PROTEIN VARIATION IN CILIATES

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SUMMARY

1. The ciliate Paramecium aurelia has been used as an experimental organism for the study of enzyme variation, which has been examined in (a) samples from two natural populations (b) single samples from a large number of widespread separate populations and (c) samples from each of the fourteen known syngens. The variants found in stocks of a single syngen have been used for genetic studies with special reference to (a) the inheritance of mitochondrial enzymes and (b) the relationships between isoenzymes.

2. Measurements of enzyme activity have shown that paramecium contains the enzymes of the hexose-shunt, Emden-Meyerhoff pathway and TCA cycle. Variations in the structure of some of these enzymes (mainly dehydrogenases) have been detected using starch gel electrophoresis. The subcellular localisation of the bands of activity observed on starch gels has been determined by cell fractionation using the techniques of sucrose density gradients and differential centrifugation.

3. Isocitrate dehydrogenase has been shown to occur in two distinct forms, one in the mitochondria and the other in the supernatant. Genetic studies have shown that the mitochondrial form is controlled by nuclear genes which were shown not to be closely linked to the genes controlling the supernatant form. The possible sub-unit structures and loci controlling their synthesis have been discussed in the light of these studies.

4. 3-hydroxybutyrate dehydrogenase has been shown to be located in the mitochondrion. Genetic studies have shown that this enzyme is controlled by nuclear genes and by observation of starch gel zymograms this enzyme has been

found to be a tetramer.

5. Malate dehydrogenase has been shown to occur in mitochondrial and supernatant forms. The mitochondrial form occurs as a smear of activity on starch gels and attempts to resolve this smear into discrete bands have failed. No variants of the mitochondrial form have been detected, but variants of the cytoplasmic form have been found and this form of the enzyme has been shown to be a dimer.

6. The linkage between the genes controlling isocitrate dehydrogenase (mitochondrial form) and 3-hydroxybutyrate dehydrogenase has been investigated but no close linkage could be demonstrated.

7. Clones derived from single separate isolations of paramecium from two natural populations of syngen 9 have been screened for variation in iso-citrate dehydrogenase, glucose-6-phosphate dehydrogenase, malate dehydrogenase and 3-hydroxybutyrate dehydrogenase. No variation was detected in any of the enzymes except 3-hydroxybutyrate dehydrogenase. Two alleles of the latter enzyme were detected in one of the populations and only one allele in the other population.

Single samples isolated from separate populations of syngens 1 & 2 in different parts of the world were screened for variation in succinic dehydrogenase, glutamate dehydrogenase, fumarase, iso-citrate dehydrogenase, 3-hydroxybutyrate dehydrogenase and malate dehydrogenase. Two alleles for malate dehydrogenase were found to occur at almost equal frequency in syngen 1 and a low frequency of variation in isocitrate & 3-hydroxybutyrate dehydrogenases of syngen 2. All the other enzymes were found to be invariant.

8. Comparison between stocks belonging to the fourteen syngens have been made for variation in the six enzymes used in the intrasyngen survey. Inter-

syngen variation has been shown in all enzymes except succinic dehydrogenase. All the syngens, except 1 and 5, are different if all five enzyme mobilities are considered. Comparison of the type and frequency of intrasyngen variation suggested that the enzyme patterns observed for each syngen are typical. These differences have been used to classify stocks of unknown syngen and could be used in place of classification by mating reaction.

9. (i) The lack of close linkage between the cytoplasmic and mitochondrial forms of iso-citrate dehydrogenase were discussed in relation to immunological and biochemical data obtained with the mammalian enzyme. The results obtained here are in agreement with these findings.

(ii) A critical account of the research which has been undertaken on the genetics of mitochondrial proteins has been given. It was concluded that none of the approaches used gave any information about specific proteins determined by the mitochondrion. The results obtained in this study were discussed in relation to this work and were considered important as they provide information about the control of specific proteins.

(iii)The practical value of the intersyngen enzyme differences have been considered. Comparison of these differences to other more physiological differences shows that there is no correlation. The possible nature of the enzyme differences in intra- and intersyngen comparisons has been considered.

INTRODUCTION

For the purpose of this study protein variation is concerned with alterations in protein structure caused by mutations in the DNA of the cell. In ciliates, the study of this type of variation has, in the past, been limited to work on enzyme variation in Tetrahymena pyriformis (Allen et al. 1963a & b; Allen, 1964, 1965, 1968) and antigenic variation in Paramecium aurelia (Beale 1957; Pringle 1956; Jones 1963, 1964). This investigation was undertaken to extend the range of proteins studied and to apply the variation found to certain genetic and biochemical problems. The genetics and reproductive systems of Paramecium aurelia have been extensively studied and, together with certain special features, make this ciliate an obvious choice as an experimental organism. In fact, the technical difficulties encountered in studying the genetics of ciliates such as Tetrahymena and Euplotes, make paramecium the only ciliate with which the genetic studies undertaken here are feasible.

Before outlining the problems investigated, a brief description of the reproductive processes, organisation and biochemistry of paramecium is necessary. Paramecia can undergo three different reproductive processes, namely conjugation, autogamy (self-fertilization) and binary fission. Conjugation occurs when two stocks of complementary mating type are mixed; the animals adhere to each other in pairs, undergo nuclear reorganisation and exchange of haploid nuclei occurs between the two conjugants (Sonneborn 1954). After this process the conjugants separate and, if each is isolated into fresh medium, undergo binary fission to produce two clones of genetically identical

animals. The process of conjugation allows one to discriminate between nuclear and cytoplasmic inheritance as the two exconjugants are identical as regards their nuclear genes but their cytoplasm is derived from one or other parent. Thus, if the conjugants differ in a certain factor, comparison of the clones derived from each exconjugant should show whether the factor is inherited by nuclear genes (both exconjugants identical) or through the cytoplasm (exconjugants different).

Under certain growth conditions clones of paramecia go into autogamy. During this process the macronucleus degenerates and a process identical to that seen in conjugation occurs, except that no fusion of nuclei from different animals occurs. Instead two nuclei from the same animal fuse together and a new macronucleus develops from them. It has been shown (Sonneborn 1947) that the two haploid nuclei which fuse are derived mitotically from the same product of meiosis and therefore autogamy produces animals homozygous at all loci.

Paramecium occurs abundantly in separate populations all over the world and therefore potentially provides a large amount of material to examine for variation. A stock can be defined as a collection of organisms derived from a single wild isolation from one of these populations. The species Paramecium aurelia is divided into fourteen genetically isolated groups which are called syngens (Sonneborn 1938). In general no mating (conjugation) occurs between stocks of one syngen and stocks of another syngen. Certain intersyngen cross-mating reactions have been found (Sonneborn & Dipell 1946) but a low F_1 survival is always observed.

Paramecium is a eucharyotic cell, possessing typical cell inclusions, such as mitochondria, endoplasmic reticulum, ribosomes, golgi apparatus etc. It differs from other eucharyotic cells in having two types of nucleus, namely the macronucleus and the micronucleus. The macronucleus is polyploid and generally considered to control the same processes of the cell as the typical eucharyotic nucleus does. The micronucleus is small and, as far as is known, is only involved in the reproductive processes of autogamy and conjugation. The macronucleus is derived from division products of the micronucleus after conjugation and autogamy.

Biochemical research on paramecium has been very limited, being largely restricted to whole cell respiration studies. The studies reported here and elsewhere (Kung 1968) show that paramecium contains the enzymes of the hexose-shunt, Emden-Meyerhoff pathway and tricarboxylic acid cycle, although lactic dehydrogenase activity is absent. The work of Kung (1968) has shown the presence of several cytochromes comparable to those found in mammalian mitochondria. These findings are difficult to reconcile with the nutritional requirements shown by paramecium in axenic medium. In a recent study (van Wagtendonk 1969) it was found that paramecium required eighteen common amino acids and certain fatty acids but no other carbon source, suggesting that the enzymes of the Emden-Meyerhoff pathway are not being used. These results imply that considerable metabolic changes occur when paramecium is transferred from the normal bacterized culture into an axenic medium.

Paramecium, when compared to other eucharyotic organisms, has several advantages for the study of protein variation and the application of

this variation to genetic studies. In any study of protein variation the availability of material to screen for variants is an important consideration; the wide distribution and occurrence of an immense number of separate populations provides a potentially large source of material for this study. The property of binary fission and cloning allows rapid sampling of wild collections and their maintenance in the laboratory. In contrast the maintenance of samples of a given genotype, isolated from wild populations of other eucharyotes involves breeding with standard strains which is laborious. For example if wild collections of drosophila are made, crosses with a standard strain must be undertaken before the wild isolate can be sacrificed for examination which makes analysis of the original population complex. The genetic analysis of variants in mammals is hindered by factors such as long immaturity periods, small numbers of progeny, lack of homozygous strains and,in Man,the impossibility of undertaking selected matings. With paramecium few of these difficulties arise, since homozygous clones can be obtained by autogamy, crosses can be made rapidly and large numbers of progeny can be obtained for genetic analysis. Also conjugation can be used for determining whether nuclear or cytoplasmic inheritance is present. The main object of this study has been to exploit some of these advantages to investigate various aspects of protein variation, as outlined below.

Specific proteins must be studied if genetic analysis of variants is to be undertaken. The ease with which enzymes can be detected by specific assay mixtures make these proteins an obvious choice for this study. The above mentioned advantages of paramecium were exploited in the study of the following topics:

1. The genetics of certain mitochondrial enzymes;
2. The structural and genetic relationships between isoenzymes, and
3. The variation of enzyme structure in natural populations, between stocks from different populations and between stocks from different syngens.

These are the topics which can be most usefully studied using paramecium. Each problem and the approach taken in this study will be separately outlined.

A large amount of research has been directed towards the elucidation of the function of mitochondrial DNA. The studies of Work et al (Roodyn, Suttie and Work 1962; Haldar, Freeman and Work 1966) measuring the incorporation of radioactive amino acids into isolated mitochondria, have demonstrated that the only protein fraction showing significant incorporation was the insoluble structural protein of the mitochondrial membrane. From this work and other studies the view has arisen that synthesis of the soluble proteins of the mitochondrion is controlled by the nucleus, although there is little direct evidence for this. With this background, some more evidence was sought using variants of mitochondrial enzymes of paramecium as markers for genetic analysis.

Extensive biochemical studies, but relatively little genetic work, have been made on isoenzymes in higher organisms and considerable information could be obtained about the structure and relationships between isoenzymes using a genetic approach. As paramecium is a single celled organism only the following types of isoenzyme can be considered:-

1. multiple forms of an enzyme which arise from different subcellular localisations
2. multiple forms of an enzyme which result from protein-protein or protein-small molecule interactions.

Malate dehydrogenase illustrates both types of isoenzyme, exhibiting mitochondrial and supernatant forms (Thorne 1960) and several bands of activity in the mitochondrial form after starch gel electrophoresis (Thorne, Grossman & Kaplan 1963). In the present work it was planned to investigate the genetic relationship between the mitochondrial and supernatant forms of an enzyme. Information as to the structural relationships between different zones of activity of the same enzyme can be obtained by the observation of the variants and of the heterozygote patterns obtained by crossing the normal with the variant form. This sort of analysis is simplified by starting with homozygous clones thus avoiding the possibility of the observed multiple banding being due to heterozygotes of two alleles coding for the enzyme.

The study of enzyme variation in natural populations has been undertaken with many organisms (Man, Harris 1969; voles, Semeonoff & Robertson 1968; Drosophila, Gillespie and Kojima 1968; etc.) largely with the object of establishing the existence of polymorphism and measuring the frequency of different alleles in different populations. Enzyme polymorphism has not been established in paramecium before and so isolates were made from natural populations and screened for variation. As such a large number of populations occur, the existence of any gene flow between them could be investigated. Enzyme variation was also studied by comparison of stocks from all fourteen syngens, firstly to see if they could be distinguished and secondly to provide information on the relationships between the syngens. The division of paramecium into syngens might also provide a useful system for the study of protein evolution, by comparing the structure of corresponding enzymes in different syngens.

There are two practical points to be considered in the study of enzyme variation, firstly the method used to find or produce variation and

secondly the property of the enzyme which is examined for variation. The large amount of material available with paramecium makes it possible to screen stocks isolated from different parts of the world. In the present investigation, considerable numbers of existing laboratory stocks and a number of new collections made from natural populations were examined.

The structure of an enzyme determines most, if not all, of its properties which can readily be measured, such as activity, heat stability and electrophoretic mobility. The techniques of complement fixation and peptide mapping can also be used. The properties mentioned are affected by variation in structure to a greater or lesser degree and although ideally one would like to detect all variation, the techniques involved are not completely practicable. Peptide mapping, activity and heat stability all require relatively large quantities of material, the production of which is very laborious with paramecium. The technique of complement fixation is very sensitive, but because of this will tend to give large experimental errors resulting in low precision. In a study of variation of lactate dehydrogenase of Rana pipens (Salthe 1969) this technique was used and showed a large number of indistinct differences which could be grouped into a few fairly distinct categories. This finding, together with the lack of suitability of the differences for genetic studies, made it seem impracticable to use this technique.

Electrophoretic mobility is by far the most widely used of the techniques applied to enzyme variation (Shaw 1965). It is applicable to enzyme studies and can be used to give information about the sub-unit structure of the enzymes studied, which is relevant to the proposed isoenzyme

studies. The main disadvantage is that electrophoretic mobility does not detect all types of variation, but only those in which a change in charge occurs. If starch or acrylamide gels are used uncharged amino acid substitutions may be detected if conformational or other changes result. Various supporting media are available for zone electrophoresis but in the present work only starch gels were used. Large gels can be poured making direct comparisons between extracts very easy and eliminating the need for marker proteins or completely standard procedure; the length of run can be reduced to 3-4 hrs by using high voltages and an efficient cooling system.

In general the object of this investigation was to study variation in enzyme structure and to establish paramecium as an experimental organism suitable for this purpose. Initial studies were undertaken to assay for various enzyme activities and establish the optimum conditions for starch gel electrophoresis of the enzymes used in this study. Large numbers of stocks were screened for variation and any variants found used in the studies outlined. In the course of this work a considerable number of unforseen problems arose, some of which were studied and found to give interesting new information.

MATERIALS AND METHODS

1. Chemical Reagents.

Hydrolysed starch for gel electrophoresis is a product of Connaught Medical Research Laboratories, Toronto, Canada. Sephadex G.100 is a product of Pharmacia, Uppsala, Sweden. Whatmans DEAE-cellulose (DE.22) and No. 4 grade chromatography paper are a product of Reeve and Angel Co. Ltd. Melinex polyester film (Type O) was a gift from ICI. Biochemical reagents, enzymes and tris (Sigma 7-9) were obtained from Sigma Chemical Company Ltd., London. Inorganic chemicals and sucrose (Analar Grade) were obtained from BDH Ltd.

2. Stains.

The following stains were used for examination of nuclei in whole cells.

a) Acetocarmine - Fast green

10.5 parts saturated solution of acteocarmine in 45% acetic acid.
4.5 parts 45% (v/v) acetic acid
2 parts 1N. HCl
1 part 1%(w/v) Fast green in 95% ethanol

b) Lacto-orcein

1 gm of synthetic orcein was dissolved in 22 mls lactic acid, 36 mls of glacial acetic acid and made up to 200 mls with distilled water.

Acetocarmine - Fast green was used routinely for detecting autogamy; approximately 20 animals were isolated in the minimum fluid onto a glass slide and to this 1 drop of stain was added. This preparation was examined at a magnification of 80X, autogamy being detected by the fragmentation of the normal dark staining nucleus.

The lacto-orcein stain was used to detect post autogamous macronuclear

fragments in order to check that autogamy had occurred in a particular clone. 1-3 animals from a clone were isolated onto a slide in the minimum of fluid and fixed in osmic acid vapour for 30 seconds. A drop of the stain was added to the fixed animals and the preparation examined under phase contrast microscopy.

3. Culture media.

a) Lettuce infusion: baked lettuce was boiled in distilled water (1.8 gms/L) for 15-20 mins, filtered and autoclaved in stoppered flasks. The medium was buffered with solid calcium carbonate and the pH adjusted to 6.8 with 0.5N sodium hydroxide before use.

b) Grass infusion: dried grass was boiled with distilled water (120 g/L) for 15 mins, filtered through muslin and centrifuged at 7000g for 15 mins. 1L aliquots of the infusion were dispensed into Thomson bottles and autoclaved (15 mins at 15lbs/psi).

c) Axenic: the medium was made up from its constituents as described by Soldo and van Wagtendonk (1966). The cultures used in this work were a gift from Dr. I. Gibson of the University of East Anglia. The constituents were as follows:-

CONSTITUENT	mg/ml	µg/ml
Proteose peptone	10	
Trypticase	5	
Yeast nucleic acid	1	
$MgSo_4$ $7H_2O$		500
TEM-4T		100
Stigmasterol		5
Calcium pantothenate		5
Nicotinamide		5
Pyridoxal HcL		5
Pyridoxamine HCl		2.5
Riboflavin		5
Folic acid		2.5
Thiamine HCl		15
Biotin		0.00125
DL-thiotic acid		0.05

The pH of the medium was adjusted to 7.0 with sodium hydroxide. TEM-4T is a mixture of fatty acids (0.5% lauric, 3% myristic , palmitic, 27% stearic, 18% linoleic and 43% oleic). This medium was mixed with the quantities described and autoclaved in stoppered flasks.

4. Stocks

A stock of paramecium is a culture derived from a single animal isolated from a natural population. The stocks used in this study were collected near Edinburgh by G.H. Beale and the author, obtained from T.M. Sonneborn, Indiana, collected in Europe and U.S.A. by G.H. Beale and sent by H. Kosciuszko from Poland, Czechoslovakia, Hungary and Roumania. A complete list of the stocks used is given in Table I. Those referred to by an initial followed by a number were collected from natural populations in the Edinburgh area; the initial refers to the location and the number refers to the clone e.g. Hu 34/1 is a clone isolated from Humbie Quarry near Edinburgh, 43 denoting the collecting vial and 1 denoting the clone isolated from that vial. The numbers given to the other stocks are arbitrary.

5. Collection and isolation of stocks

Samples of water and decaying vegetable matter were taken from the edge of a pond in 40 ml collecting vials with steel caps. About 10 mls of bacterized lettuce medium was added to each vial and the vials were incubated at 18°C for 24-48 hrs. Samples from each vial were pipetted into depression slides and examined under the microscope. Single animals (Paramecium aurelia being identified by its morphology) were isolated by micropipette into depression slides containing bacterised lettuce medium. These clones, after being left to divide for a few days were transferred by pipette into

SYNGEN	STOCKS
1	P (Maryland), S (Cold Spring Harb.), Z (California), 27 (California) 33 (Maryland), 41 (Georgia), 61 (Massechussets), 62 (Mass.), 90 (Pennsylvania), 103 (Philadelphia), 119 (Pennsylvania), 129 (Florida), 143 (Scotland), 144 (France), 145 (France), 147 (Japan), 153 (Connecticut), 156 (Conn.), 168 (Japan), 171 (Japan), 175 (Peru), 177 (Chile), 180, 181, 182 (Japan), 217 (Florida), 220 (Hawaii), 271 (Oklahoma), 241 (Mississippi), 244 (Florida), 266 (Florida), 285 (California), 313 (California), 320 (Poland), 332 (USSR), 335 (USSR), 336 (Michigan), 337 (Kamchatka), 513, 514, 515, 516, 520, 521 (France), 523 (Switzerland), 535 (Australia), 540 (Mexico), 544 (Louisianna), 548, 550, 551, 555 (California), 558 (Mexico), 559 (Mexico), 561 (Italy), 575 (Scotland), 576, 577, 579, 581, 584, 585, 586, 587, 588, 589, 590, 592 (Poland), 593 (Czechoslovakia), 595 (Italy), 596 (Italy), 597, 598, 599 (Bulgaria), 600 (Roumania), 601 (Roumania), Hun -1, 2, 3, 4, 6, 7, 8, 9, 10, 11, (Hungary).
2	1 (New York), 4 (New York, 5 (Maryland), 7 (New York), 8 (Maryland), 9 (New York), 11 (Maryland), 12 (Vermont), 21 (Maryland), 28 (Massechussetts), 34, 35, 36 (Conn.), 49 (New Haven), 50 (Oregon), 53 (Indiana), 71 (Indiana), 72 (Texas), 86 (N. Jersey), 88, 91, 93 (Penn.), 100 (N.Jersey), 104 (Penn.), 114 (Indiana), 115 (Indiana), 122 (Vermont), 149 (Florida), 160 (Minnesota), 179 (Chile), 187 (Tennessee), 193 (Germany), 197 (Germany), 206, 207, 208 (Norway), 235 (California), 239 (Florida), 249 (Florida), 259 (Colorado), 260 (Colorado), 291 (Lebanon), 292 (Lebanon), 304 (Germany), 305 (Arizona) 310 (New Zealand), 318 (California), 333 (USSR), 339 (USSR), 511 (Scotland), 517 (Scotland), 526, 527, 537, 562 (Italy), 566, 567, 569, 570 (USSR), 576 (England), 578 (Poland), 583 (Poland), 1009, 1010 (Tennessee), 1011 (Indiana), Bl 163/1, 164/1, 169/1, 180/5 (Scotland), Can-1 (Scotland), Cr 8/1, 9/2 (Scotland), Cur 2, 4/1 (Scotland), Du 35/1, 41/2 (Scotland), Fi 159/2 (Scotland), Genoa - 3b, Pisa - 5b (Italy), Hu 1/3, 10/2, 16/1, 20, 35/1, 40, 43/2 (Scotland), Hun - 5 (Hungary), Bal (Scotland), Rav 1/3, 2/1 (Scotland), Rieff-2 (Scotland), Sed 3/1 (England), Serp 1/1 - 1/18, 2/1, 2/2 (England).

