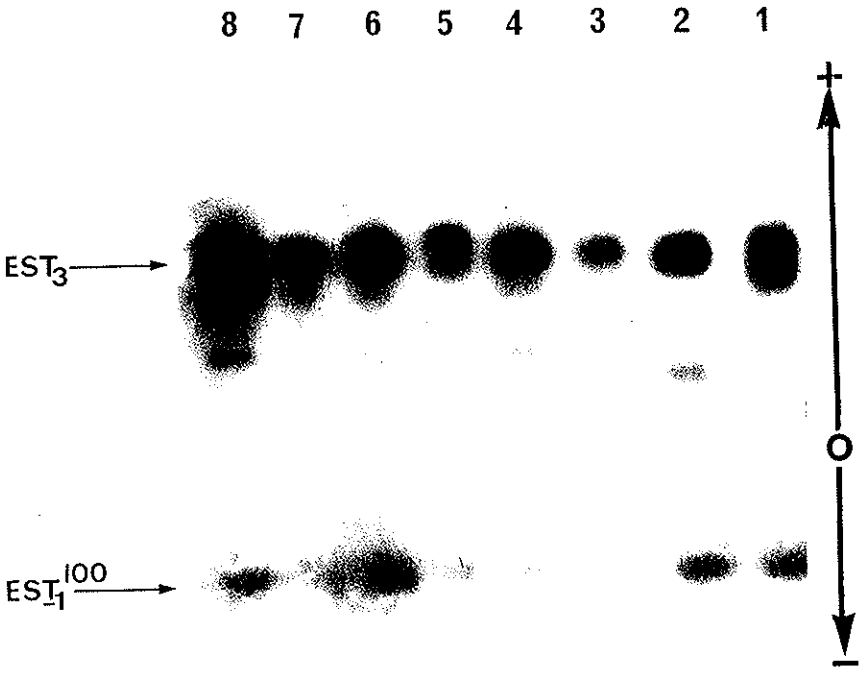

8 7 6 5 4 3 2 1
+
EST3
O
EST1100
−

Figure 33:

Intra-syngenic variations between stocks of syngen 1 for phospho-gluco isomerase.

1 :	Ms25	syngen 1	4 :	Ih4	syngen 1
2 :	Isn	" "	5 :	Ih2	" "
3 :	NS2	" "	6 :	KOK	syngen 3

Figure 34:

Intra-syngenic variations between stocks of syngen 1 for hexokinase.

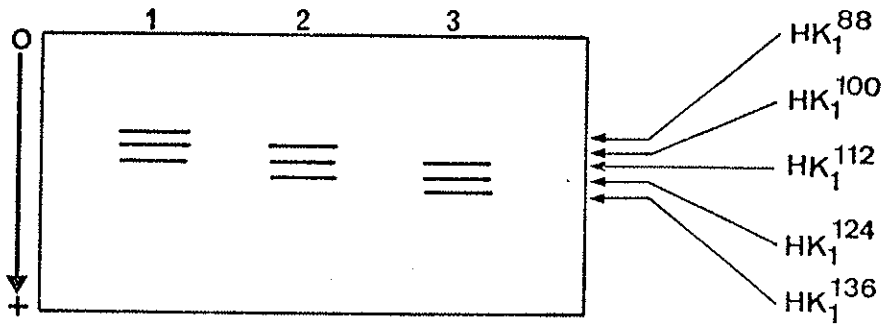
1 : MS25, Isn, Ih2

2 : NS2

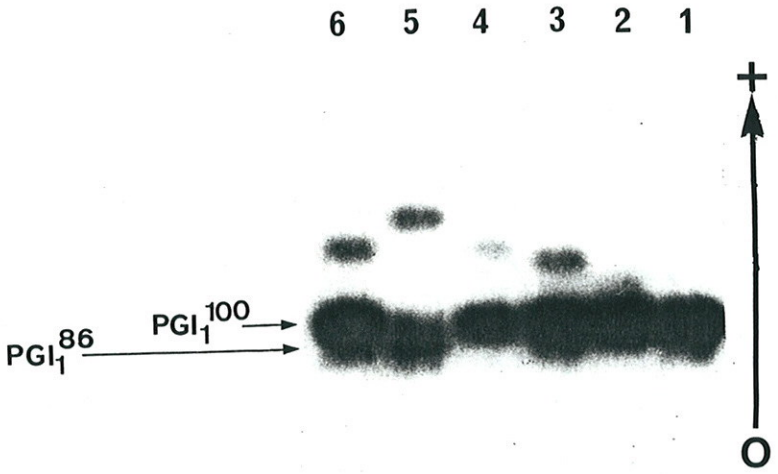
3 : Ih4

Figure 35:

Three different patterns of hexokinase activity in five stocks of syngen 1.



O : Origin


6 5 4 3 2 1
+
PGI1 100
PGI1 86
O

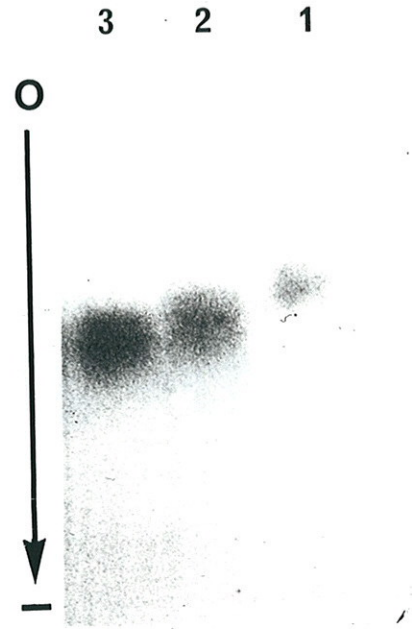
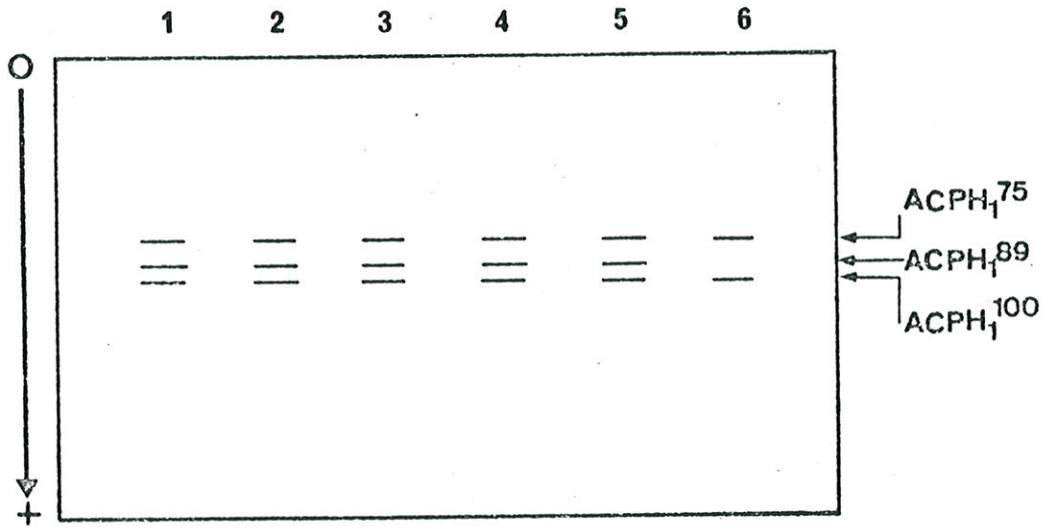

3 2 1
O
–

Figure 36:

Intra-syngenic variations between stocks of syngen 1 for acid phosphatase.

1 :	MS25 syngen 1	4 :	Ih4 syngen 1
2 :	Isn " "	5 :	Ih2 " "
3 :	NS2 " "	6 :	KOK syngen 3

O : Origin


1
2
3
4
5
6
O
+
ACPH1 75
ACPH1 89
ACPH1 100

DISCUSSION

The purpose of the experiments reported here was to determine if there were differences between syngens in their enzymic patterns of activity. First, however, variation between stocks of the same syngen had to be assessed. This could vary depending on the particular enzymes and number of stocks examined as well as on evolutionary pathways in this genus.

The following characterization of the enzyme variation within syngens 1, 3, 12 and 13 can be made.

1) Enzymes without intra or inter-syngenic variation

There are enzymes which do not show intra-syngenic variation, and also do not show any inter-syngenic variation. These enzymes are considered to be monomorphic in the four syngens of P. caudatum and include EST_2; EST_{-1}, MDH_1; Me_1; $ASAT_{-1}$; SOD_1 and SOD_{-1}.

2) Enzymes with low level of intra-syngenic but without inter-syngenic variation

Only esterase 3 (EST_3) is categorized in this group. This enzyme does not show any inter-syngenic variation. However, a low level of intra-syngenic variation is observed in this enzyme. It should be noted that in inter-syngenic comparisons, the most common form of an enzyme is compared with respect to any two syngens.

3) Enzymes with high level of intra-syngenic but without inter-syngenic variation

Phosphogluco isomerase is the sole representative of this group. Although the level of intra-syngenic variation is high relative to EST_3, this enzyme does not show any inter-syngenic variation.

4) Enzymes with intra and inter-syngenic variation

The two enzymes $AcPh_1$ and HK_1 have some degree of intra and inter-syngenic variation.

Comparisons in degree of enzyme variation between P. caudatum and other protozoa

Some of the enzymes described above have also been examined in other protozoa. Comparisons will now be made between the degree of their variation in the four syngens of P. caudatum and other protozoa.

1) Esterases

The monomorphism of the two esterases (EST_2, EST_{-1}) and the low level of polymorphism in EST_3 in syngens 1, 3, 12 and 13 of P. caudatum was a surprise. Esterases are known to be generally variable in both plants and animals (Johnson, 1974; Selander et al., 1973).

Intra and inter-syngenic variation in esterases have also been readily found in T. pyriformis and P. aurelia complex (Allen et al., 1971a, 1971b). In T. pyriformis complex, Allen and Weremiuk (1971a) examined esterase variation in at least two strains in each of the syngens and found extensive variation both within and between syngens. Therefore, it was difficult to assess the relationship between syngens based on their esterases. On the other hand esterases in the P. aurelia complex rarely showed intra-syngenic variation except in species (syngen) 2, although inter-syngenic variation was observed in this protozoan. Utilizing esterases as a means of syngens identification with the exception of the grouping 1, 3, 5 and 7, 13 it was possible to distinguish the rest of the syngens (species) of P. aurelia (Allen et al., 1971b).

Comparing the esterase variability in the three mentioned protozoa, it can be seen that the extent of esterase variation was much higher in T. pyriformis complex than in either P. aurelia or P. caudatum. The extent of esterase variation was higher in P. aurelia than in P. caudatum. It should be noted however, that lack of esterase variation in syngens 1, 3, 12 and 13 of P. caudatum might only apply to these four syngens,

rather than being a general characteristic of P. caudatum. Esterases variants have been detected in unknown stocks (see Chapter Three).

2) Malate dehydrogenase

Malate dehydrogenase showed neither intra nor inter-syngenic variation in the four syngens of P. caudatum. However, a variant form of this enzyme was observed in the unknown stock Cal1 (see Chapter Three).

Intrasyngenic variation has been reported in different stocks of syngen 1 in T. pyriformis complex for this enzyme (Borden et al., 1973a). Malate dehydrogenase has also been reported to vary in different syngens of the former complex of species (Borden et al., 1977). Williams and Smith (1980) studied malate dehydrogenase as a genetic marker for use in clonal ageing in Paramecium tetraurelia. They reported that two of the eleven examined stocks each exhibited bands of unique mobility. This species, formerly called syngen 4 of P. aurelia, has not shown a particular propensity for enzyme variation, when compared with the other species of P. aurelia (Allen et al., 1975). It is postulated that malate dehydrogenase shows a low level of polymorphism in the latter complex of species.

In the rodent parasites, Plasmodium berghei and P. vinckei, two forms of malate dehydrogenase (cathodal and anodal) were detected (Charter, 1973). The cathodal form of the enzyme was invariant. However, two different patterns were found with the anodal form of the enzyme. More recently this enzyme has been examined in three species of Crithidia brasiliensis (a trypanosomatid) and it was reported that on the basis of malate dehydrogenase variation, it was possible to distinguish one of the species from the other two (DE SA et al., 1980).

3) Malic enzyme (Me)

Malic enzyme showed neither intra nor inter-syngenic variation in

the four syngens of P. caudatum. When unknown stocks were included, the enzyme proved still to be monomorphic (see Chapter Three).

In spite of complete monomorphism in P. caudatum this enzyme was reported to be polymorphic in T. pyriformis complex (Borden et al., 1977). Some other parasitic protozoa, such as Leishmania species and six trypanosomatid species, have also been reported to show variation in the electrophoretic pattern of malic enzyme activity (Al-Taqi et al., 1978; Goncalves De Lima et al., 1979).

4) Aspartate aminotransferase

The enzyme, aspartate aminotransferase, was also monomorphic in the four syngens of P. caudatum. When the unknown stocks were included, only one stock showed variation for this enzyme (see Chapter Three). Aspartate aminotransferase has not been examined in P. aurelia or in T. pyriformis complex. However, it has been reported that of six species of trypanosomatids, only two were indistinguishable on the basis of this enzyme (Goncalves De Lima et al., 1979).

5) Superoxide dismutase

The two systems of superoxide dismutase (SOD_1 and SOD_{-1}) both were monomorphic in the four syngens of P. caudatum. However, including the unknown stocks a single variant form of SOD_{-1} was detected (see Chapter Three).

Superoxide dismutase has been reported in T. pyriformis complex (Borden et al., 1977; Nanney et al., 1980). More bands were reported in the recent study than in the earlier study. However, variation was observed in all 13 species of T. pyriformis complex for this enzyme in both studies. Nanney et al., 1980, also reported that of the three newly isolated mating groups 5, 13 and 14, it was possible to distinguish mating group 5 from the other two, on the basis of the superoxide dismutase electrophoretic mobilities of the new strains.

6) Phosphogluco isomerase

The types of intra-syngenic variations were specific in the three syngens - 1, 3 and 12. However, the most common form of the enzyme was identical in these three syngens as well as the two stocks of syngen 13.

In P. aurelia, phosphogluco isomerase showed high intra and inter-syngenic variation in electrophoretic mobility (Tait, 1978). The degree of polymorphism in P. aurelia for this enzyme was such that one syngen was often indistinguishable from the variants in other syngens. But, surprisingly, by screening this enzyme in P. aurelia, it was possible to distinguish syngens 1 and 5. These two species were indistinguishable following the screening of eight enzyme loci in previous studies, ICD_s, ICD_m, HBDH, FUM, GDH, EST A, EST B and EST C (Tait, 1970a; Allen et al., 1971b).

Inter-species variation of phosphogluco isomerase has also been reported in four trypanosomes species - Trypanosome lewisi, T. congolense, T. vivax and T. brucei brucei (Bagster and Parr, 1973). However, the two trypanosomatid species of insects, Crithidia deanei and Herpetomonas samuelpessoai, did not show any variations in their phosphogluco isomerase pattern of activity (Gonclaves, De Lima et al., 1979).

7) Hexokinase

Intra and inter-syngenic variations were also observed for the enzyme, hexokinase. The amount of intra-syngenic variation was so extensive making it difficult to distinguish a common pattern in any of the syngens, with any confidence. Furthermore, there was an overlap in the pattern of intra and inter-syngenic variation. It was not possible

therefore, to differentiate the syngens on the basis of their hexokinase activity. It should be noted that poor resolution, the existence of smearing and also the closeness in relative mobility of bands, can be a major problem in identification of this enzyme in different stocks. Attempts to run the gels for longer periods of time, in order to increase bands resolution, resulted in some of them being lost. It might be possible to obtain better resolution by changing conditions of gel electrophoresis (e.g. buffers and pH).

This enzyme has not been examined in T. pyriformis complex or in P. aurelia. Two variant forms of the enzyme were observed in strains of the parasite Plasmodium berghei (C arter, 1973).

8) Acid phosphatase

A low level of intra-syngenic variation was observed for acid phosphatase in syngen 3. The two stocks of syngen 13 also showed variations. The most common form of the enzyme, however, was identical in syngens 3 and 12. One of the variant forms in syngen 13 (stock Hj 6) had an identical pattern to the most common form in syngens 3 and 12. Syngen 1 showed no intra-syngenic variation but inter-syngenic variation was observed between this syngen and the others.

Acid phosphatase showed extensive variation both within and between syngens of the Tetrahymena pyriformis complex (Allen et al., 1971a). A similar pattern within syngens was observed for three out of 12 syngens. The acid phosphatase pattern of activity proved to be too variable for any syngenic assessment in this protozoa. Acid phosphatase has not been screened in P. aurelia or any other protozoan.

The results discussed above indicated that enzymes have different degrees of variation in different protozoa. For example, malic enzyme is monomorphic in P. caudatum but polymorphic in the T. pyriformis

complex and Leishmania (Borden et al., 1977; Al-Taqi et al., 1978). Tait (1978) has suggested that in order to utilize enzyme variation as a means of species identification one requires sets of enzymes which are relatively invariant within species and clearly variant between species. The problem of syngen identification, at least for the four available syngens of P. caudatum, arises from the fact that although intrasyngenic variation has been observed the most common form of each enzyme within a syngen is almost always similar to the common form in other syngens. Therefore, much more intra-syngenic than inter-syngenic variation is observed.

Another important point in syngen identification is the kind of variation which has been detected in intra and inter-syngenic comparisons. For example, intra-specific (intra-syngenic) variation have been found at six loci in P. aurelia (Tait, 1968, 1970; Allen and Golembiewski, 1972; Cavill and Gibson, 1972), but the variant forms observed were unique and did not coincide with those observed in inter-species comparisons. Later, Tait (1978) as a result of screening phosphogluco isomerase (see page 63), argued that in P. aurelia, the amount of intra and inter-species variation which may be observed is very irregular and unpredictable depending on which enzyme is chosen for study as well as the number of available isolates of a given species.

Comparison of the degree of similarity in stocks of different syngens

When the mean coefficient of identity was calculated in each of the syngens from Tables IV, VI, VIII, the values 88, 82 and 89 per cent were obtained respectively in syngens 1, 3 and 12. Syngen 13 was excluded from these comparisons since only two stocks were available in this syngen. The values in syngens 12 and 1 were very close. It

should be noted, however, that only five stocks have been examined in syngen 1 in comparison with eleven stocks in syngen 12. This observation indicated that increasing the number of stocks did not affect the amount of variation, at least in the two syngens 1 and 12. On the other hand, comparing syngen 3 to syngens 1 and 12, it is seen that the mean value of similarity differs significantly between syngen 3 and the other two syngens (82 compared to 88 and 89 respectively). Here again, the number of stocks examined in each of these syngens did not have any effect on the observed degree of variation within each syngen, i.e. six stocks in syngen 3 and five and eleven in syngens 1 and 12. These results indicate that syngen 3 is more variable than syngen 1 and 12. Further evidence for this conclusion comes from the study on lactate dehydrogenase variation in P. caudatum (Agatsuma and Tsukii, 1980). When this enzyme was screened in four syngens, 1, 3, 12 and 13, with the exception of one variant in syngen 3, no other variation was observed in the other syngens.

In P. aurelia, it has also been reported that species 2 (syngen 2) was more variable than the other 13 species (syngens) of this ciliate (Tait, 1970a; Allen et al., 1971b). The reasons for this exceptional variability are not known (Allen et al., 1975).

