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UNIVERSITY OF GLASGOW, April, 1962.

Summary of Thesis:

Constitutive and Adaptive Enzymes in Mammalian Cells.

Presented by P.F. Fottrell, M.Sc. (N.U.I.)

for the Degree of

Doctor of Philosophy in the Faculty of Science.

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Functionally similar proteins from different species were examined by starch gel electrophoresis to determine the extent and nature of the molecular variation between homologous proteins from various sources. Marked variations were encountered when esterases, phosphatases, catalases and peroxidases from different species were compared. The electrophoresis patterns were so distinct that the species of origin could be recognised from the type of pattern produced. Organ specific esterase patterns were also demonstrated in several animals including mouse, rat, guinea pig and hen. In contrast esterase zymograms from different human organs were remarkably similar to each other. Species characteristic esterase patterns persisted in cultured cell lines which in some cases had been growing for many years in heterologous media. The esterase pattern were not altered by the presence of organic esters in the growth medium. Therefore these patterns almost certainly reflect genetic differences. To explain how molecular heterogeneity is compatible with similarity in function, it was suggested that species variations were confined to those areas of protein molecules which were not essential for function. Several possibilities were considered to explain the occurrence of the large number of proteins displaying esterase activity, e.g. at least 16 in mouse liver.

Investigations into the existence and mechanism of

adaptive enzyme formation in animal cells were also undertaken. Animal cells maintained in culture were employed for this study because they provided a viable system, free from hormonal and nervous influence. Attempts were made to alter the levels of several enzymes in cultured cells by including substrates, products or related compounds in the growth medium. Under these conditions the levels of several enzymes including esterases, phosphatases, lactic dehydrogenase, glucose-6-phosphate dehydrogenase etc. were stable. On the other hand several examples of adaptive enzyme systems which provide more conclusive evidence for the existence of the phenomenon in animal cells were encountered. Glutamyl transferase activity in strains L and HLM was found to increase several fold when glutamine was removed from the culture medium. Active protein and RNA synthesis was necessary for induction of glutamyl transferase. Addition of glutamine to the medium of 'induced' cells resulted in a rapid disappearance of glutamy transferase activity. In cell free extracts glutamyl transferase activity disappeared during incubation at 37° in an atmosphere of air. Various substances including nitroge ascorbic acid, $NADH_2$, glutamic acid and glutamine protected the enzyme from inactivation. The glutamyl transferase system represents an example of enzyme repression and the rapid disappearance of glutamyl transferase from induced

cells after addition of glutamine may involve mechanisms for oxidizing thiol linkages.

Preliminary studies on the β-glucuronidase in strain L suggest that this system may be analogous to the β-galactosid in E.coli in that both are repressed by products of glucose metabolism. Arginase activity has been increased some 15-20 fold in HeLa cells after growth in the presence of arginine and citrulline (or ornithine). Indirect evidence for the accumulation of an inducing substance which is responsible for arginase induction has been obtained. The mechanism involved in adaptive enzyme formation in animal cells was discussed with reference to some recent hypotheses on the nature of adaptive enzyme formation in bacteria.

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Variation of Alkaline Phosphatase Activity Among Cells of Inducible and Constitutive Strains of Human Fibroblasts.* (29287)

GEORGE M. MARTIN (Introduced by Earl P. Benditt)
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Investigators concerned with enzyme induction in microorganisms commonly assume that all of the cells in a genetically homogeneous population behave similarly. Evidence is presented here that this may not be true for an instance of enzyme induction in cultures of diploid human fibroblasts.

Cox and Pontecorvo(1) reported differences among established strains of human fibroblasts with respect to the activity of alkaline phosphatase of lysates of the mass cultures. Under the conditions of their experiments, most strains had no measurable alkaline phosphatase activity unless permitted to grow in the presence of small amounts of a substrate of that enzyme. Two more unusual types were noted: "constitutive" strains which demonstrated relatively high enzyme activity and "non-inducible" strains, which had no measurable enzyme activity even after treatment with substrate.

Methods. Histochemical stains for alkaline phosphatase were originally performed (on coverslip monolayers) for the purpose of conveniently classifying strains with respect to their enzyme activity when grown with or without an inducing substrate (1 mM phenyl phosphate). The diploid strains, of typical "fibroblast" morphology, had been established for periods of 2-10 months by explant or trypsinization techniques and were grown in Waymouth's medium with 10% calf serum. Cultures for pleuropneumonia-like organisms were negative. A modification of the method of Fredricsson(2) was employed for the alkaline phosphatase stains. Fredricsson discovered that diffusion of enzyme could be greatly diminished when the Gomori technique was performed in an incubation medium of 40% acetone; only slight inhibition of enzyme activity resulted. Our experience confirmed this; aqueous incubations yielded a more diffuse precipitation of calcium phosphate, with less sharp localization and considerably more nuclear staining, generally accepted as an artifact(3). Fixation in formalin (at 4°C, pH 7.0 for 15 min) did not prevent this diffusion, which was quite evident when aqueous incubations were carried out in the presence of di Na p-NO_2 phenyl PO_4; even after the coverslip was removed from the incubation medium, enzymic hydrolysis continued at a rapid linear rate (as measured by the optical density of p-NO_2 phenol at 410 mμ). Markedly improved preparations resulted even with final concentrations of acetone of only 16%; the higher concentration recommended by Fredricsson was less preferable because a variable degree of precipitation of substrate was observed. A second modification was a doubling of the calcium concentration, designed to favor the "capture reaction" (precipitation of the enzymically liberated phosphate as calcium phosphate). Finally, in order to prevent resolubilization of the precipitated phosphate, all rinses subsequent to the enzyme incubation were in 40% acetone buffered with 10 mM 2 amino-2-methyl 1-propanol-HCl, pH 9-10. Controls

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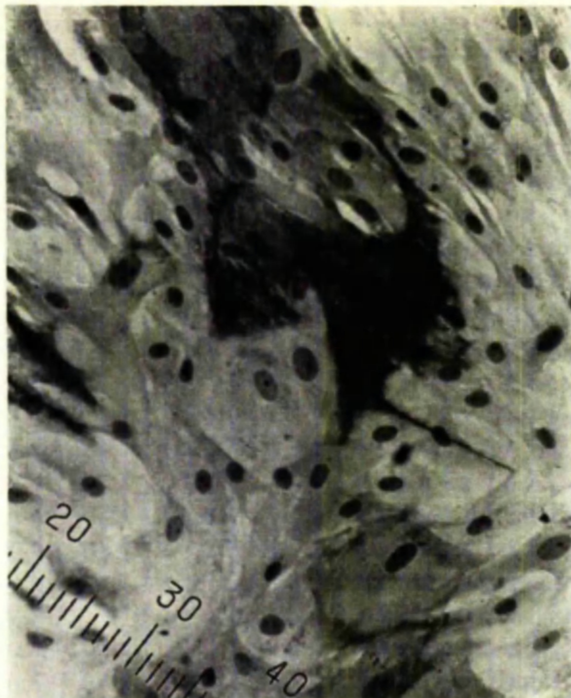

FIG. 1. Alkaline phosphatase stain of monolayer of human fibroblasts grown in medium containing 2 mM phenyl PO_4 for 6 days. Fixed in neutral buffered formalin at 4°C for 5 min. Incubated in 2.5 ml 2% Na β-glycerophosphate, 2.5 ml 2% Na veronal, 0.5 ml 4% $Ca(NO_3)_2$, 0.5 ml 0.8% $MgCl_2$ and 4 ml of 40% acetone for 5 hr at 37°C. Calcium phosphate precipitate converted to CoS by treatment with 2% $Co(NO_3)_2$ and 0.2% $(NH_4)_2S$, both in 40% acetone. Nuclear staining is due to light counterstain with safranin. Scale = 0.01 mm per division.

incubated without substrate and treated with enzyme inactivating agents (heat and methyl alcohol) failed to show intracellular deposits of cobalt sulfide.

Results. It was surprising to find that all preparations examined (over 100 coverslip monolayers from 12 strains) showed marked variation in enzyme activity from cell to cell. The heterogeneity could not be solely attributable to the histochemical "all or none" artifact(4). The appearance varied from no discernible precipitate of cobalt sulfide to jet black deposits covering the entire cytoplasm, with all gradations in between. A typical field is shown in Fig. 1, which was from a monolayer which had grown for 6 days in the presence of 2 mM phenyl PO_4 (without change of the medium). As is suggested in the illustration, clusters of cells (? small clones) with high enzyme activity could occasionally be noted, but more commonly, the distribution of "active" cells appeared to be random. Fields identical to that of Fig. 1 could be found in a "constitutive" strain classified by the method of Cox(1) and in a strain tentatively classified as constitutive by a method developed by the author(5). With long incubation times (up to 16 hours), definite enzyme activity could be demonstrated in one non-inducible and in all non-induced strains having low specific activities; here again, one could find all gradations of activity from cell to cell.

By far the greatest enzyme activity was observed in monolayers which had been grown for periods of up to 3-4 weeks without change of medium. This "self-induction" phenomenon(1) is now thought to be related to the degradation of cystine and cysteine, which repress the synthesis or activation of alkaline phosphatase in human fibroblast cultures(6). Degree of enzyme activity varied markedly from cell to cell. There was an apparently artifactual deposition of cobalt sulfide along the loose margins of partially detached cells, but enzyme activity was not otherwise related to any discernible morphologic differences. Enzyme activity was not related to the degree of cytoplasmic degenerative change as evidenced by fine and large droplet fatty alteration (stainable with neutral fat stains such as Fettrot 7B and Oil Red O) or accumulations of lipochrome pigments. Apparently phagocytized material was occasionally noted, but could not obviously be related to the degree of enzyme activity. The degree of confluence of various groups of cells within a given monolayer was definitely not correlated with enzyme activity. Enzyme activity did not appear to be correlated with the number of nucleoli or with the size of cells, nuclei and nucleoli. The distribution of enzyme activity within monolayers grown on 22 mm square cover slips vertically arrayed in Columbia staining jars containing 10 ml of medium could not be differentiated from monolayers grown in the conventional horizontal position; therefore, oxygen and other concentration gradients did not appear to influence enzyme activity.

Using a quantitative microfluorometric assay developed by Rotman(7), based upon

the micro-droplet spray technique of Collins (8), we have successfully measured the enzyme activity of single human fibroblasts grown in culture. The results substantiate the histochemical observations of heterogeneity; up to 9-fold differences in enzyme activity have been observed among cells derived from the same monolayer.†

Discussion. Synchronization and cloning experiments are in progress to evaluate further the cellular heterogeneity of alkaline phosphatase activity. Preliminary results using the dilution plating method of cloning (9) have been inconclusive, since in our hands "clones" of diploid human fibroblasts derived with this technique usually can be shown to arise from small clumps of cells rather than from single cells. Attempts to establish clones from isolated single cells (using micro-pipetting techniques, micro-capillaries and feeder layers) have so far been unsuccessful. We hope that others more skilled in cloning techniques will also pursue this line of investigation.

Our knowledge of the frequency of mitotic recombinational events in organisms such as *Aspergillus nidulans*(10) suggests that such mechanisms are not at all likely entirely to account for the marked degree of heterogeneity observed. While there is still no information concerning the occurrence of mitotic crossing-over in mammalian cell cultures, mitotic non-disjunction is not likely to be a sufficiently frequent event in diploid human fibroblast cultures in view of the reports of prolonged karyotype stability from several laboratories(11). Should the variation in enzyme activity and/or synthesis prove to be "physiologic," these observations would still be significant, especially in view of the availability of quantitative techniques for assay of enzyme activity in single cells. We are profoundly ignorant of the detailed biochemical events which occur during the mitotic cycle of a cell, especially under conditions of enzyme adaptation. It would be of great importance to establish the stage of the cycle during which alkaline phosphomonoesterase activity and/or synthesis is enhanced and/or repressed and to discover the responsible mechanisms. Such investigations could clarify the ultimate function(s) of this group of enzymes.

It should be emphasized that even under apparently optimal conditions for induction of alkaline phosphatase *in vitro* ("self-induction"), substantially longer incubation times (60-120 minutes) were required to obtain results comparable to those which are regularly observed (using similar or identical techniques) with certain tissues (renal tubular and intestinal epithelia, healing wounds) from humans and other mammals, in which incubation times of 5-15 minutes suffice to demonstrate marked enzyme activity. If we assume that the enzyme activity which we observe histochemically is related to enzyme concentration and in turn, to rates of enzyme synthesis, this suggests that even when "induced," the genome of the cultured fibroblast is still in a state of relative repression with respect to the synthesis of alkaline phosphatase(s). Two regulatory loci and one structural locus have been described for the alkaline phosphatase of *E. coli*(12). Several genetic loci are also known to control the synthesis of the enzyme(s) in *Aspergillus nidulans* (13). The situation in man is likely to be even more complex, especially in view of the apparent heterogeneity at the cellular level.

Summary. Histochemical studies of 12 strains of diploid human fibroblasts derived from newborn foreskins revealed marked variation of alkaline phosphatase activity within all populations, whether classified as constitutive for the enzyme or inducible by substrate. Variation was also apparent in non-induced populations having low activity and in "self-induced" populations having high activity.

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CONSTITUTIVE AND ADAPTIVE ENZYMES IN MAMMALIAN CELLS.

A Thesis Submitted for the Degree of Doctor of Philosophy in the Faculty of Science.

by

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The word 'homeostasis' was first proposed by W.B. Cannon (1926) to describe "the ability of living things to maintain their own constancy". As an eminent mammalian physiologist Cannon was referring in particular to the stability in composition of blood and other body fluids. However in present day biology the term has been extended to include such phenomena as the adaptation of organisms to environmental changes and the regenerative ability of organisms to revert to their original size following partial destruction. Although the occurrence of these phenomena is well known practically no information is available about the mechanisms involved.