TABLE 1: Stocks of syngens 1 and 2 used in this study. Those underlined are from the U.S.A.

SYNGEN	
3	231 (Vermont), 92 (Philadelphia), 152 (Connecticut)
4	32 (Maryland), 47 (California), 51 (Indiana), 127 (Florida), 139 (Florida), 139 (Florida), 174 (Chile)
5	87 (Philadelphia), 314 (Illinois)
6	101 (Philadelphia), 165 (India), 225( )
7	38 (Florida), 227 (Florida), 325 (Florida)
8	214 (Florida), 299 (Panama), 565 (Uganda)
9	317 (France), 338 (Leningrad), Du 51/3, 76/5 (Scotland)
10	223 (Florida)
11	219 (Florida), 247 (Florida)
12	274 (Louisianna)
13	209 (France), 238 (Madagascar), 321 (Mexico)
14	328 (Australia)

TABLE 1 (cont.): Stocks of syngens 3 - 14 used in this study. Those underlined are from the U.S.A.

N.B. Only 3 stocks are known for syngen 13 and only 1 for syngen 14.

sterile, plugged test tubes. These cultures can then be stored at 11°C with periodic (4-6 weeks) additions of bacterised lettuce medium, until required for enzyme studies.

6. Culture of animals

A detailed description of methods used for the culture of paramecia are given by Sonneborn (1950) but a brief outline is given here as some of the methods used differ from those previously described.

Genetic work and the maintenance of stocks was carried out using bacterised lettuce medium. The lettuce medium was innoculated with Aerobacter aerogenes (cultured on agar slants) and incubated for 12-18 hrs at 25°C to allow growth of the bacteria, before being used to culture paramecium. Cultures for enzyme studies, cell fractionation and columnwork were grown on grass medium which gives greater density of growth. Two similar methods for such mass culture were used and are described below:-

a) 250 ml cultures for electrophoresis:

A tube of lettuce-grown animals was innoculated into 100 mls of grass medium contained in sterilized, cotton wool stoppered 250 ml conical flasks and incubated at 25°C or 31°C (depending on the syngen under study). The culture became cloudy due to bacterial growth after 24 hrs and then after a further 2-3 days the culture cleared as the paramecium ingested the bacteria. To such a 'cleared' culture 100 mls of bacterised grass medium was added and the culture incubated again until it had cleared the bacteria (final density 4×10^4 cells/ml). The culture was then ready for harvesting and preparation of extracts for electrophoresis.

b) 4 - 25L cultures:

A tube of lettuce grown animals was inoculated into 1L of grass medium in a

stoppered 2.5L Thomson bottle and incubated at the appropriate temperature until the culture cleared, as described in (a). This 1L seed culture was then used to sub-inoculate further 1L aliquots of grass medium which were incubated, as before, until the bacteria had cleared.

7. Harvesting of paramecium:

Cultures of either type were first filtered through 2 layers of muslin to remove debris formed in the culture. Small cultures (4L and less) were centrifuged in pear-shaped vessels in an M.S.E. oil testing centrifuge at 1300 rpm for 5 mins. The pellets of concentrated animals obtained from the same culture were pooled and pipetted into conical centrifuge tubes which were centrifuged at 600g for 5 - 10 mins. A hard packed pellet of cells was obtained, from which culture debris and fluid were removed by pasteur pipette. Large cultures (5L and more) were first concentrated by centrifugation in an Alfa-Laval Cream Separator which concentrated the culture to 1-2L. These concentrated cultures were then centrifuged as described for the small cultures. The cells from large cultures were washed with M.S. solution (0.013M NaCl, 0.003M KCl, 0.003M $CaCl_2$, 0.004M phosphate, pH = 6.8) and sedimented.

8. Preparation of extracts for electrophoresis

The pelleted animals (see 7) were mixed with 1.5 volumes of 0.1M phosphate buffer (pH = 7.4, 0.75M sucrose) and transferred to glass homogeniser tubes. This buffer was modified for isocitrate dehydrogenase by adding 0.1 mg/ml NADP to stabilize the enzyme. Homogenisation was carried out at $4^{\circ}C$ using a Tri-R-homogeniser with a close fitting teflon pestle. 25-30 strokes at setting 4.5 was found to be sufficient to break most of the cells, with the minimum dmaage to mitochondria, although most of the enzymes weakly bound to this organelle are liberated.

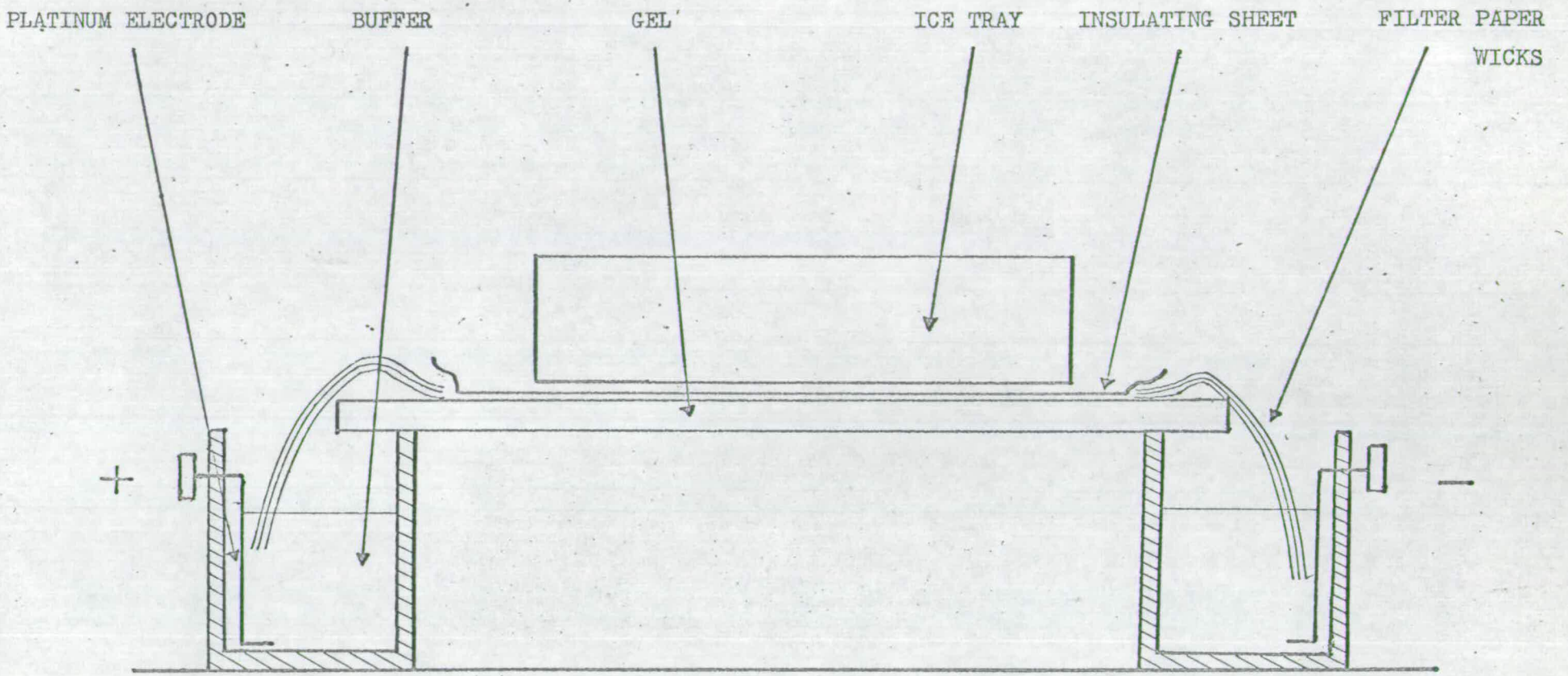


FIGURE 1 Electrophoresis apparatus

Diagram showing the apparatus used for starch gel electrophoresis at 4°C in the cold room. The ice is replenished at hourly intervals. The two platinum electrodes (marked + and −) are connected to a power pack with a stabilized voltage output.

The homogenate was then centrifuged for 30 mins at 75,000g at 4°C and the supernatant decanted into serum tubes. The pellet was homogenised in 1% Triton X.100, left at 4°C for 15 mins, and then centrifuged at 75,000g for 10 mins; the pellet from this centrifugation was discarded. The two fractions obtained in this manner are referred to as supernatant (SN) and triton X.100 extracted mitochondria (MTX) and were then ready for electrophoresis.

9. Starch gels.

The method used was similar to that described by Smithies (1955) and Smith (1968). Dry hydrolysed starch was added to Buchner flasks, followed by buffer appropriate for the enzyme being studied. The flask was swirled to mix the starch and heated over a bunsen until a clear viscous liquid was obtained. The flask was left to cool for 2-3 mins and then evacuated at the pump to remove air bubbles. The gel was poured into glass moulds (4 x 17 x 20 mm & 4 x 12 x 16 mm) until just proud of the edges. The gels were left, uncovered at room temperature (18-20°C) for $1\frac{1}{2}$ - 3 hrs and then transferred to the cold room (4°C) for 30 mins before electrophoresis. The concentration of starch used was as directed by Connaught Research Laboratories (9-11%), so that reproducible results were obtained from one batch of starch to the next.

10. Electrophoresis (methods and apparatus).

Pieces of 8 x 2 mm Whatmans 4 mM chromatography paper were soaked in the paramecium extracts and inserted into slits in the gel, previously made by a razor blade.

After inserting all the extracts in this manner, the gel was placed between two troughs (see Fig. 1) containing platinum electrodes, buffer and 4-5 thicknesses of Whatmans No. 4 Chromatography paper. The thicknesses of paper, soaked in buffer were then pressed firmly along both ends of the gel and a

ENZYME	SUBSTRATE	COENZYME	OTHER ADDITIONS	BUFFER	ELECTROPHORESIS BUFFER	
					GEL	ELECTRODES
ISOCITRATE DEHYDROGENASE	sodium isocitrate 15 mg	NADP 4 mg	$MnCl_2$ 20 mg MTT-tetr. 6 mg PMS 0.5 mg	0.1M tris-HCl pH 8.4 25 mls	0.01M phosphate -citrate pH = 7.0	0.05M phosphate -citrate pH = 7.0
FUMARASE [1]	sodium fumarate 0.125g	NAD 5 mg	0.1 ml MDH 6 mg MTT 0.5 mg PMS	0.1M phosphate pH = 7.4 25 mls	"	"
3-HYDROXY-BUTYRATE DEHYDROGENASE	sodium-3-hydroxy-butyrate 0.25g	NAD 5 mg	6 mg MTT	"	0.076M tris-citrate pH = 8.9	0.3M borate-NaOH pH = 8.9
SUCCINIC DEHYDROGENASE [2]	sodium succinate 4.5g	-	12 mg ATP 8 mg MTT 20 mg PMS	0.5M pottasium phosphate pH = 7.0 25 mls	5mM succinic-8.7mM tris pH = 6.0	0.1M succinic 0.186M tris pH = 6.0
GLUTAMIC DEHYDROGENASE	sodium glutamate 0.3g	NAD 5 mg	6 mg MTT 0.5 mg PMS	0.1M phosphate pH = 7.4 25 mls	0.05M tris-borate-EDTA pH = 8.0	0.5M tris-borate-EDTA pH = 8.0
MALIC DEHYDROGENASE	sodium malate 0.27g	NAD 5mg	6 mg MTT 0.5 mg PMS	0.1M tris-HCl pH = 8.4 25 mls	0.01M phosphate -citrate pH = 7.0	0.05M phosphate citrate pH = 7.0
GLUCOSE-6-PHOSPHATE DEHYDROGENASE	sodium glucose 6-phosphate 15 mg	NADP 4 mg	6 mg MTT 0.5 mg PMS	"	"	"
HEXOKINASE	1.8mg glucose	0.5iu G-6-PDH NADP 10 mg	20 mg $MgCl_2$ 50 mg ATP 4 mg MTT 0.5 mg PMS	0.1M tris-HCl pH = 7.4 25 mls	0.02M veronal pH = 8.6 + 1.0mmole EDTA 5mmole mercpt.	0.2M veronal pH = 8.6 +1.0mM EDTA 5mM mercapt.

TABLE 2. Showing the composition of assay mixtures used for developing zymograms. Also shown are the buffer systems used routinely for electrophoresis.
1. Malate dehydrogenase activity = 475 µmolar units/ ml. 2.Gel incubated in 30 ml sodium succinate K_2HPO_4 solution for 20 mins prior to adding stain.

sheet of Melmex polyester film was laid over the surface of the gel and wicks. Two cooling systems for the gel were used:-

(i) flat bottomed tin trays filled with ice chips were placed on the gel (insulated by the Melinex sheet) in the cold room. The ice was replenished at regular intervals.

(ii) brass cooling plates were placed above and below the gel and were connected to a Grant cooling unit and waterbath from which water at 4°C was circulated.

This system was run on the bench in the laboratory.

Using either of these systems electrophoresis was carried out with a voltage of 20v/cm across the gel. Migration of the enzymes studied was achieved with runs of 3-4 hrs duration. At the end of this period the gel was removed from the electrophoresis apparatus and a thin slice (approximately 1 mm thick) was removed from the upper surface of the gel and discarded. Thegel was placed in the mould and the enzyme staining mixture poured over the cut surface of the gel, which was then incubated at 35°C, in the dark, for 1-2 hrs or until bands of enzyme activity appeared. After developing the stain, the gel was rinsed overnight with cold tap water and then photographed. Using this method of staining, small volumes (25-30 mls) of the assay solution can be used.

11. Assay of enzymes and buffer systems for electrophoresis.

The dehydrogenase staining systems depend on the reduction of the dye MTT-tetrazolium (3(4,5 dimethylthiazole-2) - 2,5 diphenyltetrazolium) by reduced coenzyme generated by the oxidation of substrate by the enzyme. MTT-tetrazolium was found to give more reproducible results than nitro-blue tetrazolium. The solutions used for staining are given in Table 2; all reagents (except PMS) were dissolved in buffer and mixed 1-2 hours before use and PMS was added just

ENZYME	SUBSTRATE	COENZYME	OTHER ADDITIONS	BUFFER	ABSORBTION MONITORED
ISOCITRATE DEHYDROGENASE [1]	0.006M sodium isocitrate 0.1 ml	0.00135M NADP 0.1 ml	0.018M $MNCl_2$ 0.1 ml	0.25M tris-HCl pH = 7.4	$E_{340m\mu}$
SUCCINIC DEHYDROGENASE [2]	0.2M sodium succinate 0.2 ml	-	0.3 ml 0.1M KCN 0.3 ml 0.01M $K_3Fe(CN)_6$	0.1M sodium phosphate pH = 7.4	$E_{400m\mu}$
MALATE DEHYDROGENASE [3]	oxaloacetic acid (1mg/ml) 0.1 ml	NADH (1mg/ml) 0.1 ml	-	0.1M sodium phosphate pH = 7.4 0.8 ml	$E_{340m\mu}$
GLUTAMATE DEHYDROGENASE [4]	0.2M sodium glutamate 0.2 ml	0.02M NAD 0.1 ml	-	0.05M sodium phosphate pH = 7.6	$E_{340m\mu}$
3-HYDROXYBUTYRATE DEHYDROGENASE [5]	0.1M sodium dl-3-hydroxybutyrate 0.1 ml	0.015M NAD 0.1 ml	0.1M KEDTA pH = 8.1 0.1 ml	0.1M tris-HCl pH = 9.0 0.5 ml	$E_{340m\mu}$
ACONITASE [6]	0.03M sodium citrate (in buffer)	-	-	0.05M sodium phosphate pH = 7.4 2.9 mls	$E_{240m\mu}$
MALIC ENZYME [7]	0.03M malate 0.05 ml	0.000675M NADP 0.2 ml	0.05M $MNCl_2$ 0.06 ml	0.25M tris-HCl pH = 7.4 0.3 ml	$E_{340m\mu}$

TABLE 3. Assay mixtures used for estimating enzyme activity. All mixtures were made up to 2.9 ml with distilled water and 0.1 ml of extract added to start the reaction.

References: 1. Ochoa (1955a); 2. Slater & Bonner (1952); 3. Ochoa (1955b); 4. Strecker (1955); 5. Robinson & Coon (1957); 6. Anfinsen (1955); 7. Ochoa (1955c).

before incubating the solution with the gel. The mixtures used have been modified from Fine and Costello (1963) and Smith (1968) except the 3-hydroxy-butyrate dehydrogenase assay mixture which was adapted from a histochemical stain (Seligman 1963) by the author. The electrophoresis buffers (gels and electrode) used for the separation of the enzymes listed are also given in Table 2.

Certain parts of this study have involved the spectrophotometric assay of various enzymes, particularly those not detected on gels, and the details of the assay mixtures used are given in Table 3. The volumes of each solution given are for use in 1 cm light path spectrophotometer cells (volume = 3.5 ml) and the assays were carried out using a Beckman DB Spectrophotometer and a Beckman pen recorder. From the trace the change in absorbtion over the first minute was calculated and taken to be a measure of the initial velocity. Specific activity measurements were made by measuring the amount of protein by the method of Lowry et al. (1951), using bovine serum albumin as a standard. In the assays detailed in Table 3, 0.1 ml of extract was added to each cuvette, and diluted until an almost linear trace of transmittance with time was obtained.

12. Cell Fractionation.

With paramecium, considerable difficulties are encountered in the isolation of cell organelles, uncontaminated with other cell inclusions. The reasons for this are twofold, firstly the large number of intracellular particulates and secondly the bacteria which are present in considerable numbers even in stationary phase cultures. Paramecium contains the normal cell fractions found in metazoan cells namely nuclei, mitochondria, lysosomes, ribosomes and

"cytosol" (i.e. supernatant fraction) but also a homogenate will contain trichocysts, cilia, fragments of cell wall and gullet, glycogen granules and cytoplasmic crystals. These other inclusions nearly always contaminate any preparation of a particular cell organelle.

Differential centrifugation yields very impure preparations of cell organelles (Preer & Preer 1959). Mitochondria and macronuclei have been prepared in a relatively pure state in this study, for the purpose of localising the various bands of enzyme activity observed on starch gels.

Macronuclei were prepared by differential centrifugation followed by flotation of contaminating material in sucrose solutions according to the method of Stevenson (1967). This method involves homogenisation in 0.1% Tween 80, followed by centrifugation at 600g and then centrifugation through 2.4M sucrose to remove unbroken cells, cytoplasmic debris and nucleate remains. Throughout the procedure, purification was monitored by staining with aceto-camine fast green.