Nanney and McCoy (1976), on the basis of electrophoretic variation in eight enzymes (hydroxybutyrate dehydrogenase, malate dehydrogenase, malic enzyme, isocitrate dehydrogenase, glutamate dehydrogenase, tryosine amino transferase, aldolase and superoxide dismutase) in T. pyriformis, have suggested that isolates must be alike in at least 67% of their enzymes as judged by electrophoretic mobility, to be in the same syngen. However, most stocks within a syngen usually have higher percentages than 67.

As is shown in Tables IV, VI and VII, the coefficient of identity

between stocks within a syngen of P. caudatum is well above the value of 67%. Comparing the coefficient of identity between stocks of different syngens (Table II), the value varies from 87% to 100%. These figures are as high as the coefficient of identity between stocks within one syngen. Consequently, it is not possible to differentiate the four syngens of 1, 3, 12 and 13 from one another on the basis of their variation in the nine examined enzymes.

One important point which should be taken into consideration in inter-syngenic comparisons, is to choose the stock which possesses the most common pattern of enzyme activity in each syngen. Coincidently stocks used in Chapter Three for inter-syngenic fulfil this requirement.

It has been reported that most species of P. aurelia show a low coefficient of identity and so could easily be distinguished (Tait, 1978). This report was based on ten enzyme loci in the 14 species (syngens) of P. aurelia. However, in spite of the low coefficient of identity between species of this ciliate, some species had a high coefficient of identity. For example, species 1 and 5 had a value of 90% and species 7 and 14 had a value of 70%. In both cases the calculated value for the coefficient of identity is above the 67% which was suggested by Nanney and McCoy (1976). According to the above observations, Tait has concluded that using the degree of similarity between isolates when no further morphological information or other criteria of species identification are available (see Chapter One), the number of species would be underestimated. This conclusion is certainly valid in the case of four syngens of P. caudatum (1, 3, 12 and 13). These syngens have also been classified on the basis of their mating reactivity. In the absence of this criterion, they would have been clustered in one group, if enzyme comparisons were the sole method of identification.

It has also been reported in other protozoa such as trypanosomatids that for a more comprehensive classification, physiological characteristics (e.g. nutritional requirement) and isozyme patterns should be considered as well as the structure and host specificity (Goncalvas De Lima et al., 1979).

Classification of the unknown stocks on the basis of coefficient of identity

1) The three unknown stocks 2C, 2F and 42

As shown in Table II, the coefficient of identity between these three stocks and known stocks of different syngens varied from 69% to 80%. These values again were above the 67% value. If the degree of similarity is taken into consideration, then these three unknown stocks would be clustered in one group with the four examined syngens. In order to clarify this position attempts have been made to classify these three stocks according to their mating reactivity with the known stocks. The results of these attempts will be discussed in a separate chapter on mating type.

2) The unknown stock Call

The degree of similarity between this unknown stock and the other stocks is very low (Table II). This stock can be differentiated as a new syngen, on the basis of its electrophoretic variation. There is a coefficient of identity ranging between 18% to 26% in comparisons made between Call and the other stocks.

These results indicate that the high degree of similarity between the four syngens (1, 3, 12 and 13) must be limited only to these syngens rather than being a general rule for all the syngens of P. caudatum. For this reason further stocks have been collected from different geographical sites in England and Scotland, hoping to find greater

inter-syngenic variation. The results of this investigation will be reported in the following Chapter.

CHAPTER FIVE

ENZYME VARIATIONS IN NATURAL STOCKS OF P. CAUDATUM

Introduction

In the preceding chapters, the degree of variation between various proteins was estimated both within and between stocks in four known syngens of P. caudatum (syngens 1, 3, 12 and 13). The results of these studies indicated that the degree of variation was low and that it was impossible to distinguish any of these syngens on the basis of these enzymes. All the stocks in these four syngens were collected from different parts of Japan. It has also been reported (Chapter Three) that the four unknown stocks 2C, 2F, 42 and Cal1 had a greater range of variation when they were compared to the known stocks than that between any of the known stocks. The three unknown stocks of 2C, 2F and 42, which were collected from different parts of England did not show variations between each other. However, the unknown stock Cal1 was variable in comparison with the known and the three unknown stocks. This stock was collected from Manhattan beach, California. These results indicated that geographical isolation probably plays a role in the degree of enzymatic variation in P. caudatum (i.e. the stocks which were collected from relatively close geographical distance had less variation than the ones which were collected from widely separated geographical sites).

In order to determine (1) if the observed variation is really geographically dependent and (2) if the lack of intersyngenic variation among the four syngens 1, 3, 12 and 13 (as was reported in Chapters Three and Four) is specific to these four syngens or if it is a general phenomenon in P. caudatum and also (3) to obtain more reliable estimates of enzymatic variation in natural population of P. caudatum, collections of stocks were isolated from different parts of Scotland and England.

These stocks, as well as 10 stocks collected from California, were examined for their enzymatic variations. These stocks and their geographical origins are listed in Table IX.

Table IX: Stocks collected from nature and their geographical origins

Stocks	Geographical Origin
No1 - No14	Hethersett, Norfolk
No15 - No24	U.E.A. Broad, Norfolk
No25 - No45	Marlingford, Norfolk
Mar1 - Mar10	Margate, Kent
ALSC01 - ALSC010	Auchencrieff Loch, Scotland
LLSC01 - LLSC010	Lochmaben Loch, Scotland
Cal1 - Cal10	Manhattan Beach, California

Enzymes used in this study are as follows:

Esterases
- (1) α-Naphthyl propionate esterase.
- (2) α-Naphthyl butyrate esterase.

(3) Malate dehydrogenase.

(4) Malic dehydrogenase.

(5) Aspartate: 2-oxoglutarate aminotransferase.

(6) Superoxide dismutase.

(7) Phosphogluco isomerase.

Hexokinase and acid phosphatase were not examined in this study. The first enzyme (hexokinase) was very variable in different stocks and also the bands of activity of this enzyme had a poor resolution. It was difficult therefore to score any clear pattern of hexokinase activity

in different stocks, especially when a large number of samples were involved in the comparisons. Hexokinase, as well as the second enzyme acid phosphatase, was not detectable in some stocks. It is not known whether lack of hexokinase and acid phosphatase activity in some of the stocks is due to the existence of "null" allele in these stocks or if the enzymes are produced normally but are not detectable under buffer conditions which have been used in the present study. Other possibilities are that these two enzymes are produced normally but are very unstable or they are produced at such a low level and are not detectable by the technique of gel electrophoresis. It was reported in P. aurelia (Tait, 1970a) that stocks of syngen 6 and 8 showed no hydroxybutyrate dehydrogenase activity on the gels. When extracts were assayed spectrophotometrically, however, activity was detected but at a lower rate than extracts of other syngens.

With these ambiguities it was decided to exclude hexokinase and acid phosphatase from the present study.

Materials and Methods

Samples were collected in 500 ml containers, from different sites in each pond. The samples were checked under a binocular microscope for P. caudatum cells. Usually the density of cells was very low. In these cases about one third of volume of each container was poured out and grass medium inoculated with Klebsiella pneumoniae (pH: 7.0) was added. The samples were left for 24 hours and after this period, the single cells were isolated and transferred to depression slides containing grass medium inoculated with Klebsiella (pH: 7.0). Usually 60% of isolated cells died. The live cells were grown for a few days in the slides and then transferred to test tubes containing the same medium. The preparation of extracts and the procedures of gel

electrophoresis have been described earlier (see Materials and Methods, Chapter Two).

Results

Esterases

(1) α-Naphthyl propionate esterase

In the anodal part of the gel three different patterns of enzyme activity were detected (Figure 37). Most of the stocks of No1-No45 had a fast banding pattern identical to the pattern of α-naphthyl propionate esterase activity in the unknown stock 2C. These stocks will be referred to as the group one - No1-No45. The pattern of activity in stocks Mar1-Mar10 was also identical to the group one - No1-No45 (samples 1, 2 and 4 in Figure 37). The other stocks of No1-No45, which will be referred to as group two - No1-No45, consist of stocks No9, No35 and No37. The pattern of activity in the latter group was a slower banding pattern than the pattern in group one. However, it was identical to the pattern of activity in stocks LLSC01-LLSC010 and ALSC01-ALSC010 (samples 3, 7, 8 and 9 in Figure 37). The third pattern of enzyme activity which migrated slightly faster than the pattern in group two - No1-No45 and stocks from Scotland (compare samples 6 and 7 in Figure 37) was detected in stocks Cal1-Cal10 (samples 5 and 6 in Figure 37).

In the cathodal part of the gel a single band of activity was observed in each of the stocks (Figure 37). This band had three different mobilities in different stocks (slow, intermediate and fast). The slow band was detected in stocks Cal1-Cal10 (samples 5 and 6 in Figure 37). The band of intermediate mobility was detected in group one - stocks No1-No45 and also stocks Mar1-Mar10. This band was also identical in mobility to the cathodal band in stock 2C (samples 1, 2 and 4 in Figure 37). Finally, a fast mobility band was detected in

group two - stocks No1-No45 as well as stocks LLSC01-LLSC010 and ALSC01-ALSC010.

(2) α-Naphthyl butyrate esterase

In the anodal part of the gel, three different patterns of α-naphthyl butyrate esterase were observed (Figure 38). The pattern of enzyme activity was identical in group one - stocks No1-No45, Mar1-Mar10, and stock 2C (sample 1 and 10 in Figure 38). The second pattern was observed in group two - stocks No1-No45, stocks LLSC01-LLSC010 and stocks ALSC01-ALSC010 (samples 3, 4, 5, 8 and 9 in Figure 38). The third pattern of activity was observed in stocks Cal1-Cal10 (samples 2, 6 and 7 in Figure 38).

In the cathodal part of the gel, the pattern of α-naphthyl butyrate esterase were identical to the observed pattern when α-naphthyl propionate was used as substrate.

When the anodal bands of activity were compared in two different substrates (α-naphthyl propionate and α-naphthyl butyrate), it was shown that most stocks had the same pattern of enzyme activity with both substrates (compare samples 2 and 3 in Figure 39a to samples 2 and 3 in Figure 39b). The exceptions were group one stocks No1-No45, Mar1-Mar10 and stock 2C. They had different patterns in different substrates, i.e. the first anodal band of activity from the origin in α-naphthyl propionate was not detectable in α-naphthyl butyrate (compare sample 1 in Figure 39a to sample 1 in Figure 39b).

(3) Malate dehydrogenase

Three different patterns of enzyme activity were detected among the 85 stocks (Figure 40).

1) The most common pattern was a single fast band and was observed

in stocks No1-No45, LLSCO1-LLSCO10 and ALSCO1-ALSCO10. This band also had identical mobility with the band in stock 2C (samples 1 and 2 in Figure 40).

2) The second pattern was a single slow band and was observed in stocks Cal1-Cal10 (samples 3 and 4 in Figure 40).

3) The third pattern appeared as a double band in stocks Mar1-Mar10. This double band consisted of both slow and fast forms of the enzyme (samples 5 and 6 in Figure 40).

It should be noted that all the stocks collected from a single pond had identical patterns of malate dehydrogenase activity.

(4) Malic enzyme

This enzyme appeared as a single band with identical mobility in all the stocks.

(5) Aspartate: 2-oxoglutarate aminotransferase

A single cathodal band of enzyme activity was detected in all the stocks. This band had three different mobilities in different stocks (Figure 41). Stocks Cal1-Cal10, LLSCO1-LLSCO10 and ALSCO1-ALSCO10 had the slowest band of mobility. The band which had intermediate mobility appeared in stocks No1-No45 as well as stock 2C, and the band of fastest mobility was detected in stocks Mar1-Mar10.

(6) Superoxide dismutase

In the anodal part of the gel a single monomorphic band of activity was detected in all the stocks (Figure 42).

In the cathodal part of the gel a single band of activity was detected. This band had two different mobilities (slow and fast) in the different stocks. The slow band was detected in stocks No1-No45 (except

No9, No11, No35, No43) and stocks Mar1-Mar10 (sample 1 in Figure 42). The fast band appeared in stocks No9, No11, No35, No43; LLSC01-LLSC010; ALSC01-ALSC010 and Cal1-Cal10 (samples 3, 5, 6 in Figure 42). The slow band of activity also had identical mobility as the band in stock 2C.

The cathodal band of samples 2 and 4 were not clear in Figure 42.

(7) Phosphogluco isomerase

Five different patterns of the enzyme activity were observed among the stocks (Figure 43). Three of these five patterns were detected in stocks No1-No45. The most common pattern among these stocks was a single band that had identical mobility to the band of activity in stock 2C (samples 2 and 3 in Figure 43). The faster band of mobility in samples 2 and 3, because of its inconsistency in appearance in different experiments, was not considered in this study. Three of the stocks No15, No16 and No32 had a double band of activity (sample 1 in Figure 43) and two stocks, No37, No41, had three bands of enzyme activity (sample 4 in Figure 43).

Stocks Mar1-Mar10, LLSC01-LLSC010 and ALSC01-ALSC010, all had a single band of identical mobility. This band migrated at a slower rate from the origin than the single band in stock 2C (samples 7 and 8 in Figure 43). Finally stocks Cal1-Cal10 had a single band of activity. This band migrated at a faster rate than the single band in stock 2C (samples 5 and 6 in Figure 43).

Summary of results and discussion

With the exception of stocks No1-No45, no variations were observed within stocks that were collected from each of the following sites: Lochmaben Loch (LLSC01-LLSC010); Auchencrieff Loch (ALSC01-ALSC010); Margate (Mar1-Mar10) and Manhattan beach, California (Cal1-Cal10). The

lack of variation within stocks from the same geographical site, in most of the cases, might be due to limited number of stocks which were used for detecting enzyme variations. Considering the high rate of mortality (about 60%) among the isolated cells from nature, it is not known if the survivors do infact represent all the possible existing patterns of variation within a pond. It is possible that by increasing the sample size, the number of variant stocks would also be increased. The natural populations of syngens 3, 4, 9 of P. aurelia did not show any variations. Stocks of these three syngens as well as syngen 2 of P. aurelia were all collected from a single pond (Gibson and Adams, 1974).

In addition to the lack of variation within a single pond, absolute similarity in the pattern of enzyme activity was also observed between stocks collected from different ponds. For example, stocks LLSC01-LLSC010 (Lochmaben Loch, Scotland) compared with stocks ALSC01-ALSC010 (Auchencrieff Loch, Scotland) and stock 2C with majority of stocks No1-No45. Lack of geographical differentiation was also reported in P. aurelia between geographically isolated stocks from any one syngen with regard to enzyme polymorphism (Adams and Allen, 1975). For example stocks of syngen 1 which were collected from locales as far apart as Japan and Scotland had an identical pattern of enzyme activity.

Stocks Mar1-Mar10 (collected from Kent), whilst having identical patterns with each other, did not have identical patterns for all the enzymes with stocks collected from other ponds. However, the degree of similarity between these stocks and stocks No1-No45 from Norfolk was much higher than the observed degree of similarity between stocks which did not have absolute degree of similarity (see later for discussion on coefficient of identity).

Rare variant stocks were observed between stocks No1-No45. These stocks were collected from three closely located ponds around Norwich

(see Table IX). The few variant stocks were as follows. Stocks No9, No35 and No37 had the group two pattern of esterase activity. Stocks No9, No11 and No35 had the fast cathodal band of superoxide dismutase activity and finally stocks No15, No16, No32 had double bands and stocks No37, No41 had three bands of phosphogluco isomerase activity. The other stocks No1-No45 had the group one pattern of esterase activity, the slow cathodal band of superoxide dismutase and a single band of phosphogluco isomerase activity.

The conclusions from this study were (1) almost all the stocks collected from a single pond had identical patterns of enzyme activity; (2) a few variant stocks were found in some of the collections. The enzymes contributing to these variations were esterases, superoxide dismutase and phosphogluco isomerase; (3) detection of variations in stocks were irregular. For example, stock No9 had variant forms of esterases and superoxide dismutase activity, but the common form of phosphogluco isomerase activity. On the other hand, stocks No15 and No16 had the variant form of phosphogluco isomerase and common forms of esterases and superoxide dismutase; (4) the only enzyme which was monomorphic in all the stocks was malic enzyme.

In order to avoid any confusion when comparison was made between stocks from different geographical sites, the rare variant stocks within stocks No1-No45 were not considered. An interesting phenomenon which should be noted was the absolute similarity in patterns of enzyme activity between stock 2C and stocks No1-No45. The significance of this identity became obvious when it was disclosed that stock 2C was collected from Devon in 1954. This indicated that the pattern of the enzyme activity has not been changed under laboratory conditions over a long period of time (other stocks in this study were collected in 1977-1978).

Presumably, all the stocks with identical patterns of enzyme activity

do belong to the same syngen. However, the possibility of them representing two or more different syngens cannot be ruled out completely (see later for further explanation). These stocks which may represent one or more syngens and which have identical patterns of activity in ten out of 10 stocks represent then a greater degree of similarity than that reported earlier for intra and inter-syngenic variations of syngens 1, 3, 12 and 13. In syngen 1, one of the 10 possible pairwise comparisons between the stocks had an absolute degree of similarity (100% coefficient of identity). In syngen 3, one out of the 15 possible pairwise comparisons had absolute degree of similarity. In syngen 12, nine out of 55 possible pairwise comparisons had an absolute degree of similarity. Finally in syngen 13, the only possible pairwise comparisons had a high degree of similarity (93%), but two stocks in this syngen did not have identical patterns of enzyme activity.

In inter-syngenic variations involving the four syngens, one out of 10 possible pairwise comparisons had an absolute degree of similarity, i.e. syngen 13/syngen 12. The stocks used for inter-syngenic comparisons represented the most common pattern of the enzyme's activity for their syngen.

An important point which should be taken into consideration was that in assessing intra and inter-syngenic variations in syngens 1, 3, 12 and 13, nine enzymes were screened whereas in the present study seven enzymes were screened. The two enzymes hexokinase and acid phosphatase, which were excluded from the present study, contributed to some extent to the degree of intra and inter-syngenic variations. Re-calculating the coefficient of identity within and between syngens, for seven instead of nine enzymes, the following results were obtained. In syngen 1, three out of 10; in syngen 3, three out of 15; in syngen 12, twenty-six out of

55 and in syngen 13, one out of 1, possible pairwise comparisons had 100% coefficient of identity. These stocks were not necessarily collected from the same pond or the same area. For example, from the three stocks of syngen 1 which had an identical pattern of enzyme activity, two were collected from Iwate (Isn and Ih4) and the third one from Miyagi (Ms25). It can be seen that by excluding two enzymes, hexokinase and acid phosphatase, the number of stocks within a syngen which had absolute similarity in enzymatic patterns increased. However, intra-syngenic variations were still observable, even when the number of stocks within any syngen was as low as 5 or 6.

When the degree of inter-syngenic variation was calculated for seven enzymes, ten out of 10 pairwise comparisons had absolute degree of similarity (100% coefficient of identity). These data indicated that the stocks collected from nature, even when they showed absolute degree of similarity could have still represented different syngens. The only possible and reliable way of categorizing these stocks into one or two syngens was through conducting the mating type test. Considering the time and labour involved, this test was beyond the scope of the present study. For the sake of simplicity, all the stocks with identical pattern of enzyme activity were considered to represent one syngen. However, it is realized that this assumption might underestimate the number of syngens that are involved in this study.

Assessment of similarity between stocks collected from different ponds

The coefficient of identity was calculated for the stocks which were collected from different ponds. The results are listed in Table X.

Stocks	No1-No45*	Mar1-Mar10	LLSC01-LLSC010	ALSC01-ALSC010	Ca11-Ca110	2C
No1-No45*	-	72	36	36	27	100
Mar1-Mar10		-	33	33	25	72
LLSC01-LLSC010			-	100	44	36
ALSC01-ALSC010				-	44	36
Ca11-Ca110					-	27
2C						-

Table X: shows the coefficient of identity between stocks collected from nature.