It was at one time predicted (Cannon, 1929) that lower forms of life were not capable of controlling their environment. Within the last decade however, it has been recognised that micro-organisms possess very precise regulatory mechanisms, and that our knowledge of homeostatic control systems in higher animals can benefit from similar studies on micro-organisms.

<u>Metabolic Control Systems in Micro-organisms:</u> One particular aspect of the metabolic control systems in micro-organisms, which has been of considerable interest

over the last decade, involves the regulation of enzyme activity and enzyme formation. It is now appreciated that the levels of many enzymes in micro-organisms are dependent, to a large extent, on the composition of the growth medium. The process whereby substrates and products (and related compounds) of enzyme reactions regulate the formation of their respective enzymes has been termed enzyme adaptation. Two distinct but related phenomena, enzyme induction and enzyme repression are involved in this process.

Enzyme Induction: Although reports of induced enzyme formation appeared in the literature towards the end of the last century it is not clear whether these represented examples of true enzyme induction or not. Most biologists at that time were not convinced that alterations in the enzyme content of micro-organisms could occur without concommitant changes in genotype. This is not surprising in view of the many examples of adaptation by selection which were known at that time. The classical experiments of Dienert(1900) were aimed at demonstrating that induced enzyme formation was possible in the absence of cell growth. Dienert showed that a strain of yeast which usually did not ferment galactose could be 'trained' to do so. Within 24 hours, in the absence of cell growth, the yeast which previously could not utilize galactose

became so acclimatized as to depend on galactose as the sole source of carbon. Furthermore the 'galactozymase' activity was found to disappear when glucose was substituted for galactose in the growth medium. This type of adaptation was clearly different from the irreversible and more slowly developing type of adaptation which involved the selection of a mutant, with a fixed quantity of enzyme, from a growing population. Although it had not been conclusively proved that cell division had not taken place in Dienert's experiments, his work was of great value in stimulating interest in the subject of induced enzyme formation.

During subsequent years this experiment was repeated many times by various investigators (Harden & Norris, 1910) However no conclusive evidence was forthcoming to demonstrate that cell division did not occur during the course of the experiment. In the intervening years Harden & Norris (1923) disproved the possibility that enzyme adaptation simply involved a change in cell permeability when they showed that both 'galactozymase' and 'glucozymase' activity could be detected in the adapted yeast cells.

It was not until 1936 that it was finally demonstrated (Stephenson & Yudkin) that enzyme induction could take
1936

place in the absence of cell growth. These workers repeated Dienert's original experiments and from controlled experiments in which total and viable cell counts were carried out, concluded that increased galactozymase activity was not dependent on increased growth rate. Recently more conclusive evidence has demonstrated that induced enzyme formation in E. coli was not due to the formation of a mutant or dependent on increase in cell number. With the aid of a bacteriophage which lysed only those cells that contained β-galactosidase it was found (Benzer, 1953) that all the enzyme was liberated only when all the cells were lysed. This eliminates any possibility of the enzyme being present in some cells and absent in others as would occur if the increased β-galactosidase activity in E.coli was due to a mutation. As further proof that cell multiplication was not necessary for induced enzyme formation, it was shown (Sher & Mallette, 1954) that the inducible lysine decarboxylase of E.coli was formed in the presence of the inducer when cell division was inhibited by ββ'-dichloro-diethylsulphide.

When enzyme induction had been recognised as a phenomenon clearly distinguishable from 'selection' many workers became interested in the field. The use of induced enzyme systems for investigating protein bio-

synthesis was undoubtedly a great attraction. An even greater attraction however was the possible implications of adaptive enzyme formation in growth and differentiation, as stated by Dienert (1900) and Spiegelman (1948).

The 'Inducer': Much of the early work to determine the nature of induced enzyme formation was hampered because of the complexity of the systems used. The inducible 'galactozymase' of yeast first described by Dienert (1900) has been studied by many investigators (Spiegelman, 1946). However it was later demonstrated (Caputto et al. 1950) that 'galactozymase' consisted of two distinct enzymes (galactokinase and galactowaldenase) and it could not have been known in previous studies whether these two enzymes behaved similarly under the experimental conditions employed. A similar situation exists with the inducible yeast 'maltozymase' which was employed in many investigations on induced enzyme formation (Spiegelman & Dunn, 1947; Spiegelman, Reiner & Cohnberg, 1947). 'Maltozymase', which catalyses the production of CO_2 from maltose anaerobically, has recently been shown (Robertson & Halvorson, 1957) to consist of three enzyme systems, (a) a permease which is responsible for the entry of maltose into the cell, (b) a hydrolytic enzyme, α-glucosidase, (c) finally the glycolytic enzymes which oxidize the glucose released by glucosidase to CO_2 and

H_2O. It is obvious that factors such as the availability of co-enzymes would influence the resultant 'maltozymase' activity in this system and therefore accurate interpretation of this type of study is difficult.

A further discrepancy in the earlier work was in the type of inducer used to bring about the formation of the induced enzyme. The most common type of inducer was either a substrate of the enzyme or some similar substance. In some cases however the inducer was made to serve a dual function, i.e. in addition to its role as inducer it was often the sole source of carbon and energy to the cell. Thus in experiments on the adaptation of 'galactozymase' (Spiegelman, 1946) and 'maltozymase' (Spiegelman & Dunn, 1947) where galactose and maltose were the sole source of carbon it is difficult to separate the inducing ability of these substances from their importance to the cell as supplies of energy and carbon. This difficulty can be overcome if the inducer is not appreciably metabolized by the cell and is therefore gratuitous in its action (Monod, 1947). When an inducer is gratuitous and providing a suitable supply of carbon and energy is available it is then possible to study more precisely the nature of the mechanism of enzyme induction.

The inducible β-galactosidase and penicillinase in E.coli and B.cereus respectively are particularly suitable

for investigating induced enzyme formation because gratuitous inducers are available and the details of the enzyme reactions have been elucidated. Indeed it is due to studies on these systems that most of our knowledge of enzyme induction has been derived. The type of inducer required for enzyme induction in these systems has been thoroughly investigated and certain generalisations are beginning to emerge. It has been demonstrated (Monod, 1956) for instance that not all substrates of β-galactosidase were capable of inducing the enzyme in E.coli. Neither were all the compounds capable of inducing β-galactosidase substrates of the enzyme. Methyl-β-D-thiogalactoside and melibiose were inducers but not substrates while phenyl-β-D-galactoside and neolactose were substrates but not inducers. Although all these compounds are structurally related to each other it is apparent that there are no rigid structural requirements for the inducer. It is possible that these various substances may be converted into a 'common' inducer which would account for the ability of galactose, which exists in solution as a mixture of α and β isomers, to induce both α and β-galactosidase in E.coli Stain B (Holmes, Sheinin & Crocker, 1959). This is unlikely however, since it has been shown (Monod, 1956) that the rate of induction of β-galactosidase in E.coli was six

times greater when methyl-β-galactoside was the inducer than with galactose, the natural substrate of the enzyme.

Similarly various side chain derivatives of penicillin are able to induce penicillinase in B.cereus to almost the same extent (Pollock, 1957) and β-glucuronidase induction can be increased by increasing the number of carbon atoms in the aglycone group (Stoeber, 1957). During their investigations on the inducing ability of various substances with the β-galactosidase system in E.coli, Monod and his co-workers (1956) reported an interesting finding. Phenyl-β-D-thiogalactoside which is a competitive inhibitor of β-galactosidase did not induce the enzyme in E.coli and was actually found to inhibit β-galactosidase induction. The observations of Rickenberg et al. (1956) suggested that phenyl-β-D-thiogalactoside acted by preventing the inducer from entering the bacterial cell by inhibiting a specific 'galactoside-permease' system which was also inducible and was responsible for the transport of the inducer across the cell membrane. Other inducible permease systems have more recently been described for β-glucuronidase in E.coli (Stoeber, 1957) and for citric acid transport in Aerobacter (Davis, 1956). The induction of penicillinase does not require a permease system (Pollock, 1959). Similarly induction of β-galactosidase in B.megaterium by phenyl-β-D-thiogalactoside has been described (Landman,1957).

and it appears that no permease is required in this system. Not all induced enzyme systems are therefore controlled by permeases and it will be interesting to see if permease systems exist in animal cells. Evidence such as that of Paul & Mendelsohn (1960) on the interaction of leucine, isoleucine and valine in strain L cells could be interpreted as resulting from competition for a common permease by the three amino acids.

From what has already been said it is apparent that enzyme induction and enzyme action appear to be independent entities. Inducers and substrates however are generally structurally similar and many inducers have been found to combine with the inducible enzyme. Although Halvorson (1960) has demonstrated that all inducers of α-glucosidase in S. cerevisea are complexants of the enzyme, two inducible enzyme systems where the inducer does not combine with the enzyme have been reported. They are the induction of β-galactosidase in E.coli by melibiose (Schlenk, Shapiro & Parks, 1958) and the induction of protocatechuric acid oxidase in Neurospora by vanillic acid (Lavine & Floyd, 1954). The precise role of the inducer has not yet been fully established, however the recent theory of Jacob & Monod (1961) is particularly interesting. This and some of the various other theories will be discussed in a later section.

Kinetics of Enzyme Induction: Over the last decade the kinetics of several induced enzyme systems in bacteria and yeasts have been extensively studied and many of these systems have been found to have certain features in common. For instance, the addition of the inducer to a growing population of cells was usually followed by an increase in enzyme activity, after a brief lag period (in some cases). When enzyme activity was plotted against time following the addition of methyl-β-thiogalactoside (5.10^{-4}M) to a culture of E.coli, strain ML-30, β-galactosidase activity increased almost immediately (Mandelstam, 1960). By plotting β-galactosidase activity against increase in bacterial mass under similar conditions and in a medium containing succinate, it was demonstrated (Monod, 1956) that the rate of increase in enzyme activity was constant from the moment of addition of the inducer. The plot of β-galactosidase activity against bacterial mass allowed the differential rate of increase in enzyme activity to be determined as expressed by Monod (1956)

$$\frac{\Delta z}{\Delta x} = \varphi$$

where z = β-galactosidase activity and x = bacterial mass. The advantage of determining the differential rate of increase lies in the fact that interference from non specific factors was considerably reduced. The rate of increase in enzyme activity when plotted against time was

dependent on several factors such as the availability of a source of nitrogen, and whether a gratuitous inducer was employed.

A similar situation to that observed in E.coli for β-galactosidase has been described (Duerksen & Halvorson, 1959) for the inducible α-glucosidase system in yeast. When the differential rate of α-glucosidase increase was determined, a linear rate of increase was observed, immediately following the addition of the inducer, ethyl-β-thioglucoside (10^{-2}M). When the concentration of the inducer was decreased the linear rate of increase of α-glucosidase was maintained in marked contrast to the situation in E.coli where the initial rate of increase of β-galactosidase activity decreased with decreasing concentrations of inducer (Monod, 1956). The reason for this interesting difference lies in the presence of an inducible permease in certain strains of E.coli and the absence of an inducible permease in yeast for the α-glucosidase system (Duerksen & Halvorson, 1959). The galactoside permeases of E.coli have been extensively studied by Monod and his colleagues (Monod, 1956). These workers have clearly demonstrated the inducible nature of the permeases by the absence of any lag in the rate of β-galactosidase increase when 'preinduced' cells are treated with low concentrations of inducer.

The studies of Pollock and co-workers (Pollock, 1959) on the inducible penicillinase of B. cereus have revealed some interesting differences from the two systems already described. Although no hindrance to the passage of penicillin into the cells has been demonstrated, a 15-minute lag period occurs before the rate of penicillinase activity increases linearly. No inducible permease has been demonstrated and it is unlikely that the 15 minute lag period can be explained as the time required to synthesize the new enzyme since incorporation periods for ^{14}C-amino acids and ^{35}S-methionine into the enzyme, were less than 3 minutes and 30 seconds respectively (Pollock, 1959; Pollock & Kramer, 1958). An interesting finding emerging from these studies was that exposure of the cells for 1 minute to the inducer (penicillin), followed by treatment with penicillinase, was sufficient to maintain a linear increase in penicillinase for several generations afterwards. Pollock (1959) demonstrated that up to 200 molecules of penicillin became irreversibly attached to a single cell during a short exposure to penicillin and that the number of molecules fixed per cell determines the subsequent rate of increase of enzyme activity. From further observations by Kogut, Pollock & Tridgell (1956) who showed that each molecule of bound penicillin induced the synthesis of 40 molecules of penicillinase per hour,

it was concluded that penicillin was acting as a catalyst in bringing about increased penicillinase activity.

The penicillinase system was different from some other induced enzyme systems where removal of the inducer was followed by a gradual decrease in enzyme activity as 'induced' cells were diluted out during subsequent growth. The penicillinase activity of B.cereus for instance disappeared far more slowly than the β-galactosidase activity of E.coli when the inducer was removed.

The nature of induced enzyme formation: Despite some differences in the kinetics of various induced enzyme systems the fundamental principles were obviously very similar. Two aspects of the phenomenon to which the attentions of many investigators were aimed, were (a) to determine whether the increased enzyme activity was due to synthesis of new enzyme molecules or if activation of an existing precursor occurred, and (b) how the inducer stimulated increased enzyme activity.

Since the early reports of induced enzyme formation it was generally believed that de novo protein synthesis was involved although there was very little evidence available to support this. Yudkin (1938) proposed a 'precursor' theory based on the law of mass action to

explain the formation of induced enzymes. Enzymes, according to Yudkin, were present in cells as inactive precursors which were in equilibrium with the active enzymes. This equilibrium was said to obey the law of mass action and following the addition of a substrate, the formation of an enzyme-substrate complex displaced the equilibrium in favour of the forward reaction.

The 'precursor' hypothesis is applicable to some enzymes such as pepsin, trypsin and chymotrypsin which exist as zymogens. Similarly under the action of glucagon or epinephrine the inactive phosphorylase a can be converted into the enzymically active phosphorylase b (Sutherland, 1956).