Mitochondria were prepared by differential centrifugation followed by sucrose density gradient centrifugation (Stevenson 1967), similar to the method described by Luck (1963, 1965). A variety of homogenisation buffers were used to determine which gave the best preservation of mitochondria; the medium used was 0.1M sodium phosphate buffer pH = 7.4, 0.75M in sucrose, and the cells were homogenised as described in section 8. The homogenate was centrifuged at 600g for 10 mins to sediment macronuclei, large pieces of cell wall, pieces of gullet and whole animals. The supernatant from this centri-fugation was then centrifuged at 10,000g for 15 mins and the supernatant containing ribosomes and cytosol was discarded. The pellet was resuspended and resedimented twice in the buffered sucrose solution before finally being

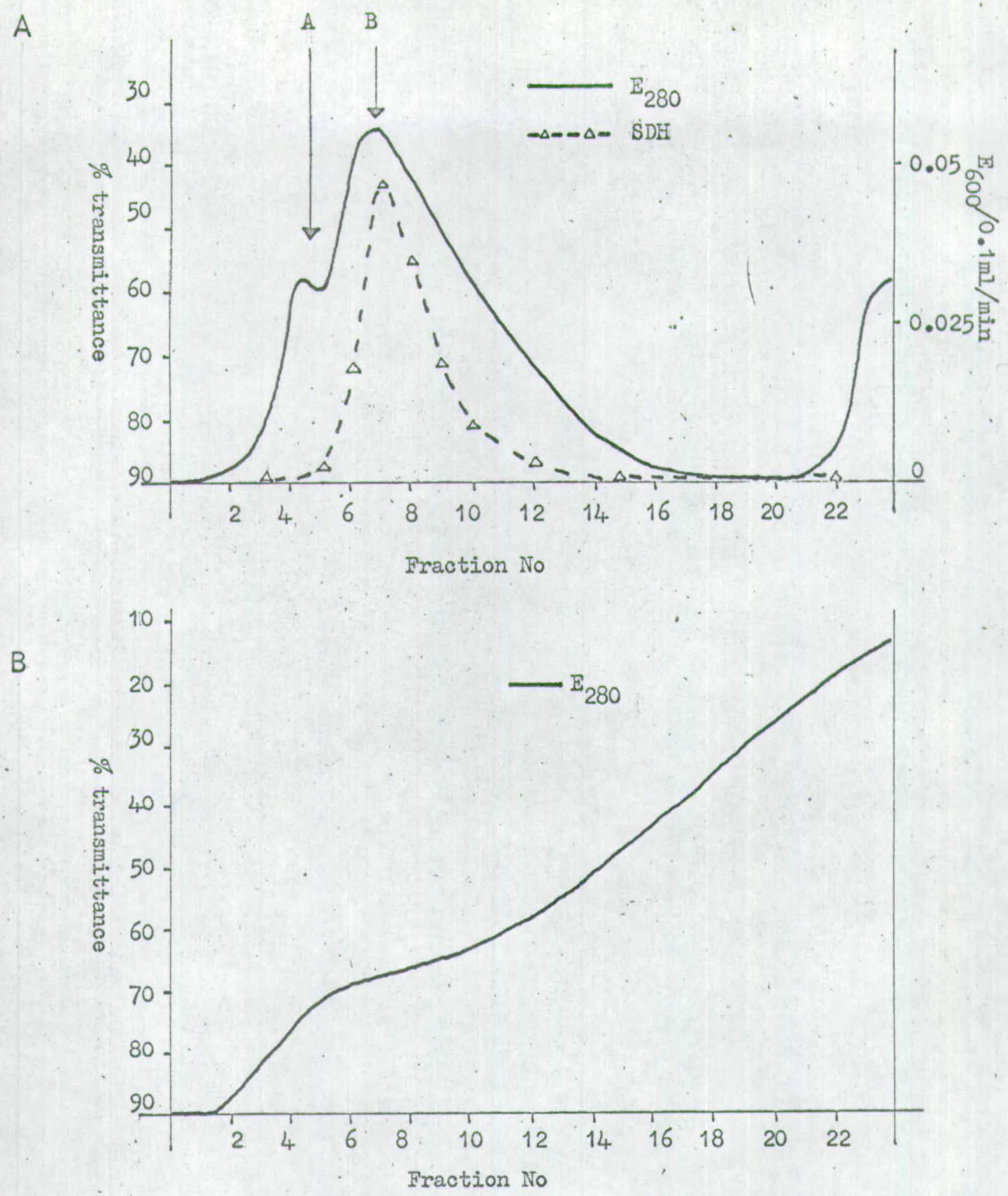


FIGURE 2 Sucrose density gradient fractionation of mitochondria.

A. Fractionation of a sucrose density gradient of a 2X washed 10,000g mitochondrial pellet, obtained by differential centrifugation of a paramecium homogenate. A suspension of the crude mitochondrial pellet was layered on a 20ml linear gradient (35%-65%) and centrifuged at 40,000g for 2hrs. Traces of transmittance at 280mμ and succinic dehydrogenase activity in the fractions are shown.

B. Identical sucrose density gradient of a bacterial homogenate prepared under similar conditions. A trace of transmittance at 280mμ is shown.

suspended in a volume of buffer equal to that of the original homogenate. This suspension was then layered on 20 ml linear (35% - 65%) sucrose density gradients which had been prepared previously. The gradients were centrifuged for 2 hrs at 40,000g in a M.S.E. 3 x 20 ml swing out head, and after coasting to a stop were fractionated from the bottom, in 1 ml cuts using an M.S.E. tube piercer. As the gradient was fractionated the effluent was monitored at 280mμ with an LKB Uvicord attached to a recorder.

A typical result from such a preparation is shown in Fig. 2a; an initial low peak (A) of protein is observed, followed by a larger peak (B). This first peak (A) was thought to be due to bacteria, but on running a bacterial preparation on the same type of gradient (see Fig. 2b) no peaks were observed in the positions shown in the mitochondrial preparation. Each 1 ml fraction from the gradient was assayed for succinic dehydrogenase (Slater & Bonner 1952) which is known to be located exclusively in the mitochondria. This enzyme was shown to be present in the second peak (B) only, which suggests that the first peak (A) is not due to mitochondria. Examination of material from each of the peaks by oil immersion phase contrast microscopy showed that the first peak contained bacteria and large pieces of pellicle with mitochondria attached, while the second peak contained almost exclusively mitochondria with some broken trichocysts.

Assays were performed directly on samples from the gradient fractions, but before running on starch gels, the fractions containing material were diluted (1:2) with distilled water and centrifuged at 10,000g for 10 mins, the pellet of mitochondria was then homogenised in 1% TX.100 (or 0.1M sodium phosphate buffer pH = 7.4) and used for starch gel electrophoresis.

13. Chromatography.

Work with columns was restricted to attempts to purify and separate the different bands of malic dehydrogenase observed on starch gels; both Sephadex G.100 (gel filtration) and DEAE-cellulose (Whatmans DE 22 ion exchanger) were used to determine whether the differences in mobility observed were due to charge or size differences and also to try and obtain better resolution of the mitochondrial form.

Sephadex G.100 was equilibrated at 4°C with 0.1M KCl in 0.5M tris-HCl buffer pH = 7.5 for 48 hrs. The equilibrated Sephadex was packed in a 1 x 1 x 25 cm column at a flow rate of 10 ml/hr. A 1 ml sample of extract was applied to the column which was run at 25 ml/hr., 3 ml fractions being collected. The effluent was monitored at 254 mμ using an LKB Uvicord and the fractions were assayed for MDH activity as described in Table 3.

A similar extract was run on DEAE-cellulose, equilibrated and recycled as described in Whatmans Laboratory Manual. A 1.5 x 25 cm column was packed at a flow rate of 78 ml/hr and run at 20 ml/hr for 12 hrs. before applying a 1 ml sample of extract. The sample was eluted with a linear gradient from 0.02M phosphate to 0.2M phosphate pH = 7.0 at a flow rate of 20 ml/hr and the effluent from the column was monitored at 280 mμ, using an LKB Uvicord. The fractions were then assayed for MDH activity as shown in Table 3.

Fractions were concentrated for starch gel electrophoresis by pipetting into visking tubing which was sealed at both ends and placed in 50% polyethylene glycol at 4°C. When the volume had been reduced to 0.1 - 0.2 ml the visking tubing was removed from the polyethylene glycol and the concentrated fraction removed by pipette, ready for electrophoresis.

14. Crossing, cloning and induction of autogamy.

The techniques for these three processes are described in detail elsewhere (Sonneborn 1950). Cloning involves the isolation of single animals by micropipette into depression slides containing bacterised lettuce medium. The slides were then incubated until approximately two hundred animals were observed in a single depression, transferred to tubes and then grown up for harvesting.

Autogamy was induced by rapid growth followed by starvation (Sonneborn 1937). A single animal was introduced into a slide depression containing culture medium, after 24 hrs one of its progeny was transferred to another slide depression and the process repeatedly daily. As the animals starved down, they were tested for autogamy by staining a sample with Aceto-carmine - Fast green. Autogamy was recognised by the disintegration of the macronucleus into several fragments; using this technique slide depressions with 90-100% autogamous animals were found. Exautogamous clones were then established and autogamy was backchecked after one or two fissions.

15. Genetic analysis of enzyme variants.

Crosses were made between clones with differing enzyme electrophoretic mobilities and the two exconjugants from such a cross grown up as separate clones. Comparison of two such clones derived from the same cross indicates whether the enzyme studied was controlled by a nuclear gene: if both clones were identical a nuclear gene was implicated, but if they were different, i.e. each expressed their respective parental types, a cytoplasmic "gene" would be implicated. If nuclear control was implicated in this manner, a further check was undertaken by obtaining an F_2 from such a cross. An F_1 clone was

passed through autogamy and the resulting exautogamous F_2 clones were screened for the two parental types. A segregation into the two enzyme types approximating to a 1:1 ratio, would indicate control by a nucear gene. Thus using both techniques the location (nuclear or cytoplasmic) of the genes controlling a particular enzyme can be indicated and nuclear control, if it occurs, established conclusively.

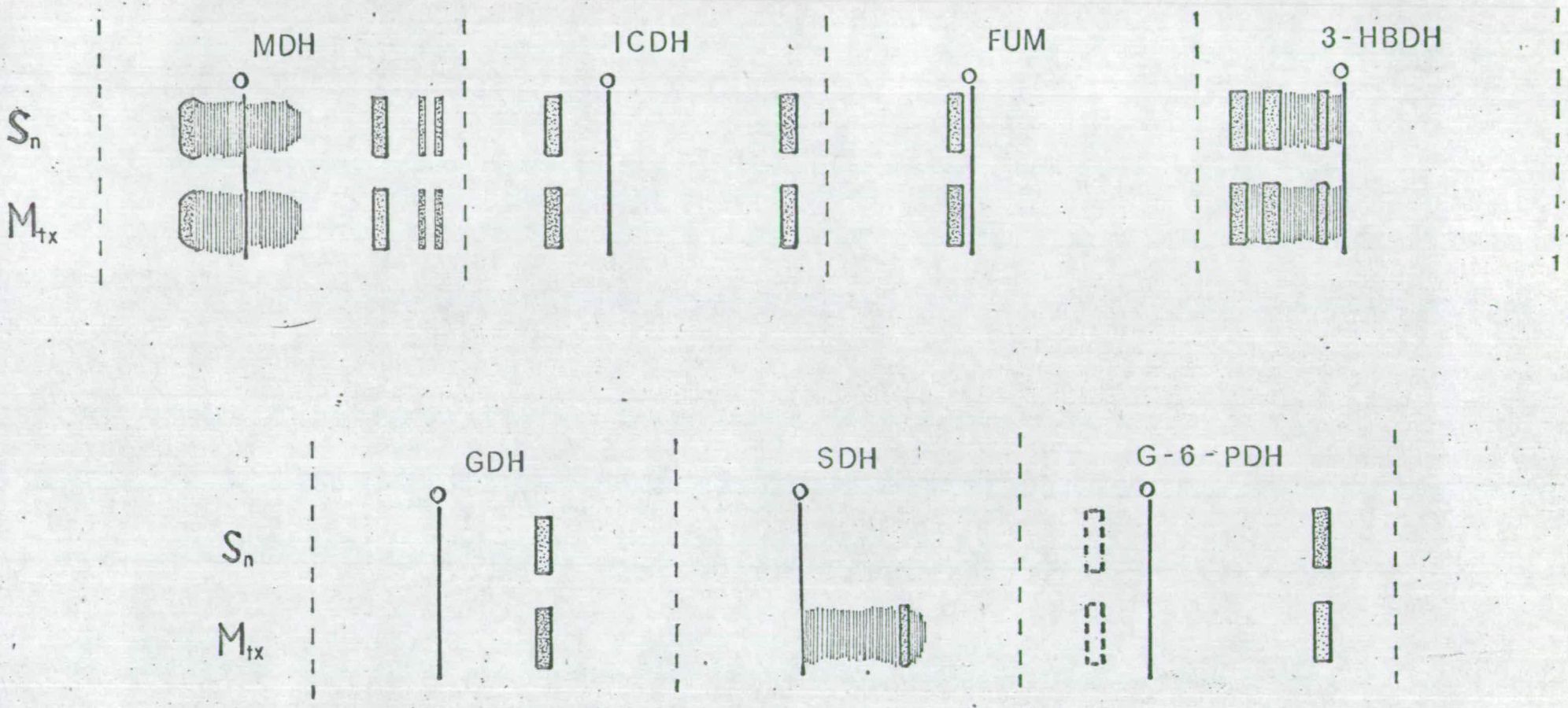


FIGURE 3

Diagramatic representation of starch zymograms of malate dehydrogenase(MDH),iso-citrate dehydrogenase(ICDH),fumarase(FUM),3-hydroxybutyrate dehydrogenase(3-HBDH),glutamate dehydrogenase(GDH),succinic dehydrogenase(SDH) and glucose-6-phosphate dehydrogenase (G-6-PDH). The buffer systems and enzyme staining solutions are those shown in Table 2. Two inserts are shown for each enzyme,a 70,000g supernatant fraction (S_n) and a 1% triton X-100 extract of a 70,000g pellet fraction.

RESULTS

Part I. Preliminary study of electrophoretic patterns and buffer systems.

1. Substrate dependence and buffer systems.

Tests were made for a range of enzyme activities after starch gel electrophoresis of paramecium extracts, firstly to detect the presence of the enzyme in paramecium and secondly to find the optimum conditions for their separation. The enzyme activities found were then used for the studies outlined in the introduction. Typical results for stocks of syngen 1 are shown diagrammatically in Fig. 3; each gel has two inserts, one being the supernatant fraction and the other being a crude mitochondrial extract prepared by differential centrifugation. The mitochondrial localisation was later confirmed by electrophoresis of mitochondria prepared by sucrose density centrifugation.

Each enzyme system was tested for substrate dependence by omitting substrate from the assay mixture before incubation. In this way malate dehydrogenase (NAD), fumarase, 3-hydroxybutyrate dehydrogenase (NAD), glutamate dehydrogenase (NAD) and succinic dehydrogenase were shown to be completely substrate dependent i.e. no stain was obtained in the absence of substrate. Glucose-6-phosphate and isocitrate dehydrogenases each showed two bands of activity on electrophoresis, one migrating towards the cathode and the other towards the anode. In the absence of substrate neither of the anodal bands corresponding to each enzyme showed any staining reaction but the cathodal band showed a staining reaction in both cases. Two explanations

were considered for the lack of substrate dependence of the cathodal band: either the band indicated the presence of a NADP 'nothing' dehydrogenase (Wilkinson 1965, Scandalios 1968) or there might have been activity due to bound substrate. In order to test the latter explanation extracts were dislysed against 0.1M phosphate buffer pH = 7.4 (+ 0.1 mg/ml NADP) at 4°C for 12 hrs. and then subjected to starch gel electrophoresis followed by staining. After this treatment the band was found to be wholly dependent on the presence of isocitrate in the assay mixture and so was presumably isocitrate dehydrogenase. Thus glucose-6-phosphate dehydrogenase occurs in a single form, namely the anodal cytoplasmic band.

A variety of buffer systems have been used for the enzymes studied with the object of obtaining optimum conditions for the detection of variants. Isocitrate dehydrogenase was subjected to electrophoresis using three buffer systems other than that shown in Fig. 3. 0.01M tris-citrate at pH = 8.0 and pH = 6.0 both gave greater migration rates but the sharpness of the cathodal band was considerable reduced and the anodal band was completely inactive. The discontinuous system of Poulik was also used but gave a considerable loss of activity. Although the migration given with 0.01M phosphate-citrate pH = 7.0 was not great for the cathodal band, this system was routinely used due to the stability of the enzyme in this system as compared to the other systems.

Five alternative buffer systems were used for the electrophoresis of fumarase, namely 0.01M phosphate citrate pH = 7.0 and pH = 6.0, 0.01M tris-citrate pH = 6.0, 0.01M tris-HCl pH = 8.4 and the discontinuous system of Poulik. Using the first four systems similar migration distances were observed, with reduced activities in the last three of these. The discontinuous system of

Poulik gave anodal migration of this enzyme but the activity and sharpness of the bands were considerably reduced, so 0.01M phosphate-citrate pH = 7.0 was routinely used.

3-hydroxybutyrate dehydrogenase was examined using the discontinuous system of Poulik and 0.01M phosphate-citrate pH = 7.0. No migration from the origin was observed with the latter system and so the discontinuous system of Poulik was routinely used.

The cathodally migrating zone of activity of malic dehydrogenase has not been resolved into discrete bands by the use of a wide range of buffer systems (0.01M phosphate-citrate pH = 7.0, 0.01M tris-HCl pH = 8.4, 0.01M tris-citrate pH = 8.0 and pH = 6.0, discontinuous system of poulik and 0.01M glycine-NaOH pH = 9.0). Using any of these systems the cathodal band appeared as a smear and so 0.01M phosphate-citrate pH = 7.0 was routinely used for this enzyme because the anodal bands were well resolved.

Assays of several other enzymes (aconitase, puruvic dehydrogenase, α-ketoglutarate dehydrogenase, α-glycerol phosphate dehydrogenase, choline oxidase, malic enzyme, NAD-dependent iso-citrate dehydrogenase and hexokinase) were made after stach gel electrophoresis, but no activity was detected, except in the case of hexokinase. Spectrophotometric assays were attempted with α-ketoglutarate dehydrogenase, malic enzyme and aconitase, but activity was only detected in the latter case. The negative results obtained with aconitase after starch gel electrophoresis could have been due to the assay mixture used; this consisted of sodium citrate, NADP, isocitrate dehydrogenase and manganese chloride and was designed by the author. Possibly by varying the concentrations of reactants a workable system could be found. Both

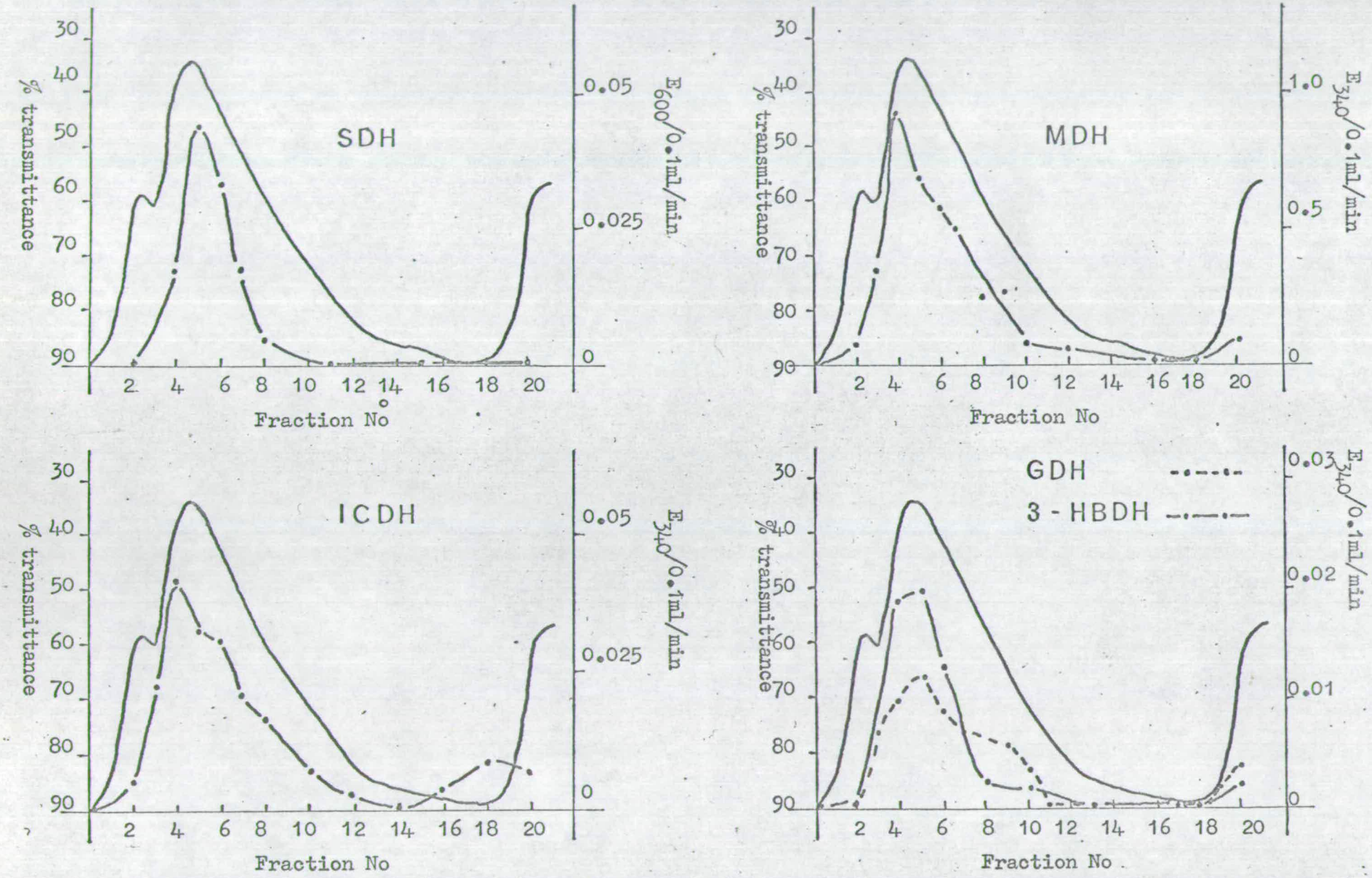


FIGURE 4 Mitochondrial Localisation of Enzyme Activity:

The graphs(all referring to the same gradient) show the fractionation of a 2X washed 10,000g mitochondrial pellet on a linear sucrose density gradient(35%-65%) centrifuged at 40,000g for 2hrs. The transmittance at 280mμ is shown by the continuous trace and the various enzyme activities by the discontinuous traces. The fractions,3&4,5&6,and 7&8 were pooled in the pairs given and centrifuged to sediment the

α-ketoglutarate and pyruvate dehydrogenases require several cofactors which were assumed to be in sufficient concentration in the extract but may not have been. Thus the lack of detectable activity may have been merely a reflection on the assay system used.