* Stock No_1-No_{45} were considered as samples from a single geographical site, because of the close location of the three ponds where the samples were collected from and also the most common pattern of enzyme activity in these collections were identical.

By considering the coefficient of identity as a sole criteria of syngen identification and by also accepting the figure 67% as the minimum figure for any stocks to be clustered in the same syngen, three different syngens can be distinguished from Table X.

1) A syngen represented by stocks No1-No45, Mar1-Mar10 and 2C.

2) A syngen represented by stocks LLSC01-LLSC010 and ALSC01-ALSC010.

3) A syngen represented by stocks Ca11-Ca110.

The low level of similarity between different stocks can be a very promising observation in regard to the problems of syngen identification in P. caudatum. If stocks with a low coefficient of identity do represent different syngens, then even allowing for some degree of intra-syngenic variation it will be possible to distinguish at least some of

the different syngens of P. caudatum on the basis of their electrophoretic patterns of enzyme activity. It was reported earlier (Chapters Three and Four) that by using the technique of gel electrophoresis, it was not possible to distinguish the four syngens 1, 3, 12 and 13 of P. caudatum from each other . The question to be asked is that, what mechanisms keep the degree of enzyme variations low in some syngens, and high in others? It was reported (Sonneborn, 1957) that syngens 1, 3, 12 and 13, have been found in the warmer regions, whereas other syngens had wider geographical distributions. However, how geographical distribution of the syngens can be effecting the degree of enzymatic variations is not known. Without having more accurate information on electrophoretic patterns of the other 12 syngens of P. caudatum, the answer to the above question remains unsolved.

In P. aureliaahigh coefficient of identity was reported in two of the species comparisons, species 1 compared to species 5 (90%), and species 7 compared to species 14 (70%) (Tait, 1978). In contrast a low level of enzymatic similarity was reported in most of the syngens in the T. pyriformis complex. Borden et al. (1977) reported that syngen 4 of the latter ciliate had 0% coefficient of identity with syngens 1, 3 and 5. On the basis of electrophoretic patterns in these two ciliates, Adams and Allen (1975) reported that the genetic distances were greater amongst syngens of T. pyriformis than syngens of P. aurelia. They also calculated the mean divergence time for the two ciliates and reported that the mean divergence time for P. aurelia species was similar to the figure for Drosophila sibling species. For the Tetrahymena species it was similar to the figure for Drosophila non-sibling species. Similar pattern of results was found in comparisons of nucleotide sequence divergence between Drosophila sibling and non-sibling species, P. aurelia and Tetrahymena species (Allen and Li, 1974). A more recent report on

molecular polymorphism of ciliary proteins from five different species of Tetrahymena, also indicated vast molecular distance between species of this ciliate (Seyfert and Willis, 1981).

It should be noted, however, when comparing different organisms on the basis of their enzymes' variation, it is important to have the same sets of enzymes in the organisms. It is known that different enzymes have different degrees of variability (for more detail see General discussion). For example, esterases and phosphogluco isomerase were reported to be quite polymorphic, whereas succinate dehydrogenase was monomorphic in different species of P. aurelia. However, in detecting enzyme variations by the technique of gel electrophoresis in protozoa, the technical problems limit the researcher to certain sets of enzymes, which are not necessarily the same in other protozoa.

Figure 37:

Variations in α-naphthyl propionate esterase, between 85 stocks isolated from nature.

1 : stock 2C

2 : group one stocks NO1-NO45

3 : group two stocks NO1-NO45

4 : stocks Mar1-Mar10

5,6 : stocks Ca11-Ca110

7,8,9 : stocks LLSCO1-LLSCO10 and ALSCO1-ALSCO10

10 : stock 2C

Figure 38:

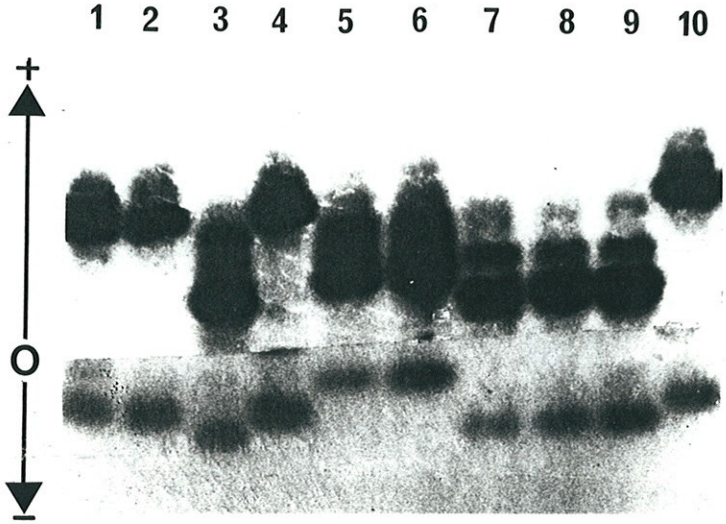
Variations in α-naphthyl butyrate esterase, between 85 stocks isolated from nature.

1,10 : group one stocks NO1-NO45, Mar1-Mar10, 2C

2,6,7 : stocks Ca11-Ca110

3,4,5,8,9 : group two stocks NO1-NO45, LLSCO1-LLSCO10, ALSCO1-ALSCO10

O : Origin


1 2 3 4 5 6 7 8 9 10
+
O
−

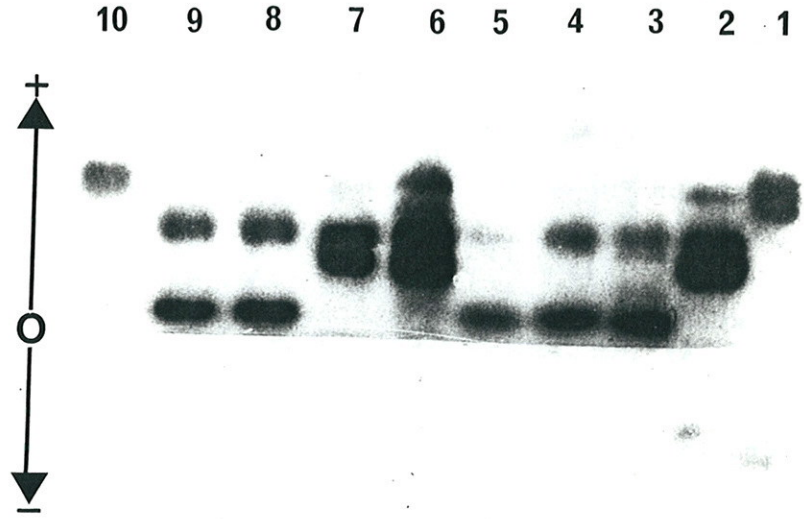

10 9 8 7 6 5 4 3 2 1
+
O
−

Figure 39a:

Variations in α-naphthyl propionate esterase.

Figure 39b:

Variations in α-naphthyl butyrate esterase.

1 : group one NO1-NO45, Mar1-Mar10, 2C

2 : stocks Cal1-Cal10

3 : group two NO1-NO45, ALSCO1-ALSCO10, LLSCO1-LLSCO10.

Figure 40:

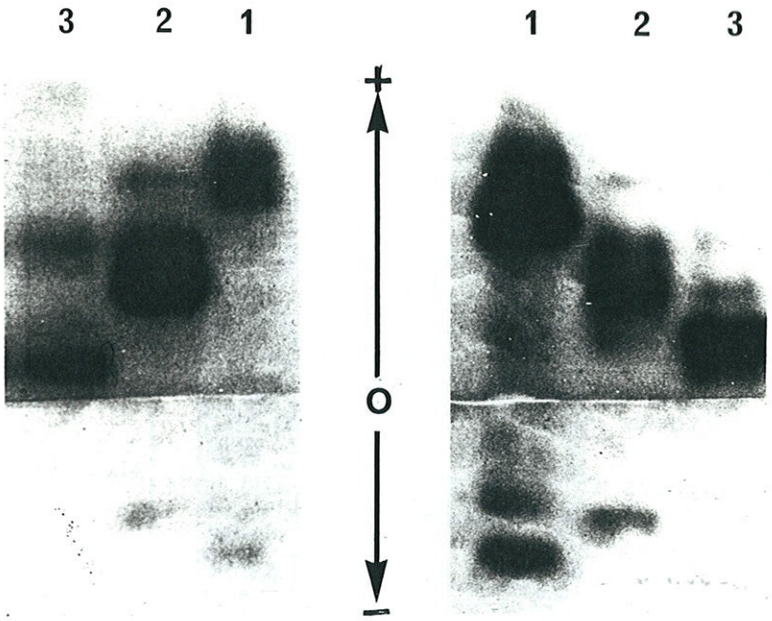
Variations in malate dehydrogenase between 85 stocks isolated from nature.

1,2 : stocks NO1-NO45, LLSCO1-LLSCO10, ALSCO1-ALSCO10

3,4 : stocks Cal1-Cal10

5,6 : stocks Mar1-Mar10.

O : Origin



39 b

39 a

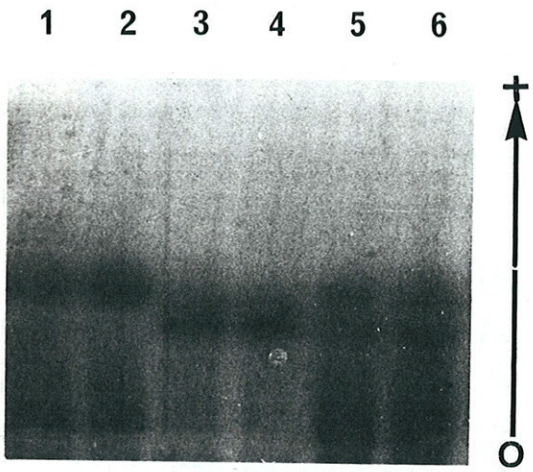


Figure 41:

Variations in aspartate aminotransferase between 85 stocks isolated from nature.

1 : stocks NO1-NO45

2,3 : stocks Cal1-Cal10, LLSCO1-LLSCO10, ALSCO1-ALSCO10

4 : stocks Mar1-Mar10.

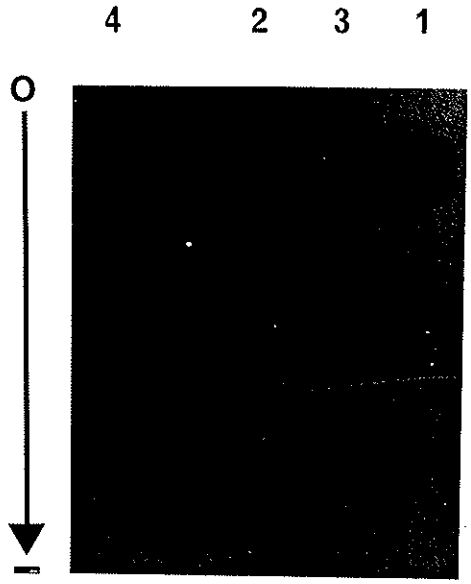
Figure 42:

Variations in superoxide dismutase between 85 stocks isolated from nature.

1 : stocks NO1-NO45 (except stocks NO9, NO11, NO35 and NO43), Mar1-Mar10

3,5,6 : stocks LLSCO1-LLSCO10, ALSCO1-ALSCO10, Cal1-Cal10, NO9, NO11, NO35 and NO43.

O : Origin


4
2
3
1
O
−

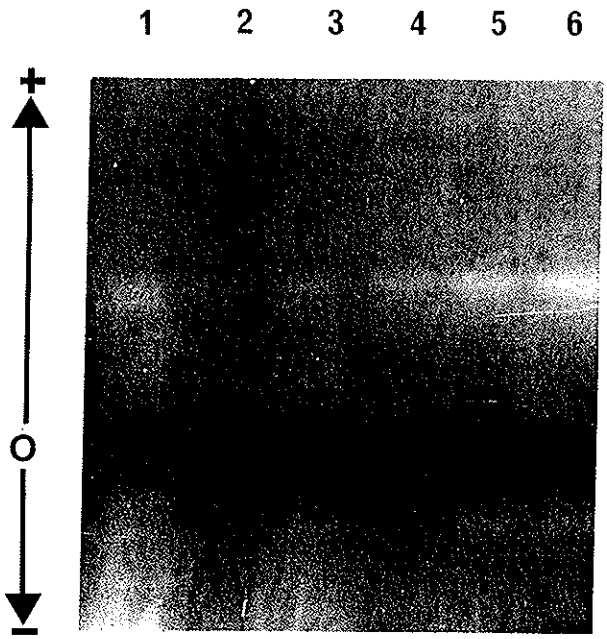

1
2
3
4
5
6
+
O
−

Figure 43:

Variations in phosphogluco isomerase between 85 stocks isolated from nature.

1 : double band in stocks NO15, NO16, NO32

2 : single band in stock 2C

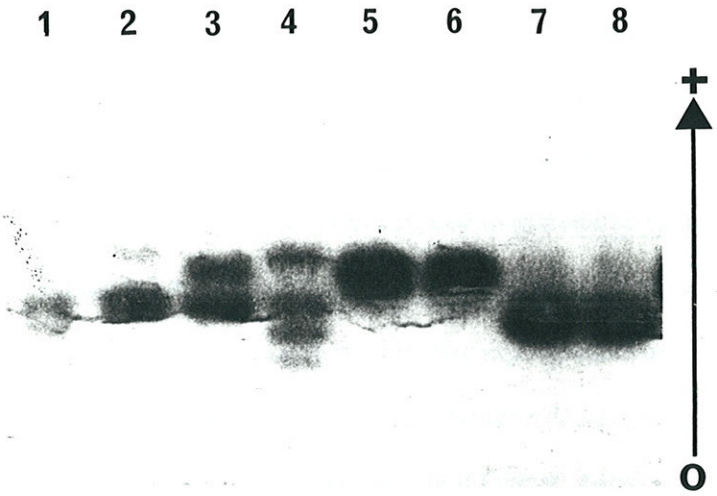
3 : single band in stocks NO1-NO45 except stocks in 2 and 4

4 : three banding patterns in stock NO37-NO41

5,6 : single band in stocks Cal1-Cal10

7,8 : single band in stocks Mar1-Mar10, LLSC01-LLSC010, ALSC01-ALSC010

O : Origin


1
2
3
4
5
6
7
8
+
O

CHAPTER SIX

MATING TESTS IN P. CAUDATUM

Introduction

Mating types of P. caudatum

As described in the first chapter, each of the 16 syngens of P. caudatum (Sonneborn, 1957) has two complementary mating types, which can be referred to as odd (O) and even (E). The mating reaction occurs specifically between a cell of type "O" and one of type "E" both belonging to the same syngen. In some special cases the mating reaction occurs between a cell of type "O" of one syngen and "E" of another, but invariably the hybrid between this intersyngenic mating is inviable.

The inheritance of mating type in P. caudatum

The inheritance of mating type has been reported in two syngens (3 and 12) of Paramecium caudatum (Hiwatashi, 1968). These two syngens have a synclonal mode of mating type inheritance, i.e. both the exconjugants from a conjugating pair express identical mating type, indicating direct gene control of mating type. Mating type in the two syngens 3 and 12 is determined by a pair of alleles with simple dominance. Clones homozygous for the recessive gene express the odd mating type during the course of clonal life history. Clones, with the dominant mating type allele, either homozygotes or heterozygotes, express the even mating type during the first months of the clonal period of maturity (for definition see later), but become unstable in the later period of clonal life cycle. It has been reported that change in the expression of the dominant mating type allele is controlled by a modifier gene, which interacts only with the dominant allele at the mating type locus (Hiwatashi et al., 1976). It has also been suggested

(Butzel, 1955) that the odd mating type substance is a precursor of the even mating type substance.

Because of this change in the expression of the mating type locus, intraclonal conjugation (see also selfing) has been observed in stocks of even mating type (Hiwatashi, 1960 and 1968).

Genes are also involved in mating type determination in Euplotes crassus. Five different mating types have been reported, each being determined by a different allele at the mating type locus (Heckmann, 1967). There is a complete dominance among these alleles, so that in diploid cells only one of them is phenotypically expressed. Other examples where nuclear genes are involved include the four mating types in syngen 1 of Paramecium bursaria (Bamford, 1966) and seven mating types in Tetrahymena (Nanney, 1964).

Background to fertilization events in P. caudatum

In the genus Paramecium a clonal cycle begins with fertilization ((1) conjugation, (2) autogamy, (3) selfing, (4) cytogamy), passes through some stages ((I) immaturity, (II) maturity and (III) senility), and ends in clonal death (Sonneborn, 1974).

(1) Conjugation

Conjugation is normally induced by interaction between cells of complementary mating types (Sonneborn, 1937) under appropriate conditions (e.g. clonal age and nutritive state). The clone must be in the period of maturity (see later) and the culture should be neither overfed nor overstarved. Cells in logarithmic growth phase or late stationary phase are not reactive. The optimum temperature for mating reactivity is 20-25^{o}C.

The various stages of conjugation are as follows: (A) the cells mate, (B) prezygotic and zygotic nuclear events, and (C) postzygotic

events and restoration of the vegetative nuclear state.

(A) Mating of cells

When paramecia of complementary mating types are brought together under appropriate conditions for conjugation, the cells stick together by reactive cilia located on the ventral surface of the cell (Hiwatashi, 1961). This event results in the formation of large agglutinates of cells (mating reaction). The union of the animals in the mating reaction is highly specific (Sonneborn, 1937). If paramecia of complementary mating types are marked with different vital stains, it can be clearly observed that the animals in the clump always consist of two different mating types (Hiwatashi, 1951).

The specific substances for each mating type are called mating type substances and are known to be proteins (Watanabe, 1977), since the mating activity of cells or cilia was easily lost by treatment with proteolytic enzymes.

Role of cilia in cell mating

The mating reaction involves cilia of complementary mating types and the paramecia in the reaction adhere to the tips of cilia (Sonneborn, 1937; Jennings, 1939; Viver, 1960). The term mating reaction refers only to the ability of the cilia to adhere to one another and is not synonymous with conjugation. The role of cilia in the mating reaction is further confirmed by the observation that detached cilia from reactive cells of one mating type can agglutinate the reactive cells of a complementary mating type and even induce conjugation between cells (Fukushi and Hiwatashi, 1970; Miyake, 1964). Furthermore, agglutination has been observed between detached cilia from complementary mating types (Takahashi et al., 1974). More recently Kitamura and Hiwatashi (1980) have succeeded in isolating mating reactive membrane vesicles from cilia

of P. caudatum which have an ability to induce conjugating pairs between cells of the complementary mating type.

As the mating reaction proceeds, cilia and trichocysts at the anterior tip and on the ventral surface just behind the tip of the cell disappear, and pairs of cells unite at the anteroventeral surface (hold fast union). Cilia continue to disappear on the vent ral surface and the cells unite more firmly, especially in a region just posterior to the mouth, the "paroral union". In this step of the union, the cytoplasmic membrane of the conjugants disappears at the contact points and the membrane fuses together (Figure 44).

There is a relation between mating reactivity and the degeneration of cilia, since ciliary degeneration occurs only in cells physiologically competent to participate in the mating reaction. In cells with a higher degree of mating reactivity, ciliary degeneration extends more rapidly (Watanabe, 1978). This author has suggested that ciliary degeneration is due to resorption, since before complete disappearance of the cilia, many short cilia are observed. Resorption of cilia has been reported in other ciliates, during conjugation of Oxytricha (Hammer Smith, 1976) and during oral replacement and partial deciliation in Tetrahymena (Williams and Nelsen, 1973; Rannestad, 1974).