Because of the absence of any opposing evidence Yudkin's hypothesis was not contradicted for several years. A 'stabilizer' hypothesis was proposed in 1947 by Monod who suggested that the inducer functioned by stabilizing the enzyme molecule which would otherwise disappear. This theory was later abandoned but recent evidence particularly from investigations on the tryptophan pyrrolase (Dubnoff & Dimick, 1959) and the thymidine kinases (Hiatt & Bojarski,1960) systems, suggests that the stabilizing of enzyme molecules by substrates may be important, in animal cells if not in bacteria. According to Spiegelman (1946) enzyme formation was

induced by cytoplasmic granules or plasmagens which were probably identical to molecules of RNA since he demonstrated that inhibition of RNA synthesis prevented induced enzyme formation. Appreciation of the true nature of induced enzyme formation had to await further developments in the field of protein and ribonucleic acid biosynthesis.

Removal of RNA by RNAase or inhibition of RNA synthesis by purine and pyrimidine analogues and in some cases by irradiation was found to prevent the formation of several induced enzyme systems. Thus Spiegelman (1956) demonstrated that while 99% of the DNA of protoplasts from B.magaterium could be removed without affecting the induction of β-galactosidase, a loss of 30% of the RNA caused cessation of the enzyme forming ability. The protoplasts were prepared by treating sensitive cells of B.magaterium with lysozyme under hypotonic conditions and had previously been found capable of nucleic acid and protein synthesis (McQuillen, 1955) as well as supporting the growth of viruses (Brenner & Stent, 1955; Salton & McQuillen, 1955). Similarly inhibition of RNA synthesis in yeast by ultraviolet irradiation prevented the formation of the inducible α-glucosidase (Halvorson & Jackson, 1954). In this instance a 95% suppression of α-glucosidase synthesis was observed when 22% of RNA synthesis was inhibited. Penicillinase induction in B.cereus was

likewise inhibited by ultraviolet irradiation (Torriani, 1956).

Many workers have used purine and pyrimidine analogues to demonstrate the dependence of induced enzyme formation on concommitant RNA synthesis. Creaser (1956), for example, demonstrated inhibition of inducible β-galactosidase and catalase in S.aureus by 8-azaguanine. This finding was in agreement with the observations of Ben-Ishai & Spiegelman (1955) who demonstrated that the induction of β-galactosidase in E.coli was inhibited by 5-hydroxy-uridine which had previously been reported (Roberts & Visser, 1952) to prevent the incorporation of uracil into RNA. A small quantity of 5-hydroxy-uridine (5 ug/ml) was sufficient to inhibit β-galactosidase formation and the compound was effective even after induction had begun. It was further shown (Ben-Ishai & Spiegelman, 1955) that while 5-hydroxy-uridine inhibited β-galactosidase formation the synthesis of other cellular proteins was hardly affected at all.

The information obtained from the use of analogs of purines, pyrimidines and amino acids strongly suggested a close relationship between RNA and protein synthesis and the concommitant participation of both in induced enzyme formation. Evidence of a more conclusive nature, demonstrating the fabrication of a new protein molecule

during enzyme induction emerged from subsequent investigations. For instance Rotman & Spiegelman (1954) used the inducible β-galactosidase system of E.coli to determine if de novo protein synthesis occurred during enzyme induction. The bacteria had previously been grown on ^{14}C-lactate until uniform labelling was obtained. They were then transferred for a short period to a nonradioactive medium in the presence of an inducer of β-galactosidase. The induced enzyme was isolated, purified and its radioactivity determined. The results revealed that less than 1% of the carbon content of the induced β-galactosidase had been derived from carbon present in the cells before induction. This finding was later verified (Hogness, Cohen & Monod, 1955) in a similar experiment using ^{35}S-sulphate. The amino acid pool size in E.coli is sufficiently small to allow accurate interpretations to be drawn from this type of experiment (Spiegelman, 1956). More recently it has been shown (Pollock & Kramer, 1958) in an identical type of experiment that less than 2% of the sulphur of induced penicillinase in B.cereus was derived from sulphur present in the cells prior to addition of the inducer.

These general findings have been further confirmed by the use of immunological techniques. Thus evidence for the formation of a new protein molecule was provided by

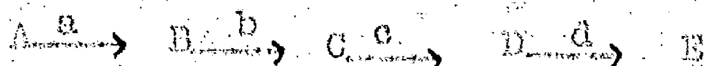
Monod & Cohen (1952) during investigations on the β-galactosidase in E.coli. Similarly the formation of penicillinase in B.cereus was demonstrated immunologically and was further found to be identical to penicillinase present in other strains of B.cereus (Kogut, Pollock & Tridgell, 1958).

In this section the control of enzyme formation by substrates and related compounds has been discussed. In recent years another mechanism for regulating the synthesis of specific bacterial enzymes has been described. In this instance the product of the enzyme reaction has been shown to affect the enzyme responsible for its synthesis. The phenomenon has been termed feedback control and two distinct mechanisms have been recognised. The product of the enzyme reaction can either inhibit the activity of the enzyme (feedback inhibition) or suppress its formation (repression). Together with enzyme induction these mechanisms are probably of paramount importance in maintaining a steady state in metabolic pools in bacteria.

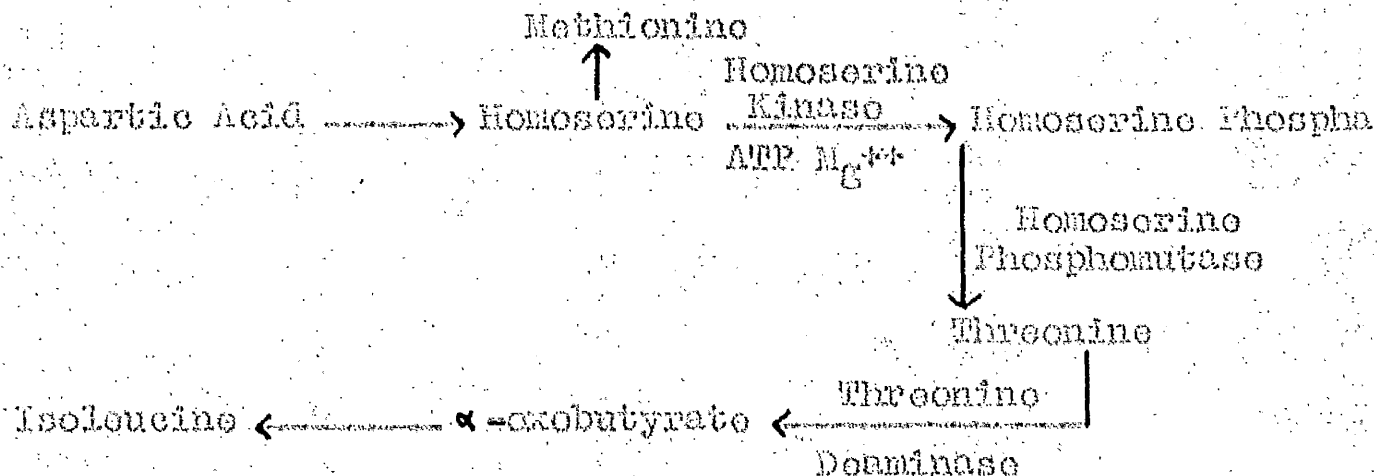
Feedback inhibition and repression as metabolic control systems in micro-organisms: The regulation of enzymic reactions by products of the reaction have aroused considerable interest in recent years and preliminary findings have indicated that this system is widespread in bacteria and involves many biosynthetic

pathways. 'Feedback' in the field of electronics has been used to describe a system where the output regulates the input. An analogous situation in an enzyme system controlled by feedback would be one where as a reaction sequence proceeded conditions were created that were unfavourable to further progress and would cause the system to slow down. Although regulation by feedback involves inhibition of a specific enzyme system there are two distinct mechanisms whereby this can operate. The ultimate product of a reaction can either directly inhibit a specific enzyme along its synthetic pathway or the final product may suppress the synthesis of one or more of the enzymes concerned with its formation. In the latter type of mechanism, termed enzyme repression (Vogel, 1957) the effect of the product is exerted on the enzyme forming system whereas the former type of mechanism involves a direct inhibition of the catalytic function of the enzyme. In some cases of feedback control both types of mechanisms may operate. Since earlier reports of feedback control were described by, for instance, Monod & Cohen-Bazire (1953) who demonstrated that the formation of tryptophan synthetase in E.coli was inhibited by tryptophan and indole, many similar systems have been investigated. These studies have revealed the similarity of the operative mechanism in

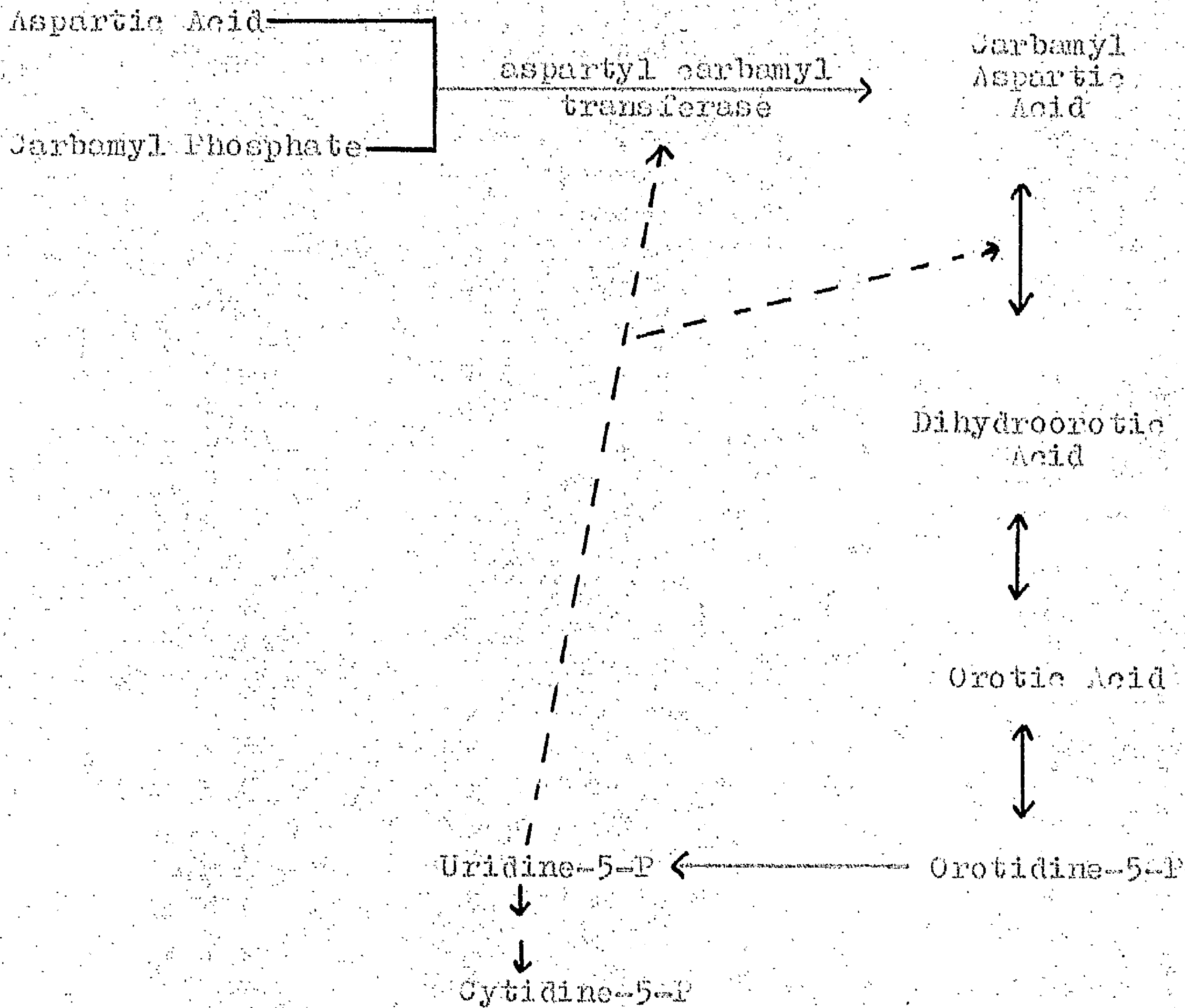
many feedback systems and have demonstrated how important such a phenomenon could be in metabolic control. Thus in a reaction sequence where a compound such as A is converted into B, C, etc. to give a product E,



the inhibitory effect of E is usually maximal on the enzyme responsible for converting A→B. When the sequence is controlled by repression, the synthesis of several of the enzymes is suppressed. Inhibition of the first step in the reaction sequence by E allows compound A to be utilised for other biosynthetic pathways since many biosynthetic systems utilize common intermediates. A single metabolic pathway can therefore be regulated without interference with other reaction sequences. An example of this is the inhibition of threonine biosynthesis in E.coli and yeasts by threonine (Wormser & Pardee, 1958). Threonine is synthesized in E.coli from aspartic acid as follows:

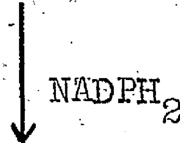


Feedback Control of Pyrimidine Biosynthesis in E. coli
(Yates & Pardee, 1957).

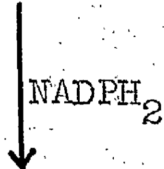


Formation of lysine and threonine from aspartate in E.coli (Stadtman et al. 1961).

Aspartate —Aspartokinase, ATP→ Aspartyl Phosphate



Aspartic semialdehyde → → → Lysine



Homoserine → → → Threonine

Methionine

Homoserine kinase was found to be specifically inhibited by threonine without affecting the synthesis of methionine. Similarly isoleucine specifically inhibited threonine deaminase. Other enzymes such as homoserine phosphomutase were not inhibited by threonine or isoleucine and neither did amino acids structurally similar to these inhibit homoserine kinase or threonine deaminase to the same extent.