2. Mitochondrial localisation.

Although the enzymes described have been shown to be localised in the mitochondria by crude cell fractionation the possibility of contamination of the mitochondrial fraction with other cell organelles cannot be excluded. To establish the localisation of these enzymes more rigorously, mitochondria were prepared by density gradient centrifugation (as described in Materials and Methods). Fig. 4 shows such a gradient analysed for protein (absorbtion at 280 mμ), malate dehydrogenase, succinic dehydrogenase, isocitrate dehydrogenase, 3-hydroxybutyrate dehydrogenase and glutamate dehydrogenase. The enzyme activities show peaks corresponding to the second peak of protein, which has been shown to be mitochondrial. Using an identical gradient, mitochondria from the same homogenate were pelleted by centrifugation from the fractions and subjected to starch gel electrophoresis after homogenisation in 0.1M phosphate buffer pH = 7.4.

Three fractions A, B and C (Fig. 4) were obtained by pooling the pairs of fractions 3 and 4, 5 and 6 and 7 and 8. The zymograms of these fractions, together with the homogenate and supernatant fraction are shown in Fig. 5. Before electrophoresis for succinic dehydrogenase, 0.05 ml of 1% triton X.100 was added to each fraction in order to release the enzyme from the mitochondria. Activity was only observed at the origin (Fig. 5); it is thought that this was due to the lack of release of enzyme from the mitochondria and does not indicate that the bands normally observed are not

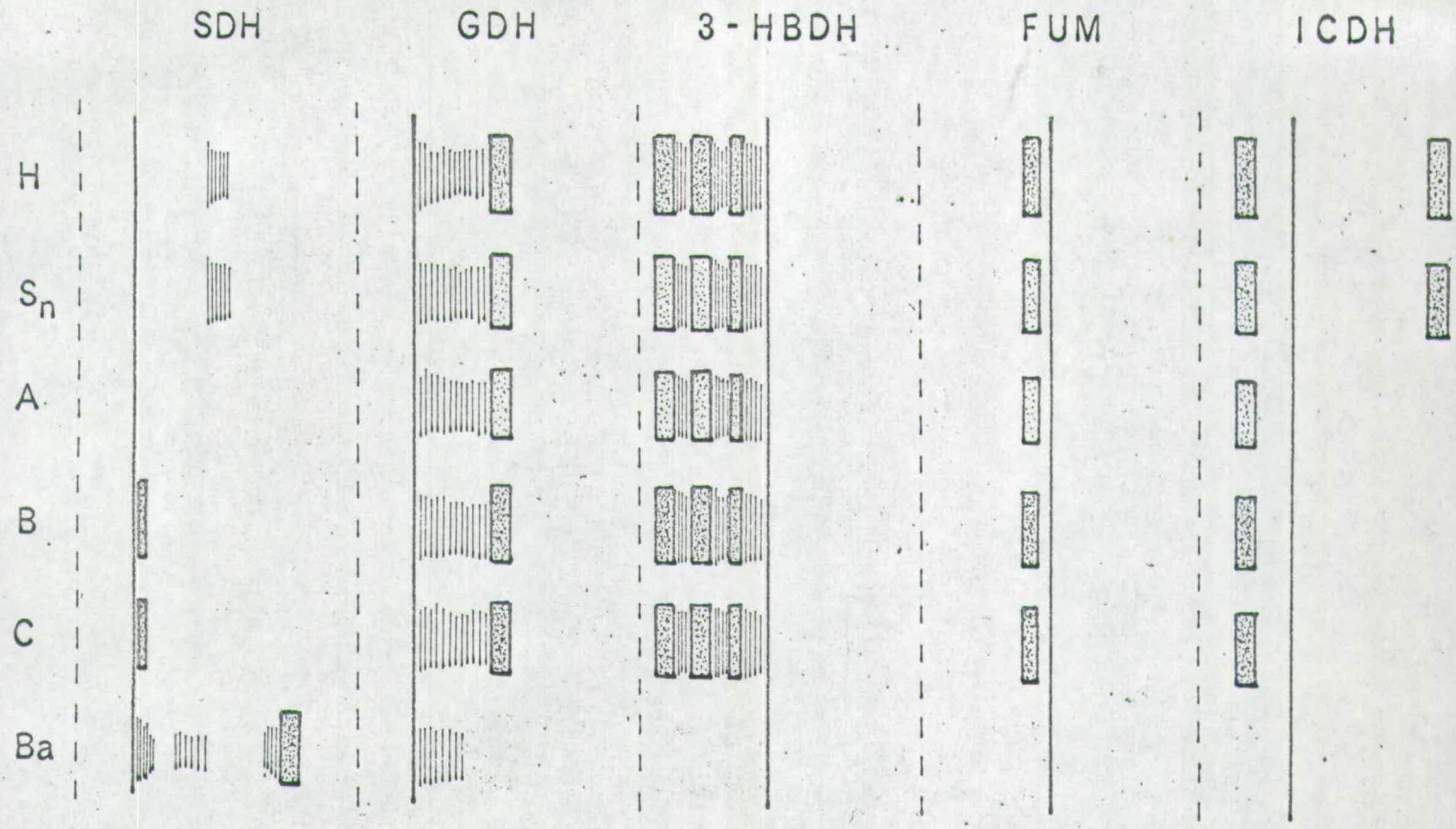


FIGURE 5 Localisation of Zones of Activity on Starch Gels.

Diagrammatic representation of zymograms showing the banding pattern of various cell fractions, including mitochondria prepared by sucrose density gradient centrifugation (Fig.4). The buffer systems and assay solutions are shown in Table 2. These results were obtained using a homogenate of paramecia from stock 513 (Syngen 1).

Key to Symbols: H - homogenate; S_n - supernatant from centrifugation of H at 10,000g for 20mins; A- fractions 3 & 4 from density gradient (Fig.4); B - fractions 5 & 6; C - fractions 7 & 8; Ba. - bacterial homogenate prepared in the same way as the paramecium homogenate.

mitochondrial. Fractions 3 and 6 (Fig. 4) were examined by phase contrast microscopy as described previously and together with the enzyme profile indicate that peak (2) contains mitochondria and that the bands observed on starch gel are due to mitochondrial enzymes.

Although peak (2) contains broken trichocysts these are not present in large amounts. The enzyme activities show a distribution along the gradient similar to that of succinic dehydrogenase which is exclusively mitochondrial. It therefore seems unlikely that the trichocysts are responsible for the observed enzyme activities. The peaks of enzyme activity are divided between fractions 5 and 6, malate and isocitrate dehydrogenase appearing in fraction 5 and the remaining activities in fraction 6. The reason for this is not known. Although this variation provides an interesting point for investigation, it does not invalidate the mitochondrial localisation of the enzyme activities.

3. Bacterial contamination.

The possibility of bacterial contamination in the preparation of extracts is a very real one as there will be considerable numbers present in the culture fluid. In order to eliminate the possibility that any of the bands observed on starch gels were bacterial in origin, cultures of axenically grown cells were compared with those grown on bacterised medium. Stocks 51 (syngen 4) and 540S (syngen 1) were compared for fumarase, isocitrate dehydrogenase, glutamate dehydrogenase and succinate dehydrogenase. These enzymes were shown to be identical in both axenic and bacterised cultures and this demonstrates that these enzymes originated from paramecium.

As regards malate dehydrogenase, the mitochondrial and supernatant forms were identical in bacterised and axenic cultures, but the third set of bands (Fig. 3) were absent in the axenically grown cultures, suggesting that these bands were bacterial (see Part III for details). To demonstrate that the 3-hydroxybutyrate dehydrogenase observed on the gels was not due to bacteria, an extract of Aerobacter aerogenes was prepared and compared with an extract from a bacterized paramecium culture. The activities and electrophoretic patterns were totally different, the paramecium enzyme migrating towards the cathode and the bacterial enzyme towards the anode. This result establishes that the enzyme pattern obtained with paramecium extracts is due to the paramecium and not the bacteria.

4. Conclusion.

In this section it has been shown that Paramecium aurelia contains the following enzyme activities, isocitrate dehydrogenase, succinic dehydrogenase, malate dehydrogenase, glutamate dehydrogenase, glucose-6-phosphate dehydrogenase, 3-hydroxybutyrate dehydrogenase, fumarase, hexokinase and aconitase. All but the latter have been detected after starch gel electrophoresis and the subcellular localisation of the zones of activity has been established by cell fractionation. The possibility that some of the zones of activity detected on starch gel electrophoresis were bacterial in origin has been considered and by using axenically grown cultures it has been shown that all the zones of activity, except for some in malate dehydrogenase, are due to paramecium. Assays for several other enzymes have been made without positive results, possibly due to technical difficulties rather than the absence of these enzymes.

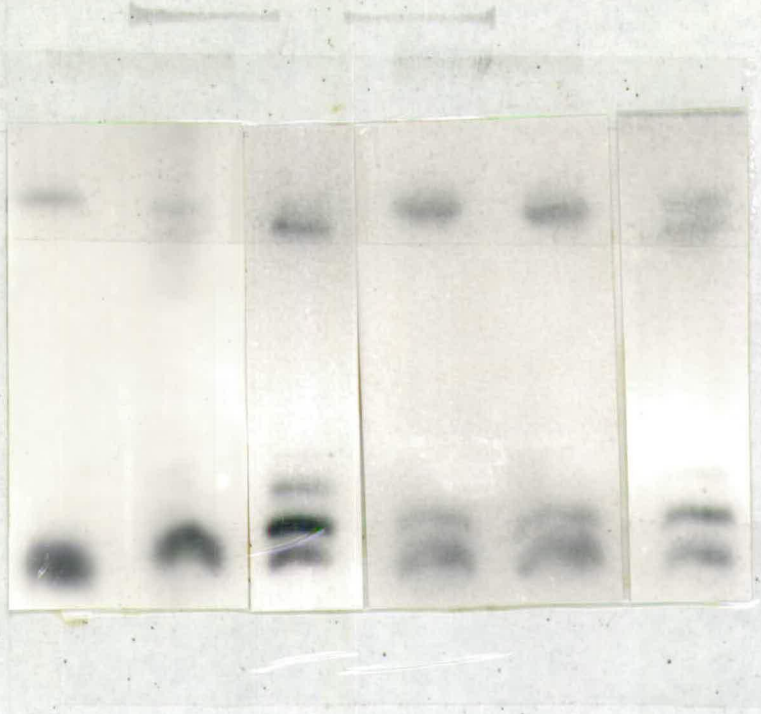
Part II. Isocitrate dehydrogenase.

The object of studying this enzyme was to examine the genetic relationship between the supernatant and mitochondrial forms of the enzyme and to study the nature of the genetic control of the mitochondrial enzyme. The genetic control of the supernatant form has been studied in the mouse (Henderson 1965), but no studies have previously been made on the mitochondrial form. Genetic variants of both forms have been found in paramecium and have been used for the studies outlined.

1. Electrophoretic variants.

Electrophoresis of extracts from two different stocks of paramecium (Syngen 2) have shown two variants, one being in the cathodal band and the other in the anodal band (Fig. 6(a)). The great majority of stocks, however, are alike in regard to the positions of both anodal and cathodal bands and will be referred to as the normal types. Preparation of mitochondria by sucrose density gradient centrifugation, followed by starch gel electrophoresis of mitochondrial extracts, confirmed that the cathodal band was mitochondrial and the anodal band is cytoplasmic.

The homozygous pattern of the cytoplasmic variant (ICD_c-1) shows two bands of activity while the normal form (ICD_c-2) shows only a single band. The slow migrating (Y) band of ICD_c-1 is of lower activity than the fast migrating band (X), which has the same mobility as the normal form. The homozygous 'normal' pattern of the mitochondrial enzyme (ICD_m-1) shows a single band, whereas the variant homozygous form shows three bands of activity (a, b and c). The central band (b) has the greatest intensity of staining while bands a and c are of lower and almost equal intensity. The possible



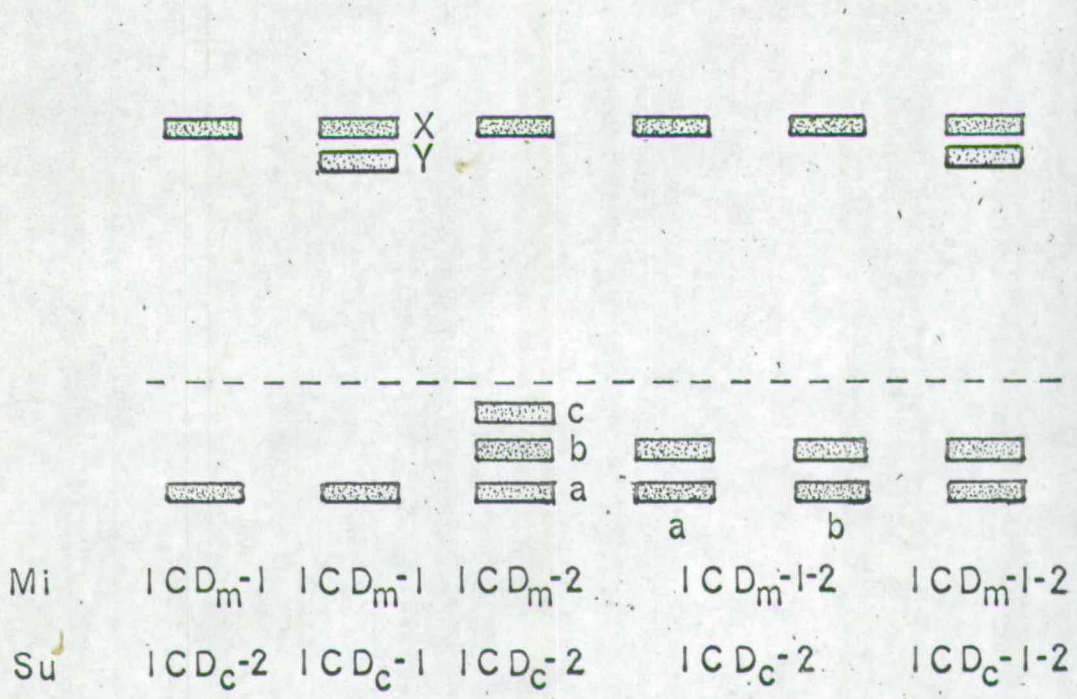


FIGURE 6

Starch gel zymograms (photograph & diagram) showing common and variant forms of both supernatant and mitochondrial iso-citrate dehydrogenase. Also shown are the heterozygote patterns of two crosses: (i) the common mitochondrial form (ICD_m-1) X the variant mitochondrial form (ICD_m-2) and (ii) the common mitochondrial form (ICD_m-1) and the variant supernatant form (ICD_c-1) X the variant mitochondrial form (ICD_m-2) and the common supernatant form (ICD_c-2). For cross (i) the two exconjugant clones (a & b) from one pair of conjugants are shown

The stocks used were 1010 (ICD_m-1,ICD_c-2) ,Du 41/2 (ICD_m-1,ICD_c-1) and 58 (ICD_m-2,ICD_c-2),all of Syngen 1.

A.

ICD_M-1	ICD_M-2	TOTAL
25	32	57

χ^2 = 0.86 not significant at the 5% level

B.

ENZYME	ICD_C-1	ICD_C-2	TOTAL
ICD_M-1	5	10	15
ICD_M-2	8	13	21
TOTAL	13	23	36

ICD_M - χ^2 = 1.0

ICD_C - χ^2 = 2.76

not significant at the 5% level.

TABLE 4:

A. Segregation of the alleles for mitochondrial iso-citrate dehydrogenase obtained by passing a heterozygote (ICD1-2) through autogamy. The value of χ^2 given is for the expected 1:1 ratio.

B. Segregation of the alleles for the supernatant (ICD_C) and mitochondrial (ICD_M) forms of iso-citrate dehydrogenase obtained by passing an F_1 (heterozygous for both sets of alleles) through autogamy. The values of χ^2 are given for the expected 1:1 ratio.

explanations for these patterns will be discussed in detail in a later section (see p.33).

2. Genetics of mitochondrial and supernatant forms

The genetic studies consisted simply of crossing normal and variant forms and then passing the resultant heterozygote through autogamy to obtain an F_2 which was then screened for the segregation of the two types. Two crosses were made, one involving only the 'normal' and variant forms of mitochondrial isocitrate dehydrogenase (ICD_m-1 x ICD_m-2) and the other involving both types of variant (ICD_m-1, ICD_c-2 x ICD_m-2, ICD_c-1).

The pattern shown by the two exconjugant clones from a given pair of the first cross (ICD_m-1-2) is shown in Fig. 6 . Both are identical, suggesting that the enzyme is not controlled by a cytoplasmic genetic factor. The segregation of the two alleles into the two parental types after passing the heterozygote through autogamy is shown in Table 4(a). The ratio of the two types is not significantly different from the 1:1 ratio expected and indicates that this mitochondrial enzyme is controlled by nuclear genes. The heterozygote pattern obtained in the second cross (ICD_m-1, ICD_c-2 x ICD_m-2, ICD_c-1) is shown in Fig. 6 . Heterozygotes of this type were passed through autogamy and the resulting F_2 screened for the four possible phenotypes (ICD_c-1, ICD_m-1; ICD_c-2, ICD_m-1; ICD_c-1, ICD_m-2; ICD_c-2, ICD_m-2). The segregation is shown in Table 4(b) and although the numbers examined are small it can be seen that the mitochondrial and supernatant forms are controlled by different gene loci. No close linkage occurs between the two loci.

3. Sub-unit structure.

On starch gel electrophoresis, both the variant forms show multiple bands which cannot be due to the heterozygous state as both are homozgous

clones. By considering the homozygouse and heterozygous patterns obtained on starch gel electrophoresis deductions can be made about the sub-unit structure of this enzyme.