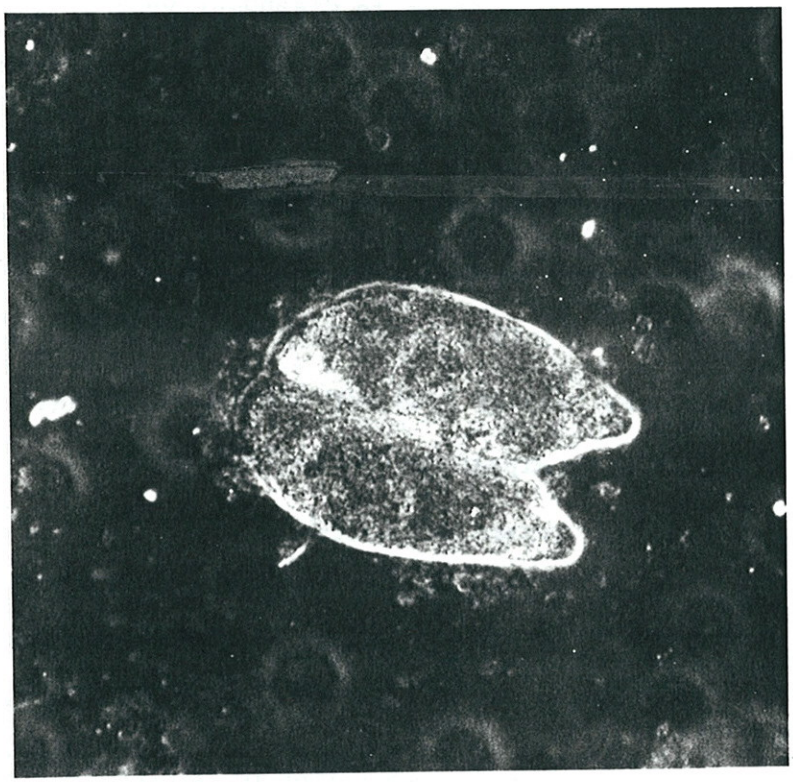
Degeneration of cilia have been observed during chemically induced autogamy in P. caudatum. However, no degeneration of cilia occurs during the autogamy process in P. tetraurelia (Watanabe, 1978).

(B) Prezygotic and zygotic nuclear events

Early micronuclear migration (E.M.M.)

The earliest nuclear change that has been observed in the conjugation process is called early micronuclear migration (E.M.M.). This phenomena occurs within 30 minutes, after mixing of the complementary mating types

Figure 44



A Conjugant Pair

or when cells are chemically conjugated. E.M.M. occurs before the formation of hold fast union and during this period the micronucleus leaves the concavity of the macronucleus. The proportion of cells undergoing "E.M.M." is the same as the proportion of conjugants (Fujishima and Hiwatashi, 1977). E.M.M. does not occur in immature cells.

Prezygotic nuclear phenomena

At the final stage of union, three micronuclear divisions usually occur. The first two are meiotic. After the meiotic divisions, all but one of the four products disintegrate. The surviving nucleus lies near the region of the mouth, i.e. in the paroral cone region. Sonneborn (1954a) has suggested that the fate of any given micronucleus, whether it disintegrates or survives, depends upon the cytoplasmic region where the micronucleus comes to lie. All the micronuclei lying outside the paroral region are doomed to disintegrate. The third micronuclear division is mitotic and produces two genetically haploid gamete nuclei (Figure 45).

Zygotic phenomena

During this period, mutual nuclear exchange occurs between the conjugants. One of the haploid gamete nuclei moves into the other mate and fusion takes place in each individual between the two gametic nuclei.

In normal conjugation a synkaryon is formed by union of the migratory and stationary gametic nuclei. The third micronuclear division is equational. The two synkarya formed in the pair therefore have the same genotype. After the formation of the synkarya the macronucleus starts to degenerate, becomes more and more irregular in shape and number and eventually disintegrates.

Figure 45

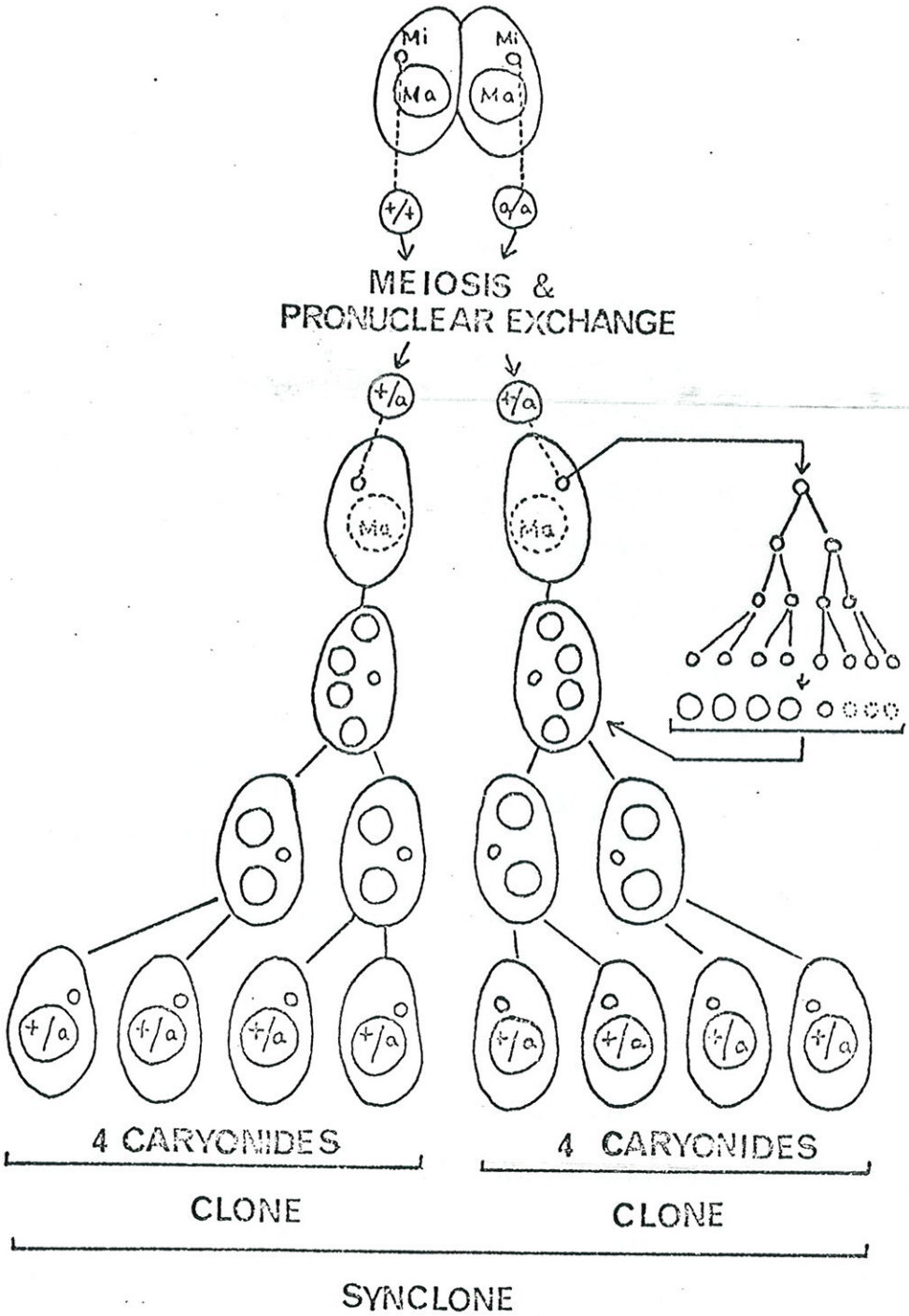


Diagram showing nuclear reorganization in conjugation of Paramecium caudatum. Dotted circles in diagram represent degenerating macronuclei (Ma) and degenerating postzygotic division products (small circles). Ma, macronucleus; Mi, micronucleus. Taken from Hiwatashi (1968).

(C) Postzygotic events and restoration of the vegetative nuclear state

The nuclear phenomena that follows the formation of the synkaryon involves the division of the synkaryon. This varies according to the species of ciliate. These divisions will create the new micronucleus and macronucleus. At the same time, both parents have by now separated and are referred to as "ex-conjugants". They divide once or twice according to the species. These divisions ensure the normal distribution of the newly formed nuclei to the individuals.

In P. caudatum, the synkaryon undergoes three successive divisions, and 8 nuclei are formed. After the third postzygotic division, four nuclei form new macronuclei (macronuclear analgen). One forms the new micronucleus and the other three disintegrate. Two divisions of the ex-conjugated cell occur and this re-establishes the normal vegetative nuclear situation, i.e. one macronucleus and one micronucleus in each sub-carynoides (Figure 45).

(2) Autogamy

Essentially the same series of nuclear events occurs as in conjugation, but in a single (unpaired) cell. Fusion of the two haploid nuclei in autogamy produces individuals homozygous for every gene. Autogamy is known to occur naturally in species of the P. aurelia complex, P. jenningsi and some other ciliates (Hertwi g, 1914; Sonneborn, 1939a). P. caudatum has no autogamy process. However, it has been possible to induce autogamy in P. caudatum artificially (Mikami et al., 1979; Tsukii et al., 1979).

Complete homozygosity which is brought about by autogamy, is very useful for genetic studies. For example, autogamy makes the screening of recessive mutants possible.

(3) Selfing

This sexual process is known to be very common in P. caudatum. Selfing occurs due to temporary change of the expression of mating type (Hiwatashi, 1960 and 1969). The occurrence of the two mating types within a clone results in self conjugation (for more details see the inheritance of mating types). The nuclear events which occur are similar to those of conjugation. Selfing has also been observed in P. aurelia (Kimball, 1939; Bleyman, 1967) and Euplotes (Heckmann, 1967). As with autogamy, selfing produces homozygous individuals.

(4) Cytogamy

If the nuclear migration from one mate to another does not occur during conjugation, then the synkarya are formed by union of two gametic nuclei produced within the same animal (Diller, 1936; Wichterman, 1940; Sonneborn, 1947). This is called cytogamy.

The occurrence of cytogamy has been reported in P. caudatum (Wichterman, 1940). In P. aurelia the frequency of cytogamy is usually less than 5 per cent of all conjugants, but its frequency is increased at both high and low temperatures (Sonneborn, 1941; Hallet, 1972 and 1973).

Cytogamy usually occurs in crosses involving mis-shapen mutants or in cells differing greatly in size, and in crosses between species. In general, cytogamy occurs when the paroral cones of the two mates fail to make or to maintain contact (Sonneborn, 1974). The genetic consequences are that the ex-conjugants, unlike in normal conjugation, have different genes after an apparent mating.

Macronuclear regeneration

This is the process of development of functional macronuclei from

fragments of the prezygotic macronucleus after any of the "sexual" processes described above, instead of from products of the synkaryon (macronuclear anlagen) (Sonneborn, 1940 and 1947a).

In P. caudatum, Mikami and Hiwatashi (1975) have reported that the frequency of macronuclear regeneration increases when conjugating pairs or ex-conjugants cells are placed directly in fresh nutrient medium, instead of being placed into exhausted culture medium. Excessive food induces early cell division without division of its macronuclear anlagen. The genetic consequences of macronuclear regeneration is that although all ex-conjugants have the same genes in their micronuclei, they differ phenotypically. The macronucleus whose genes are responsible for determining phenotypic traits during vegetative growth, is inherited directly from one of the parents and not from a fertilized nucleus.

Following the initiation of a new life cycle after fertilization, clones of paramecia pass through these stages: (I) immaturity, (II) maturity and (III) senility.

(I) Immaturity

During this period the cells have no mating reactivity and are also unable to undergo autogamy. The duration of the immature period is measured in terms of the number of fissions, rather than in actual days. Temperature and quality of nutrients limits the fission rate and therefore the duration of the immature period (Sonneborn, 1957; Kroll and Barnett, 1968; Takagi, 1970; Miwa and Hiwatashi, 1970; Bleyman, 1971).

It has been reported that the cytoplasm of P. caudatum (syngen 3) during the period of sexual immaturity was effective in inducing immaturity when injected into mature cells of other syngens of P. caudatum as well as P. tetraurelia and P. multimicronucleatum. However, it was not effective when injected into mature cells of P. bursaria (Miwa, 1979). It was concluded that the immaturity substances differ specifically

between paramecia of the "aurelia" group and those of the "bursaria" group. The immaturity substances have been isolated and partially characterized by Haga and Hiwatashi (1980). The substance is a single protein which is called "immaturin", and is contained in the soluble fraction of immature cells and is heat-labile.

(II) Maturity

During this period, paramecia have strong mating reactivity. In maturity, cells show the ability to mate if they are not overfed or extremely starved, and if the temperature is appropriate (20-25°C). The duration of maturity varies between different stocks and syngens of P. aurelia and P. caudatum (Sonneborn, 1957).

Mutants that show early maturity have been found in P. caudatum (Myohara et al., 1978), P. multimicronucleatum (Takagi, 1971) and Tetrahymena thermophila (Bleyman, 1971). These mutants have a shorter immaturity period than the wild type. However, no mutant without an immaturity period has been found.

(III) Senility

If during the maturity period cells fail to mate (i.e. because of the lack of appropriate partner), they eventually lose their capability of mating and the cell division time increases. It is followed by death (Sonneborn, 1957, 1974).

Outbreeders and inbreeders

Sonneborn (1957) has divided syngens according to the duration of their maturity and immaturity periods into two groups, outbreeders and inbreeders. The life process of some syngens favour and prolong the possibility of cross conjugation. These syngens have long periods of immaturity and maturity (outbreeders). The life process of the other

syngens makes them obligatory inbreeders. These syngens have no immaturity or very short immaturity and short maturity periods. Sonneborn assumed that outbreeding might be considered advantageous for long-time survival of diploid free-living organisms, and this could have been the ancestral breeding system of the current species.

Studies on mating in P. caudatum

The study to be reported here involves mating between different strains of P. caudatum. In the first section of this chapter, attempts have been made to ascertain relationships between stocks 2C, 2F, 42, which are unclassified as to syngen and the known syngens 1, 3, 12 and 13. The second section of this study deals with the analysis of crosses where intra-syngenic variations occur between stocks, and the inheritance of phosphogluco isomerase will be discussed in intra-syngenic cross between stock YT1 and YT3 in syngen 3.

Genetic crosses become essential since minor variations in experimental conditions can in some cases cause enough variation in electrophoretic mobility to obscure or mimic genetic variation (Johnson, 1971). Prakesh et al., (1969) have called the different enzyme forms produced by different alleles at the same locus, allozymes, to distinguish them from isozymes which are different forms of an enzyme arising from several causes (Market and Møller, 1959).

MATERIALS AND METHODS

In the first section of the following study, the known stocks [Hj6, AK1 (syngen 13); Hj1 (syngen 12); KOK (syngen 3); Isn (syngen 1)] and the unknown stocks of 2C, 2F, 42 were used. Each of these stocks were grown in 250 ml of bacterized medium (pH: 7.0), at 26°C for about 4-5 days. Then, they were filtered separately, centrifuged in an oil testing centrifuge in 100 ml pear-shaped vessels at 600 g for 10 minutes. The concentrated paramecia including 10 ml of the culture medium were collected after centrifugation. A few drops of each stock were transferred to a depression slide and then a few drops of the other stock were added. All possible combinations of the unknown stocks were set-up with each other. Depression slides were kept at 20°C for about 10 hours. The mixtures were checked at various intervals. If no mating pairs were observed, the mixtures were discarded. After this period of time pairs, where they occurred, were separated from the mixture and kept in a depression slide containing exhausted bacterized medium. Exhausted medium was used in order to avoid the occurrence of macronuclear regeneration (see Introduction).

The pairs were checked at various intervals and those which had not formed firm connections in the first few hours were discarded. The process of conjugation took 10-15 hours. After this period the conjugant pair separated. Each ex-conjugant was placed in a different depression slide and was kept in exhausted bacterized medium. Four carynoides formed and each cell was transferred to a new depression slide and kept for a few days in bacterized medium. After cells divided a few times they were transferred to a test tube containing 2 ml of fresh bacterized medium. Each day a few mls of the medium were added and when cells had grown densely, they were transferred to a two litre Thompson bottle, containing one litre of bacterized medium.

The preparation of samples, the buffer and staining conditions used for gel electrophoresis have already been described in Chapter Two.

SECTION I

MATING REACTIONS OF UNKNOWN AND KNOWN SYNGENS

Table XI shows the results of mating reactivity of all possible combinations between known/unknown, known/known and unknown/unknown stocks.

Stocks	Hj6	AK1	KOK	Hj1	Isn	2C	2F	42
syngen 13 { Hj6		++	-	-	-	+	++	++
syngen 13 { AK1	++		-	-	-	++	+	+
syngen 3 → KOK	-	-		-	-	-	-	-
syngen 12 → Hj1	-	-	-		-	-	-	-
syngen 1 → Isn	-	-	-	-		-	-	-
unknown { 2C	+	++	-	-	-		++	++
unknown { 2F	++	+	-	-	-	-		+++
unknown { 42	++	+	-	-	-	++	+++	

Table XI: shows mating reactivity between different stocks.

\- : No mating reactivity.

\+ : Weak mating reactivity.

++ : Stronger mating reactivity than +.

+++ : Stronger mating reactivity than ++.

The summary of results tabulated in Table XI are as follows:

(1) No inter-syngenic mating reactivity was observed between the known syngens - 1, 3, 12 and 13.

(2) Mating reactivity was observed and conjugating pairs were also

formed between the two stocks of syngen 13 (AK1 and Hj6).

(3) The two stocks of syngen 13 showed mating reactivity with the unknown stocks - 2C, 2F and 42. Conjugating pairs were observed between these stocks.

(4) The three unknown stocks 2F, 2C and 42 showed mating reactivity with each other. Conjugating pairs were observed between these stocks.

The results were obtained after repeating these experiments many times. Each time when a pair of conjugants was formed, it occurred between the two stocks of syngen 13 or between these stocks and any of the three unknown stocks or between the three unknown stocks. Conjugating pairs were not formed in all the experiments even between the two stocks of syngen 13 of complementary mating types which would be expected to mate freely. The reason for this may be due to slight variation in conditions under which different experiments were conducted. For example, the amount of light can be effective. However, I have not found any specific environmental conditions which ensure the occurrence of conjugation even between stocks of the same syngen.

The number of conjugants which formed between stock Hj6 and either of the two stocks 2F and 42 was greater than the number of pairs formed between stock Hj6 and stock 2C. On the other hand, stock AK1 formed more pairs with stock 2C than with the other two unknown stocks 2F or 42. In general, the percentage of pairs formed in relation to the total number of paramecia in the mixture was very low (approximately 20-30 per cent).

Studies on ex-conjugants from mating pairs

Merely observing that the conjugant pairs have been formed in a mixture would not be sufficient to place the two stocks involved in

the conjugation in a single syngen. It is essential to show genetic exchange has really occurred between the apparent conjugants. One way to demonstrate that genetic exchange has occurred between the pairs is to screen an enzyme which has different mobility in the parents and to follow its inheritance. In true conjugation, it is expected that all the ex-conjugants would have an identical pattern of enzyme activity, corresponding to both forms of the enzyme in the parents, e.g. in the case of monomeric enzymes. If the enzyme is a polymer, an intermediate band or bands of activity would be observed in addition to the parental bands. In the case of selfing, because the two parents have the same genotype, the exchange of genes between the two parents would not lead to variant forms of enzymes in the progeny.

In cytogamy, two different patterns of enzyme activity would be detectable among the progenies, one pattern corresponding to the one of the parents and the other to the alternative parent.