Another example of feedback control by the product of a reaction on the enzyme catalyzing the first step in the reaction sequence has been demonstrated (Yates & Pardee, 1956; 1957) for pyrimidine biosynthesis in an E.coli mutant with a requirement for uracil. If uracil was omitted from the growth medium the level of aspartylcarbamyl transferase, which is responsible for the formation of carbamyl aspartate from aspartic acid and carbamyl phosphate, increased in activity some 500-fold. Two other enzymes immediately following aspartylcarbamyl transferase in the reaction sequence also displayed increased activity, though not nearly to the same extent as aspartylcarbamyl transferase. Uracil was found to repress the formation of these enzymes and both uracil and cytidylic acid also competitively inhibit aspartylcarbamyl transferase.

An interesting type of feedback inhibition, involving

the aspartokinase system in E.coli has recently been demonstrated (Stadtman et al., 1961). Cell free extracts of E.coli have been found to contain two distinct aspartokinases which catalyze the formation of aspartyl-phosphate from aspartate and ATP. L-lysine specifically inhibited one aspartokinase: the inhibition was non competitive and was the result of repression of enzyme formation. The other aspartokinase was specifically and competitively inhibited by L-threonine which suppressed the catalytic function of the enzyme. The existence of two aspartokinases permits regulation of the synthetic pathway of one of the amino acids without affecting the other, since both are formed from a common intermediate aspartyl phosphate (as demonstrated in the accompanying diagram).

Since feedback control has been found to be so widespread and to involve many biosynthetic pathways it is obvious that the phenomenon must form an important part of the general mechanism of metabolic control in bacteria. The phenomenon of enzyme repression is of particular interest since this is the converse of enzyme induction and evidence is accumulating to suggest that the mechanism in both cases may be similar. Since both types of phenomena must involve specific mechanisms for regulating protein biosynthesis some current findings

in this field will now be considered.

Protein biosynthesis: Although our knowledge of protein synthesis has increased enormously since Caspersson (1941) and Brachet (1942) first suggested a possible connection between protein and RNA synthesis, the complete details of the process are still not clearly understood. Early observations (Claude, 1943; Brachet, 1942) that most of the cytoplasmic RNA was associated with small particles (microsomes) in the endoplasmic reticulum suggested that microsomes were possible sites for protein synthesis. When suitable methods for fractionating various cell components were devised it was found (Keller & Zamecnik, 1956; Hoagland et al. 1957) that microsomes could incorporate amino acids in the presence of ATP and a soluble cell fraction. Although the bulk of protein synthesis takes place in the microsomes other cellular components are capable of forming polypeptide chains. Recent investigations into the mechanism of protein synthesis in these organelles have revealed a striking similarity in the fundamental mechanism (Chantrenne, 1961).

Most of the essential components in the soluble fraction of the cell have now been defined so that it is possible to formulate some of the steps in protein biosynthesis. The initial reaction involves the activation of amino acids by ATP (Hoagland, Keller &

Zamecnik, 1956; Berg, 1956; De Moss & Novelli, 1956) resulting in the formation of amino acyl adenylates (Hoagland et al. 1957; Karasek et al. 1958; Kingdon Webster & Davie, 1958). Some amino acyl adenylates have been isolated (Karasek et al. 1958) and their formation from ATP and amino acids has been demonstrated by the transfer of ^{18}O-labelled carboxyl group to AMP after activation (Bernlohr & Webster, 1958). The enzymes catalysing the activation process have been isolated from soluble cell fractions by precipitation at pH5 (Hoagland et al., 1957). Some of these enzymes have been purified (Berg, 1958; Novelli & De Moss, 1957; Preiss et al., 1959) and the interesting finding has emerged that each amino acid has a specific activating enzyme. Each amino acyl adenylate apparently remains firmly bound to the activating enzyme (Karasek et al., 1958) which also catalyses the next step in the sequence (Berg & Offengand, 1958) i.e. the transfer of the amino acid to a soluble RNA molecule in the cytoplasm. The specific amino acid activating enzymes have therefore a dual role in activating the amino acids and then transferring the activated amino acids to sRNA. The sRNA has a low molecular weight (25,000 - 30,000) containing some 80-90 nucleotides (Herbert & Canellakis, 1960; Tissures, 1959; Brown & Zubay, 1960; Dillig,

Schachtschabel & Krone, 1960) as compared to the ribosomal RNA which has a molecular weight of $0.5-1.10^6$ (Kurland, 1960). There is now a considerable amount of evidence to suggest that each activated amino acid reacts with its own specific s-RNA acceptor (~~Brown, Brown & Gordon, 1959;~~ Zamecnik, ~~Stephenson & Scott,~~ 1960; Hartman & Lipman, 1961). The activated amino acid has been shown to form an ester linkage with the 2'- or 3'-hydroxyl of the terminal adenosine nucleotide of s-RNA (Hecht, Stephenson & Zamecnik, 1959; Kreis et al., 1959; ~~Hecht, Ito & Lipmann, 1959~~). After the activated amino acids are joined to their specific s-RNAs they are then transferred to the ribosomal RNA where the polypeptide chain is fabricated. The main function of these small RNA molecules therefore appears to be the transfer of the activated amino acids to the protein forming site. The studies of Lipmann (1961) on the synthesis of glutathione (GsH) from γ-glutamylcysteine and glycine suggests that transfer RNA functions catalytically in this reaction. Further support for the catalytic role of transfer RNA has come from investigations on haemoglobin synthesis in reticulocytes (MORRIS & Schweet, 1960) and a ribosomal system in E.coli (~~Nathans &~~ Lipmann, 1961). Also the existence of cytoplasmic enzymes whose sole function appears to be the replacement of the

Cytosine-Cytosine-Adenosine terminal sequence (which is common to all transfer RNAs so far isolated) has been demonstrated (Chantrenne, 1961) and this suggests that ample provision is made to maintain the chemical configuration of the transfer RNA. Transfer RNA therefore probably functions in a cyclic fashion but so far the configurations of these molecules have not been elucidated and it is therefore not possible to predict how each transfer RNA molecule recognises its own particular amino acid.

Similarly the final steps in the synthetic sequence have not yet been formulated. The mechanism by which the amino acids are removed from the transfer RNAs to the ribosomal RNA has not been clarified. Some progress is being made however, e.g. Hultin (1961) has succeeded in isolating from cell sap several enzymes which catalyse the transfer of amino acids from transfer RNA to the ribosomes. These transferring enzymes are distinct from the amino acid activating enzymes and require the presence of guanosine triphosphate in the reaction medium. The stimulatory effect of sulphydryl compounds and inhibition by compounds such as ρ-chloromercuribenzoate suggests that sulphydryl groups are necessary for the function of this reaction. A further interesting observation concerning these enzymes was that the transfer

of different RNA bound amino acids was catalysed by different enzymes which might indicate a degree of specificity comparable to the activating enzymes.

Except for the synthesis of haemoglobin there is scarcely anything known about how the amino acids are incorporated into the polypeptide chain on the ribosomes. Studies on haemoglobin synthesis in reticulocytes (Bishop, Leahy & Schweet, 1960; Dentzis et al., 1958) have revealed that a sequential addition of amino acids occurred from the N-terminal end of the chain to the other end. It is not known at present if this mechanism is typical of other systems. Once the amino acids have condensed to form the protein, it leaves the ribosomes by some mechanism still unknown. There is at present insufficient evidence available to indicate whether this final step is enzyme-catalysed or spontaneous.

The interesting findings of Von Ehrenstein and Lipmann (1961) provide evidence for the similarity of the mechanism of protein synthesis in bacteria and animal cells. Using amino acyl-sRNAs derived from E.coli these workers were able to demonstrate the formation of haemoglobin on ribosomes obtained from rabbit reticulocytes. The important implication of this finding is that the acyl-sRNAs are not species specific but can be interchanged, suggesting a common coding system for

amino acids may exist for all forms of life (Lipmann, 1961). In contrast to these observations was the finding (Hartmann & Lipmann, 1961) that although the threonine activating enzyme from calf liver would react with sRNA from other animal species, no reaction was observed with sRNA from E.coli.

The mechanism of protein synthesis has been reasonably defined up to the point of attachment of the activated amino acids to the transfer RNA. Beyond this step there are several questions still to be answered satisfactorily; the method of coding involved in the formation of the polypeptide chain being of paramount importance. A great deal of evidence is now available, which strongly suggests that the specific configuration of the amino acids in the protein molecule is controlled by the DNA in the cell nucleus. Studies on transformation (Averry, McCarty & McLeod, 1944) and mutation have indicated that DNA is the ultimate carrier of all genetic information in the cell. Thus such properites as drug resistance and capsular formation can be easily transferred among bacteria by means of DNA transfer (Hotchkiss, 1956). Evidence for the participation of DNA as a determinant of protein structure has come from studies on mutations where in some cases the incorporation of a single base analogue into the DNA molecule can give rise to altered

protein structure (Gros, 1959; ~~[illegible]~~, ~~1959~~) attributable to the substitution of one amino acid for another in some instances (Freese, 1959). Similarly the recent studies with human haemoglobin (Ingram, 1959) and tobacco mosaic virus (Tsugita & Fraenkel-Conrat, 1960) where alterations in the sequential arrangement in the polypeptide chain have been demonstrated as a result of mutations. Since alterations in base sequence of the DNA molecule are reflected in changes in the amino acid sequence of specific proteins, the sequential arrangement of the amino acids in a polypeptide chain is probably controlled by the linear sequence of nucleotides in DNA. Studies are now in progress in many laboratories to decipher how a sequence of 4 bases in the DNA molecule can arrange the sequence of 20 amino acids in a protein molecule (Crick et al., 1961).

Another intriguing problem is to determine how the ribosomes obtain the information from DNA to manufacture proteins to a set pattern. Although the amino acids are assembled, to form a specific protein, on the ribosomes, recent evidence suggests that the information for protein synthesis is not contained in the ribosomes per se. For instance the similarity in size (Hall & Doty, 1959; Littauer & Eisenberg, 1959; Kurland, 1960) and base composition (Belozersky, 1957) of ribosomal RNA

from various sources is not compatible with the large variations in size and amino acid sequences of polypeptide chains or the heterogeneity in the nucleotide sequence of DNA from different species of bacteria (Chargaff, 1956). Recent work (Volkin & Astrachan, 1958; Nomura, Hall & Spiegelman, 1960) which indicated that only part of the ribosomal RNA carries the information has given rise to the possible existence of a messenger RNA which would be responsible for transporting this information from the nucleus. This type of RNA has been shown to exist in E.coli (Gros et al. 1961; Monod, 1961) and has apparently a very rapid turnover rate as it only remains on the ribosomes for a relatively short period of time (Nomura, Hall & Spiegelman, 1960). In animal cells the messenger RNA must be more stable and remain on the ribosomes for a longer period since reticulocyte ribosomes (almost free of DNA) can form 50-100 molecules of haemoglobin (Dentzis, Borsook & Vinograd, 1958) and liver microsomes continue to make serum albumin when separated from the cells (Campbell, 1960). Perhaps the best evidence, so far, demonstrating the existence of messenger RNA, emerged from the recent findings of Brenner, Jacob & Meselson (1961) who investigated the mechanism of protein synthesis in E.coli following infection with T_2 bacteriophage.

When E.coli had been infected with T2 most of the protein synthesized was phage protein (Koch & Hershey, 1959). Therefore phage DNA takes over from the bacterial DNA after infection and determines the type of protein that will be manufactured. Although no new ribosomal RNA synthesis could be detected after phage infection, the presence of new RNA with a very rapid turnover rate and a base composition complementary to phage DNA was demonstrated. In confirmation of previous observations of Nomura et al. (1960), Brenner et al., (1961) found that this RNA was associated with the pre-existing bacterial ribosomal RNA and furthermore that it could be separated from the ribosome by differential centrifugation in a caesium chloride gradient. These findings indicated that while protein synthesis takes place on the ribosomes they are dependent on a supply of messenger RNA from the nucleus for the formation of specific protein sutructures.

Mechanism of Enzyme Induction and Repression in Bacteria:

The configuration of amino acids in proteins is apparently controlled by functional units of DNA which are arranged linearly on the chromosomes. The existence of genes whose sole function is the supply of information (probably mediated by messenger RNA) to the ribosomes has

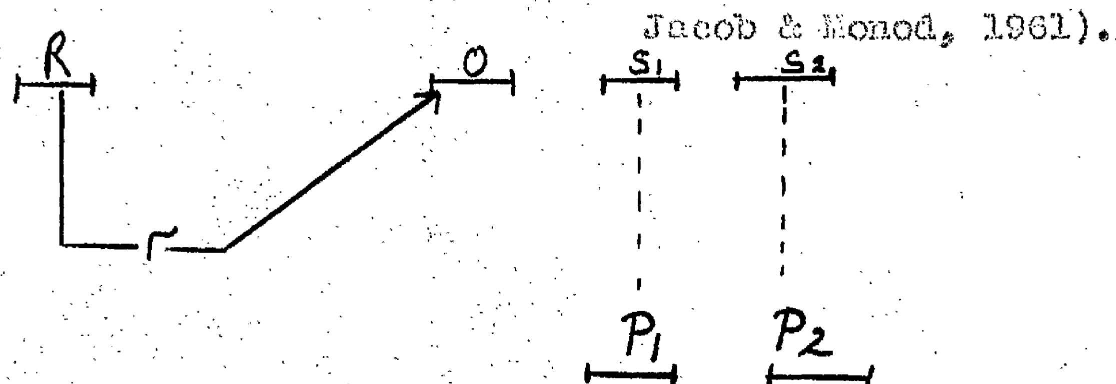
been further supported by recent findings on inducible alkaline phosphatase and β-galactosidase in E.coli. Levintal and co-workers (1961) demonstrated that structural alterations of alkaline phosphatase in E.coli following mutations induced by irradiation could be correlated with changes in a particular genetic locus on the chromosome, i.e. a gene responsible for molecular configuration of alkaline phosphatase. Similarly the gene responsible for the structural integrity of the inducible β-galactosidase in E.coli has been identified (Pardee, Jacob & Monod, 1959; Monod, 1961). It was demonstrated that the activity of the β-galactosidase structural gene (Z) was under the influence of a controlling gene (i) lying immediately adjacent to it on the chromosome. Mutations involving gene i resulted in β-galactosidase being produced in fixed amounts (i.e. the formation of a constitutive mutant from an inducible strain). Other inducible systems from which constitutive mutants have been derived include the penicillinase of B.cereus (Knight, Pollock & Tridgewell, 1956), the α-glycosidase of yeast (Kihara et al., 1959) and the amylomaltase of E.coli (Cohen-Bazire & Jolit, 1953). If these mutations are also due to a loss in the function of gene i the difference between the inducible and constitutive strains depends on the

presence of i^+ or i^-. The inducible strain is characterized by z^+i^+ while a constitutive strain possesses z^+i^-. The presence or absence of a permease enzyme (Y) will also indirectly alter the situation.