The cytoplasmic variant form shows two bands of activity and when crossed to the common, single banded form the heterozygote shows two bands as well, with the slower migrating form (Y) giving a lower intensity of staining than in the homozygous state. This pattern can be accounted for in three ways:

1. the slower moving band is due to a separate protein not present in most stocks,
2. the pattern is due to an alteration in the structure of the common form allowing two conformations of the enzyme to exist, or
3. the alteration in the structure of the variant form allows the binding of a small molecule which alters the charge of the enzyme so that a band of slower mobility is produced.

The following pieces of evidence suggest that the first explanation is unlikely and either of the other explanations could be correct. When clones heterozygous for the variant and common forms were passed through autogamy, the two bands of the variant segregated together and not independently. This suggests that the second band of activity in the variant is not a separate protein unless very closely linked to the isocitrate dehydrogenase locus. Also the second band has the same substrate, coenzyme and cofactor requirements which would again suggest that this band of activity is due to the same protein as that in the first band of activity. Without further evidence it cannot be conclusively stated that the second band of activity is due to the same protein but it seems to be very probable.

The three banded pattern observed with ICD_m-2 can be explained in

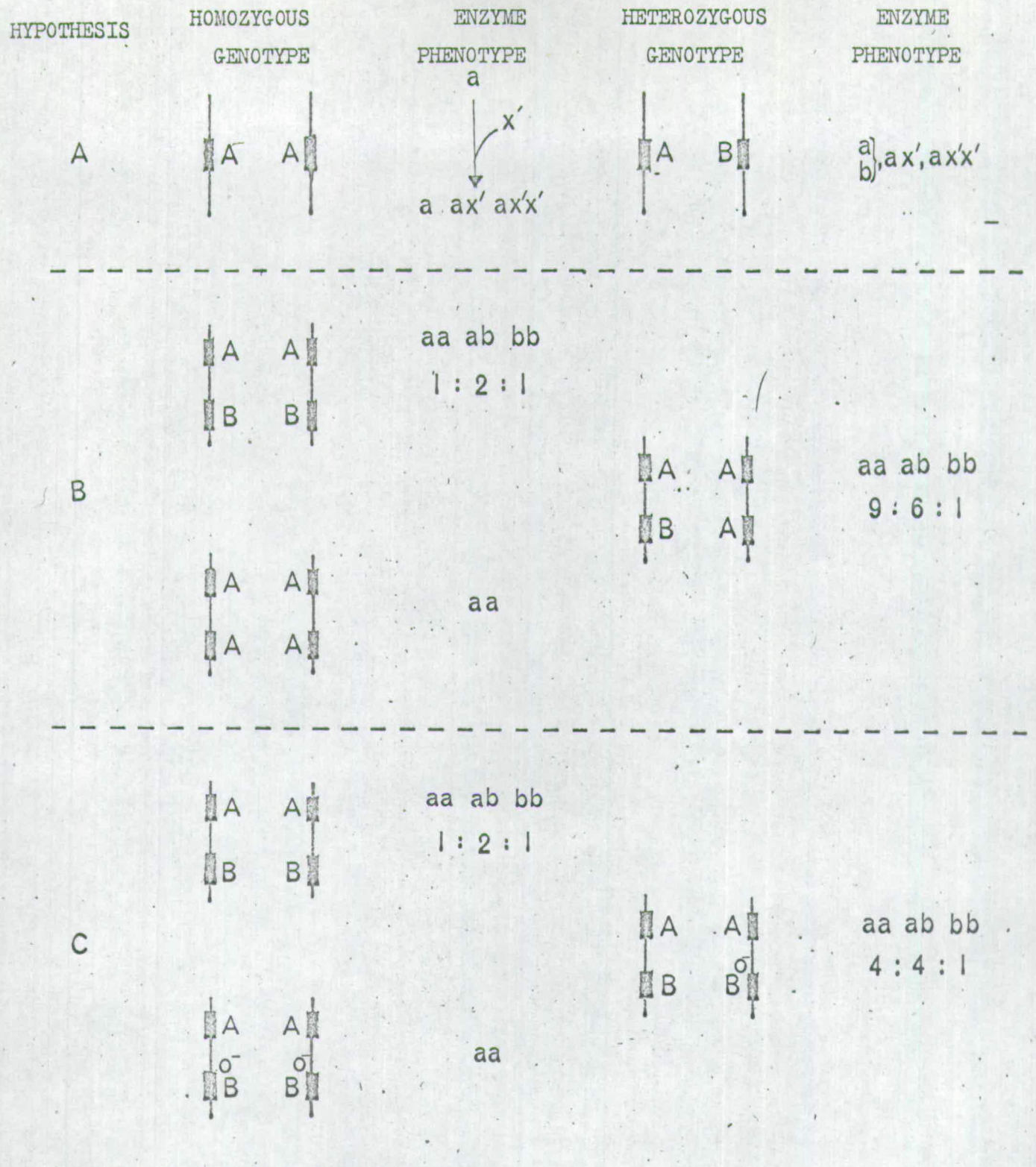


FIGURE 7

Diagram of the possible explanations for the electrophoretic pattern of isocitrate dehydrogenase. The enzyme phenotypes (homozygous and heterozygous) predicted by each explanation are shown, together with the ratios of the different types of dimer produced in each case.

Symbols: o^- denotes a non-functional operator locus and X denotes a charged coenzyme or other small molecule.

several ways and from these explanations the expected heterozygote pattern can be predicted. These will be described and then considered in relation to the observed heterozygote pattern and other relevant data.

Multiple banding has been observed with other enzymes including mitochondrial malate dehydrogenase. Several explanations have been put forward, namely that the different bands represent conformers (Kitto et al. 1966), or in the case of alcohol dehydrogenase that they are due to binding by the protein of coenzymes or other small molecules (Unsprung & Carlin 1968; Kaplan 1968). Both hypotheses when applied to the pattern of ICD_m-2 will predict a smilar banding pattern for the heterozygote formed when ICD_m-2 is crossed with ICD_m-1 and so only the small molecule binding theory will be considered. ICD_m-1 and ICD_m-2 are considered as different in structure, in that ICD_m-2 is able to bind one or two charged molecules (X') thus giving the three bands a, b and c (Fig. 7(A)). In the heterozygous state half the quantity of bands b and c would be expected as only one allele for ICD_m-2 is present. An identical situation would arise if ICD_m-2 was structurally altered in such a way that if could exist in three conformations of differing mobility.

An analogous explanation to that verified for lactate dehydrogenase (Shaw & Barto 1963) can be considered, i.e. two loci determine the different polypeptides. If it is assumed that isocitrate dehydrogenase is a dimer, each polypeptide being coded for by a separate locus, a three banded pattern for ICD_m-2 would be expected, namely the two pure types and an intermediate hybrid type (Fig. 7(B)). The ICD_m-1 type could be due to identical or different polypeptides which do not differ in charge at this pH and so produce

only a single band of activity. In the heterozygote, assuming random association of the polypeptides, one would again expect three bands with intensities in the ratio 9:6:1, as there will be 3 polypeptides of identical charge to every one of different charge.

A third explanation (Fig. 7(C)), similar to the last, is that ICD_m-1 and ICD_m-2 are each controlled by two loci and are identical except that the operator locus for polypeptide B in ICD_m-1 is nonfunctional so that only one type of polypeptide is produced and therefore one band on starch gel electrophoresis. In the heterozygote one would expect three bands but with ratios of intensity of 4:4:1 as there would be two polypeptides of identical charge to every one of a different charge.

The heterozygote (ICD_m1-2 Fig. 6) shows only two bands, but when the extract was concentrated approximately threefold band c could just be detected. The presence of band c in lower concentration in the heterozygote is predicted by all three explanations, but the relative amounts of band c are different in each case. Explanation A predicts that band c will be reduced by a factor of 2 compared to the homozygote, explanation B predicts a reduction by a factor of 4 and explanation C by a factor of 2.25. The absence of band c was used as a quantitative assay in the following way: extracts of ICD_m-2 and ICD_m1-2 were adjusted to equal total protein concentrations, ICD_m-2 was diluted 1:2, 1:4 and 1:6 and all five extracts were then subjected to starch gel electrophoresis. Band c was detected in ICD_m-2 in all cases except at a dilution of 1:6 and was not detected in ICD_m1-2. If hypothesis A or C was correct activity should not have been detected at dilutions of 1:2 or 1:4 as none was detected in the heterozygote, but as activity was detected in these

dilutions, this argues against either of these hypotheses. The absence of activity in the 1:6 dilution is predicted by hypothesis B and so presents evidence in favour of this hypothesis.

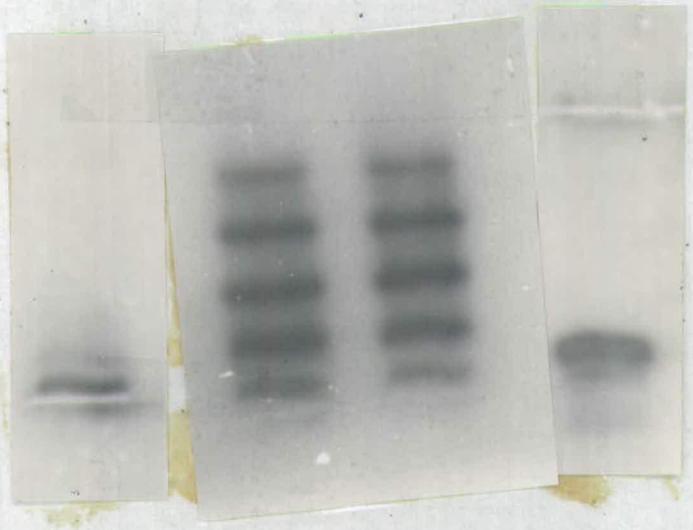
4. Conclusion.

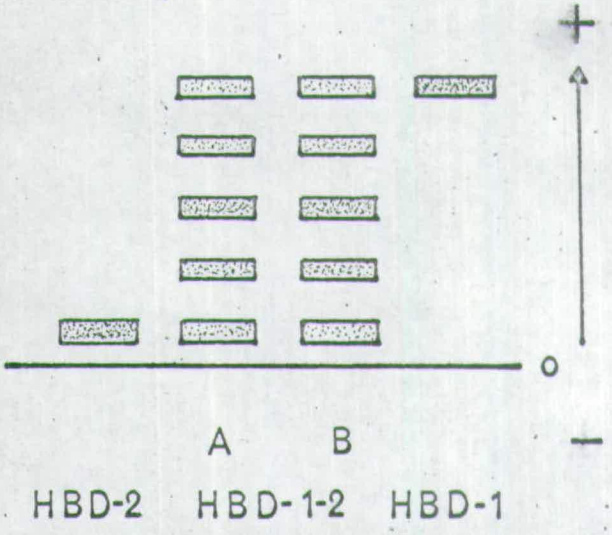
The results presented here for the inheritance of mitochondrial isocitrate dehydrogenase confirm the current view that the soluble proteins of the mitochondria are controlled by nuclear genes. The lack of close linkage between mitochondrial and cytoplasmic forms of this enzyme provide genetic evidence for the separate control of these two forms, a view which has been held for some time on the basis of immunological differences between the two forms. Although conclusive evidence for the sub-unit structure of mitochondrial isocitrate dehydrogenase has not been obtained, circumstantial evidence suggests that it is a dimer, each polypeptide being controlled by a different locus. Further evidence would be required to establish conclusively that this enzyme is a dimer and a variant at the polypeptide A locus would provide firm evidence for the existence of two loci determining the structure of this enzyme.

Part III. 3-hydroxybutyrate dehydrogenase.

The enzyme 3-hydroxybutyrate dehydrogenase has been shown by cell fractionation to be located exclusively in the mitochondrion. This study was undertaken to determine whether this enzyme was controlled by a nuclear gene or by cytoplasmic determinants, and to gain information of the sub-unit structure of this enzyme.

A





B

HBD-1	HBD-2	TOTAL
24	34	58

$\chi^2 = 1.72$ (not significant at the 5% level)

FIGURE 8 Genetic control of 3-hydroxybutyrate dehydrogenase.

A. Starch gel zymogram (photograph and diagram) of the two types of 3-hydroxybutyrate dehydrogenase (HBD-1 and HBD-2) found amoung stocks of Syngen 9. Also shown are the heterozygote patterns of both ex-conjugant clones from a cross between animals of different enzyme type (HBD-1-2,A and B).

B. Table showing the segregation,into the two parental types,of an F_2 obtained by passing an F_1,of the type shown in A.,through autogamy.

1. Variant forms and genetic control.

Electrophoretic variants of this enzyme have been detected in stocks of syngens 2, 4 and 9, although the variant in syngen 4 has not been studied. The results for syngen 9 (Tait 1968) are described here and those for syngen 2 in Part V (p.45). Two forms of the enzyme were detected in isolates made from a natural population and have been designated HBD-1 and HBD-2 respectively (Fig. 8(A)). The two forms appear as single bands with 1-2 minor bands migrating slightly further towards the anode in each case. The nature of these minor bands is not known.

Clones of the two different enzyme types were crossed and heterozygotes deriving cytoplasm from either parent were shown to be identical when subjected to starch gel electrophoresis (Fig. 8(A) HBD 1-2A and B). These heterozygotes were passed through autogamy and the resulting F_2 scored by starch gel electrophoresis for the two parental types. The segregation was not significantly different from a 1:1 ration as shown in Fig. 8(B). This result together with the identical patterns of F_1 clones deriving cytoplasm from either parent, indicates that this enzyme is controlled by nuclear genes which segregate in the expected Mendelian fashion.

2. Sub-unit structure.

The five banded pattern observed in the heterozygote (HBD1-2) can be explained if the enzyme is a tetramer of identical sub-units (Shaw 1964). Thus if HBD-1 consists of four sub-units A and HBD-2 of four sub-units B, the heterozygote, assuming random mixing of the polypeptides, will have five types of molecule A_4, A_3B, A_2B_2, AB_3 and B_4 which will be in the ratio 1:4:6:4:1. Initial observations of the heterozygote pattern showed almost equal intensities of the five bands; this could be explained by one of several possibilities,

namely "phenotypic drift", presence of exautogamous animals, low activity of the intermediate forms or non random association of the polypeptides.

Phenotypic drift has been observed in heterozygotes of esterase and phosphatase variants of Tetrahymena (Allen 1968)and results in the expression of predominantly one or other parental type after several fissions of heterozygote sub-clones. The mechanism underlying this phenonema is obscure, but it seemed the most likely explanation of the observed band patterns as it had been observed in ciliates before. Heterozygotes (of paramecium) were sub-cloned at intervals of 3 days and the initial heterozygote and subsequent sub-clones were examined by starch gel electrophoresis. All sub-clones showed five bands of activity and the initial sub-clones showed ratios of intensity of the five bands which approximated to a 1:4:6:4:1 ratio. The increased intensity of the parental bands in subsequent sub-clones was correlated with an increased percentage of autogamy and therefore formation of a proportion of homozygotes, thus accounting for the initial observation.

3. Conclusion.

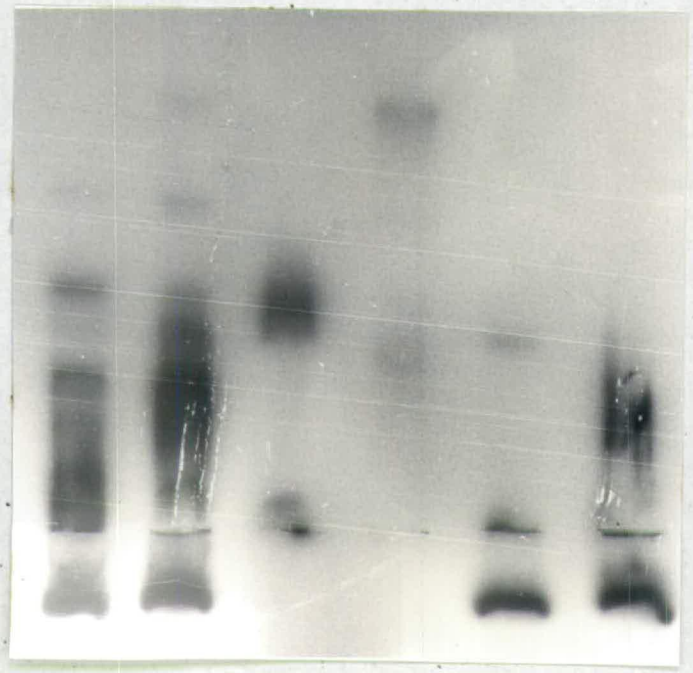
The results presented here have shown that 3-hydroxybutyrate dehydrogenase is controlled by nuclear genes and that the enzyme consists of four sub-units of identical charge. The relative staining intensities of the five bands observed in the heterozygote have been shown to agree with the intensities expected if random association of the two different polypeptides occurred. The initial observation of equal intensities of these bands has been shown to be due to some of the heterozygotes passing though autogamy.

Part IV. Malate dehydrogenase.

Malate dehydrogenase was studied with the object of investigating the genetic relationship between the supernatant and mitochondrial forms of this enzyme. On the basis of immunological studies and amino acid composition of the mammalian enzyme (Thorne 1960; Grimm & Doherty 1961), it has been suggested, though without any direct genetic evidence, that the supernatant and mitochondrial forms are under independent genetic control. For a study of this type, genetic variants are required and in the present study were obtained by screening for electrophoretic variants in stocks of the same syngen. It was originally hoped that variants of the mitochondrial form of this enzyme could be obtained, so that information about its sub-unit structure and genetic control could be gained. With these objectives in mind, the electrophoretic pattern of malate dehydrogenase was examined and a large number of stocks screened for variants.

1. Zones of activity.

The pattern observed after starch gel electrophoresis of paramecium extracts in 0.01M phosphate-citrate buffer pH = 7.0 has been described in Part I (Fig. 3). Three zones of activity were observed, a cathodal smear, a sharp anodal band and 2-3 faster moving anodal bands. Preparations of nuclear, mitochondrial and supernatant fractions were made and extracts from these subjected to starch gel electrophoresis. This experiment showed that the cathodal smear (designated Mm) was mitochondrial, the intermediate anodal band (designated Mc) occurred in the supernatant fraction and the group of rapidly migrating anodal bands (designated M^A) were derived from a cell fraction which



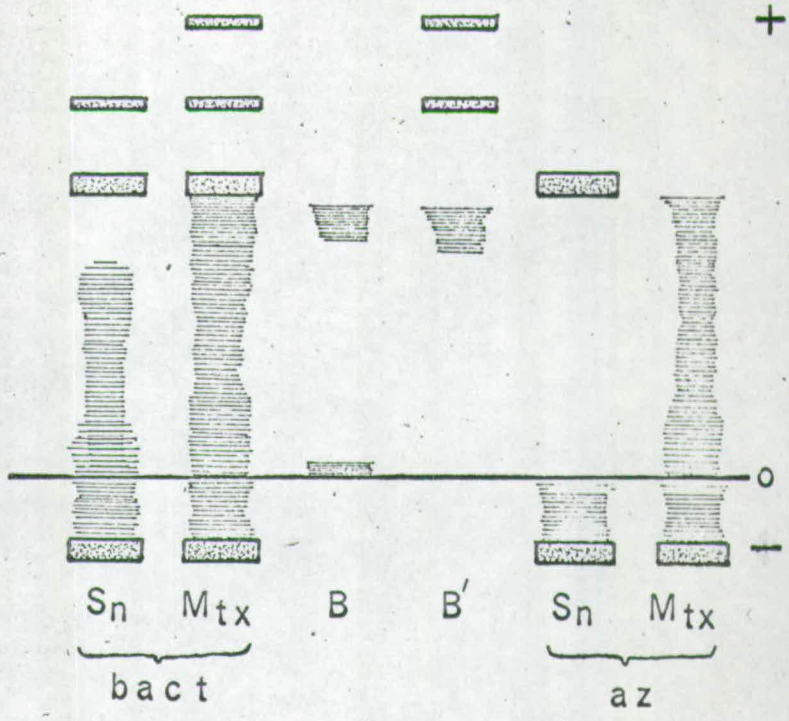


FIGURE 9 Zones of Malate Dehydrogenase activity due to Bacteria.

The photograph and diagram show the starch gel electrophoresis of various extracts, stained for malate dehydrogenase(for electrophoresis conditions see Table 2).

Key to Symbols:

S_n - 75,000g supernatant from a homogenate of paramecia.

M_{tx} - 1% Triton X 100 extract of the 75,000g pellet.

B - homogenate of bacteria spun down from a culture after removal of the paramecium.

B - homogenate of bacteria from a bacterial culture used to feed paramecium.