Amongst the stocks which were reported earlier to form conjugating pairs, the two stocks of Hj6 (syngen 13) and 2F (unknown) were chosen and screened for phosphogluco isomerase and α-naphthyl propionate esterase following conjugation. These two stocks were chosen because of the higher percentage of pair formation and consequently the greater number of pairs which could be isolated. Phosphogluco isomerase and α-naphthyl propionate esterase were chosen for screening since clear variations in their patterns of activity had been detected between the unknown stocks (2C, 2F, 42) and the stocks of syngen 13 (AK1 and Hj6) (see also Chapter Three).

Results

1) Phosphogluco isomerase

It was mentioned earlier (see Chapter Three) that the first anodal

band of activity from the origin had a faster mobility in stock Hj6 than in stock 2F. Figure 46 shows the band of phosphogluco isomerase activity in stocks Hj6, 2F and the hybrid F_1 between them. The enzyme had two bands of activity in the hybrid cells. Each band corresponded to one of the parental bands. All the eight carynoides which resulted from a conjugant pair had identical patterns. This indicates that true conjugation had occurred between the parental stocks (2F and Hj6).

The fast band of activity in stock Hj6 was attributed to allele pgi_1^{100}, and the slow form of band in stock 2F to allele pgi_1^{86}. The heterozygotes possessed both alleles pgi_1^{100}/pgi_1^{86}. For designating the alleles the same principle followed as reported earlier (see Chapter Three, Section II). The only difference was that in designating alleles the abbreviation used for bands would be represented by small letters.

The apparent lack of any intermediate band in the hybrids indicated that this enzyme is a monomer in syngen 13 of P. caudatum.

The nature of this enzyme in syngen 3 of P. caudatum will be discussed in the second section of this chapter. For the present purpose, suffice it to say that results of the study on phosphogluco isomerase indicates that true conjugation has occurred between the two stocks Hj6 and 2F.

2) α-Naphthyl propionate esterase

Only the slowest anodal band of activity from the origin, in each of the parental stocks (2F and Hj6), was considered in this study (Figure 47). The single cathodal band of activity did not vary in these two stocks and the same band was observed in the ex-conjugants following mating between the two stocks.

α-Naphthyl propionate esterase in the anodal part of the gel appeared as a single intense band of activity in the parental stocks

Figure 46:

Phosphogluco isomerase in stocks Hj6 (syngen 13), 2F (unknown) and the ex-conjugants between them.

1,8 : stock Hj6

2,9 : stock 2F

3,4,5,6,7 : ex-conjugants between 2F and Hj6

Figure 47:

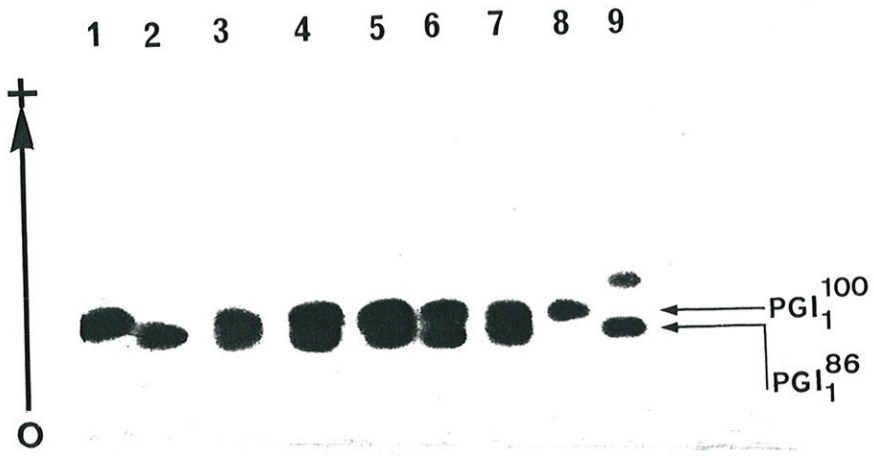
α-Naphthyl propionate esterase in stocks Hj6 (syngen 13), 2F (unknown) and the ex-conjugants between them.

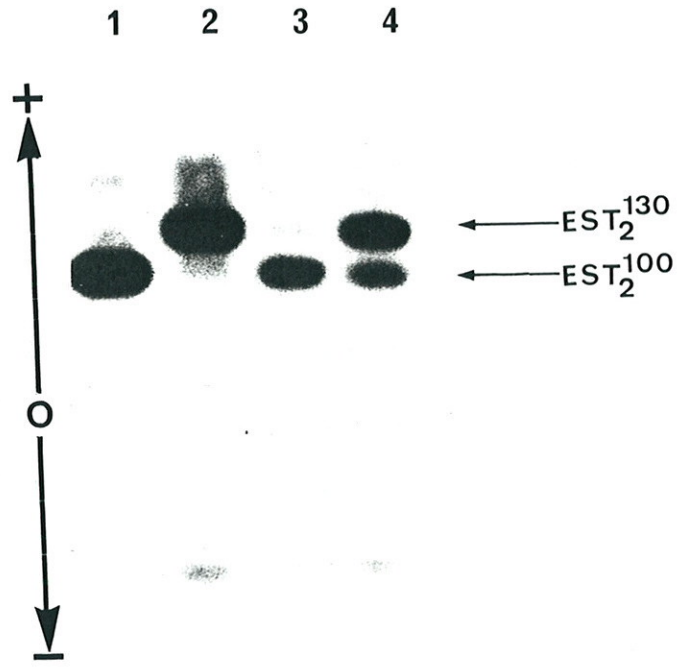
1,3 : stock Hj6

2 : stock 2F

4 : ex-conjugants between 2F and Hj6

O : Origin


1 2 3 4 5 6 7 8 9
+
O
PGI_1^{100}
PGI_1^{86}


1 2 3 4
+
O
EST_2^{130}
EST_2^{100}

(2F and Hj6). This band had slower mobility in stock Hj6 than in stock 2F. Figure 47 shows the α-naphthyl propionate esterase in the parental stock and the hybrid between them. All the 8 carynoides resulting from a single pair had an identical enzyme pattern indicating that true conjugation had occurred between the parental stocks. The enzyme of the hybrid cells had two bands of activity, each corresponding to one in the parental stocks.

To designate the alleles and bands, the same principles were followed as described with phosphogluco isomerase. The slower band of activity in stock Hj6 (syngen 13) can be attributed to the allele est_2^{100} and the faster form of the band in stock 2F to allele est_2^{130}. The heterozygotes (hybrids) possess both alleles est_2^{100}/est_2^{130}. The absence of an intermediate band suggests that this enzyme is a monomer in P. caudatum.

The observed pattern of α-naphthyl propionate esterase in the ex-conjugants of Hj6 and 2F was compatible with the reported pattern of inheritance in species 2 (syngen 2) of P. aurelia (Allen et al., 1972).

Discussion

Any isolates of P. caudatum could have been classified on the basis of their mating type characteristics,* to any of the 16 syngens of this ciliate, if stocks of complementary mating types of these syngens were readily available. Of the 16 originally classified syngens of P. caudatum (Sonneborn, 1957) only four were available. These four syngens (1, 3, 12 and 13) were collected and classified

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* Mating type characteristic is the ability of any single stock to mate and produce viable progeny only with stocks of complementary mating type of the same syngen.

in Japan. The three unknown stocks 2F, 2C and 42 have been collected from different parts of England and most probably they belong to the same syngen, judging by the observed coefficient of identity (100%) between them (see Table II, Chapter Three) as well as their mating reactivity (see Table XI).

These three stocks (2C, 2F, 42) showed mating reactivity with the two stocks of syngen 13 (AK1 and Hj6). Conjugant pairs were formed between them and the formation of hybrids have been proved by screening of two enzymes, phosphogluco isomerase and α-naphthyl propionate esterase in the parents and the ex-conjugants. The eight carynoides resulting from a single pair had identical patterns of enzyme activity. These results indicated that true conjugation had occurred between stock Hj6 (syngen 13) and 2F (unknown syngen). This phenomenon can be explained in two ways:

(1) the formation of hybrids represents intra-syngenic crosses in which case the unknown stock 2F as well as stocks 2C and 42 are members of syngen 13, or

(2) the formation of hybrids represents inter-syngenic crosses and the three unknown stocks - 2C, 2F, 42, should be located in another syngen.

These two possibilities will be discussed at greater length in the following part.

Considering the coefficient of identity in the patterns of enzyme activity from the electrophoretic data (Table II, Chapter Three), it can be seen that the coefficient of identity between the three stocks 2C, 2F, 42 and the two stocks of syngen 13 (AK1, Hj6) is either 73 or 80 per cent. The average figure between them although, is slightly lower than the average figures for coefficient of identity in intra-syngenic comparison between different stocks of syngens 1, 3, 12 (see Tables IV,

VI, VIII, Chapter Four). They fall within the range of intra-syngenic variation for the stocks of the least similarity, e.g. comparisons of stock YT3 to stocks KOK, KT2 or YT1 in syngen 3 or of stock Nn2a with stocks TK 306 or Wa 33 in syngen 12. It is also significantly higher than the calculated value for the coefficient of identity between stocks collected from nature (25 to 44 per cent) and which probably represent different syngens (see Table X, Chapter Five). The 73%-80% coefficient of identity between stocks of syngen 13 (AK1, Hj6) and the three unknown stocks 2C, 2F, 42 is not low enough to rule out the possibility of these stocks belonging to the same syngen (syngen 13).

Out of nine enzymes which were examined, four showed variation between stocks in syngen 13 and the three unknown stocks. The variant enzymes were α-naphthyl propionate esterase, α-naphthyl butyrate esterase, phosphogluco isomerase and hexokinase.

Hexokinase proved to be polymorphic and the occurrence of intra and inter-syngenic variation was quite common. Any two stocks can show variation for this enzyme but still belong to the same syngen. Phosphogluco isomerase was less polymorphic than hexokinase. However, intra-syngenic variation was observed in syngens 1, 3 and 12. In syngen 1, one out of four, in syngen 3, two out of five, and in syngen 12, two out of eleven stocks showed variation for this enzyme. α-Naphthyl butyrate esterase was less polymorphic than the other two enzymes, but some intra-syngenic variation was observed in syngens 1, 3 and 12.

The most interesting enzyme in respect of the type of observed variations was the α-naphthyl propionate esterase. This enzyme was monomorphic both within and between stocks of syngens 1, 3, 12 and 13. No intra and inter-syngenic variation was observed with this enzyme. The lack of variation was observed within stocks collected from Japan and also within the three stocks of 2C, 2F, 42, was thought to be

dependent on the geographical origin of stocks. Stocks from the same geographical origin had the same pattern, which differed from the pattern in the stocks of another geographical origin. Further study on the stocks collected from nature ruled out this hypothesis. Stocks collected from Scotland had an identical pattern of α-naphthyl propionate esterase activity with the stocks collected from England (see Chapter Five). It should be noted, however, that the esterases which did not show any geographical differentiation were different from those where the variation was thought to be geographically dependent. In the first case an identical pattern of esterase activity was observed with the two substrates, α-naphthyl propionate and α-naphthyl butyrate. And in the second case, the first anodal band of α-naphthyl propionate esterase designated as EST_2 only interacted with α-naphthyl propionate and did not interact with the α-naphthyl butyrate.

In P. aurelia, with the exception of syngen 2, intra-syngenic variation were reported to be rare for esterases (Allen et al., 1971b). This was despite the fact that the stocks came from different geographical sources. Adams and Allen (1975) suggested that the lack of differentiation amongst stocks within a syngen suggested that in nature, selection for certain esterase genotypes must be high.

In the present study, it is not known if selection is involved in preserving the est_2^{100} allele in the Japanese stocks, although they belong to four different syngens. If selection is involved it is not known why it is only operating in the cells collected from Japan. However, it should be noted that it may be possible to find variant forms of the α-naphthyl propionate esterase by collecting and examining more samples from Japan.

As described in the earlier chapters, by comparing the isozyme patterns of ten loci in P. aurelia, it was possible to distinguish 14

species of this group (Tait, 1978). However, in two cases, the species' comparisons yield a high coefficient of identity, 90% (for species 1 and 5) and 70% (for species 7 and 14). Both values were within the range of the coefficient of identity observed in intra-species variation. Sonneborn (1975) and Tait (1978) both suggested that matching the isozyme patterns should be used as a supplementary method of identification to that of mating type.

Butzel (1974) demonstrated the genetic isolation of the syngens (species) of P. aurelia by several means.

(1) Whilst mixtures of sexually reactive mating types within a syngen may yield up to 90% conjugants, mating between syngens never yield more than 40% conjugants, and frequently yield only a few or no pairs.

In P. caudatum, the mixture of any of the three unknown stocks (2C, 2F, 42) and the two stocks of syngen 13 (AK1, Hj6) at the maximum did not yield more than 30% conjugants. However, the rate of conjugant formation between the two stocks of syngen 13 (AK1, Hj6) with the complementary mating types was not any greater than 30%.

(2) The second means of distinguishing intra and inter-syngenic (species) crosses in P. aurelia is the rate of survival, which was reported to be as high as 90% in intra-syngenic crosses. Haggard (1974) has exhaustively examined crosses between syngen 4 and 8 of P. aurelia. He reported that 32% of the ex-conjugants survived but only 9% were true hybrids, i.e. involved reciprocal cross fertilization followed by normal nuclear reorganization. In intra-syngenic crosses 94% and 80% of intra-syngenic ex-conjugants survived in syngens 4 and 8 respectively.

In P. caudatum, the crosses between the three unknown stocks (2C, 2F, 42) and the two stocks of syngen 13 (Hj6, AK1) did not yield more than 20% survivors. The same figure was observed for the rate of survivals in crosses between the two stocks of syngen 13 (Hj^6 and AK1).

In crosses between stocks Hj 6 and 2F, about 10% of survivors passed through selfing.

Haggard (1974) also reported that the ex-conjugants between syngens 4 and 8 of P. aurelia had a reduced fission rate and were very difficult to maintain. In P. caudatum the ex-conjugants between the three stocks (2C, 2F, 42) and stocks of syngen 13 (Hj6, AK1) did not show either a lower rate of fission or any greater difficulty in their maintenance than the ex-conjugants of the two stocks of syngen 13 (Hj6 and AK1). Some earlier reports by Pringle (1955) and Johnson (1955) indicated that mating between members of the same local population usually gave a fair proportion of viable F_1 cells, whereas crosses between representatives from populations of different localities gave highly variable results. However, they usually had a less viable F_1. This might be due to variation in micronuclear chromosome number in different stocks (36 to 165) in P. caudatum (Diller, 1940). It was suspected that there were greater differences in chromosome numbers between stocks of different localities than between stocks in the same local population.

Differences in chromosome number were also found to be correlated with low fertility in crosses of P. aurelia. Crosses between stocks from the same population were found to produce more viable progenies than the crosses from different populations (Kosciuzko, 1965; Dipple, 1954). Chromosome numbers vary in different stocks of P. aurelia between 30-63 (Kent W.S., 1882). Chromosome incompatibilities could be the most important factor bringing about the high rate of mortality in the future generations.

In P. bursaria, where the number of chromosomes varies between 80 to several hundred (Chen, 1940), the F_1's between syngens were unable to survive (Bomford, 1966).

(3) The F_1 hybrids between syngens of P. aurelia may exhibit a number

of cytological abnormalities including loss of micronuclei, abnormal macronuclei and changes in size and shape (Butzel, 1953). No information is available in this respect for P. caudatum.

(4) In P. aurelia, inter-syngenic crosses can be demonstrated by an almost absolute failure of the F_1 of the possible inter-syngenic crosses to give rise to viable F_2 cells either by autogamy, back crossing or by crossing F_1's (Butzel, 1953; Sonneborn, 1957).

In P. caudatum, Hiwatashi (1968) reported the poor survival of F_1 hybrids in intra-syngenic crosses and extremely poor survival in the F_2 generation. However, no figures have been given in this study. The results of intra-syngenic crosses between stocks of syngen 3 and 12, to be reported in the next section of this chapter, reinforce Hiwatashi's observations. Therefore the relatively poor survival in crosses between stocks of syngen 13 (AK1, Hj6) and the unknown stocks (2C, 2F, 42) cannot be a means for separating the unknown stocks into a new syngen.

There is no information available on the rate of F_2 survival in the present study since it has not been possible to mate any of the ex-conjugants. Lack of mating reactivity between the ex-conjugants might be due to a long immaturity period.

The last point which should be mentioned is that the four syngens 1, 3, 12 and 13 of P. caudatum have been categorized into group one of P. caudatum. Different syngens of this group showed no sexual reaction other than that between stocks of complementary mating type within each syngen (Sonneborn, 1957). Any stocks therefore which show mating reactivity with stocks of syngen 13 are considered to belong to this syngen.

Conclusion

Considering all the available data, it is concluded that most probably the three unknown stocks of 2C, 2F and 42 belong to syngen 13 of P. caudatum. The only hesitation in making a definite statement is due to lack of information in the second generation of crosses between these three unknown stocks and the two stocks of syngen 13. Otherwise all the other features of mating between these two groups were consistent with intra-syngenic crosses in P. caudatum. The only point was the low percentage of pair formation in the mixtures of stocks of these two groups. It was also mentioned that the percentage of pair formation between the two stocks of syngen 13 (AK1, Hj6) was low. This phenomenon might be due to the long duration that these stocks have been kept under laboratory conditions without undergoing any conjugation, i.e. there is no renewal of life cycle. The results of mating studies in syngen 3 and syngen 12 of P. caudatum reinforce this view. When newly isolated stocks with complementary mating types of any of these syngens were mixed together the percentages of conjugants in most cases were as high as 90% or more (see Section II of this chapter). The frequency of conjugation is not known in nature but cells which have been kept under laboratory conditions without any sexual formation for a long period of time (years), could be in the late maturity period of their life cycle. The manifestation of this period is the low formation of pairs in the mixture of stocks with complementary mating types.

The two stocks of syngen 13 (AK1, Hj6) have complementary mating types. Both were able to conjugate with any of the three stocks 2C, 2F, 42. It was not therefore possible to identify the mating type expressed by these three stocks. This phenomenon was thought to be the result of clonal aging in these cells. It was reported that clones with even mating type become unstable in the later period of their life cycle, i.e. toward the end of maturity and the beginning of senility (for more detail see Introduction, Chapter Six).

SECTION II

MATING IN SYNGENS 3 AND 12 OF P. CAUDATUM AND GENETIC CONTROL OF PHOSPHOGLUCO ISOMERASE IN SYNGEN 3

Introduction

A breeding analysis has been carried out between variant stocks in some species of P. aurelia and the T. pyriformis complex of species. Genetic control of the molecular basis of some of these enzymes in these two ciliates have been reported.

In P. aurelia genes have been reported to determine esterases in species 1 and 8 (Cavill and Gibson, 1972; Allen et al., 1972); malate dehydrogenase (NAD-dependent) in species 4 (Williams and Smith Sonneborn, 1980); hydroxybutyrate dehydrogenase in species 9 (1968, Tait); isocitrate dehydrogenase in species 2 (Tait, 1970b) and phosphogluco isomerase in species 1 (Tait, 1978).

In the Tetrahymena-pyriformis complex genes have been disclosed for tyrosine amino transferase; malate dehydrogenase (NADP-dependent); isocitrate dehydrogenase and tetrazolium oxidase (superoxide dismutase) in species 1 (Borden et al., 1973b); esterases and acid phosphatase in species 1 (Allen, 1960, 1961; Allen et al., 1963).

In syngen 3 of P. caudatum, genetic control of lactate dehydrogenase was reported and breeding data indicated that this enzyme is a dimer coded for by two codominant alleles at a single locus (Agatsuma and Tsukii, 1980).