More direct genetic evidence for the presence of genes which regulate the function of the structural genes has emerged from investigations on the repressible alkaline phosphatase and the inducible β-galactosidase of E.coli (Levinthal, Green & Rothman, 1961; Jacob & Monod, 1961). A number of hypothesis have recently been put forward to explain the mechanism of enzyme induction and repression in bacteria in terms of their effect on these controlling genes. Jacob & Monod (1961) have provided genetic evidence which suggests that enzyme biosynthesis in bacteria is controlled by specific genes - 'the regulator' genes. These genes are distinct from the structural genes which are responsible for the sequential arrangement of the amino acids in the polypeptide chain. The information contained in the structural genes is thought to be conveyed to the ribosomes by an unstable RNA fraction (the messenger RNA). Regulator genes are thought to act directly on the structural genes by means of specific cytoplasmic compounds (repressors) which interfere with the flow of messenger RNA from the structural genes. Structural genes controlled by a single repressor and

grouped together would constitute a genetic functional unit termed an 'operon'. In this instance a special gene, associated with the operon, called the 'operator' would co-ordinate the synthesis of the various enzymes within the operon. The repressor would then act directly at the site of the operator gene. Enzyme induction and enzyme repression would then result from the interaction of specific chemical substances with the products of the regulator genes.

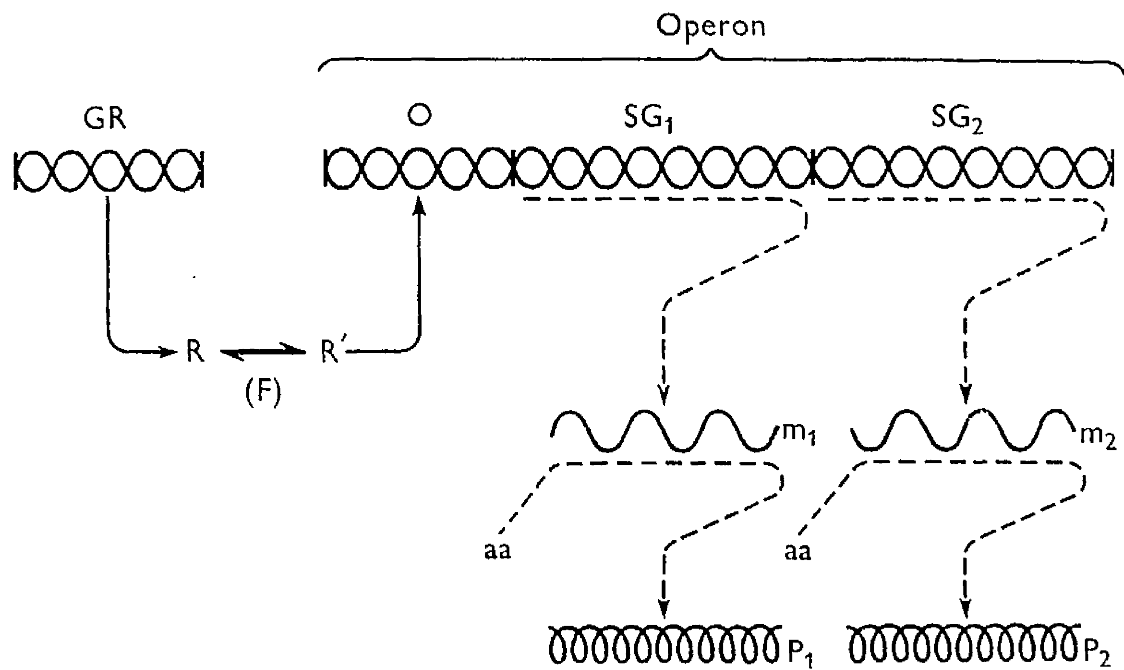
Regulation of Enzyme Synthesis (Scheme proposed by Jacob & Monod, 1961).



R = regulator gene
r = repressor

O = Operator gene
S_1 & S_2 = structural genes
P_1 & P_2 = proteins structurally controlled by S_1 & S_2.

An alternative hypothesis was proposed by Szilard (1960) as to how the repressor might operate at the ribosomal level of protein synthesis. He pictures the repressor



General scheme of the regulation of enzyme synthesis. GR = regulator gene, O = operator, SG_1, SG_2 = structural genes, m_1, m_2 = messengers made by SG_1 and SG_2, P_1, P_2 = proteins made by m_1 and m_2, R = repressor converted to R′ in presence of effector (F).

molecule being synthesized in the cytoplasm under the control of the regulating gene i and combining reversibly with the enzyme molecule on the ribosomes. Through the formation of a covalent bond between the ribosomal RNA and the enzyme molecule the repressor keeps the enzyme attached to the ribosome. The repressor is thought to combine with a specific controlling site which is distinct from the catalytic site, on the enzyme molecule. Stimulation of enzyme formation by the inducer could be explained by its ability to compete with the repressor for this controlling site. The concept of a controlling site on the enzyme molecule would explain the induction of enzymes by substances which are not substrates of the enzymes. It is also suggested that repressor compounds at the terminal end of biosynthetic pathways would have a specific chemical affinity for this controlling site only. An analogous model has been proposed (~~Rickenberg, 1960~~; Halvorson, 1960) where the inducer is pictured as catalytically increasing the rate of removal of the enzyme molecule from the enzyme forming site. The existence of specific enzymes which remove the polypeptide chain from the ribosomes has recently been reported (Chantrenne, 1961) and it is possible that the function of these enzymes could be influenced by the inducer or the repressor. Finally, as suggested by Chantrenne (1961),

since there is practically no information available on the mechanism that determines the secondary and the tertiary structure of the fabricated protein the possibility that inducers and repressor might function at this level cannot be eliminated at present.

A clear understanding of the mechanism of enzyme induction and repression must obviously await further developments in the field of protein biosynthesis. It is obvious however that together with the process of feedback inhibition, enzyme induction and repression represent important parts of the normal metabolic regulatory mechanism in bacteria.

Metabolic Control Systems in Animal Cells.

(a) Induced Enzyme Formation: Interest in induced formation of enzymes in animal cells was probably stimulated by the results obtained from microbial systems. Some of the pioneering work in this field such as that of Weinland (1899; 1906) who reported that the levels of invertase and lactase in dogs increased following administration of diets rich in sucrose and lactose, is difficult to assess because the experiments were not properly controlled. After the work of these early investigators little appears to have been done on induced enzyme formation in animals until relatively recently.

Studies on induced enzymes in animal cells have followed the pattern of similar studies in bacteria in that enzyme substrates were administered in increased amounts and the enzyme levels were measured at various time intervals afterwards. Thus Lightbody & Kleinman (1939) argued that in animals fed on diets with a high protein content there should be an increased need for the urea cycle. This was tested experimentally and a twofold increase in the levels of arginase was demonstrated in the livers of animals after several days on a high protein diet. Similarly Freedland & Harper (1957; 1958a, b) having reasoned that if glucose was substituted by fructose and galactose in the diets of rats the levels of the enzymes, responsible for converting these substances into glucose and for maintaining a normal level of blood glucose should increase. Thus increased levels of glucose-6-phosphatase and fructose-1-6-phosphatase were observed. These findings have recently been confirmed by Fitch & Chaikoff (1959) who further demonstrated increased levels of several glycolytic and Krebs cycle enzymes in the livers of rats fed diets high in fructose or glucose.

The increase in the activity levels of the enzymes concerned with carbohydrate metabolism was not of the same order as that observed with induced enzyme systems

in bacteria. The increases in most cases although less than twofold were statistically significant and the experiments appear to have been adequately controlled. Nothing is known concerning the nature of these increased enzyme levels although the inhibition of the increase in some cases by ethionine and its reversal by methionine (Freedland & Harper, 1958a) suggested that protein synthesis may be necessary for induction to take place. The possible influence of hormones on this system has so far not been sufficiently investigated and in view of the findings with other induced enzyme systems it will be interesting to see to what extent hormonal influence is involved.

Since Knox & Mehler (1951) first reported the induction of rat liver tryptophan pyrrolase this system has attracted the attention of many investigators. The level of this enzyme has been shown to increase approximately tenfold, 4 to 10 hours after intraperitonial injection of tryptophan and returned to normal 15 to 20 hours afterwards (Knox & Mehler, 1951). It was soon realized from further studies (Knox, Auerbach & Lin, 1956) that the phenomenon was not a simple straightforward induction of the enzyme by tryptophan. The level of the enzyme was also influenced by numerous other substances notably cortisone, hydrocortisone (Civen & Knox, 1959;

Knox, Auerbach & Lin, 1956) and insulin (Schor & Frieden, 1958). The ability of hormones to elicit a response in this system is a considerable handicap to the efforts being made to elucidate the mechanism involved. The effects of cortisone and hydrocortisone are probably non-specific since it is well known that these hormones can influence the levels of several other enzymes in liver (Knox, Auerbach & Lin, 1956; Rosen, Roberts & Nicol, 1959). A possible explanation of the effect of tryptophan on the tryptophan pyrrolase system emerged from findings of Ephemotchkina (1954) and Dubnoff & Dimick (1959). It was demonstrated by the latter workers that the rapid loss in the activity of tryptophan pyrrolase which occurred during incubation of rat liver slices in vitro could be prevented if tryptophan was present in the medium. In the light of these findings a possible interpretation of the effect of tryptophan would be that it functioned in vivo by preventing the breakdown of existing enzyme rather than enhancing enzyme formation. Further studies are obviously necessary before the true nature of the tryptophan pyrrolase system is understood.

The knowledge obtained from studies on the tryptophan pyrrolase system demonstrated that great caution must be exercised when interpreting some of the recent examples

of induced enzyme systems in intact animals. Because many of the reported cases (Knox, Auerbach & Lin, 1956) of induced enzyme formation in intact animals have not been investigated as thoroughly as the tryptophan pyrrolase system they are therefore difficult to assess at present. The increased activity of tyrosine -α-ketoglutarate transaminase in rat liver after tyrosine was injected (Knox & Goswami, 1960) resembles the tryptophan pyrrolase system in the rate and extent of the increase and by the fact that hydrocortisone elicited a comparable response. Further studies on this system should demonstrate the extent of this resemblance. A further interesting observation from studies of the tyrosine-α-ketoglutarate system was that tyrosine administration caused a fivefold decrease in the activity of liver hydroxyphenylpyruvate oxidase which degrades the hydroxyphenylpyruvate from tyrosine by the action of tyrosine-α-ketoglutarate transaminase (Knox & Goswami, 1960). This inhibition could be prevented and partially reversed in in vivo and in vitro by vitamin C, glutathione and 2,6-dichlorophenol-indophenol (Zannoni & LaDu, 1960), indicating that sulphydryl groups might be involved in the activity of hydroxyphenylpyruvate oxidase. It has not been stated whether the inhibition is specific to tyrosine alone and

it is difficult to ascertain what part such a mechanism would play in tyrosine metabolism since it is not similar to feedback control systems that have already been described in bacteria. It may however partly explain the abnormal tyrosine metabolism that usually accompanies scurvy. Furthermore this finding demonstrates that other factors excluding hormones can influence enzyme activity particularly so when sulphydryl groups are necessary for maximum enzymic activity.

Studies on carbohydrate metabolism in extracts of mammalian cells have demonstrated the existence of control mechanisms which operate independently from hormonal control. For instance, the availability of ADP has a profound effect on the respiratory rate of tissue extracts such as muscle. ADP stimulates the rate of respiration in tissue homogenates (provided suitable substrates such as pyruvate or succinate are available). Similarly the increased production of ADP, during muscular activity when the chemical energy in the terminal phosphate bond of ATP is hydrolysed for the mechanical energy of contraction, provides the necessary stimulus for increased respiration.

Examples of metabolic control by the products of reaction sequences through direct feedback inhibition of enzyme activity or by enzyme repression are not as

numerous in intact animal cells as in micro-organisms. The few examples that have been reported appear interesting, e.g. the threefold decrease in the level of rat liver phenylalanine hydroxylase 5 hours after an intraperitoneal injection of tyrosine (Auerbach & Weissman, & Wyckoff 1958). Since phenylalanine hydroxylase (in the presence of $NADH_2$ and oxygen) catalyses the conversion of phenylalanine to tyrosine a reasonable explanation for the decreased enzyme activity would be that a feedback inhibition or repression comparable to that described in micro-organisms had occurred. The enzyme was not inhibited in vitro by high concentrations of tyrosine so it is unlikely that the system resembles the inhibition of hydroxyphenylpyruvate oxidase (Knox & Goswami, 1960) by tyrosine. The evidence so far available suggests that this is an example of enzyme repression and since the phenomanon has also been demonstrated in animal cells (HeLa) maintained in culture (Eagle, Piez & Fleischman, 1957) it is unlikely that hormonal action is primarily responsible for the repression. Auerbach, Weissman & Wyckoff (1958) further demonstrated that if rats were maintained for longer periods on a diet containing high concentrations of phenylalanine and tyrosine, phenylalanine was metabolised through a secondary pathway because of the decrease in activity of phenylalanine

hydroxylase. Phenylpyruvate was thus formed,(by transamination from phenylalanine) and was excreted in the urine. Psychological tests revealed that the mental ability of these animals was considerably reduced and a situation analogous to the genetic disease phenylketonuria in humans was produced.