Bact - extracts from paramecium cultured on bacteria.

az - extracts from azenically grown paramecium.

was particulate. The M^A bands varied in mobility from one preparation to another, sometimes being absent altogether. In the cell fractionation studies these bands were found not to be consistently associated with any one cell component, although they were usually in the mitochondrial fraction when this was prepared by differential centrifugation.

Several pieces of evidence suggest these bands (M^A) originate from bacteria present in the culture medium. Repeated crude fractionation of paramecium homgenates into supernatant and mitochondrial fractions showed that M^A when present, was associated with the mitochondrial fraction, which could contain bacteria. Bacterial extracts prepared in the same way as paramecium extracts showed the presence of bands of malate dehydrogenase activity and in one such preparation the zones of activity were of identical mobility to M^A (Fig. 9). Crosses of stocks of paramecium exhibiting M^A of differing mobility showed that this band was not associated with any genic variation in paramecium. All these findings together with the absence of M^A in axenically grown cultures, suggest but do not prove conclusively that M^A originates from bacteria present in the culture medium.

2. Resolution of the mitochondrial form.

Before screening stocks for variation in mitochondrial malate dehydrogenase, attempts were made to resolve the cathodal smear into discrete bands so that variation might be detected more readily. Alterations in the pH and buffer systems did not produce any improved resolution, as described in Part I and so the following explanations for the pattern observed were considered:-

(1) there was absorbtion of the enzyme onto other proteins migrating towards the cathode.

(2) there was incomplete extraction from the mitochondria so that the enzyme was still attached to fragments of the mitochondrial membrane which differ in charge.

(3) there was binding of small molecules such as sialic acid residues or coenzymes.

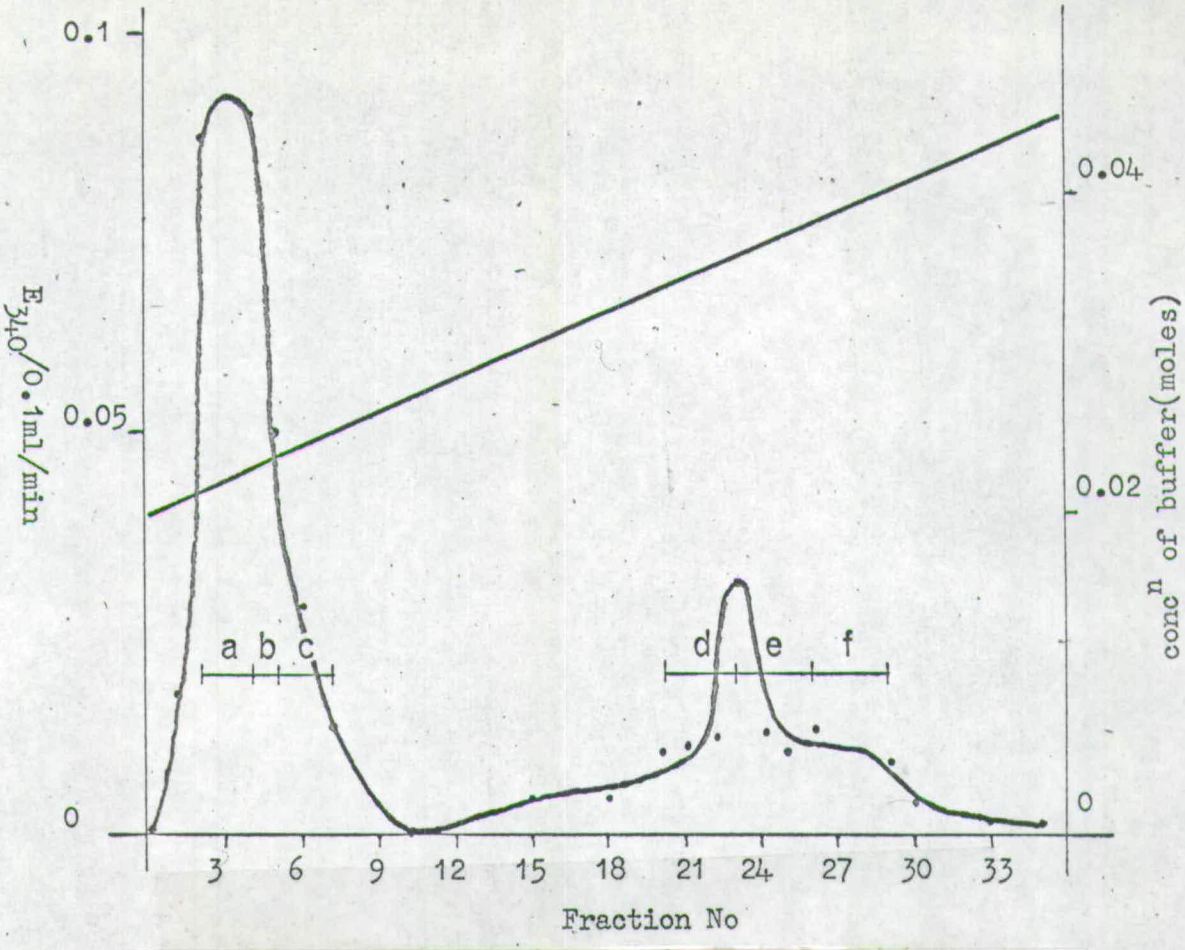
(4) there were various oxidation states of the -SH groups (known to be present in the mammalian enzyme) resulting in a series of enzyme molecules of differing charge.

(5) the smear was due to a basic property of the enzyme molecule.

Experiments were undertaken to test whether any of these possibilities occurred, so that treatments could be developed to allow resolution into discrete bands.

Purification of the crude paramecium extracts was undertaken using both Sephadex G.100 and DEAE-cellulose to remove other proteins which might be migrating with the enzyme. The effluent from the columns was assayed for activity and the active fractions concentrated against polyethylene glycol and then run on starch gels. In the case of DEAE-cellulose, two peaks of activity were detected (Fig. 10) and the fractions shown were run on starch gels; the supernatant and mitochondrial forms were separated, but no improved resolution of the mitochondrial form was observed. A single broad peak of activity was observed using Sephadex G.100, and fractions concentrated from this peak showed no improved resolution on starch gel electrophoresis . These two results suggest that the smeared pattern of the enzyme is not due to its absorbtion onto other proteins.

Two experiments were undertaken to examine the possibility that the enzyme was attached to small membrane fragments. Firstly the crude mitochondrial fraction was treated with 1% deoxycholate and 5% triton X.100 (separately) and the resulting extracts were subjected to starch gel electrophoresis, but no further resolution was observed. Secondly, homogenates were centrifuged



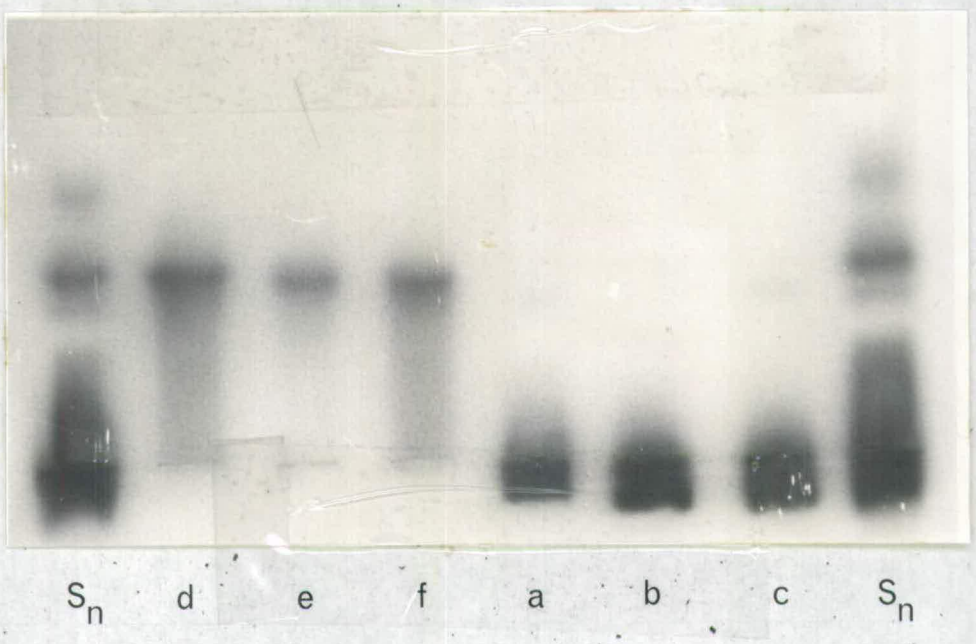


FIGURE 10 DEAE-cellulose chromatography of malate dehydrogenase

The graph shows the fractionation of malate dehydrogenase from a homogenate. The continuous line shows the profile of malate dehydrogenase in the column effluent after elution with a linear gradient of increasing phosphate buffer concentration (for details see Materials and Methods).

The photograph shows the electrophoretic patterns obtained when the fractions a,b,c,d,e and f were run on starch gels.These fractions were obtained by concentrating,against ethylene glycol,the various fractions shown on the graph. These results were obtained using a homogenate of stock 513 (Syngen 1). For electrophoretic buffer systems and assay solutions see Table 2.

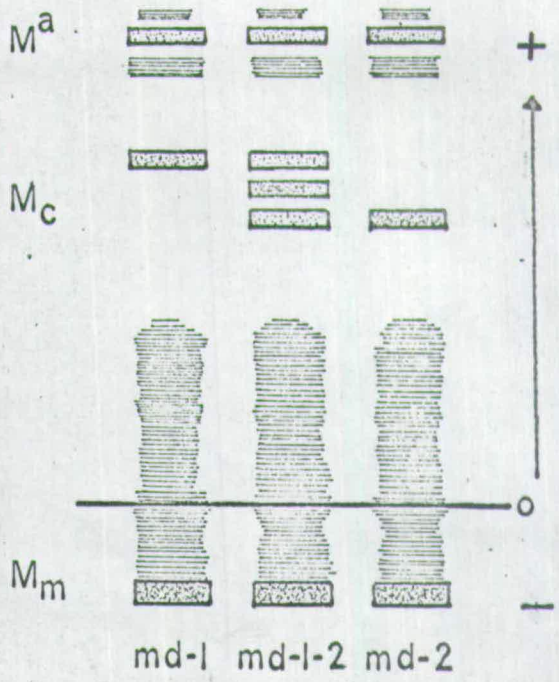
for 1 hr at 120,000g which would sediment any fragments of the same order of size as ribosomes. On electrophoresis of the supernatant from such a run, no further resolution was observed. Although these experiments are not absolutely conclusive, if the smear is due to binding of mitochondrial fragments, these must be very small and bound very firmly to the enzyme.

Dialysis of paramecium extracts against the homogenisation buffer for 12 hrs. at 4°C were undertaken to test whether any loosely bound, small molecules were involved in the smearing. Comparison of dialysed and undialysed extracts by starch gel electrophoresis showed that these extracts were identical. This result indicated that if the lack of resolution was due to the binding of some small molecule, then these molecules were firmly bound to the enzyme.

The binding of NAD to alcohol dehydrogenase has been shown to cause isoenzyme formation (Jacobson & Murphy 1967, Jacobson 1968) and a similar possibility was considered for malate dehydrogenase. Addition of NAD (0.01M final concentration) to extracts for 24 hrs. at 4°C prior to electrophoresis produced no change in electrophoretic pattern, although with alcohol dehydrogenase this concentration did produce an effect. Neuraminidase is known to change the electrophoretic pattern of phosphatases (Butterworth & Moss 1966, Ghosh & Fishman 1969). Incubation of paramecium extracts (with this enzyme) at two different pH's (5.5 and 7.4) at 37°C produced no change in pattern. The possibility of different oxidation states of the -SH groups in the protein was examined by pretreating extracts with a range (100mM, 50mM, 20mM & 10mM) of mercaptoethanol concentrations for 24 and 48 hours prior to electrophoresis, but no change in pattern was observed with any of these treatments.

These negative results suggest that the cathodal smear may be due to

a.



b.

	MD-1	MD-1-2	TOTAL	χ^2
Backcross				
	38	41	79	0.114

	MD-1	MD-2	TOTAL	χ^2
Autogamy				
	22	32	54	1.86

FIGURE 11. Genetic control of supernatant malate dehdrogenase

a. Zymogram of malate dehydrogenase (photograph and diagram) showing the two enzyme types (MD-1 and MD-2) found in stocks of Syngen 1. Also shown is a heterozygote (MD-1-2) obtained by crossing animals of different enzyme type.

b. This table shows the segregation of malate dehydrogenase alleles when an F_1 (MD-1-2) is either backcrossed to MD-2 or passed through autogamy. The values for χ^2 are not significant at the 5% level when the figures obtained are compared to the expected 1:1 ratio.

some structural property of the enzyme molecule such as conformational isomerism or to some other factor not considered here. A similar lack of resolution of malate dehydrogenase has been observed in Tetrahymena pynformis (Allen 1967), where neither supernatant nor mitochondrial forms could be resolved. Screening of different Paramecium stocks within the same syngen and also comparison of stocks from all 14 syngens (of Paramecium aurelia) have shown that no variation in the pattern (of mitochondrial MDH) occurs and so no genetic studies have been possible.

3. Genetics and sub-unit structure of the supernatant form.

Stocks of syngen 1 isolated from different parts of the world were screened for variation in electrophoretic mobility of the supernatant band (M_c) of malate dehydrogenase. Two forms were found and have been designated MD_c-1 and MD_c-2 (Fig. 11a). Clones of the two types were crossed and the resulting heterozygote (MD_c1-2) is shown in Fig. 11a. Heterozygotes of the type shown (MD_c1-2) were backcrossed to one of the parental types and, in a separate experiment, passed through autogamy to obtain two sets of F_2's. The segregation of the alleles controlling this enzyme is in accordance with Mendelian inheritances as shown by the figures in Fig. 11(b).

The heterozygote (MD_c1-2 Fig. 11(a)) shows three bands of activity consisting of the two parental bands with a band of intermediate mobility. The intensity of staining of the bands approximately to a ratio of 1:2:1 (the intermediate band being the most intense). If it is assumed that the activity is proportional to the amount of enzyme present the banding pattern can be explained if malate dehydrogenase is a dimer. Thus, if MD_c-1 consists of two identical sub-units A, and MD_c-2 of two identical sub-units B, then the

heterozygote (MD_c 1-2) will have three molecular species AA, AB and BB. This accounts for the three banded pattern and for the ratio of intensities observed.

4. Conclusion.

The study of the interrelationship between the mitochondrial and supernatant forms of malate dehydrogenase and the structure of the mitochondrial form has been unsuccessful owing to the lack of resolution or variation of the latter form. Many attempts have been made to resolve the mitochondrial form but have, in all cases, failed. The third zone of activity (M^A) has been shown to probably be bacterial in origin, although this is not certain. The genetics and sub-unit structure of the supernatant form (M_c) have been elucidated and illustrate the ease with which such studies can be made using paramecium as an experimental organism.

Part V. Independence of genic loci for iso-citrate dehydrogenase and 3-hydroxybutyrate dehydrogenase.

Variant froms of both isocitrate dehydrogenase and 3-hydroxybutyrate dehydrogenase have been found in syngen 2 and provide material for studying any linkage between these two enzymes. Linkage has previously been found only once in paramecium (Beisson and Rossignol 1969). It is worth noting that the two enzymes studied are bound to the mitochondrial membrane and it was thought that this similar localisation might be reflected in the genotype.

A clone showing the common 3-hydroxybutyrate dehydrogenase phenotype, but the variant mitochondrial iso-citrate dehydrogenase phenotype was crossed with a clone showing the variant 3-hydroxybutyrate dehydrogenase phenotype, but the common mitochondrial isocitrate dehydrogenase phenotype. The resulting

ENZYME	ICD_M-1	ICD_M-2	TOTAL
HBD-1	17	21	38
HBD-2	8	11	19
TOTAL	25	32	57

ICD_M χ^2 = 0.86 not significant at the 5% level.

HBD χ^2 = 6.34 not significant at the 1% level.

TABLE 5:

Segregation of the alleles for 3-hydroxybutyrate dehydrogenase (HBD) and iso-citrate dehydrogenase (ICD) obtained by passing an F_1 (heterozygous for alleles at both loci) through autogamy.

Values of χ^2 are given for the expected 1:1 ratio. HBD-2 and ICD_M-1 are the common forms and HBD-1 and ICD_M-2 are the variant forms of these enzymes in syngen 2.

heterozygote was passed through autogamy to obtain an F_2 which was screened for the four parental phenotypes. The results of this experiment are shown in Table 5 and no close linkage is evident. The figures for the segregation of 3-hydroxybutyrate alleles are in the ration 2:1, the common form being the least frequent. Application of the χ^2 test to this segregation shows that these figures do not differ significantly from a 1:1 ration at the 1% level of significance. This is in contrast to the segregation of iso-citrate dehydrogenase alleles which gives a much lower value of χ^2 which is not significantly different from a 1:1 ration at the 5% level. It is possible that the different segregation ratios obtained are due to sampling errors. The segregation of 3-hydroxybutyrate dehydrogenase alleles in syngen 9 (Part III) were shown not to differ from a 1:1 ration at the 5% significance level.

Part VI. Enzyme polymorphism.

Populations in which more than a single allele at a structural locus occur, each allele occurring relatively frequently, are referred to as polymorphic. In this study the structural loci examined all determine certain enzymes. The different alleles, when they occur, have been studied both in natural populations and in single isolates from different widely separated populations. Natural populations were studied in order to establish the existence of enzyme polymorphism and to measure the frequency of the different alleles found in separate populations. In the study of small samples from isolated populations, stocks which had been isolated from different parts of the world were compared to examine whether different alleles

occurred in different, widely separated populations. It is not known to what extent mixing occurs between populations of paramecium and both these studies could provide information on this point.

The initial observations on stocks, allegedly belonging to the same syngen, and originating from different parts of the world showed considerable variation in enzyme structure. On detailed examination, using the test for mating reaction, many of the so called variants were found to be stocks of syngens other than that under study. This suggested the possibility of distinguishing syngens on the basis of the electrophoretic mobility of their enzymes. The practical value of this would be considerable and such a study could also provide information about the evolutionary and taxonomic relationships between different syngens.

1. Enzyme variation in two natural populations of syngen 9.

Two natural populations were studied both located about 10 miles from Edinburgh and within 500 yards of each other, one being a lake in a disused quarry (Humbie) and the other a natural loch (Dundas). There was no connection by water between the two populations. They therefore provide a useful system for comparing two populations and the evidence of any movement of paramecium between them. Single isolates of paramecium from each location were collected and screened by starch gel electrophoresis for glucose-6-phosphate, malate, isocitrate (NADP) and 3-hydroxybutyrate dehydrogenases by the methods previously described. In any population several syngens are usually found. In these populations syngens 1, 2 and 9 were identified and in addition a few members of an unidentified syngen. The syngens were identified by using the test for mating reaction with standard stocks of

known syngen. It was found that syngen 9 predominated and so results are given for this syngen only.

A total of 53 clones, each derived from a single separate isolate, from Dundas and 40 clones from Humbie were screened. No variation was found in isocitrate, malate or glucose-6-phosphate dehydrogenases. The Humbie population was found to be polymorphic for 3-hydroxybutyrate dehydrogenase, two forms of this enzyme being detected (HBD-1 and HBD-2 Fig. 12(a)); HBD-1 was found to predominate, 90% of the isolates being of this type. In contrast HBD-1 was not detected at all in the Dundas isolates, these being exclusively of the HBD-2 type. The numbers of individuals examined in this study were small but indicate that variants of iso-citrate, malate and glucose-6-phosphate dehydrogenases would, if present, occur with a relatively low frequency. The interesting feature of these results is the great difference between the two populations as regards 3-hydroxybutyrate dehydrogenase despite their proximity. The low frequency of HBD-2 in Humbie could represent a mixing of the populations, but one would expect to find a low frequency of HBD-1 in Dundas, unless the mixing is in one direction only or HBD-1 has a very low survival rate in Dundas. As the effect of the different forms on the whole organism is unknown, it is impossible to state definitely whether the difference between the populations occurred by chance or is the result of different selective forces in each location. These questions require further investigation before any firm conclusions can be drawn, but could provide a useful system for an experimental approach to understanding the difference.