Results on mating reactivity and percentage of pair formation

All the possible combinations of stocks with complementary mating types have been made within syngen 12 and syngen 3. The mating reaction

between the stocks was very strong. In most of the cases more than 90% of cells formed conjugant pairs in the mixture. However, the rate of survival was on average very low. In syngen 12, the conjugant pairs of the following crosses were isolated and the percentage of ex-conjugant survivors have been calculated. In crosses between the stocks Nn2a/Nn4 the rate of survival was 20%; between stocks TK-306/Hj1 17%; between stocks Wa33/Wa16, 16%; between stocks Nn4/TK306, 22% and between stocks TK306/TK305, 18%. On average then the rate of survival between stocks of syngen 12 was about 18%. The rate of survival was not dependent on the extent of the initial mating reactivity. In some mixtures of the two stocks, such as TK306/TK305, mating reactivity was very strong and almost all the cells were involved in conjugation. The union of three or four cells was frequently observed. In other crosses such as TK306/Hj1, mating reactivity was not very strong and not more than 80% of the cells in a mixture formed pairs. However, the rates of survival did not vary in these two cases. It was not known what percentage of survivors in any of these cases were the results of processes other than conjugation (e.g. selfing). No further studies have been carried on to determine the percentages of true ex-conjugants.

Strong mating reactivity was also observed between stocks of complementary mating types in syngen 3 of P. caudatum. In the case of stock KT2, although many attempts have been made to mate this stock with the other stocks of syngen 3, especially with stock YT3, they were all unsuccessful. The reason for this failure is not known. The rate of ex-conjugants' survival in syngen 3 was not significantly different from the rate of survival in syngen 12. For example in different experiments the following results were obtained when stocks YT1 and YT3 were crossed. Experiment (1) 150 pairs were isolated and 20 survived (13% survival); (2) 139 pairs were isolated and 19 survived (13% survival); (3) 78 pairs

were isolated and 20 survived (25% survival); (4) 66 pairs were isolated and 14 survived (21% survival). Mating reactivity was very strong between these two stocks (YT_3 and YT_1) and quite frequently the union of 3 or 4 cells was observed. This phenomenon has been previously reported in Paramecium (Chen, 1946; Siegel, 1954; Sonneborn, 1955; Vivier, 1960). The union of more than two cells occurs when the mating reactivity is very strong.

The ex-conjugants from mating between stocks YT1 and YT3 were chosen to study the nature of phosphogluco isomerase inheritance in syngen 3 of P. caudatum. These two stocks had discrete differences in their pattern of phosphogluco isomerase activity and also to carry out conjugation between them was very easy. Any time the cells of these two stocks were mixed together, they conjugated readily if they were not overfed or grossly starved. The enzyme phosphogluco isomerase was chosen because it was easily detectable in P. caudatum. The other two enzymes which showed differences in mobility between the two stocks YT1 and YT3 were hexokinase and α-naphthyl butyrate esterase. The first enzyme (hexokinase) did not have a very clear pattern. When the patterns in ex-conjugants were compared to the parental patterns, it was difficult to deduce any results without some ambiguity. Therefore, these results were not considered in the present study. The second enzyme (α-naphthyl butyrate esterase) had very slight differences in mobility between the two stocks YT1 and YT3. This made it difficult to compare the ex-conjugants and the parents without ambiguity.

The molecular structure of phosphogluco isomerase has been discussed in the first section of this chapter. It is desirable to look at the nature of this enzyme in another syngen of P. caudatum.

The inheritance of phosphogluco isomerase in syngen 3 of P. caudatum

The first anodal band of phosphogluco isomerase activity in stock YT3 migrated at a slower rate than the band in stock YT1. The pattern of enzyme activity in the ex-conjugants was different from either of the parents. All the real ex-conjugants had three bands of activity, two corresponding to the band of each of the parents and one intermediate band (Figure 48). All the eight carynoide which resulted from a single pair had identical patterns. Selfing was also observed in 7% of ex-conjugants. In these cases, the ex-conjugants had identical patterns of enzyme activity which was identical to one of the parents (YT_3).

These results indicated that this enzyme is a dimer in syngen 3 of P. caudatum, and is coded for by two alleles at a single locus. The fast and slow bands of activity are designated as $PGI_1^{100/100}$, $PGI_1^{75/75}$ respectively and the hybrid band is designated as $PGI_1^{75/100}$ (Figure 48).

Attempts have been made to cross the real ex-conjugants, up to six months after the occurrence of conjugation. However, no mating reactivity has been observed between the ex-conjugants. The lack of mating reactivity in the ex-conjugants might be due to long periods of immaturity in these stocks.

Discussion

Phosphogluco isomerase had three bands of activity in the ex-conjugants of syngen 3, and two bands of activity in the ex-conjugants between stocks 2F and Hj6 of syngen 13. These differences can be explained in two ways.

(1) The nature of the enzyme is different in different syngens. It is monomeric in syngen 13 and dimeric in syngen 3.

Amongst stocks which have been reported in the previous chapters,

some had two bands of phosphogluco isomerase activity, e.g. stocks Nn2a, Nn1a (syngen 12) and stocks NO15, NO16, NO32 collected from nature (Figures 27 and 43). The existence of these stocks also indicated the monomeric nature of this enzyme. Two of the stocks collected from nature, NO37, NO41, had three bands of phosphogluco isomerase activity (Figure 43), indicating the dimeric nature of this enzyme. If the nature of phosphogluco isomerase is really different in different syngens of P. caudatum, can it then be used as a means of syngen differentiation? This can only be answered by examining the enzyme in other syngens of P. caudatum.

In P. aurelia, two stocks of species 1 (syngen 1) were crossed, one with a single band and the other with three bands of activity. It was concluded that this enzyme was dimeric in species 1 of P. aurelia (Tait, 1978). However, there is no other report available on phosphogluco isomerase in other species of P. aurelia. It is especially important to look at the nature of this enzyme in species 5 of this ciliate, where the enzyme appears either as one band or two bands of activity in different stocks.

(2) The other way to explain the existence of the two bands in the ex-conjugants between stocks Hj6 (syngen 13) and 2F is that the enzyme may be a dimer, as in syngen 3, but the subunits in the case of syngen 13 are physically constrained from interacting.

In Drosophila pseudoobscura, it was reported that all the heterozygous females had a three banded phenotype for esterase-5 locus (Hubby and Lewontin, 1966). This was attributed to a dimeric structure of est-5 enzyme. The enzyme in males and homozygous females was a monomer. Phosphogluco isomerase has been reported to have a dimeric structure in 18 species of marine Molluscus (Wilkins, 1975).

Recently, Williams and Smith Sonneborn (1980) reported that isozymes

and allozymes (for definition, see Introduction), because of their Mendelian inheritance and also the ability to be quickly assayed by electrophoresis, provide excellent genetic markers. They reported that malate dehydrogenase could be used as a genetic marker to monitor aging cells for rejuvenating sexual processes (autogamy) in P. tetraurelia (formerly syngen 4 of P. aurelia).

For any genetic work true cross-fertilization conjugants must be distinguished from false conjugants due to selfing, autogamy, cytogamy and macronuclear regeneration. With the exception of cytogamy, all types of false conjugations can be distinguished by the lack of an immature period in their clones after conjugation (Hiwatashi, 1968). However, by screening an enzyme such as phosphogluco isomerase, which is very easily detected, all forms of false conjugation can be distinguished from true conjugation. Only in the latter case will the ex-conjugants have an identical pattern of enzyme activity, which is different from the pattern of activity in any of the parents. Phosphogluco isomerase can be used as a genetic marker for any genetic experiments in P. caudatum.

Figure 48:

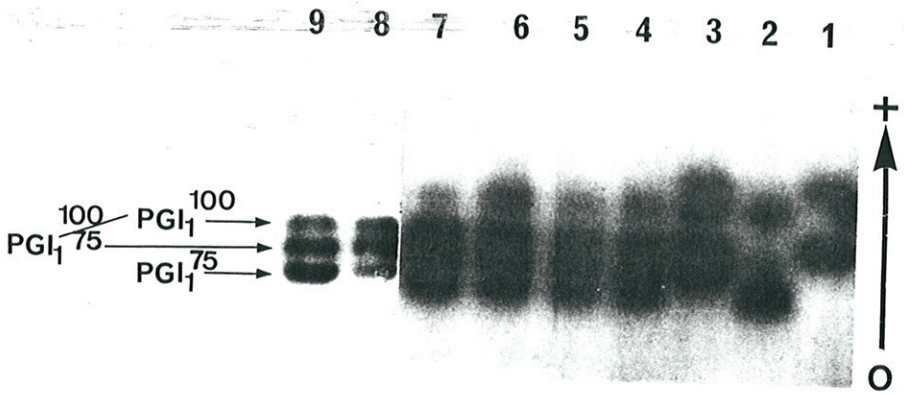
Phosphoglucoisomerase in stocks YT1, YT3 (syngen 3) and the ex-conjugants between them.

3,4,5,6,7,8,9 : ex-conjugants between YT1 and YT3

1 : stock YT1

2 : stock YT3

O : Origin


9
8
7
6
5
4
3
2
1
+
O
PGI1 100
PGI1 75
PGI1 100
PGI1 75

CHAPTER SEVEN

EFFECT OF BACTERIA ON THE ENZYME PROFILES OF P. CAUDATUM

Introduction

It was reported by Rowe et al. (1971) and Gibson and Cavill (1973) that the presence of bacteria in the growth medium did effect the esterase patterns of activity in P. aurelia. They found that bacterial enzymes might contribute directly to the esterase pattern or else be associated with the disappearance of enzyme in cells growing in axenic media. Esterases were, however, the only enzymes examined in P. aurelia (see General Introduction) which were influenced by the presence of bacteria.

In the preceding studies, stocks of P. caudatum cultured in bacterized medium were used. Measures were taken to ensure that cells were grown in the presence of one species of bacterium, in this case Klebsiella pneumoniae. Different stocks were washed and then transferred to mono-axenic medium (grass medium + Klebsiella pneumoniae) (for the washing procedures see the following Materials and Methods). This process was very important in order to ensure that only one kind of bacterium could be involved in modulating the banding patterns of enzymes in Paramecium grown in bacterized medium. Since the bacterial enzyme might influence the banding pattern of Paramecium, it was essential to determine if the observed bands on starch and acrylamide gels were due to enzymes produced by Paramecium rather than by the bacteria themselves.

Stock KOK (syngen 3) was transferred to two different sterile media, axenic and adaptation. Extracts of this stock growing in these two media were compared with an extract from cells growing in bacterized medium, also with an extract of Klebsiella pneumoniae on its own. The results of this study are reported in the following part of this chapter.

MATERIALS AND METHODS

Stock KOK (syngen 3) of P. caudatum was transferred from bacterized medium (monoaxenic medium) to axenic and adaptation media.

Axenic medium

The axenic medium used was that of Soldo et al. (1966). The concentrations of the components are shown in Table XII.

Table XII: Component of axenic medium

Component	Final Concentration	
Proteose peptone	10	mg/ml
Trypticase	5	mg/ml
Yeast nucleic acid	1	mg/ml
$MgSO_4 \cdot 7H_2O$	500	mg/ml
TEM-4T	100	mg/ml
Stigmasterol	5	mg/ml
Vitamins		
Calcium panthothenate	500	mg/ml
Nicotinamide	500	mg/ml
Pyridoxal HCl	500	mg/ml
Pyridoxamine HCl	250	mg/ml
Riboflavin	500	mg/ml
Folic acid	250	mg/ml
DL-Thioctic acid (a lipoic acid)	50	mg/ml
Biotin	0.125	mg/ml

A stock solution of vitamins, 10 times the final concentration, was made up in 980 mls of distilled water plus 20 mls of 1N NaOH to

dissolve the vitamins. The stock solution had gaseous nitrogen bubbled through it for 2 hours, and then was stored in the dark at 4°C. 10 mls of the stock solution was added to 1 litre of axenic medium before autoclaving. TEM-4T is a commercial preparation of diacetyltartaric monomers of beef tallow monoglycerides. The stock solution was made by dissolving TEM-4T in absolute ethanol at 20 mg/ml. 5 mls of this stock solution were added to 1 litre of axenic medium before autoclaving. The stigmasterol stock solution was made by dissolving stigmasterol in absolute ethanol at 1 mg/ml. 5 mls of this stock solution were added to 1 litre of axenic medium before autoclaving. Both TEM-4T and stigmasterol stock solutions were stored at -20°C until use.

After addition of all components, the pH of the medium was adjusted to 7.0 with 1N NaOH, dispensed either into test tubes (1-5 ml per tube) or into 2 litre Thompson bottles (500 ml per bottle) and sterilized at 121°C, 15 lb per square inch, for 20 minutes.

Adaptation medium

The adaptation medium used was that of Allen and Nerad (1978). The components of this medium are shown in Table XIII.

Klebsiella pneumoniae was grown in one litre of sterile nutrient broth for 48 hours at 37°C. The culture of Klebsiella pneumoniae was harvested by centrifugation in a Sorval super speed RC-2 at 27,000 g for 10 minutes. The pellet of bacterial cells was resuspended in Peters solution (see Table XIII). The other component of adaptation medium, i.e. vitamins, TEM-4T and stigmasterol, was prepared as described for the axenic medium. The adaptation medium was dispensed either in test tubes (1-5 ml per tube) or into 2 litre Thompson bottles (500 ml per bottle), and then sterilized at 121°C, 15lb per square inch, for 20 minutes.

Table XIII: Component of adaptation medium

Peters solution:	
NaCl	600 mg
$CaCl_2 \cdot 2H_2O$	20 mg
$MgSO_4 \cdot 7H_2O$	10 mg
Na_2HPO_4	10 mg
KCl	10 mg
ADD:	
Vitamin stock	10 ml
Stigmasterol stock	5 ml
TEM-4T stock	5 ml
Distilled water	980 ml

+ Pellet of one litre harvested Klebsiella pneumoniae.

pH: 7.0.

The medium was autoclaved at 15 lb per square inch, 121°C for 20 minutes.

Both adaptation and axenic media were kept at 20°C in the dark until used.

Methods of establishing sterile cultures of P. caudatum

Transfer to sterile media (axenic and adaptation) was achieved in one of several ways, observing strict aseptic conditions throughout the transfer procedure. All the washing procedures were carried out in a laminar flow unit to ensure sterility.

a) Washing of cells in sterile grass medium

Grass medium was prepared as described in General Materials and

Methods (Chapter Two). The pH of this medium was adjusted to 7.0 with 1 N NaOH before sterilization. 0.3 ml of this sterile grass medium was transferred to each well of a sterilized 3 welled depression slide, contained in a sterilized glass petridish. About 50 Paramecium cells from a bacterized culture were transferred to each well and then cells of each well were washed rapidly through four wells containing sterile grass medium. The cells were left for one hour and then washed again three times. They were left in the third wash for another one hour. This procedure was carried out for six to seven hours. A sterile micropipette was used for each transfer of cells. The cells remaining alive after this procedure were transferred to a test tube containing 1 ml of either axenic or adaptation medium. 10 cells were transferred into each test tube, and were incubated at 26°C in the dark. Tests of sterility were carried out after cells had established a vigorous culture (see below).

b) Method using Chlamydomonas

This method was used exactly as it was reported by Fok and Allen (1979) with one exception. In the present study, sterile grass medium was used instead of sterile Cerophyl infusion medium. This method was more successful than that previously described. Axenically cultivated Chlamydomonas was obtained from the John Innes Institute.

Test for sterility of cultures

After paramecia had become established in axenic and adaptation media, tests for sterility were carried out using agar plates. Plates were incubated at 37°C for 48 hours and were observed for signs of contamination during the following week.

Preparation of bacterial extract

Klebsiella pneumoniae was grown and harvested as it was described in preparation of adaptation medium. The pellet of bacterial cells was resuspended in distilled water. Sonication was used for disruption of bacterial cells. The disrupted cells were harvested by centrifugation at 27,000 g in a Sorvall super speed centrifuge for 30 minutes at 4°C. The supernatant was mixed with tracking dye, then it was transferred to capillary tubes and kept at -20°C.

Effect of bacteria on esterases

Results

1) α-Naphthyl propionate esterase

The first and second anodal band in stock KOK were identical in bacterized, axenic and adaptation media. However, a third and faster band of activity was observed in the axenic and adaptation media which was not detectable in bacterized medium (Figure 49). Klebsiella pneumoniae had a single anodal band which migrated faster than the bands of Paramecium. The bacterial band was not detectable in the bacterized cultures of stock KOK (syngen 3).

In the cathodal part of the gel, the single band of activity in stock KOK was identical in the three media, bacterized, axenic and adaptation. No cathodal band was detected in the bacterial extract.

2) α-Naphthyl butyrate esterase

Stock KOK had a single band in bacterized medium in the anodal part of the gel and two bands in axenic and adaptation media. Of these two bands, one was identical to the band in bacterized medium and the other band migrated at a faster rate towards the anode than the former band (Figure 50).

The cathodal band was identical to that observed when α-naphthyl

propionate was used as substrate.

Klebsiella pneumoniae did not contribute any band to the gel with α-naphthyl butyrate.

Discussion

Of the four bands of esterase activity in axenic and adaptation cultures of stock KOK (syngen 3), three were identical to those found with paramecia grown in bacterized culture. There was a single anodal band which was only detectable in the axenic and adaptation media. This band reacted equally well with the substrates α-naphthyl propionate and α-naphthyl butyrate.

The effect of bacteria on the esterase pattern was also reported in P. aurelia (Rowe et al., 1971; Gibson and Cavill, 1972). These authors also reported an anodal band of activity which was only detectable in axenic medium and disappeared after bacteria were introduced to the axenic medium. This band which was called a D esterase since it reacted equally well with the two substrates, α-naphthyl propionate and α-naphthyl butyrate, did not disappear when dead bacteria were added to axenic medium. This observation was also reinforced in the present study and the pattern of esterase activity was identical in axenic and adaptation medium. The latter medium contains dead bacteria.

3) Effect of bacteria on malate dehydrogenase

Figure 51 shows the pattern of malate dehydrogenase activity in bacteria and stock KOK (syngen 3) grown in three different media - bacterized, axenic and adaptation. The pattern of enzyme activity in stock KOK was identical in the three different media. However, quantitative differences were observed in different media. For example, the smear which appeared close to the origin was stronger in the bacterized medium, and the fastest anodal band was stronger in adaptation and

axenic media, than in bacterized medium. The bacterial enzyme appeared as a single band which migrated at a faster rate than the first anodal band from the origin in stock KOK. The bacterial band was not detectable in bacterized cultures of stock KOK (syngen 3).

Discussion

The result of these experiments indicated that the presence of bacteria did not affect the pattern of malate dehydrogenase activity in Paramecium. This confirms the report by Williams and Smith Sonneborn (1980) in P. tetraurelia.

4) Effect of bacteria on phosphogluco isomerase activity in Paramecium

Figure 52 shows the pattern of phosphogluco isomerase activity in Klebsiella pneumoniæ and stock KOK (syngen 3) grown in axenic, adaptation and bacterized medium. The first anodal band from the origin was identical in the three different cultures of stock KOK (syngen 3). However, the second anodal band had a different mobility in the bacterized medium from the band in the axenic and adaptation media. This band migrated at a slower rate in the bacterized culture than the second anodal band of mobility in the axenic and adaptation cultures.

Two bands and a smear were observed in Klebsiella pneumoniae. The strongest bacterial band had the same mobility as the second band of activity in axenic and adaptation cultures of stock KOK (syngen 3). The second band of activity in the bacteria migrated faster than the other band of activity in the bacteria. These bands were not detected in the bacterized culture of stock KOK. A smear also existed from the origin to the first anodal band in Klebsiella pneumoniae.