A situation where a pathological condition was returned to normal, probably by means of feedback repression has been described (Huguley et al. 1959). A child with megaloblastic anaemia was excreting up to 1.5 g. of orotic acid daily in the urine and failed to respond to Vitamin B12, folic acid, iron or pyridoxine. The patient returned to normal and the orotic acid excretion fell to 100 mg. daily if a mixture of uridylic and cytidylic acids was administered. This patient has been suggested (Huguley et al.,1959) as the human equivalent to the bacterial system described by Yates & Pardee (1957). As mentioned earlier, in the latter system, the presence of uracil in the growth medium repressed the synthesis of some of the enzymes involved in the early stages of the pathway leading to orotic acid and pyrimidine biosynthesis.

Another analogous system to that described in bacteria by Yates & Pardee (1957) has been demonstrated (Bresnick & Hitchings, 1961) in extracts of Ehrlich ascites tumour

cells. A direct feedback inhibition of aspartylcarbamyl transferase and dihydroorotase occurred in the presence of various pyrimidines and related compounds. Uridine-5'-phosphate was the most potent inhibitor of aspartyl-carbamyl transferase whereas fluoro-orotic acid, orotic acid and orotidine were the most effective inhibitors of dihydroorotase.

Cell free extracts are probably the most suitable material for studying feedback inhibition and other examples cited in the literature include the inhibition of hexokinase activity by the product of the reaction glucose-6-phosphate (Crane & Sols, 1953). The usual note of caution must be exercised when extrapolating back from 'in vitro' to 'in vivo' conditions. This is particularly true now since it is known that groups of enzymes are associated together in various cellular structures. This spatial segregation of enzymes with similar functions, suchaas occurs in lysosomes (De Duve, 1959) where various hydrolytic enzymes with acid pH optima (e.g. phosphatase, ribonuclease, β-glucuronidase, etc.) are enclosed, is probably an important part of the normal metabolic control system. This structural organization of the intact cell where the release of enzymes, co-enzymes, reactants and products is carefully controlled is missing in homogenates and cell extracts.

For studies involving enzyme repression and induction it is desirable, for the time being at least, that the structural organisation of the cell is maintained. Also in view of the results of similar studies on intact animals a system free from hormonal influence is extremely desirable. The disadvantage of using tissue slices for studies of this type is that whereas the structural integrity of the cells is maintained the system is not a viable one. Animal cells maintained outside their normal habitat in tissue culture conditions provide a system free from hormonal and nervous influence and one that should be ideally suitable for investigating the effects of environmental factors on the enzyme content of animal cells.

Metabolic Control Systems in Animal Cells Maintained in Culture: The improved methods for culturing animal cells which have been developed over the last 10-15 years have been mainly responsible for the present popularity of tissue culture in virology and biochemistry. The use of antibiotics in the culture media (Lepine, Barski & Maurin, 1950; Cruickshank & Lowbury, 1952) the development of newer techniques for growing cells and for obtaining pure cell lines from single cells (Sanford, Earle & Likely, 1948; Puck & Marcus, 1955) together with the provision of almost completely defined culture media (Eagle, 1955a,b,c;

Parker, Healy & Fisher, 1954; Morton, Pasieka & Morgan, 1956; Waymouth, 1959) represent some of the advances that are responsible for the present widespread use of tissue culture. Animal cells maintained in culture provide a system for biochemical studies where the cellular environment can be altered rapidly and precisely and where all the cells in the culture are exposed to any changes in their surround. Since the cells are free from complicating factors such as hormones any alterations in the basal metabolism can usually be correlated with changes in the environment.

A few examples of enzyme induction and repression in cultured animal cells have been reported in recent years. For example, the activity level of cholinesterase in primary explants of chick embryonic intestine was decidedly influenced by the presence of acetylcholine in the culture medium (Jones, Featherstone & Bonting, 1956). Acetylcholine was found to prevent the disappearance of cholinesterase activity and since no increase in activity occurred it appeared that this was simply a case of stabilization of the enzyme by its substrate. However, Burkhalter, Jones & Featherstone (1957) later showed that a 2-6 fold increase in the specific activity of cholinesterase could be obtained if primary explants of 15 day-embryonic chick lung were incubated for 8 days in the

presence of 0.2 M acetylcholine. The increased enzyme activity appeared to be a 'true' cholinesterase induction since related compounds such as β-methyl choline were not hydrolysed to the same extent as acetylcholine. Maximum cholinesterase activity was obtained 4 days after addition of acetylcholine to the medium. It was not stated if other choline esters elicited a similar response and this does not seem too unlikely in view of the findings (Burkhalter et al., 1957) that acetyl choline, β-methyl choline and β-dimethyl aminoethyl acetate are equally effective in stabilizing the cholinesterase activity in primary explants of chick embryonic intestine.

Another example of a possible substrate induction of an enzyme in cultured cells has been recently reported (Klein, 1961). Increased arginase activity was demonstrated following the addition of arginine and RNA (from yeast) to the medium of several cell lines. Addition of arginine alone had little effect on the arginase activity unless the cells were previously grown in the presence of yeast RNA (250 ug/ml) for several days. This unusual 'RNA effect' was explained (Klein, 1961) in terms of competition for available RNA precursors between various enzyme forming systems in the cell. When the cells were supplied with a surplus of RNA precursors enzyme forming systems could then function at their

maximum rate. This line of argument however is not entirely correct because cultured cells contain relatively large pools of purines and pyrimidines (Thomson, Davidson & Paul, 1958) which would provide an ample supply of precursors for RNA synthesis. Also the addition of RNA alone to the medium resulted in a considerable increase in the arginase activity which was further slightly increased by the addition of arginine. Even though the alkaline phosphatase activity of the RNA and arginine-treated cultures was similar to that of control cultures there still remains some doubt about the specificity of this increased enzyme activity.

The induction of alkaline phosphatase in HeLa cells (Cox & Macleod, 1961; Cox & Pontecorvo, 1961) by prednisolone and by organic monophosphates appears to be a particularly interesting system. A series of six HeLa lines were tested for inducibility of alkaline phosphatase by prednisolone and only two cell lines, which originally had the lowest phosphatase activity, were found to be inducible. When the inducible cell lines were grown in the presence of high concentrations (200 ug/ml) of organic monophosphates a fivefold increase in the specific activity of alkaline phosphatase was obtained after 6-10 days compared with a fortyfold increase when prednisolone was used as the inducer. The induction appears to be

specific for alkaline phosphatase as the acid phosphatase content of the cells was not appreciably altered. In contrast to the alkaline phosphatase in E.coli (Torriani, 1960) the mammalian enzyme was not influenced by the concentration of phosphate ions in the growth medium. Increases in the phosphate ion concentration neither suppressed the activity of the 'constitutive' lines or inhibited the induction by prednisolone. The possibility of selection of a mutant which possessed the enzyme in high amounts is unlikely since, in common with some of the inducible bacterial systems, the enzyme was diluted by cell multiplication when the inducer was removed.

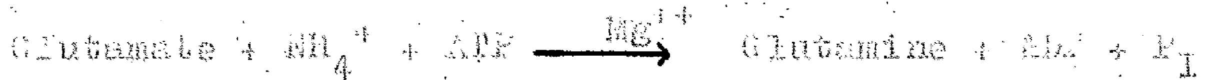
A control system possibly combining enzyme induction and repression has recently been described (Weissman, Smellie & Paul, 1960) in strain L cells. Investigations on the thymidine kinases, which in extracts of mammalian cells (in the presence of Mg^{++} and excess ATP) phosphorylate thymidine to the 5'mono, -di and triphosphates (TMP, TDP, TTP), revealed that these enzymes fluctuated during the growth cycle of the cells. The levels of TMP, TDP, and TTP-forming enzymes were found to increase during the logarithmic phase but decreased again when growth ceased. Furthermore in conformation of previous findings on rat liver, regenerating (Bollum & Potter, 1959; Mantsavinos & Canellakis, 1959) the activity of the TMP-forming enzyme

increased first followed by subsequent increases in TDP and TTP-forming enzymes. If thymidine was included in the culture medium during the growth phase, a considerable increase in the activities of the three enzymes was observed. These findings strongly suggest that a sequential induction had taken place. The fact that the levels of the three enzymes were diminished when growth ceased (independently of the presence of thymidine in the medium) suggested that some type of feedback control mechanism was operating.

Other examples of product inhibition of enzyme activity (or synthesis) in cultured animal cells include the previously mentioned tyrosine-inhibition of phenylalanine hydroxylase in HeLa cells (Eagle, Piez & Fleischman, 1957). Tyrosine is not an essential amino acid to the intact mammal, being manufactured from phenylalanine in the liver by phenylalanine hydroxylase. Most mammalian cells in culture have a requirement for tyrosine – including cells derived from liver (Eagle, 1959). One subline of the HeLa has been propagated in a medium lacking tyrosine and was found to have an increased level of phenylalanine hydroxylase (Eagle, 1959).

A similar situation involving glutamyl transferase, in HeLa cells, has been described by De Mars (1958). Glutamyl transferase catalyses a transfer reaction in which

γ-glutamylhydroxamate is formed from glutamine and hydroxylamine in the presence of catalytic amounts of arsenate, ADP and manganese (Meister, 1956). The enzyme also catalyses the synthesis of glutamine from glutamic acid and ammonia (Meister, 1956) as follows:



$$\text{Glutamate} + NH_4^+ + \text{ATP} \xrightarrow{Mg^{++}} \text{Glutamine} + \text{ADP} + P_i$$

Most animal cell strains maintained in culture have an absolute requirement for glutamine but some strains can be 'trained' to grow in its absence if the glutamic acid concentration of the growth medium is increased approx. 20-fold (to 20 mM) (Eagle, 1959). When HeLa cells were grown in a medium lacking glutamine it was found (De Mars, 1958) that the specific activity of glutamyl transferase increased considerably and gradually diminished again when glutamine was reintroduced into the culture medium. This appeared to be a straightforward feedback control mechanism where the product of the reaction controlled (by repression) the activity of the enzyme responsible for its synthesis.

An example of substrate inhibition of enzyme activity in cultured animal cells has also been described (Lieberman, 1957). It was observed that the specific activity of NADase (DPNase) was decreased considerably in strain L cells when NAD (DPN) was included in the growth medium. Since nicotinamide mononucleotide and nicotinamide riboside were more efficient inhibitors of

NADase activity than NAD itself it appeared that the NAD effect could be due to a feedback inhibition of NADase by its hydrolysis products. The system was reconstructed in cell-extracts and it was found that the amounts of nicotinamide mononucleotide and nicotinamide riboside produced by NADase action in a given period of time were not sufficient to account for the NAD effect. NAD itself was probably responsible for the 'in vivo' inhibition of NADase activity. The inhibition was specific for NADase as the activity of other enzymes such as lactic dehydrogenase, $NADH_2$-cytochrome C reductase, adenosine deaminase and glucose-6-phosphate dehydrogenase was not altered.

These findings demonstrate that in addition to hormonal and nervous control systems other regulatory mechanisms exist in animal cells. Practically no information is available at present about the operative mechanism involved in these regulatory systems. It remains to be seen for instance whether substrate induced enzyme activity in animal cells involves de novo protein synthesis or if some other mechanism is utilized.

<u>Cellular Identity of Animal Cells maintained in Culture:</u>

Animal cells maintained in culture will probably be a most useful research tool in investigating the mechanism involved in these control systems. It is sometimes

difficult to establish with certainty the origin of cells in culture because many fundamental characteristics are often lost after mammalian cells are explanted to in vitro conditions (Bloom, 1937). The pronounced morphological and metabolic alterations that accompanied the change from in vivo to in vitro conditions was interpreted by earlier workers (Champy, 1913) as being due to a process of dedifferentiation whereby the cells reverted to a common undifferentiated condition. It was later demonstrated however that many alterations, particularly metabolic ones, were not permanent and when cell multiplication was purposely slowed down the original metabolic characteristics returned. Thus Doljanski (1930) showed that a resumption of pigment formation by pure strains of iris epithelium could be obtained under conditions where cell growth was depressed. Similarly accumulation of glycogen in liver epithelial cells which were previously incapable of forming the polysaccharide has been reported (Westfall et al., 1953). Reversible changes such as those described have been referred to as modulations (Weiss, 1953), in contrast to the irreversible process of true differentiation.

Other examples of alterations in cell metabolism following explantation, such as the inability of many cell strains derived from liver, to hydroxylate phenyl-

alanine may be due to a 'selection' process. Since liver tissue is composed of many different types of cells it is not unlikely that only one cell type is able to hydroxylate phenylalanine and that this has not yet been obtained in culture. The results of Jones, Featherstone & Bonting (1956), Eagle, Piez & Fleischman (1957) and De Mars (1958) have demonstrated that alterations in metabolism of cultured cells are freely reversible.

Paul & Pearson (1957a, b) demonstrated profound changes in the oxygen consumption of chick embryonic heart and liver 48 hours after explantation to 'in vitro' conditions. For instance the initial rate of oxygen utilization of liver explants was high but decreased rapidly, remained at a low level and was not altered by renewal of the medium. The rate of oxygen utilization of the explanted heart tissue on the other hand was very low initially but increased rapidly within 48 hours to a level resembling that of liver tissue. Histological studies on the explanted material failed to correlate these metabolic changes with the death of most of the cell types and their replacement by cells possessing this type of metabolic pattern.