2. Intrasyngen variations in samples from widely separated populations of syngens 1, 2 and 4.

It is not known whether screening single stocks isolated from

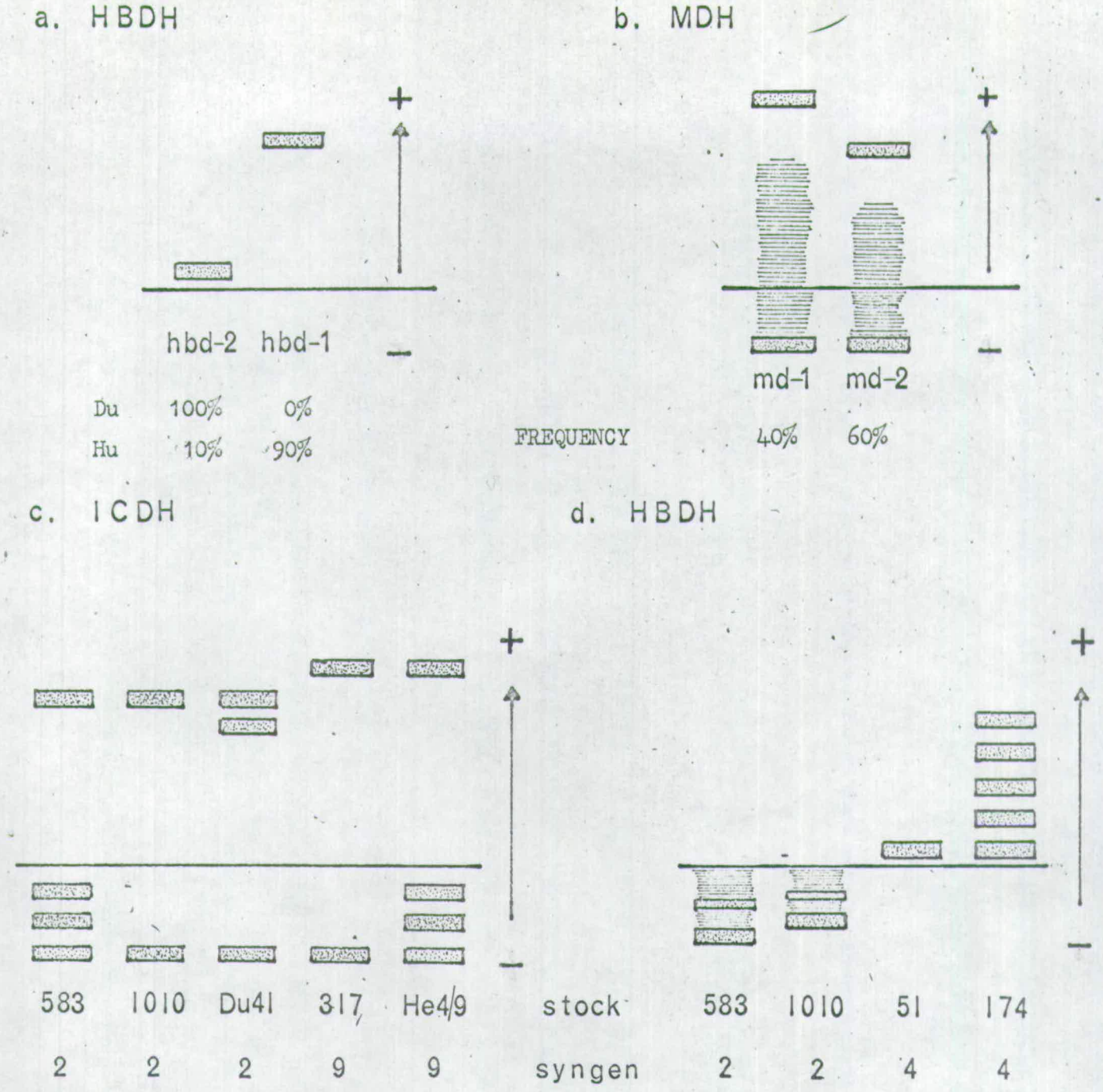


FIGURE 12 Enzyme variants

a. The two types of 3-hydroxybutyrate dehydrogenase (HBDH) found in a natural population of Syngen 9 are shown. The frequency of each allele in the Humbie (Hu) and Dundas (Du) populations are recorded.

b. The two types of malate dehydrogenase (MDH) found when stocks of Syngen 1, from all over the world, are compared. The frequency of each allele is also shown.

c. The variant forms of iso-citrate dehydrogenase (ICDH) found when stocks from different populations of the same syngen are compared. The common form in each syngen are shown by stock 1010(Syngen 2) and stock 317(Syngen 9).

d. The variant forms of 3-hydroxybutyrate dehydrogenase found when stocks from different populations of Syngens 2 and 4 are compared. The common forms in each syngen are shown by stock 583(Syngen 2) and stock 51 (Syngen 4)

separate populations all over the world increases the likeliehood of detecting variants but one might expect to find greater differences in a survey of this nature than among samples from a single population. This study could also provide information on the differences between populations and whether any movement of paramecium occurred between them. The enzymes succinic, isocitrate, glutamate, malate and 3-hydroxybutyrate dehydrogenases together with fumarase were examined for variation. This could not be done with syngen 9, as this syngen has only been found in W. Europe, but syngens 1 and 2 are found all over the world.

A total of 22 stocks of syngen 1 were screened for variation in malate dehydrogenase and 90 stocks for the other five enzymes mentioned above. The supernatant form was found to occur in two forms as described in Part IV. The patterns observed on starch gel electrophoresis are shown in Fig. 12(b) together with the frequencies of each type found. Both types have a wide distribution, MD-1 is found in stocks originating from U.S.A., France, Chile, Hungary, Japan and U.S.S.R., and MD-2 from U.S.A., Peru, Australia, Mexico and Japan. No variation was found in the other five enzymes examined.

110 stocks from syngen 2 were screened for all enzymes mentioned above except malate dehydrogenase; a total of three types of variant were found. Variants of both mitochondrial and supernatant forms of isocitrate dehydrogenase were detected (Fig. 12(c)) in single separate stocks, also a variant of 3-hydroxybutyrate dehydrogenase (Fig.12(d)) was found in two stocks from the same location. Only six stocks of syngen 4 have been screened for variation in the same enzymes as those used in the syngen 2 survey. A

FIGURE 13 Intersyngen enzyme variation

The electrophoretic patterns of iso-citrate dehydrogenase (ICDH), fumarase (FUM), 3-hydroxybutyrate dehydrogenase (HBDH), and glutamate dehydrogenase (GDH) from sample stocks of all fourteen syngens are shown diagramatically. The electrophoresis buffers and enzyme staining solutions are as described in Table 2.

variant of 3-hydroxybutyrate dehydrogenase has been detected which shows a five banded pattern (Fig. 12(d)) but no genetic studies have been undertaken.

Although more variants have been found in syngen 2 than syngen 1 the frequency of variation is still very low. Due to the small number of stocks examined in syngen 4, it cannot be said whether this represents a high or a low frequency of variation, but there is no reason to believe that the frequency of variation would differ in separate syngens. The frequency of variation appears to be very low and so casts some doubt on the ability of the technique used to detect intrasyngen variation. However, variation was readily detected when intersyngen comparisons were made (see next section) and suggests that the low frequency of intrasyngen variation is real.

3. Intersyngen enzyme variation.

Stocks from each of the fourteen known syngens were screened for variation of the six enzymes studied in the intrasyngen surveys. The results (except for malate dehydrogenase) are illustrated diagrammatically in Fig. 13. Considerable variation was found in fumarase, isocitrate dehydrogenase and 3-hydroxybutyrate dehydrogenase, but very little in malate, glutamate and succinic dehydrogenases, the latter being identical for all syngens. The number of differences between one syngen and another vary considerably, but, except for syngens 1 and 5, each syngen has a unique pattern. On the basis of these differences the syngen to which a stock, isolated from the wild, belongs can be identified (except that syngen 1 and 5 cannot be distinguished by this method). This assumes that no variation of the type observed within syngens occurs between syngens.

Paramecia belonging to syngens 6 and 8 showed no 3-hydroxybutyrate dehydrogenase activity on starch gels. Stocks from these syngens were shown to posses this enzyme by spectrophotometric assay, but the specific activity was reduced by a factor of 2 when compared to stocks from syngen 1. Assays performed at the pH used for electrophoresis showed that there was almost negligible activity at this pH. Although intrasyngen variants have been detected for this enzyme in syngens 2, 4 and 9, the patterns shown by these variants do not resemble any of the forms seen in the intersyngen comparison. The frequency of variation has been found to be low in both syngens 1 and 2, and as already mentioned probably in syngen 4. Comparison of stocks of syngen 9 from different populations has shown that the allele found in high frequency in Humbie is not found elsewhere suggesting that the frequency of variation between populations is low. As no variants have been detected in syngen 1 and only one in syngen 2 a low frequency of intrasyngen variation is suggested.

Two variants of mitochondrial isocitrate dehydrogenase were detected in syngen 2 and 9 together with a variant of the cytoplasmic form of the enzyme in syngen 2 (Fig. 12(c)). As with the intrasyngen variation of 3-hydroxybutyrate dehydrogenase, the type of variant pattern does not resemble any of the forms seen in the intersyngen comparison. No variants have been found in 90 stocks of syngen 1, only 2 in 110 stocks of syngen 2 and no variants in the syngen 9 populations examined, although 2 variants have been found in stocks from different parts of the world. The low numbers of stocks examined in the other syngens (Table 6) show no variation.

SYNGEN		NO. OF STOCKS	NO. OF VARIANTS
1		90	-
2		110	3 (HBD, 2ICDH)
3		3	-
4		6	1 (HBD)
5		2	-
6		3	-
7		3	-
8		3	-
9 WITHIN POPULATIONS	A	53	-
	B	40	36 (HBD)
BETWEEN POPULATION		6	2 (HBD, ICDH)
10		1	-
11		2	-
12		1	-
13		3	-
14		1	-

TABLE 6:

The number of stocks of each syngen examined for variation in succinic dehydrogenase, glutamate dehydrogenase, 3-hydroxybutyrate dehydrogenase, (HBDH, isocitrate dehydrogenase (ICD) and fumarase. Only three stocks of syngen 13 and one stock of syngen 14 are known. The third column records the number of variants found in each syngen and (in brackets) the variant enzymes.

N.B. The numbers of stocks examined within the two natural populations were not screened for glutamate dehydrogenase, succinate dehydrogenase or fumarase.
A - Dundas; B - Humbie.

The low frequency of intrasyngen variation (Table 6) shows that the enzyme patterns in Fig. 13 are typical for each syngen. Taken together with the type of intrasyngen variation observed, it is possible to identify the syngen to which any stock belongs by examining the electrophoretic pattern of the four enzymes in Fig. 13. In order to test this possibility a series of stocks of unknown syngen were screened for enzyme mobility and on this basis classified into syngens. This classification was then checked by testing for mating reaction and in nearly all cases shown to be correct. The results of this study are shown in Table 7, together with the classification of certain stocks which had previously been wrongly labelled. This table and the method of identification is best explained by taking an example; an extract of THI 2/3 was examined by electrophoresis, together with extracts from stocks of the various syngens. For each enzyme in Table 7 the syngens with identical enzyme mobility to THI 2/3 were recorded and then the syngen with all enzyme mobilities identical to THI 2/3 was underlined and concluded as the syngen to which this stock belonged. Two features of this table require comment, firstly the identification of the syngen 9 variants (He 2/8, He 4/9, Pisa 7A and Bl 205/2) and secondly the identification of a new syngen (X, Stock Du 79/1). The variants of syngen 9 resemble this syngen in all enzymes studied except mitochondrial isocitrate dehydrogenase/which was of a mobility unlike any other syngen. The stock Du 79/1, designated syngen X, is also identical to syngen 9 except for mitochondrial isocitrate dehydrogenase, but this enzyme differs from the variants of syngen 9 in having a mobility identical to syngens 4, 10 and 11. These results have been checked by carrying out tests for mating reaction and shown to be correct, no mating reaction being obtained with Du 79/1. This

STOCK	ICDH	FUMARASE	3-OHbutDH	SYNGEN ENZYME mobility	SYNGEN using mating
153/183	6	1,5,6,9,11,14	6,8	6(1)	6
Du79/1	M 4,10,12 S 2,3	1,5,6,9,11,14	4,9,11	X	no reactions
38	6	1,5,6,9,11,14	6,8	6(7)	6
Hun-5	2,3	2,3,7	2,3	2 or 3(1)	2
578	2,3	2,3,7	2,3	2 or 3(1)	2
583	M-N S-2,3	2,3,7	2,3	2 or 3(1)	2
THI2/3	1,5,7,8,9	1,5,6,9,11,14	4,9,11	9	9
SED-2	1,5,7,8,9	1,5,6,9,11,14	4,9,11	9	9
Fi99/4	1,5,7,8,9	1,5,6,9,11,14	4,9,11	9	9
He2/8	M-N S-1,5,7,8,9	1,5,6,9,11,14	4,9,11	9v	9
He4/9	M-N S-1,5,7,8,9	1,5,6,9,11,14	4,9,11	9v	9
B1205/2	M-N S-1,5,7,8,9	1,5,6,9,11,14	4,9,11	9v	9
Pisa-7a	M-N S 1,5,7,8,9	1,5,6,9,11,14	4,9,11	9v	9
32	6	1,5,6,9,11,14	6,8	6(4)	6

TABLE 7:

Identification of the syngen to which unidentified or wrongly labelled stocks belong, using enzyme mobilities. The syngens with enzymes of identical mobilities to those of the stock being examined are listed under each column headed by an enzyme. The syngens underlined in each of these columns are those common to all three enzymes for each stock. The underlined syngen is recorded in the column headed syngen (enzyme mobility). The figures in brackets in the latter column refer to the syngen which the stock had originally been labelled. The last column indicates whether the syngen classification has been confirmed by mating reaction.

Key to symbols: M-mitochondrial; S-supernatant; N-new form of enzyme unlike any found in other syngens; X-new syngen which does not conjugate with any syngens tested; ICDM-iso-citrate dehydrogenase; 3-OHbutDH-3-hydroxybutyrate dehydrogenase.

result brings out an important distinction between the banding pattern of a variant stock of a given syngen and that of a new syngen. The variant shows a banding pattern in one enzyme which is not like the patterns observed in the common stocks of any syngen, whereas the new syngen shows a different combination of the bands already exhibited by the other syngens. This distinction is however based on one example and therefore may not be valid in general.

The results of running stocks from all fourteen syngens for malate dehydrogenase are shown in Fig. 14. In comparison with the other enzymes studied, this enzyme shows less intersyngen variability. The cytoplasmic form of this enzyme has been shown to be polymorphic in the intrasyngen study of syngen 1 to a much greater extent than the other enzymes. As the intra-syngen variability has only been examined in one syngen this finding cannot be considered general without further screening within several other syngens.

4. Conclusion.

It has been shown that enzyme polymorphism occurs in a natural population of paramecium and that the frequency of the alleles is very different between two populations in close proximity. A study of stocks isolated from different parts of the world has shown that intrasyngen variants occur rarely with isocitrate dehydrogenase and 3-hydroxybutyrate but that malate dehydrogenase occurs in two forms which are widely distributed. The data on the polymorphism of malate dehydrogenase between populations, suggests that one population can mix with another unless one assumes that the two alleles were produced in different populations entirely independently. The more detailed study of 3-hydroxybutyrate dehydrogenase does not give an unequivocal

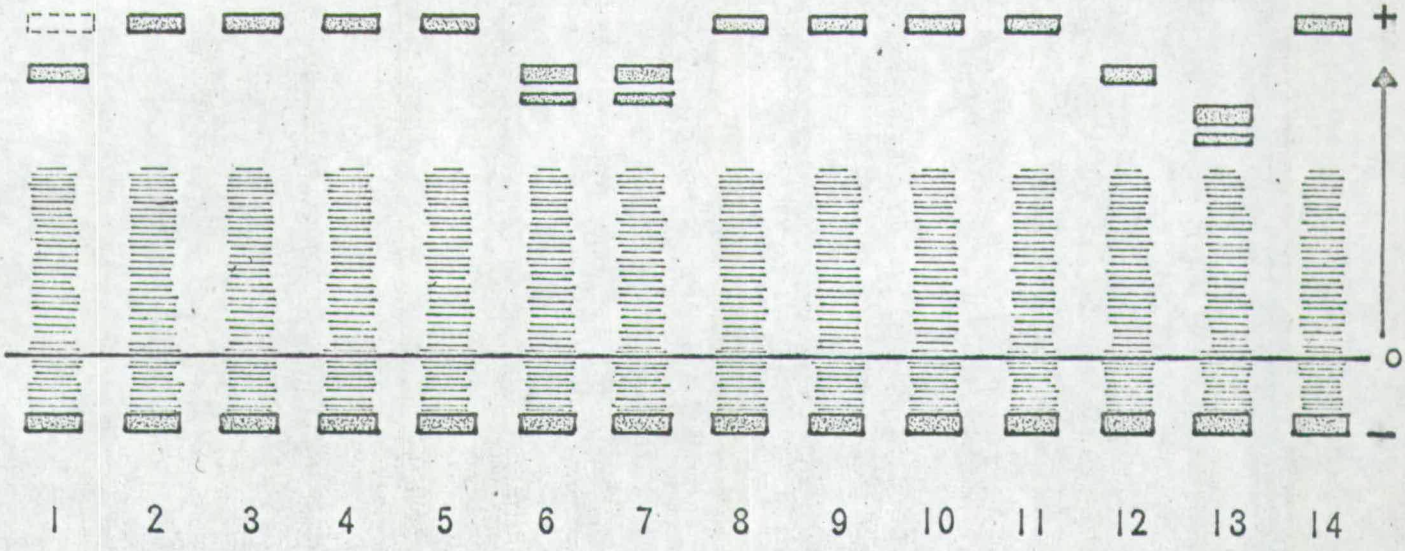


FIGURE 14 Intersyngen malate dehydrogenase variation

The electrophoretic pattern of malate dehydrogenase from sample stocks of all fourteen syngens is shown diagramatically. The dotted band for Syngen 1 shows the position of the variant form which was found in slightly lower frequency. The electrophoresis buffers and enzyme staining solution is that described in Table 2.

answer to the question of the extent of mixing between populations, but provides a system for its study.

The study of intersyngen enzyme differences has shown that considerable differences occur and that each syngen, except for syngens 1 and 5, has a unique pattern. This is of considerable practical value in the identification of stocks isolated from the wild. The identification of a syngen by mating reaction is laborious as one must have representative stocks (of both mating types) of all fourteen syngens in a reactive state. Classification by enzyme mobility may also have considerable applications for the classification of other protozoa into sub species, particularly where mating is difficult or impossible. If these differences were studied in more detail (by peptide mapping and amino acid analysis) information could be gained about the evolutionary relationships of the syngens and the enzymes themselves.

DISCUSSION.

The aim of this work was to investigate the genetic control of certain enzymes and isoenzymes in Paramecium aurelia. It has been established that variation in electrophoretic mobility of certain enzymes occurs between stocks within natural natural populations of one syngen, in different natural populations of one syngen and in different syngens. The data on the first two types of variation are scanty compared to similar data obtained with populations of other organisms (Harris 1969; Stone et al 1968) and so do not allow much discussion on the implication of these findings. Consequently this discussion has been limited to three specific topics: isoenzymes, genetics of mitochondrial proteins and intersyngen enzyme variation. The results obtained and the kind of conclusions which may be drawn from the experimental approaches used in this study will be considered in relation to work of a similar nature carried out with other organisms.

1. Isoenzymes:

The term isoenzyme is a very broad and ill-defined one and numerous classifications of different types of isoenzyme have been made on the basis of a few examples (Wilkinson 1965; Kaplan 1968). Different forms of an enzyme which are observed when different organisms or different tissues are compared will not be considered here, but only the multiple forms occurring in one cell type. This allows a broad division of isoenzymes into two classes: (i) those enzymes exhibiting multiple forms due to genetic causes and (ii) those enzymes exhibiting multiple forms due to non-genetic causes. The first class can be illustrated by considering lactate dehydrogenase and iso-citrate

dehydrogenase. The multiple bands of lactate dehydrogenase are due to the random association of two polypeptides, specified by different loci, into tetramers (Shaw & Barto 1963). The two zones of iso-citrate dehydrogenase (mitochondrial and supernatant) have been shown, in this study, to be coded for by separate loci specifying each form. Thus in both cases the multiple banding is a consequence of the presence of more than one locus affecting the enzyme. The second class consists of isoenzymes formed by the interaction of a single protein with small molecules (sialic acid, coenzymes, substrates, etc.) or with proteolytic enzymes; or by a single protein existing in more than one conformation. These two classes are most probably found in paramecium and research has been directed towards providing a better understanding of them. A genetic approach has been used, in this study, as it helps to enable the relationship between different forms of an enzyme to be established, as well as determining the sub-unit structure of the enzymes. The enzymes iso-citrate dehydrogenase, malate dehydrogenase and to a lesser extent, 3-hydroxybutyrate dehydrogenase have been studied in detail in this respect.