Discussion

These results indicated that the first anodal band of phosphogluco

isomerase activity from the origin was contributed by Paramecium and bacteria did not have any effect on it. This band was the only band which was considered in the previous studies on phospho-gluco isomerase variation in inter and intra-syngenic comparisons.

The second anodal band of the enzyme activity, which had the same mobility as that from the sterile cultures of Paramecium and the bacterial enzyme was effected by the presence of bacteria. In the presence of live bacteria this band was variable in its properties in different preparations of the same stock. For example with stock KOK (syngen 3), sometimes this band disappeared completely. At other times it migrated much faster than the first anodal band toward the anode. Finally in some preparations this band had a slightly faster mobility than the first anodal band of phosphogluco isomerase activity (Figures 9, 20, 27 and 33). This variation is thought to be dependent on the degree of starvation when the bacterized cultures of stocks are harvested. If cultures are harvested when depleted of bacteria, this band is identical to the one in sterile cultures of Paramecium. However, further experiments need to be carried out. It should be noted that because of the inconsistency of this band, it was excluded from studies reported so far.

Phosphogluco isomerase bands in P. aurelia were reported to migrate at a slower rate than the bacterial bands (Tait, 1978). In this study Klebsiella aerogenes was used for feeding paramecia.

5) Effect of bacteria on hexokinase activity in Paramecium

The patterns of hexokinase activity in the three media - bacterized, axenic and adaptation media were identical. There were three bands of activity which migrated close to the origin. The two bands of the bacterial enzyme migrated at a faster rate towards the anode (see Figures 10 and 21). There was no trace of bacterial enzymes in the extract of

stock KOK (syngen 3) grown in bacterized medium.

Discussion

These results indicated that bacterial enzymes did not alter the pattern of hexokinase activity in Paramecium. The bacterial enzymes migrated faster than the Paramecium bands, and were not detectable in the presence of Paramecium bands. However, it was shown in Chapter Three, that in stock Ca11 which did not show any band of hexokinase activity, the bacterial enzyme bands were detected. The possible explanation for this phenomenon could be that the bacterial hexokinase is only expressed when there are "null" alleles in Paramecium. In the presence of active alleles, the bacterial enzyme may be suppressed.

6) The effect of bacteria on acid phosphatase activity in Paramecium

The pattern of acid phosphatase activity in stock KOK (syngen 3) was identical in the three media - bacterized, axenic and adaptation. Klebsiella pneumoniae had a single strong band of activity which migrated very close to the origin (Figure 53). The bacterial band was not detected in the bacterized cultures of stock KOK (syngen 3).

These results indicated that the pattern of acid phosphatase did not change in bacterized and sterile cultures of Paramecium and the bacterial enzyme did not modulate the Paramecium enzyme patterns. Amongst the stocks which were reported in the earlier studies, stock Hj1 of syngen 12 had a smear (see Figure 30). This smear was consistently detectable in this stock and had faster mobility than the band in Klebsiella pneumoniae. But, because of the possibility that the smear in stock Hj1 exists as a result of bacterial interaction with paramecium enzyme, it was not considered in comparisons of stock Hj1 with the other stocks.

Aspartate amino transferase, superoxide dismutase, malic enzyme

These remaining enzymes which have been reported in the earlier chapters had identical patterns of activity in bacterized, axenic and adaptation media in stock KOK (syngen 3). Bacterial enzyme could not be detected on the same gels for these three enzymes.

Conclusions

The results of the study reported here indicate that:

1) dead bacteria do not effect the pattern of enzyme activity in Paramecium, since all the enzymes studied have identical patterns in adaptation (containing dead bacteria) and axenic media;

2) bacteria might effect the pattern of activity in some of the enzymes of the paramecia. For example in the case of esterases, the presence of bacteria inhibits the formation of one enzyme. And in the case of malate dehydrogenase the presence of bacteria has a quantitative effect on the enzyme.

These effects are, however, very minor. The general features of the enzyme patterns do not change in the presence of bacteria. It was also reported that the presence of bacteria did not have any effect on the following enzymes in P. aurelia: isocitrate dehydrogenase (both mitochondrial and soluble), β-hydroxy butyrate dehydrogenase, fumarase, glutamate dehydrogenase, malate dehydrogenase and phosphogluco isomerase (Tait, 1970a, 1978; Williams and Smith-Sonneborn, 1980).

Considering the difficulties involved in transferring Paramecium from bacterized to sterile medium it would be a sufficient control to run a bacterial extract alongside Paramecium extracts on any gels. This would distinguish the bacterial enzymes from Paramecium enzymes on the basis of their mobility. In the case where there is some ambiguity genetic studies on hybrid enzymes could be useful in

distinguishing bacterial from Paramecium enzymes, i.e. only the enzymes of Paramecia form a hybrid band or bands following conjugation.

Figure 49:

Pattern of α-naphthyl propionate esterase.

(1) Klebsiella pneumoniae.

(2) A sterile culture of P. caudatum, stock KOK (syngen 3).

(3) A bacterized culture of P. caudatum, stock KOK (syngen 3).

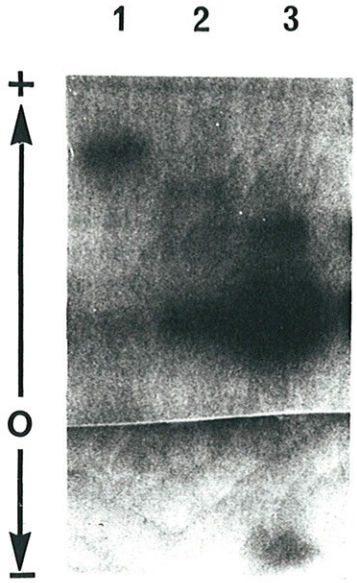
Figure 50:

Pattern of α-naphthyl butyrate esterase.

(1) A sterile culture of P. caudatum, stock KOK (syngen 3).

(2) A bacterized culture of P. caudatum, stock KOK (syngen 3).

(3) " " " " " " " " " .


1 2 3
+
O

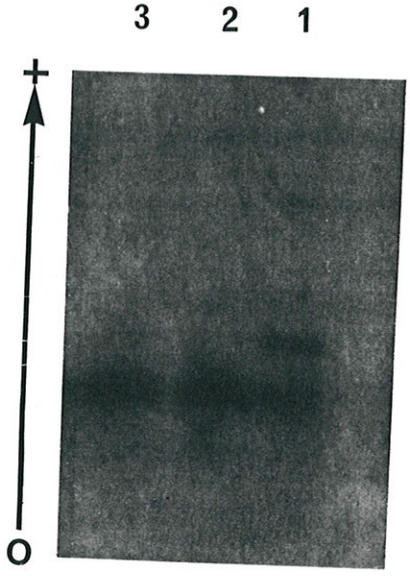

3 2 1
+
O

Figure 51:

Pattern of malate dehydrogenase.

(1) Klebsiella pneumoniae.

(2) A sterile culture of P. caudatum, stock KOK (syngen 3).

(3) A bacterized culture of P. caudatum, stock KOK (syngen 3).

Figure 52:

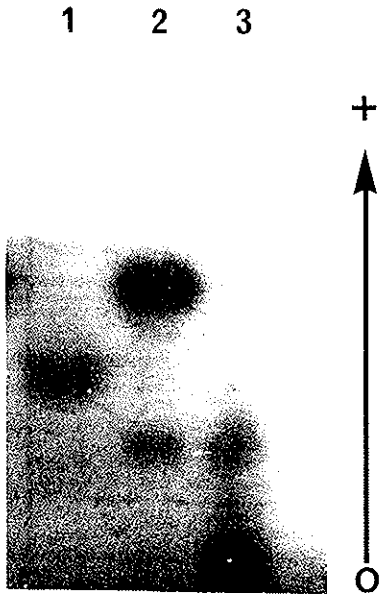
Pattern of phosphogluco isomerase.

(1) Klebsiella pneumoniae.

(2) A sterile culture of P. caudatum, stock KOK (syngen 3).

(3) A bacterized culture of P. caudatum, stock KOK (syngen 3).

O : Origin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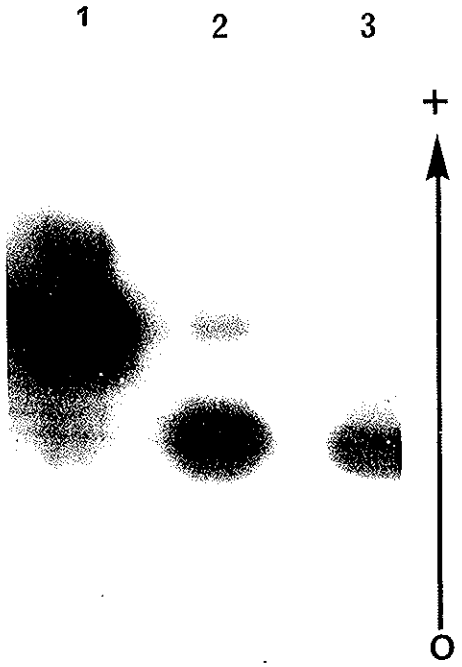
1
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+
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Figure 53:

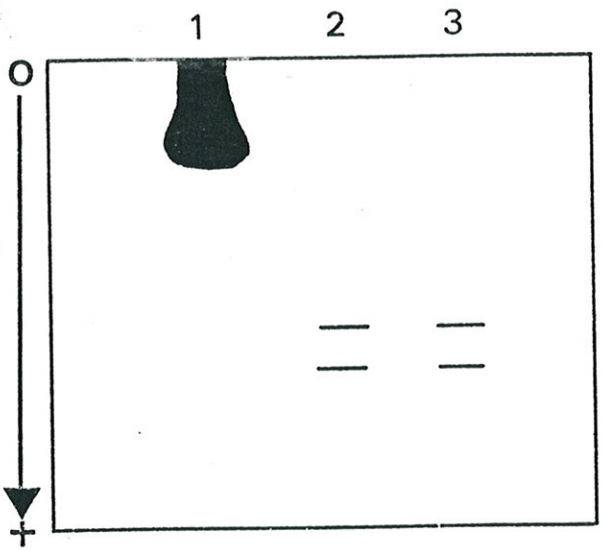
Pattern of acid phosphatase.

(1) Klebsiella pneumoniae.

(2) A sterile culture of P. caudatum, stock KOK (syngen 3).

(3) A bacterized culture of P. caudatum, stock KOK (syngen 3).

O : Origin


1
2
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CHAPTER EIGHT

GENERAL DISCUSSION

In the preceding chapters, eight enzymes (α-naphthyl propionate esterase, α-naphthyl butyrate esterase, malate dehydrogenase, malic enzyme, aspartate aminotransferase, superoxide dismutase, hexokinase and acid phosphatase) in four syngens of Paramecium caudatum (1, 3, 12, 13) and six enzymes (α-naphthyl propionate esterase, α-naphthyl butyrate esterase, malate dehydrogenase, malic enzyme, aspartate aminotransferase and superoxide dismutase) in 87 wild stocks have been studied by gel electrophoresis and their isozyme patterns elucidated. These studies have allowed an assessment of the amount of phenotypic variation in this ciliate compared to other protozoa. In the present chapter, the total variation in each of the enzymes of P. caudatum will be discussed and also comparisons will be made with enzyme variation in other organisms. This discussion will be followed by consideration of the possible mechanisms which maintain variation in any particular enzyme. This molecular variation will be assessed and compared with morphological variations. The utility of the electrophoretic technique in general and a brief explanation of the biases which might influence the observed variations will be discussed.

The overall variation of enzymes in P. caudatum

From the results reported in Chapters Three, Four, Five, it can be seen that the extent of enzyme variation increases by increasing the number of stocks which are examined. The exceptions are the two enzymes malic enzyme and the anodal system of superoxide dismutase. These two enzymes remained monomorphic. The different patterns of enzymes which have been detected among the stocks of P. caudatum are

shown in Figures 54, 55, 56, 57, 58, 59, 60, 61. The rare variants which were observed in only one of the stocks were excluded. The enzymes according to a decreasing degree of polymorphism are as follows:

(1) Phosphogluco isomerase

This enzyme was the most polymorphic. Up to six different patterns of enzyme activity were found amongst the stocks of P. caudatum (Figure 54).

A high degree of genetic variability has also been observed at the phosphogluco isomerase locus in marine species of molluscs. Individual species have between one and nine alleles at this locus (Wilkins, 1975). Gene duplication of this locus has occurred in fish. In milk fish (Chanos-chanos) (Winans, 1980), one of these loci is very polymorphic (6 alleles have been found at this locus), whilst the other locus is moderately polymorphic (up to 3 alleles have been found at this locus).

Phosphogluco isomerase was reported to be indistinguishable in the chimpanzee, the gorilla and the orangutan (Bruce and Ayala, 1979).

(2) Esterases

Four different anodal patterns of α-naphthyl propionate esterase, three different anodal patterns of α-naphthyl butyrate esterase and three different cathodal patterns were detected (Figures 55, 56). The anodal bands which had identical pattern with the two substrates were only considered as variants of α-naphthyl propionate esterase.

Esterase loci are highly polymorphic in most species of animals and plants which have been surveyed (Powell, 1975).

Amongst the primates, esterases have been reported to be under the control of two loci (King and Wilson, 1975; Bruce and Ayala, 1979). It appeared that humans and the chimpanzee share the same allele at

one of these loci. Furthermore humans and all pongids (gibbon, orang-utan, gorilla and chimpanzee) have the same allele at the other locus.

(3) Malate dehydrogenase

Three different patterns of enzyme activity were commonly detected (Figure 57). This enzyme was reported to be monomorphic in the house mouse (Mus musculus) (Selander and Yang, 1969). In the kangaroo rats three alleles have been detected for the malate dehydrogenase locus. However, two of these alleles occurred in a very low frequency (Johnson and Selander, 1971).

Malate dehydrogenase has been reported to be evolutionarily conservative (Kitto and Wilson, 1960). Malate dehydrogenase is determined by the same allele in humans, the chimpanzee and the gorilla (Bruce and Ayala, 1979).

(4) Aspartate aminotransferase

Three different patterns of enzyme activity were detected in the cathodal part of the gel (Figure 58). It should be remembered, however, that an anodal smear also existed in each of the stocks. Since the smear did not form any bands, they were not considered in this study. It was suspected that the smear was the product of a second locus, differing from the one responsible for contributing the cathodal band.

Aspartate aminotransferase has been reported to exist as two different systems, one soluble (cytoplasmic) and the other mitochondrial, in most animals, e.g. Drosophila (Grell, 1976), human (Davidson et al., 1970; Chen and Giblette, 1971), mice (Chapman and Ruddle, 1972; Delorenzo and Ruddle, 1970) and in cave dwelling fishes (Avis and Selander, 1972). In general low levels of variation have been reported for both systems of this enzyme. No variations have been detected between

mitochondrial systems of man and mouse (Delorenzo and Ruddle, 1970).

King and Wilson (1975) reported that humans and chimpanzees had different alleles for the cytoplasmic enzyme. However, Kömpf (1971) and Bruce et al. (1979) did not find any differences between humans, chimpanzees and gorillas, using different buffer systems.

(5) Superoxide dismutase

Only the cathodal system of this enzyme showed variation in P. caudatum. The anodal system was completely monomorphic (Figure 59).

In man two chemically and immunologically distinct isozymes have been found. Three alleles have been found with the cytoplasmic isozymes, whilst no variations have been observed in the mitochondrial isozyme (Beckmen and Beckmen, 1975).

(6) Hexokinase

Three different patterns of activity have been detected with this enzyme (Figure 60). In addition to these patterns, some of the stocks did not show any bands of hexokinase activity. This enzyme was not detected in 84 of the wild stocks.

The enzyme exists in multiple forms in virtually every well studied eukaryote. It has been reported that the isozymes of hexokinase change during development in Drosophila (Madhaven et al., 1972). Of the four hexokinase loci which have been found in Drosophila pseudoobscura and D. presimilis, two loci have been shown to be polymorphic, and the other two are monomorphic in both species (Parakash, 1977a and b).

(7) Acid phosphatase

Three different patterns of enzyme activity have been observed in P. caudatum (Figure 61). In addition to these patterns some stocks did

not show any bands of acid phosphatase activity. This enzyme was not screened in 84 of wild stocks.

Three different alleles have been found in human populations. One of these alleles appeared in higher frequency than the other two alleles. In the chimpanzee only one allele has been detected at the acid phosphatase locus. The same allele was reported to exist in humans (King and Wilson, 1975).

The possible mechanisms which maintain the variation in enzymes

Differences in the degree of enzymic variation have been reported in many organisms. However, the significance of differential variation and also the significance of high genetic variability at protein level remains controversial (for review, see Lewontin, 1974). Several different mechanisms have been proposed to explain the variability at each locus, although none have proved to be completely satisfactory (Ward, 1978). These mechanisms are as follows:

a) Metabolic function of the enzyme

Gillespie and Kojima (1968) and also Kojima et al. (1970) have speculated that the degree of variation of a given enzyme is related to its function. Enzymes were divided into two classes, those involved in glucose metabolism (group I) and those not involved in glucose metabolism (group II). It has been suggested that the non-glucose metabolizing enzymes, because of the greater variability of their substrate concentrations, are more variable than glucose metabolizing enzymes whose substrates remain at a constant concentration. Johnson (1974) has extended this observation and divided the enzymes into three classes, variable substrate, regulatory and non-regulatory enzymes. The first group of enzymes would have a high level of genetic variability.

Within a given metabolic pathway, the non-regulatory enzymes are less variable than the regulatory enzymes.

In P. caudatum amongst the enzymes which have been reported, esterases, acid phosphatase and superoxide dismutase have variable substrate. Malic enzyme, phosphogluco isomerase and hexokinase are regulatory enzymes. Malate dehydrogenase, lactate dehydrogenase and aspartate aminotransferase are non-regulatory enzymes. The amount of variation in these enzymes does not follow Johnson's hypothesis. For example, superoxide dismutase with variable substrate is less polymorphic than phosphogluco isomerase and hexokinase which are both regulatory enzymes. Malate dehydrogenase and aspartate aminotransferase which are non-regulatory enzymes show more polymorphism than malic enzyme, a regulatory enzyme.

Zouros (1975) has reported that some group I enzymes are consistently more polymorphic than others, while group II enzymes are sometimes extremely conservative.

b) Molecular weight of the enzyme

The other mechanism which has been proposed to control the variability of an enzyme is its molecular weight. Long chain polypeptides have on average potentially more variable amino acid sites than short chain enzymes. Koehn and Eanes (1974), also Brown and Langly (1979), have reported a positive correlation between subunit size, average heterozygosity and the average number of observed alleles per locus in Drosophila. However, Harris et al. (1977) did not find such a correlation in humans, but they have reported a negative correlation between the number of subunits and the level of polymorphism. The effect of subunit numbers on genic variation has also been claimed in other animals and plants (Zouros, 1976; Ward, 1977).

There is no information available on the molecular weight of any of the enzymes in P. caudatum. As for the subunit numbers, it is known that α-naphthyl propionate esterase is a monomer in syngen 13, lactate dehydrogenase is a dimer in syngen 3 and phosphogluco isomerase is a dimer in syngen 3 and probably a monomer in syngen 13. Lactate dehydrogenase is monomorphic, α-naphthyl propionate esterase is slightly polymorphic and phosphogluco isomerase is very polymorphic. Until more data is available on the subunit number of different enzymes, no conclusion is possible on the correlation between subunit number and variability of enzymes in P. caudatum.

c) Functional constraint of enzyme

Another factor which is likely to effect genic variation is the functional constraint imposed on the gene (Singh, 1976). Proteins with less genic variation like cytochrome c, are believed to be under more functional constraints than highly variable proteins like fibrinopeptide. Also the different evolutionary rates of change for alpha and beta hemoglobins have been attributed to differences in structural constraints (Goodman et al., 1971; Uzzell and Corbin, 1972). Singh (1979) using this concept, has suggested that enzyme systems with isozyme loci like esterases (loci with functional similarity) have fewer physiological constraints. For this reason they are more variable and more prone to having null alleles than single locus enzyme systems.