The similarity of the nutritional requirements of many cultured cells both normal and cancerous (Eagle, 1959) derived from different mammalian species has often been

proposed as an indication that a common cell type emerges under tissue culture conditions. Many of the studies on the nutritional requirements of cultured animal cells have been carried out in media supplemented with dialysed serum which contains proteolytic enzymes and peptidases and therefore accurate assessment of the amino acid requirements in such cases is difficult. Also as pointed out by Lucy (1958) similarity in amino acid requirement between different cell types does not eliminate the possibility of different rates of utilization of these compounds as indicated by the decreased utilization of arginine by organ cultures of mouse prostate glands following treatment with the carinogenic compound, 20-methylcholanthrene.

There are several important exceptions to the general uniformity in the nutritional requirements by cultured cell types of different origins. Although a minimum of 12 amino acids (in Eagle's medium) are essential for growth and survival of many different types of cell strains some cell strains such as rabbit fibroblasts have a requirement for serine (Haff & Swim, 1958). Some tumour cells (McCoy et al., 1956; 1959), require the presence of asparagine in the medium for continuous propagation. Similarly monkey kidney cells have a requirement for glycine in a medium lacking folinic

acid (Eagle, 1959). Also citrulline can replace arginine in the medium of cells of the Novikoff hepatoma but not with cells of the Walker carcinosarcoma, the Jensen sarcoma or the HDAB hepatoma (McCoy & Maxwell, 1959). Finally the mouse lymphosarcoma cell L5178 (Fisher, 1958), has more exacting nutritional requirements than the strain L cell, subcutaneous fibroblastsalso of murine origin.

The activity of specific enzymes has been used as a parameter for comparing cells in culture with the tissue of origin. In many cases marked differences have been observed as for example the absence of any activity in the Chang liver cells for the following enzymes: tryptophan pyrrolase, tyrosine transaminase, histidase (Auerbach & Walker, 1959), glucose-6-phosphatase, fructose 1-6-diphosphatase and fructokinase (Berske, Parks & Walker, 1957), together with many other enzymes which are present in normal liver. The levels of some enzymes, such as aspartylcarbamyl transferase, compared well with those found in normal rat liver. Similarly, when the levels of rhodanese and arginase of thirteen mouse strains and two human strains (Hela and epidermis) were compared (Westfall, et al. 1958) with the tissue of origin, differences in the activities of both enzymes were found to exist. The human skin cells had very low

arginase levels while three strains of mouse liver cells displayed arginase and rhodanase activity comparable to the tissue of origin. An interesting difference was observed in the arginase levels of two of the strains which were both originally derived from a single mouse tumour cell. One of these cell strains has 20 times more and the other 400 times more arginase activity than the parent strain.

Lieberman and Ove (1958) investigated the possibility that estimation of enzyme activity levels might provide an insight into the origin of cells in culture. These workers found a marked similarity in the specific activity of several enzymes such as acid phosphatase, adenosine deaminase, catalase, glucose-6-phosphatase dehydrogenase and rhodanase in cell free extracts prepared from four human cell lines derived from different sources (e.g. appendix, cervix, liver and lung - all having an epithelial-like morphology). These findings have been partly confirmed by Nitowsky & Herz (1961) who also demonstrated however that a definite dissimilarity in the alkaline phosphatase levels existed between various cell lines. For instance the alkaline phosphatase activity in extracts of the Chang liver line was 25 to 200-fold higher than that of cell lines derived from other mammalian tissues, thus resembling the situation

found in the whole animal. When several clonal lines of the Chang liver cell were obtained by the technique of Puck, Marcus & Ciecciura (1956) large variations in the alkaline phosphatase in the different clones were observed. Similarly Cox & McLeod (1961) have demonstrated 12 to 50-fold differences in the alkaline phosphatase activity of several uncloned HeLa cell lines. The acid phosphatase activity on the other hand was relatively similar in extracts of all the HeLa cell lines. The cellular identity of the cells used was established by chromosomal morphology, poliovirus susceptibility and serological properties.

These experiments indicated that the alkaline phosphatase activity of a cell line may in some instances provide a yardstick to determine its origin. The large variations in the alkaline phosphatase of various cloned and uncloned lines of two cell strains indicated however that great caution must be exercised when interpreting the results. Enzymic estimations on cultured mammalian cell lines derived from different types of tissue have not, on the whole, compared with some of the marked differences in enzyme levels that exist between organs in the intact animal.

<u>The Occurrence of Multiple Molecular Forms of Enzymes - Possible Significance in Metabolic Control Systems:</u>
Improved chromatographic, electrophoretic and immunological

techniques for resolving complex mixtures of macromolecules have contributed enormously to our knowledge of enzymes. A direct consequence of this, in recent years, has been the isolation of several distinct molecular forms of proteins that perform the same biological function. The term 'isozyme' has been suggested (Markert & Moller, 1959) "to describe the different molecular forms in which proteins may exist with the same enzymatic specificity". In the last few years a great deal of evidence has accumulated which demonstrates that not only do physical and chemical differences exist between functionally similar proteins in different species of animals but that physically and chemically distinct forms of homologous proteins exist within a single individual and even with a single tissue.

In view of the evidence demonstrating that protein structure is genetically controlled (Allison, 1959) it would be anticipated that proteins with similar functions would display chemical and physical differences in individuals of different genotype. Thus the classical studies of Sanger and his co-workers (Harris, Sanger & Naughton, 1956; Sanger, 1959), have revealed differences in the amino acid sequences of insulins derived from different species. Similarly variations in the amino acid sequences of ribonucleases obtained from various biological sources have been demonstrated (Anfinsen et al.,

1959; Aqvist & Anfinsen, 1959). The "fingerprinting" technique employed in these investigations combines separation by chromatography and electrophoresis following digestion of the protein by proteolytic enzymes.

Some indication about differences in structural configuration between functionally similar proteins can also be gained from other techniques such as immunology. An antiserum prepared against a purified enzyme from a known source will usually specifically inhibit the activity of that enzyme. An indication of molecular differences or similarities can then be obtained by comparing the degree of inhibition of homologous enzymes from various sources. Henion & Sutherland (1957) using this technique found that pronounced species differences existed in the enzyme phosphorylase isolated from chicken, cat, rabbit and dog. Besides species variations it was also observed that the phosphorylase from dog muscle and liver were not immunologically identical. Similar variations in the lactic dehydrogenases from various rabbit organs have also been demonstrated (Niesselbaum & Bodansky, 1959). Kaplan & Ciotti (1961) have employed analogues of NAD to similarly distinguish between lactic dehydrogenases from various sources.

The separation of macromolecules by electrophoresis, developed by Tiselius (~~1937~~) has proved to be a most

useful tool for investigating the physico-chemical properties of biological macromolecules. Molecular variations are almost certainly reflected in the differences in electrophoretic mobility that have been shown to exist for many homologous protein molecules. Thus the different electrophoretic mobilities of homogeneous rat and rabbit aldolase (Taylor, Green & Cori, 1948) at three different pH values and under similar conditions could be attributed to variations in chemical configuration between functionally similar proteins from individuals of different genetic make up.

The development of newer electrophoretic techniques aimed at improving the degree of resolution has considerably increased our knowledge on similarities and differences between homologous proteins from various sources. To overcome some of the limitations of paper electrophoresis various supporting media such as starch paste and starch grains have been employed. Although gels have been used as supporting media for electrophoresis for several years it was not until relatively recently that their potential was fully realized. The introduction of starch gels (Smithies, 1955) as a suitable supporting medium for electrophoresis provided a system with an extremely sharp resolving power, exceeding that of other supporting media or free boundary electrophoresis. Separation on starch

gels introduced an additional parameter to electrophoretic resolution in that the size and the shape of the molecule as well as its electrical charge determined its electrophoretic mobility. Variations in cattle (Ashton, 1957) and human (Smithies, 1955; 1959), haptoglobins have been demonstrated between different individuals of the same species by this technique. Differences in the serum proteins of several mammalian species have also been revealed (Latner & Zaki, 1957).

Starch-gel electrophoresis was employed by Markert & Hunter (1959) for separating tissue esterases from various organs of the mouse. These workers introduced a technique adopted from a well known histochemical method for locating esterase activity (Nachlas & Seligman, 1949), so that the position of the various enzymes could be revealed directly on the gel after electrophoresis. The section of the starch gel showing the enzymically active proteins has been termed a 'zymogram' (Hunter & Markert, 1957). Isozymic forms of several enzymes have been isolated by means of this technique (Markert & Moller, 1959; Tsao, 1960).

Although the existence of isozymes now appears to be well established the physiological significance of their occurrence has so far not been recognised. The recent findings of Stadtman et al. (1961) and Gregolin et al. (1961)

strongly suggest that isozymes may be implicated in metabolic control systems. The experiments of Stadtman et al. (1961) demonstrating that the activity of the two aspartokinases in E.coli was controlled independently by threonine and lysine have already been described (page 21). Two distinct forms of lactic dehydrogenase have been recognised (Gregolin et al. 1961) in yeast. One form appeared when the yeast cells were grown under anaerobic conditions and the other when the cells were placed in an aerobic environment. These findings demonstrated that the existence of chemically and physically distinct forms of these enzymes was part of the overall metabolic regulatory mechanism of the organism. It would be interesting to investigate this aspect of the occurrence of multiple molecular forms of enzymes in animal cells.

Objects of these Investigations:

This work was undertaken to study the occurrence and operative mechanism of adaptive enzyme formation in animal cells. Studies of this nature may be of special importance in view of the possible implications of adaptive enzyme formation in cytodifferentiation and homeostasis. These latter phenomena represent some of the most important problems in the entire field of biology at present. When these studies were initiated the evidence available for the occurrence of adaptive enzymes in animal cells was not convincing. This was mainly due to the fact that

investigations were usually performed on intact animals where the system was difficult to control and because of hormonal influence no definite conclusions could be drawn. The use of animal cells maintained in culture seemed a more promising system to overcome these limitations.

Concerning the mechanism of adaptive enzyme formation it is by no means certain whether this is the same in animal cells and in bacteria. Within the past year some advances have been made towards elucidating the mechanism in bacteria. The hypothesis of Jacob & Monod (1961) which was based on genetic evidence suggested that protein biosynthesis in bacteria was controlled by specific compounds which regulated the flow of an unstable messenger RNA from the structural genes to the ribosomes. Practically no information is available on the mechanism controlling protein biosynthesis in animal cells. Studies performed on the biosynthesis of haemoglobin (Dintzis, Borsook & Vinograd, 1958) and serum albumin (Campbell, 1960) suggest that the messenger RNA remains on the ribosomes for a longer period than its bacterial counterpart. The mechanism regulating protein biosynthesis may therefore differ in the two forms of life.

SECTION 2. METHODS

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Apparatus and general technique employed for separating proteins by starch-gel electrophoresis.

The apparatus and technique described by Smithies (1955) was slightly modified. Proteins were separated in starch gels by applying an electric current, horizontally across the gel, for standard times. Instead of platinum electrodes (Smithies, 1955), carbon electrodes were used and single electrode vessels at the anode and cathode-end were found adequate.

Starch: The most suitable type of starch for starch-gel electrophoresis was one which formed a firm gel and did not break during electrophoresis or subsequent handling. Several batches of 'Analar' soluble starch (British Drug Houses, Ltd.) were tested until a suitable one was found. In later studies 'starch-hydrolysed' (Connaught Laboratories) was used because this starch produced a more firm gel which considerably reduced the possibility of breaking the gel on subsequent handling. In addition a slightly better resolution of protein mixtures was obtained with this starch.

Preparation of starch-gels: A 12-15% (W/V) starch suspension was prepared in a buffer, pH 8.64, consisting of 0.076M-tris and 0.005M-citric acid. The suspension was heated over a naked flame, with constant swirling to ensure uniform heating. On disruption of the starch grains the suspension became more viscous and shortly

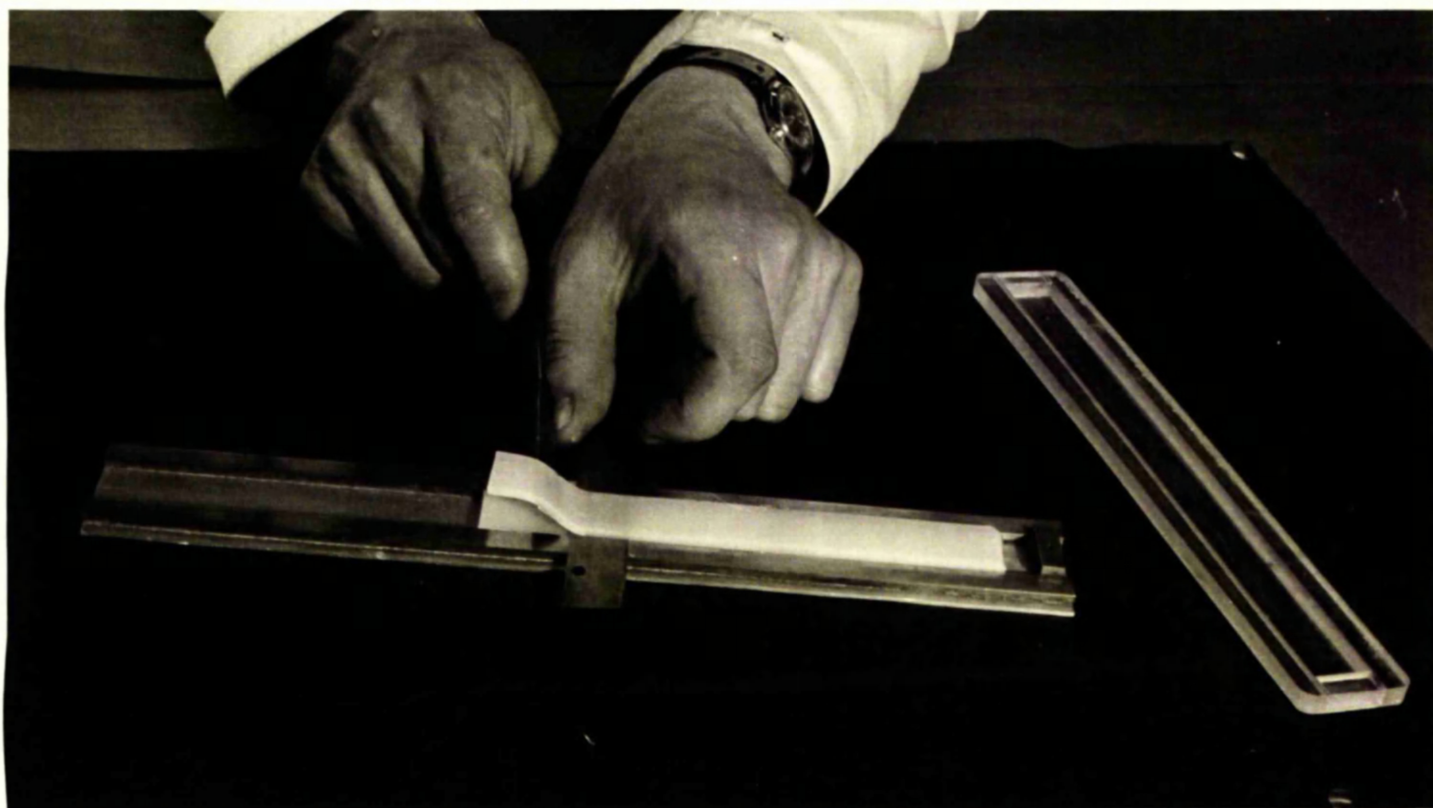
after this it was boiled for a few seconds under reduced pressure to remove air bubbles. Immediately afterwards the suspension was poured into perspex moulds (fig. 2) and covered with a thin film of polythene to prevent evaporation. Excess suspension was removed from the mould, while the gel was still fluid, by pressure with a flat piece of perspex. The suspension was allowed to form a gel by standing at room temperature for approximately 1 hour after which excess gel was removed from the sides of the tray with a razor blade.