The occurrence of two forms of several enzymes which are localised in the mitochondrial and supernatant fractions respectively, has been described e.g. malate dehydrogenase (Thorne & Cooper 1964; Grimm & Doherty 1961), as partate amino transferase (Borst & Peters 1961; Eichel and Bukovsky 1961) and iso-citrate dehydrogenase (Henderson 1965). The question has been posed (Henderson 1968) as to whether the two forms are different proteins under independent genetic control or whether they are due to modification of one protein, which results from, or gives rise to, the differences in sub-cellular localisation. Considerable biochemical and immunological work has been undertaken with both

malate dehydrogenase (Seigel & Enghard 1962; Enghard et al 1961) and iso-citrate dehydrogenase (Kornberg & Pricer 1951; Lowenstein & Smith 1962; Bell & Baron 1964). These investigations have shown that the different forms of these two enzymes show considerable difference in electrophoretic mobility, catalytic properties, immunological specificity and, in the case of malate dehydrogenase, in the peptide maps of the two forms. From such evidence, the different forms of these two enzymes have been considered to be different proteins. The observation that variation in one form is not correlated with variation in the other (Henderson 1965; Davidson & Cortner 1967 (a) & (b)) again suggests that the two forms are probably under independent genetic control and are therefore different proteins. As both forms of malate dehydrogenase have been shown to be dimers (Kitto and Kaplan 1966), the possibility of supernatant and mitochondrial forms having one polypeptide in common cannot, however, be eliminated. A similar situation could also occur with iso-citrate dehydrogenase but this is not certain as only the supernatant form has been shown to be a dimer (Henderson 1965).

The results presented in this study for malate dehydrogenase show that, in paramecium, mitochondrial and supernatant forms of this enzyme occur. The lack of variation and the poor resolution of the mitochondrial form has made it impossible to carry out any studies on its structure and relationship to the supernatant form. The genetic variation shown by the supernatant form has been used to show that the latter is a dimer, as in most other organisms studied. Suggestive evidence for the independent genetic control of the two forms is provided by the lack of concomitant variation in the mitochondrial and supernatant forms.

In paramecium, iso-citrate dehydrogenase has been shown to occur in two forms, one located in the supernatant fraction and the other in the mitochondrial fraction, as previously described in mammals. The supernatant form has not been shown to be a dimer, but there is suggestive evidence that the mitochondrial forms is dimeric. In paramecium, multiple zones of iso-citrate dehydrogenase activity are observed and can conveniently be divided into three categories:

1. Two zones of activity which are located in the mitochondrial and supernatant fractions respectively.
2. Three zones of activity in a variant of the mitochondrial form as compared to a single zone in the normal type. The variant pattern is probably due to the enzyme being determined by different loci.
3. Two zones of activity in a variant of the supernatant form as compared to a single band in the normal type. Although the double banding occurs as a result of a genetic difference in the protein, it is probably caused by interaction of the variant protein with some other molecule and thus can be referred to as non-genetic.

The genetic variants of both supernatant and mitochondrial forms have been used to show that the loci determining these two forms are not closely linked, thus confirming the suggestion by Henderson (1968) for the mouse enzyme. The electrophoretic pattern of the homozygous variant of the mitochondrial form (ICD_M-2) shows three bands and this has been explained by assuming that the enzyme is a dimer, consisting of different polypeptides. Thus the three banded pattern is thought to be due to the presence of dimers of one type of polypeptide, dimers of the other polypeptide, together with a hybrid dimer

2. Genetics of mitochondrial proteins:

A large amount of work has been undertaken on various aspects of mitochondrial autonomy (for review see Roodyn & Wilkie 1968) and this work will not be reviewed here. Only a brief summary of the main findings and a review of the various approaches which have been made towards understanding the genetic and biochemical control of the mitochondrion will be given. Before discussing these results the objectives of research on mitochondria should be defined. In my view, the object of this research is to determine the contribution of the mitochondrion to the coding of its constituent proteins and to elucidate the nuclear and cytoplasmic controls involved in this process. The research on mitochondrial inheritance can be conveniently divided into four parts:-

1. properties of isolated mitochondria, such as DNA content and DNA, RNA and protein synthesis.
2. genetics of respiratory mutants in yeast and Neurospora.
3. genetics of resistance of these organisms to the antibiotics erythromycin and chloramphenicol.
4. genetics of specific mitochondrial proteins.

These topics will be discussed largely in relation to the type of information which they can yield and in relation to the studies, reported here, on paramecium.

The idea of mitochondrial autonomy is an old one, but evidence for this idea has only been obtained in the last fifteen years. The presence of DNA in the mitochondria of a wide range of organisms (Nass, Nass and Afzelius 1965) is nowwell established. Incorporation of radioactive nucleotides into isolated mitochondria have shown that DNA synthesis occurs (Haldar, Freeman & Work 1966) and by similar techniques evidence has been found for the presence of DNA

in mitochondria from different organisms (Piko et al. 1967; Clayton & Vinograd 1967). Calculations, based on a molecular weight of 10^7 daltons, have been made for the coding capacity of such DNA (Sinclair & Stevens 1966) and a maximum of 30 proteins of molecular weight 20,000 was found. The observation that the mitochondrial RNA isolated from yeast hybridizes with the mitochondrial DNA (Wintersberger & Vierhauser 1968) suggests that the DNA codes for all the RNA of the mitochondrion and so reduces the possible number of proteins coded for even further. There are two sets of observations which make this calculation rather doubtful. Firstly it has been assumed that all molecules of DNA in the mitochondria are identical, although more than one molecule has been found in most cases and evidence has been reported for more than one type of DNA in Neurospora mitochondria (Reich & Luck 1966). Secondly in Tetrahymena and Paramecium the DNA content has been found to be larger than that in the mitochondria of other species (Suyama & Preer 1965). In a more detailed study of mitochondrial DNA in Tetrahymena (Suyama & Miura 1968) linear strands of DNA were observed, corresponding to a molecular weight of 40×10^7 daltons which would give a coding capacity for at least 100 proteins. If the coding capacity is the same in mitochondria from all organisms it must be assumed either that Tetrahymena DNA contains a large number of reiterated sequences or that each ring of DNA observed in other organisms represents a separate linkage group.

In summary, studies on protein synthesis in isolated mitochondria cannot give direct evidence concerning the control of proteins by mitochondrial DNA, but suggest that mitochondrial DNA only codes for the insoluble proteins. Calculations based on the molecular weight of mitochondrial DNA can be used to deduce the number of proteins coded for and these studies indicate that the

mitochondrial DNA can only code for some of the mitochondrial proteins. Estimations of the actual number of proteins coded for are rather dubious due to the variation between organisms, in the molecular weight of the DNA, and in the number of molecules present in each mitochondrion. Thus, although both the DNA measurements and amino acid incorporation data can yield valuable information, they do not provide unequivocal results on the contribution of the mitochondria in determining their constituent proteins.

Genetic studies of respiratory mutants in yeast (Ephrussi 1953) and Neurospora (Mitchell & Mitchell 1952) have been carried out to determine whether these characters are controlled by nuclear or cytoplasmic determinants. The 'petite' mutants of yeast are unable to respire aerobically due to the lack of various enzymes and cytochromes (Sherman & Slonimski 1964). Genetic studies have shown that some of these mutants are nuclear and some are cytoplasmic (for review see Mounlou, Jakob and Slonimski 1967). Studies of the content and properties of the mitochondrial DNA of the cytoplasmic mutants (ρ^-) have shown that the DNA is altered in buoyant density but not in content (Mounlou, Jakob and Slonimski 1966). These results suggest that the mutation is located in the mitochondrial DNA.

Both nuclear and cytoplasmic mutants of Neurospora (mi) lacking cytochromes a/a_3 and b are found (Mitchell & Mitchell 1952). No studies of mitochondrial DNA have been undertaken but microinjection of purified mutant mitochondria into wild type cells (Diacumakos et al. 1965) has indicated that these mutants are mitochondrially inherited. Therefore it seems established in both yeast and Neurospora that certain characters are determined by mitochondrial DNA and these characters could either be the cytochromes themselves or some protein determining their activity.

The proteins which are controlled by mitochondrial DNA in these mutants have not been identified. The absence of cytochromes a/a_3 and b in both nuclear and mitochondrial mutants does not make it clear whether these proteins are coded for by nuclear or mitochondrial DNA. Thus the inability to detect the primary biochemical lesion in either case does not allow one to determine which proteins are coded for by mitochondrial DNA. Evidence has been obtained that the mitochondrial structural protein in the petite cytoplasmic mutants is altered in electrophoretic mobility when compared to the corresponding structural protein in wild type and nuclear mutants (Polglase & Peel, unpublished, cited by Work 1968). Genetic analysis of this type of protein difference could be used in the future to determine whether specific proteins are coded for by mitochondrial DNA. In Neurospora it has been shown that the mitochondrial structural protein of the cytoplasmic mutants is altered (Munkres & Woodward 1967). The differences observed involve only single amino acids detected by amino acid analysis. However, in view of the low accuracy of this technique these results must be considered in some douht.

Yeast mutants resistant to chloramphenicol and erythromycin have been isolated in several laboratories and the genetics of this resistance has been studied (Wilkie, Saunders & Linane 1967; Thomas & Wilkie 1968; Slonimski, unpublished). These drugs inhibit the synthesis of cytochromes a/a_3 and b and spontaneously occurring resistant mutants can be isolated. The genetics of these mutants shows a smilar pattern to the petites, namely there are both cytoplasmic and nuclear genes determining this resistance. The cytoplasmic mutants have been shown to be mitochondrial by linking their inheritance to the p^- factor (Wilkie, Saunders and Linane 1967). Very little progress has been

made in analysing the nature of the resistance i.e. what proteins or other components of the mitochondrion are altered in the resistant strains.

The use of mitochondrial mutants, such as petites and drug resistant strains, allows the site (nuclear or mitochondrial) of the control of these mutants to be established. In contrast the study of amino acid incorporation into isolated mitochondria is unable to determine the site of the control but can be used to establish which proteins are synthesised by the mitochondrial ribosomes. The main problem of using these mitochondrial mutants is the difficulty of determining whether a protein is controlled by a nuclear structural gene and mitochondrial regulator gene or a nuclear regulator gene and a mitochondrial structural gene and so elucidate which components of the mitochondrion are determined by its DNA. Thus, neither approach can, at this time, yield complete information about the control of mitochondrial proteins, although if further biochemical research were undertaken to determine which proteins have been altered in the respiratory and drug resistant mutants, this objective might be achieved.

From this account of cytoplasmically inherited mutants it can be seen that only by genetic analysis of specific proteins can a complete description of the genetic systems controlling mitochondrial biogenesis be obtained. There are two possible approaches available at the present time, firstly to examine the drug resistant and petite strains in more detail, and secondly to examine the genetics of specific mitochondrial proteins. This latter approach has been made with cytochrome c, and structural genes for this enzyme have been shown to be nuclear (Sherman 1964; Sherman et al. 1965). Work on aconitase (Ogur et al. 1964), α-hydroxy-β-keto acid reductoisomerase and α-keto-β-hydroxy-

acid reductase (Wagner et al. 1966) has suggested that these enzymes are also controlled by nuclear genes, but as both these studies used the absence of the enzymes mentioned in mutant strains other controlling factors could be involved and so these studies do not unequivocally establish that the structural genes are located in the nucleus. Mutants of malate dehydrogenase in Neurospora have been used for genetic studies (Munkres et al. 1965) and show that this enzyme is controlled by two unlinked loci which are presumed to be nuclear. These results together with the data on amino and incorporation into isolated mitochondria, have led to the view that the soluble enzymes of the mitochondrion are determined by nuclear genes. The evidence for this generalisation is not yet sufficient, in my view, for it to be accepted. In this study evidence has been obtained for the control of iso-citrate and 3-hydroxybutyrate dehydrogenases by nuclear genes in paramecium; more recently the nuclear control of mitochondrial malate dehydrogenase in maize has been demonstrated (Longo & Scandalios 1969). These results support the idea that soluble mitochondrial enzymes are determined by nuclear genes, but still do not establish the concept as universally valid. The main disadvantage of the approach using specific proteins is the difficulty of isolating mutants for genetic work; in the studies reported here and those with maize, mutants have been obtained by screening stocks from the wild, which is very laborious. The studies on cytochorme c and malate dehydrogenase (in Neurospora) have used a selective system for obtaining induced mutants, and it is this type of approach which should be extended, if possible, to a wide range of mitochondrial proteins.

All the studies undertaken so far have disadvantages which hinder the elucidation of the problem of mitochondrial protein inheritance. The results reported in this study are considered important as they provide information on

specific mitochondrial proteins. The studies using yeast and Neurospora, on the other hand, are important in their demonstration of mitochondrial inheritance, but, as shown in the case of cytochromes a/a_3 and b, the absence of particular proteins cannot be easily used for determining the site of their structural loci. Therefore it can be concluded that the most profitable approach, at this time, is the genetic study of proteins altered by mutation and in this respect further biochemical research into the petite and drug resistant mutants of yeast could be of considerable value.

3. Intersyngen enzyme variation:

The division of the species Paramecium aurelia into syngens based on mating reaction is not really comparable to any classification of higher organisms into species and sub-species, as these divisions are largely based on morphological criteria. The classification of wild collections of paramecium into syngens will become increasingly laborious as the number of known syngens increases. This is because the classification requires the availability of representative stocks of all syngens in a suitable reactive state for mating. Thus, the ability to detect stocks of different syngens by the electrophoretic mobility of their enzymes can be of great utility. Several physiological characters of stocks of different syngens have been used to show the close relationship of certain syngens (Sonneborn 1957). Such characters together with the electrophoretic differences will be considered and discussed in relation to taxonomic and evolutionary problems.

The characteristics which have been used to relate some of the syngens are size, optimal growth temperature, inheritance of mating type, intersyngen cross-mating reactions, serotypes and others. On the basis of these characters certain groups of syngens have been found which show similarities. These groups

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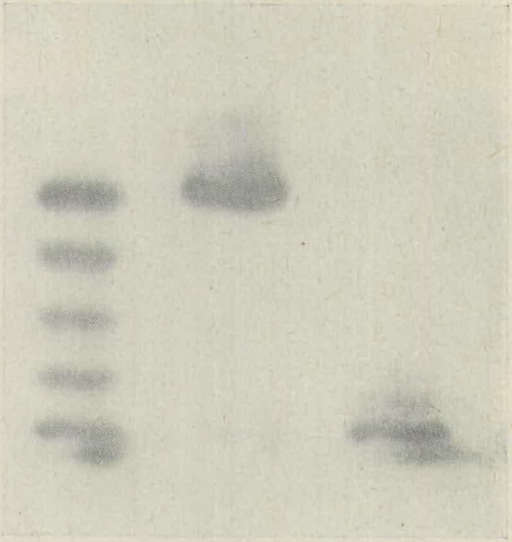
Genetic Control of β-Hydroxybutyric Dehydrogenase in *Paramecium aurelia*

MAMMALIAN β-hydroxybutyric dehydrogenase is known to be firmly bound to the outer membrane of mitochondria and can be extracted by the neutral detergent 'Triton *X*-100'[1]. In the investigation reported here the same enzyme was shown—by a similar extraction procedure—to be present in the mitochondria of the protozoan *Paramecium aurelia*. Extracts were made from a number of clones isolated from a natural population of *P. aurelia* (syngen 9), collected near Edinburgh and examined by starch gel electrophoresis. Two electrophoretically distinct forms of the enzyme were found in different clones and have been designated *HBD*-1 and *HBD*-2 respectively (Fig. 1*a*).

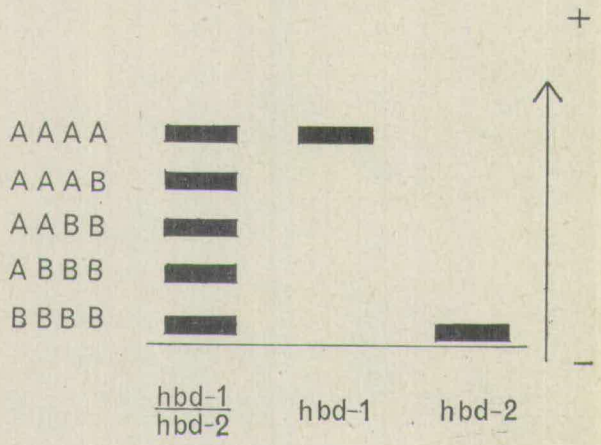
Paramecium aurelia is particularly convenient for the study of the genetics of mitochondrial proteins, for when paramecia undergo conjugation, exchange of haploid nuclei occurs and the two ex-conjugants—each containing cytoplasm (including mitochondria) from one parent only—can be isolated and grown into clones. In this way clones of paramecia are obtained which originate cytoplasmically from one or other of the parent clones, but which contain nuclear genes from both.

The mitochondrial fraction was homogenized in 1 per cent 'Triton *X*-100,' 0·1 M phosphate buffer *p*H 7·4, and kept at 4° C for 30 min. The supernatant from this extract was then run on a 10 per cent starch gel in a *tris*-citrate buffer using the discontinuous system of Poulik[2]. Electrophoresis was carried out at 18 v/cm with the gel at 4° C (cooled by an ice-pack), until the brown borate line was 3–4 cm from the anodal wick. The gel was sliced and stained for β-hydroxybutyric dehydrogenase activity using a method adapted from a histological stain[3].

Heterozygotes were obtained (from both cytoplasmic origins) by crossing paramecia containing *HBD*-1 and *HBD*-2 respectively. The hybrids containing cytoplasm from either conjugant gave identical patterns on starch gel electrophoresis. This immediately suggests that the genetic factors controlling β-hydroxybutyric dehydrogenase are nuclear and not cytoplasmic. To confirm this, heterozygotes of the type *HBD*-1/*HBD*-2 were passed through autogamy and a 1 : 1 ratio was obtained, indicating the segregation of a pair of alleles corresponding to



a



b

Figs. 1*a* and *b*. Starch gel zymogram of β-hydroxybutyric dehydrogenase. Stain developed with 0·5 g of sodium-β-hydroxybutyrate, 3 mg of nicotinamide adenine dinucleotide, 6 mg of MTT tetrazolium and 0·5 mg of phenazine methosulphate in 25 ml. of 0·1 M phosphate buffer *p*H 7·4.

Table 1

HBD-1	*HBD*-2	Total
24	34	58

($\chi^2 = 1{\cdot}72$ not significant at the 5 per cent level)

The segregation of the two alleles *HBD*-1 and *HBD*-2, after passing the heterozygote through autogamy.

the two forms of the enzyme (for details of the genetics of *P. aurelia* see ref. 4). These results are shown in Table 1, and establish that this enzyme, although firmly bound to the mitochondrial membrane, is controlled by a nuclear gene.

This raises further questions as to where the polypeptides are synthesized and how they pass into the mitochondrial membranes. Using isolated mitochondria, Roodyn, Work and Reis[5] found incorporation of radioactive amino-acids into a 'Triton *X*-100' insoluble fraction; this would suggest that synthesis of proteins soluble in 'Triton *X*-100' occurs outside the mitochondrion. This means that the polypeptides must be assembled and incorporated into the membrane from outside.

A further piece of information which has come from this work is that β-hydroxybutyric dehydrogenase is a tetramer, consisting of identical polypeptide chains. In the homozygous state the enzyme occurs as a single band, for all the polypeptides are identical, whereas in the heterozygous state a five banded pattern is obtained. This is easily explained if *HBD*-1 consists of four polypeptides AAAA and *HBD*-2 consists of BBBB (A and B differing in charge); the heterozygote will then contain a randomly assorted mixture of the two types[6] as shown in Fig. 1*b*.

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