Genetic variation in vertebrates and invertebrates

It has been reported that the degree of genetic variation in invertebrates is much more than in vertebrates (Selander and Kaufman, 1973). For example, the degree of polymorphism in human populations is 28% (Harris and Hopkinson, 1972); in Mus musculus it is 29%

(Selander, Hunt and Yang, 1969); in Scandinavian moose it is 21% (Ryman et al., 1980), whereas in Drosophila obscura it is 53% (Lakovaara and Saura, 1971); in D. willistonis it is between 81-86% (Ayala et al., 1972; Ayala, Powell and Dobzhansky, 1971); and in the snail Copaca between 52% to 63% of loci are polymorphic (Manwell and Baker, 1968).

The degree of polymorphism in P. caudatum is about 80%. This figure is quite high even among invertebrates.

Selander and Kaufman (1973a) have explained the differences in the degree of genetic variation between vertebrates and invertebrates in terms of Levins' (1968) models of evolution in changing environments. According to this hypothesis organisms with generally larger body size, greater mobility, and greater physiological and behavioural homeostatic control tend to experience the environment as fine-grained. For this reason, the optimum strategy is more often a single phenotype specialized to the most frequently encountered set of conditions. Smaller, less mobile organisms with relatively poor homeostatic control experience the environment as coarse-grained. For them the optimum strategy is more often the development of specialized morphs occurring in proportions dependent upon the frequencies of the different patches in the environment. Plants have been reported to have the same level of variation as smaller animals (Selander and Kaufman, 1973a).

Powell (1975) has elucidated two alternative theories which could explain the differences in the levels of enzyme variability between invertebrates and vertebrates. There are two ways in which the metabolic flexibility and diversity of an enzyme system could increase as a result of evolutionary polymorphism. One is to maintain multiple alleles at enzyme structural loci thereby maintaining polymorphism. Alternatively duplication of genetic loci may be selected for, so that a single genome has several loci producing a product with similar

functions, i.e. multiple loci isozymes. These different loci could then become specialized to function optimally in different tissues or developmental stages. Powell has added that these two ways of diversifying metabolic systems should not be viewed as mutually exclusive. Different evolutionary lines may exhibit various degrees of both strategies. In general, however, it appeared that invertebrates have diversified their metabolic systems more often by polymorphism rather than duplication.

Evolutionary level and molecular distance

It has been reported in the present study that some syngens of P. caudatum are genetically so similar (1, 3, 12 and 13) that it is not possible to distinguish them on the basis of their enzymic activity. On the other hand it has also been shown that some unclassified syngens of P. caudatum are genetically far apart and can easily be distinguished on the basis of their enzyme variation (see Chapter Five). All syngens of P. caudatum are by definition reproductively isolated, i.e. they are unable to mate with each other. It has also been reported that a substantial amount of reproductive isolation can arise without much genetic differentiation. Lewontin (1974) and Ayala (1976) have argued that reproductive isolation may be due to changes at only a few loci. Coyne (1976) has mentioned two factors which could be responsible for this observation. 1) The genes detected by electrophoresis may have little to do with reproductive isolations. 2) The conventional electrophoretic methods have been unable to detect more extensive genetic differences between nascent species. However, neither of these two explanations can account for the high level of similarity between some syngens and low level of similarity between other syngens of P. caudatum. There is no obvious reason to believe that the rate of molecular change

varies in the syngens with high similarity than in other syngens. The other possibility is that the four syngens 1, 3, 12 and 13 are of more recent common origin, and less time has occurred for genic changes. It is not known from the 16 syngens of P. caudatum which have a high degree of similarity and which have low levels. However, it is known that the syngens of this ciliate differ in their genetic relationship (see Chapter Five and Chapter Four, discussion).

Relative differences in genetic variation have also been reported in P. aurelia, where the two species 1 and 5 are very similar, but other species differ from each other to a high or low degree depending on the species being compared (Tait, 1970a; Allen et al., 1971b; Tait, 1978). The significance of genetic variation between morphologically identical biological species, e.g. syngens of ciliates; sibling species of Drosophila; subspecies of mouse and lizard, only become obvious when compared with the amount of genetic variation between primates.

King and Wilson (1975) have reported that genic differentiation between human and chimpanzee is similar to that observed between sibling and subspecies of other organisms. This observation has been later reinforced by Bruce and Ayala (1979).

Wilson et al. (1977) have discussed organismal evolution, i.e. morphological and concluded it is slow in frogs relative to mammals. At the molecular level, however, evolution has been very rapid. This is apparent when immunological comparisons are made for albumin, electrophoretic comparisons of many enzymes, sequencing of hemoglobin and annealing studies with DNA (Wilson, 1975).

This contrast between organismal and molecular evolution indicates that two processes are to a large extent independent of one another. King and Wilson (1975) have argued that the evolution of the biologically most significant characters, i.e. morphology, reproductive incompatibility,

and behaviour, may not be accurately reflected by changes in structural gene loci, other than they involve primarily changes in genetic regulation.

Utility of electrophoretic technique

The usefulness of the electrophoretic technique in systematic classification has been proven (for review see Avise, 1974). Avise has suggested that electrophoretic and classical approaches should be used in concert. In some cases, classical systematic criteria offer greater precision, and in other cases (e.g. sibling species), electrophoretic data are more discriminatory.

In the three human races, i.e. Caucasoids, Negroids and Mongoloids, Nei and Roychoudbury (1974) have reported genetic differences at 35 common loci between individuals from different ethnic groups. These were only slightly greater than those between individuals within the same group. These three human races have distinct phenotypic differences. However, as Lewontin (1974) put it one must be careful in evaluating these phenotypic differences.

"The apparent homogeneity within races as compared to the 'obvious' differences between them stems partly from the fact that our consciousness of racial differences is constantly being reinforced socially because racial distinctions serve economic and political ends, and partly because the very characters we used to distinguish races - skin colour and texture, hair form, eye, nose and lip shape are those to which we are most keenly attuned from the purpose of distinguishing individuals."

The reliability of reported variation in P. caudatum

One of the results of the reported study here was that the genetic

variation between syngens of P. caudatum was less than the reported variation in the P. aurelia and Tetrahymena pyriformis complexes of species (for further detail see discussions, Chapter Five). How reliable the estimation might be is questionable, however. Lewontin (1974) has suggested that a reasonably reliable estimate of genetic variation in natural populations require several considerations.

(A) Adequate sample size. It has been discussed earlier, that the degree of variation increased when 85 wild stocks were examined in addition to the stocks of four syngens - 1, 3, 12 and 13. For example, of the seven enzymes - α-naphthyl propionate esterase (cathodal and anodal systems), malate dehydrogenase, aspartate aminotransferase, superoxide dismutase (cathodal and anodal systems) and malic enzyme which did not show any variation in the four syngens, only two remained monomorphic (anodal system of superoxide dismutase and malic enzyme), when the sample size increased. The degree of variation also increased in the polymorphic enzymes (phosphogluco isomerase and α-naphthyl butyrate esterase) when additional stocks were examined.

(B) The genomes tested should be either collected directly from natural populations or a few generations removed from the original sample. In protozoan studies, it is impossible to test genomes directly from nature, since the mass culture of cells is required for any examination of enzyme variation. However, it was shown that the stock which has been kept in the laboratory for 26 years (stock 2C), did not show any variation when compared with some of the other stocks (e.g. 42) which had been collected more recently from nature. The possibility that stocks might show more variation if they are examined soon after they are collected from nature remains to be examined.

(C) Examining a large sample of loci is even more important than collecting a large sample of individuals, since different loci do not

have uniform variations. It was suggested that at least 17 loci should be considered in order to have a reliable estimate of genetic distance. In P. caudatum in addition to the reported enzymes, other proteins have been examined but the results were either incomplete or not reliable enough to allow them to be reported here. For example, hydroxybutyrate dehydrogenase has been examined in stocks of syngens - 1, 3, 13; stock Hj1 of syngen 12 and the unknown stocks - 2C, 2F, 42, Cal1. The result indicated more intra-syngenic than inter-syngenic variations. General proteins were examined in the stocks Hj6, AK1 (syngen 13); KOK (syngen 3); Isn (syngen 1); Hj1 (syngen 12); 2C; 2F; 42 and Cal1. A single monomorphic band was observed in these stocks. Isocitrate dehydrogenase, alcohol dehydrogenase, glutamate dehydrogenase and xanthine dehydrogenase were also examined in the mentioned stocks. In the case of the first enzyme (isocitrate dehydrogenase), because of the existence of the smear especially in the cathodal part of the gel, it was difficult to compare the relative mobility of the bands. The other three enzymes, however, had sharp bands of activity in 10% standard acrylamide gels. The bands were not detectable in all the stocks simultaneously. As for the other enzyme phosphogluco mutase, the bands of activity for this enzyme had different mobilities in different experiments with any one stock. It was suspected therefore that this variation was epigenetic rather than being under genetic control.

With the reported enzymes, conservative measures were taken for scoring variations. Some bands were excluded because of their inconsistency. However, by altering the conditions of gel electrophoresis, i.e. using different buffers, different pH and a different percentage of acrylamide, it should be possible to score many of these enzymes. In the present study, because of the limitation of time and money, it

was not possible to try alternative possibilities.

(D) A diverse sample of loci, not weighted heavily to one or two enzymatic functions, needs to be carried out. A survey spread over the widest possible range of enzymatic functions is preferable.

In the present study no conscious effort was made to choose any particular enzymes. The only criteria was the detectability of the enzyme system in most of the stocks as well as their reproducibility. However, the examined enzymes seemed to have a reasonably widespread range of enzymatic function. For example, esterases, acid phosphatase and superoxide dismutase are non-specific; malic enzyme, hexokinase and phosphogluco isomerase are regulatory and malate dehydrogenase and aspartate aminotransferase are non-regulatory enzymes.

(E) An unbiased sample of loci needs to be chosen not based on conscious or unconscious knowledge of their variability. The enzymes which were examined in the present study have not been reported in P. caudatum, although attempts have been made to screen the enzymes which have been examined in P. aurelia and T. pyriformis. This has been done in order to make reliable comparisons between these ciliates. With the exception of esterase and phosphogluco isomerase, however, in P. caudatum and P. aurelia, esterases, malate dehydrogenase, malic enzyme and superoxide dismutase in P. caudatum and T. pyriformis, it was impossible to screen the other reported enzymes in the other two ciliates in P. caudatum.

The results of this survey show that the reported degree of variation between the four syngens (1, 3, 12, 13) and the wild stocks of P. caudatum is a reliable estimation of genetic relationships between different syngens of this ciliate.

Figure 54:

Patterns of phosphogluco isomerase in examined stocks of P. caudatum.

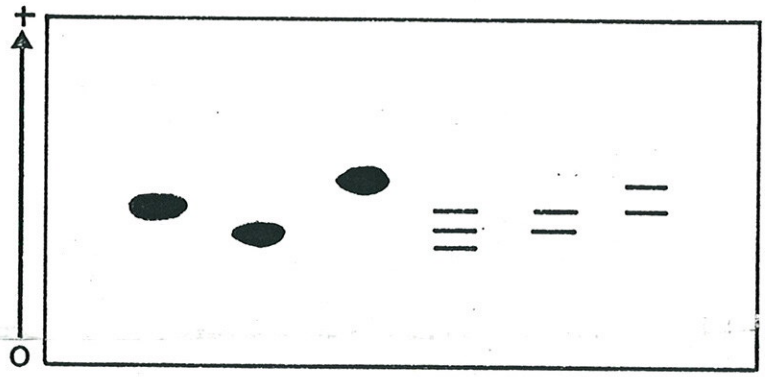


Figure 55:

Patterns of α-naphthyl propionate esterase in examined stocks of P. caudatum.

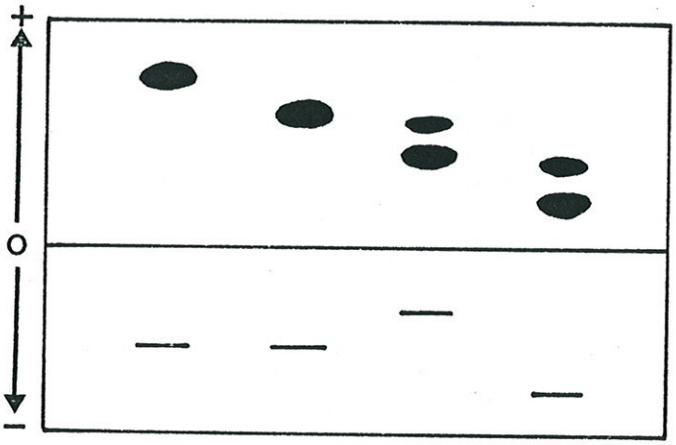


Figure 56:

Patterns of α-naphthyl butyrate esterase in examined stocks of P. caudatum.

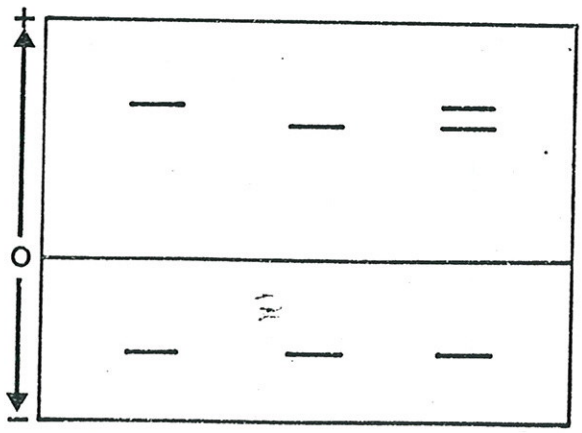


Figure 57:

Patterns of malate dehydrogenase in examined stocks of P. caudatum.

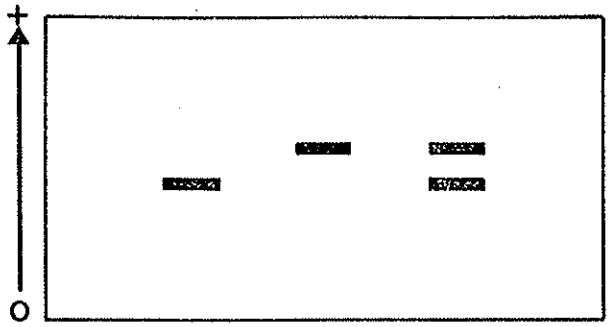


Figure 58:

Patterns of aspartate aminotransferase in examined stocks of P. caudatum.

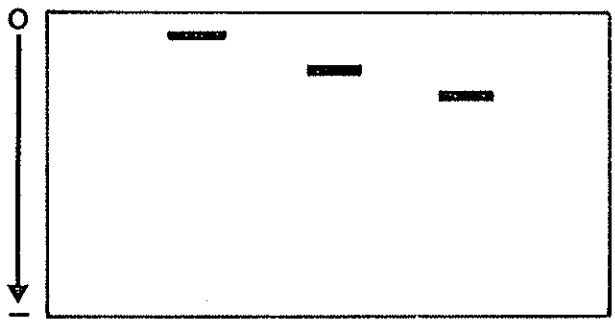


Figure 59:

Patterns of superoxide dismutase in examined stocks of P. caudatum.

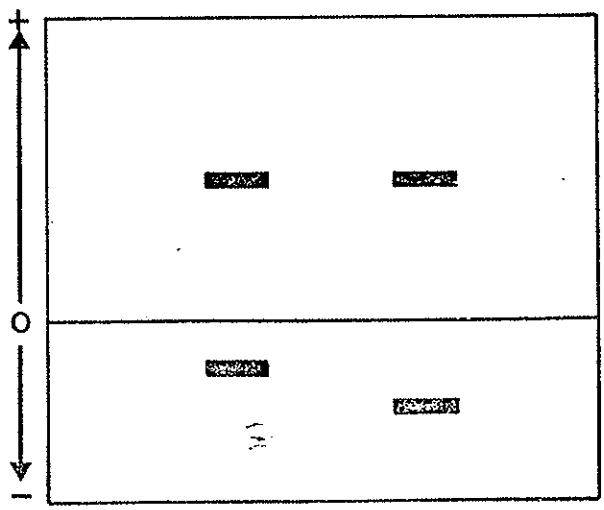


Figure 60:

Patterns of hexokinase in examined stocks of P. caudatum.

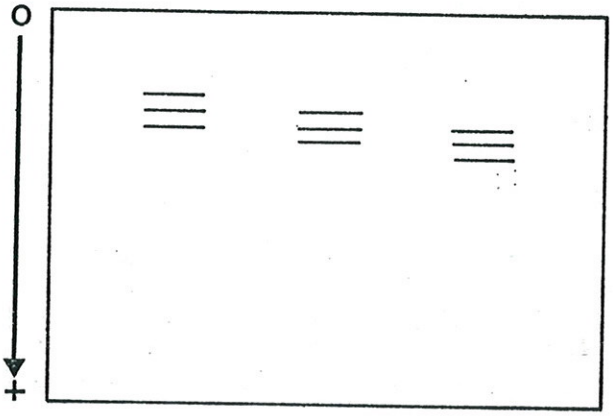
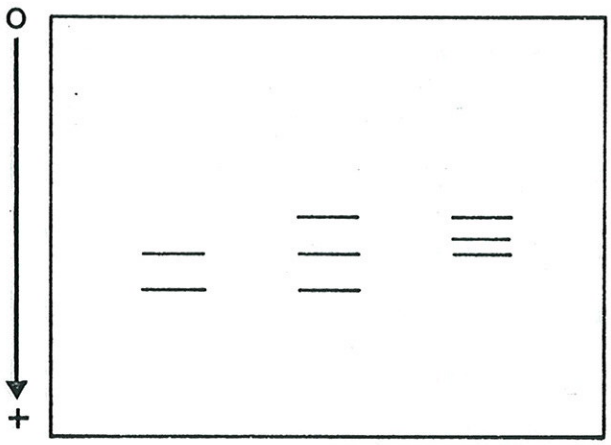


Figure 61:

Patterns of acid phosphatase in examined stocks of P. caudatum.



GENERAL CONCLUSIONS

Applying techniques of gel electrophoresis in four syngens of P. caudatum and 87 wild stocks of this ciliate, the following conclusions were made.

(1) Genetic distance was not equal between different syngens of P. caudatum. It was not possible to distinguish any of the four known syngens - 1, 3, 12 and 13. The lack of inter-syngenic variations was specific to these four syngens. On the other hand, some of the wild stocks were considered to represent different syngens, could be distinguished from each other and from the known syngens.

(2) Geographical distance did not play any role in enzyme variations. However, the possibility existed that esterase-2 variation was geographically dependent. More experiments are required to clarify this possibility.

(3) The advantages of any intra-syngenic variation as observed in the case of the enzyme "phosphogluco isomerase" was that the enzyme could be used as a genetic marker in genetic crosses with P. caudatum.

(4) The effects of bacteria were minor and in general their presence did not modulate the pattern of enzyme activity in Paramecium.

(5) Since all previously reported syngens of P. caudatum are not now available and since it is not possible to distinguish four of the syngens by the techniques of gel electrophoresis it would be premature to give species status to the syngens of P. caudatum. More experimental work should be carried out to clarify the syngen status in this ciliate and this will involve widespread collections.

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