Pieces of Whatman No.3MM filter paper, cut exactly to the cross section of the gel and then saturated with tissue extract were inserted into slits in the gel one third of the way from the Cathode-end. The electrode vessels were filled with a buffer, pH 8.0, composed of 0.3M - boric acid - 0.005M - NaOH which was renewed for each electrophoresis run. The levels of the buffer in the electrode vessels were equilibrated by means of a T-piece. Filter paper wicks were used to connect the gels to the electrode vessels (See Figure 1). The duration of electrophoresis was usually 4 hr. at 6V/cm. of gel and a current of 5 milli-amperes per gel. After electrophoresis the gels were cut longitudinally with a razor blade on a cutting block designed specially for this purpose (Fig. 2). The cut surfaces of the gel were stained for non specific proteins or enzymic activity.

Figs. 1 and 2. Apparatus used in starch gel electrophoresis.

Fig. 1.

Fig. 2.



Staining for non-specific proteins: Naphthalene Black 10B (G.T. Gurr, Ltd.) was a more sensitive stain than either bromophenol blue or bromocresol green and was used as described by Smithies (1955). To reveal the location of proteins the gel was immersed for 30 secs. in a saturated solution of the Naphthalene Black 10B in methanol-acetic acid-water (50:10:50). Background staining, which proved difficult to remove, resulted when the gel was allowed in contact with the stain for periods longer than 30 secs. Excess Naphthalene Black was removed from the gel by washing with methanol-acetic acid-water (50:10:50). Three washings were usually required, the gel being left in the final washing for 12-15 hrs.

Staining for esterases: The Gomori modification (1952) of an original histochemical method (Nachlas & Seligman, 1949) for locating esterase activity was first applied to protein mixtures resolved by starch-gel electrophoresis by Hunter & Markert (1957). In this method esterase activity was revealed with the aid of a chromogenic substrate, α-naphthyl acetate, by coupling the naphthol released with a diazo compound (tetrazotized-o-dianisidine). In these investigations the method was adopted as follows. A stock solution containing 1% (W/V) of α-naphthyl acetate in 50% (W/V) of acetone was prepared. This solution was

stable for at least one week if stored at 4°. The 'substrate-mixture' was prepared, immediately before required, by adding (slowly and with stirring) 0.8 ml. of this solution to 50 ml. of water. Tetrazotized o-dianisidine (Fast Blue Salt B, G.T. Gurr, Ltd.) (20-30 mg.) was then dissolved in this mixture.

Before adding the substrate mixture the gels were first immersed in 0.25M phosphate buffer, pH 6.25, for 15 mins. This reduced the pH of the gels to the region of the optimum pH for esterase activity and also prevented the spontaneous hydrolysis of the substrate which would occur at more alkaline pH values. The freshly prepared substrate mixture was then added to the 'buffered' gels and esterase activity was allowed to develop at 37°. The time required for adequate development varied with the activity of the preparation but was usually in the region of 20 mins. for extracts of tissues, freshly isolated from intact animals. With extracts prepared from cultured cell lines longer development periods were required (usually 12-16 hrs.). Background staining was removed by washing the gels in methanol-acetic acid-water, as before.

Classification of esterases revealed by starch gel electrophoresis: Augustinsson (1958) was able to distinguish esterases isolated from different sources on

the basis of their inhibition by specific inhibitors such as physostigmine. The inhibitory effects of physostigmine salicylate and NNN'N'-tetraisopropyl pyrophosphoramide were investigated here. The inhibitors were added to the buffer used for preparing the gel and prior to staining the gels were immersed (for 30 mins.) in 0.25M-phosphate buffer, pH 6.25, containing the inhibitor.

Location of phosphatases: This method was essentially the same as the technique used for revealing esterase activity. The substrate on this occasion was α-naphthyl phosphate. After electrophoresis longitudinal sections of the gel were submerged in an acetate buffer, (0.25M-acetic acid-sodium acetate), pH 5.8, when locating acid phosphatase activity, and in a borate buffer (0.25M-boric acid-0.10M-NaOH), pH 9.0, for revealing alkaline phosphatase activity. After 15 mins. a freshly prepared 1% (W/V) solution of α-naphthyl phosphate, dissolved in 5 ml. of appropriate buffer, was added to the buffered gels. This was immediately followed by a 1% (W/V) solution of tetrazotized o-dianisidine in 5 ml. of water. The phosphatase bands were allowed to develop to completion at 37°. Spontaneous hydrolysis of the substrate occurred at alkaline pH values and because of this, the substrate mixture was renewed from time to time when testing for

alkaline phosphatase. The gels were washed by the usual procedure when development was complete.

Locating catalase activity: The technique described by Hale & Renwick (1961) was adopted as follows. After electrophoresis the gels were immersed in a 1% (V/V) solution of hydrogen peroxide. After 5 mins. the peroxide was removed, the gels were washed several times with water to remove all traces of excess peroxide and finally submerged in 4% (W/V) potassium iodide solution, acidified with glacial acetic acid. Iodine, released from potassium iodide by peroxide, stained the starch gel a deep blue except in those arease where the peroxide was destroyed by catalase activity. By this technique catalase activity was revealed as white bands against a dark blue background. Using this method it was necessary to dilute the original tissue preparation approx.50 times. The reduced level of catalase activity in the diluted preparation prevented 'cracking' of the gel due to excess release of gas and also resulted in a sharper definition in the areas of catalase activity. The results were usually recorded diagrammatically immediately after staining because the blue background was found to fade very quickly.

Staining for peroxidase: After electrophoresis longitudinal sections of the gel were submerged in a

reaction mixture prepared as follows. A solution of benzidine in glacial acetic acid was added to 1% (V/V) hydrogen peroxide to give a final benzidine concentration of 0.01g%. A blue green colour quickly appeared at the sites of peroxidase activity.

Recording of results: Results were recorded diagrammatically as soon as possible after staining. Protein and esterase patterns were recorded by photography on Kodak microfile film. Standard exposure and development times were determined experimentally. For photography of protein patterns a red filter was used and for photography of esterase patterns a blue filter.

Preparation of tissue extracts for starch-gel electrophoresis: Several extraction procedures were initially tested, including extraction with water, 'balanced salt solution' (Hanks & Wallace, 1949) and various buffer solutions. Extraction with these solutions followed by ultrasonic treatment for various periods was also examined. Because of their ease of identification and widespread occurrence the esterases were chosen to investigate this aspect of the technique. The method used for preparing the tissue extract was found to affect the esterase pattern obtained. For instance when a tissue was extracted with water or balanced salt solution (0.15M) the resulting esterase patterns were identical and were not altered appreciably by ultrasonic treatment for periods up to 25 mins. On the other hand when tris or phosphate buffer (0.06M), pH 7.3 and pH 8.3, were used as solvents in the extraction procedure different esterase patterns were obtained.

Fig. 3. Comparison of the esterase zymograms obtained from mouse liver extracts prepared by different procedures.

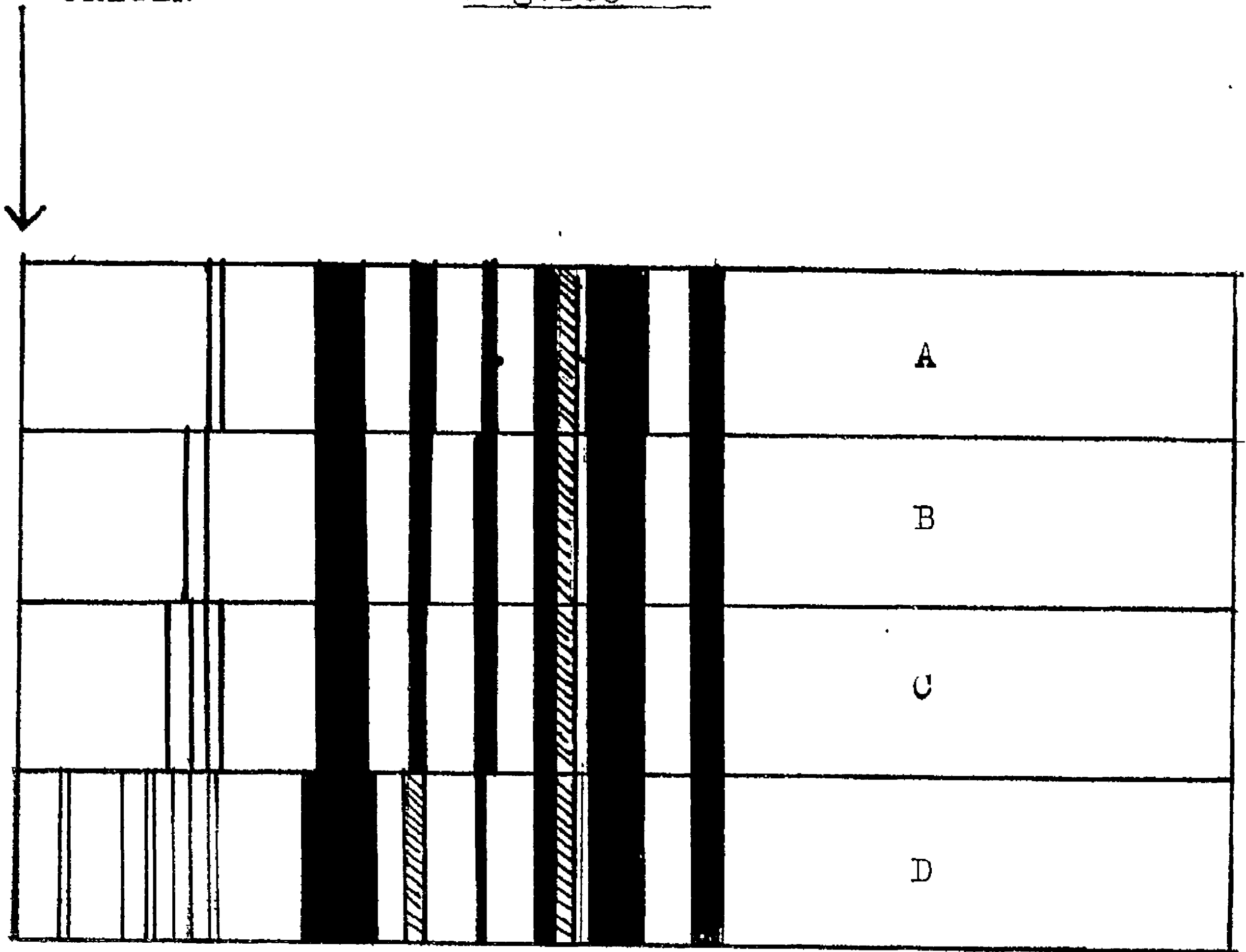
A. Homogenised in tris (0.076M); citric acid (0.005M) buffer, pH 8.64, followed by ultrasonic treatment for 20 min.

B. Homogenised in 0.06M phosphate buffer, pH 8.3 or 7.2 and subjected to ultrasonic vibrations for 20 min.

C. Homogenised in 0.06M phosphate buffer, pH 8.3 or 7.2

D. Homogenised in water or balanced salt solution and treated with ultrasonic vibrations for 20 min.

Figure3



As demonstrated in figure 3 extraction with phosphate or tris usually resulted in the loss of four or five esterase bands and an increase in the intensity of others compared with an extraction performed with water or balanced salt solution. A further loss of esterase bands and a progressive increase in the intensity of others was observed when a tris or phosphate extract was subjected to ultrasonic treatment for various periods of time. On the basis of these findings a standard extraction procedure was adopted as follows.

Extracts from tissues of intact animals: Organs were immediately removed from animals, killed by exsanguination after stunning or anaesthetizing with ether. Human tissues were obtained from the post mortem room. All tissues were thoroughly washed with NaCl (0.15M) immediately after removal and stored at -10° until required. Prior to extraction as much fatty material as possible was removed from a tissue which was then chopped up into small pieces. Grinding with 0.5 vol. of iso-osmotic salt solution or balanced salt solution in a Potter-Elvehjem homogeniser, followed, until a uniform homogenate was obtained. The homogenate was then placed in an ice bath and subjected to ultrasonic vibrations for 15-20 mins. with a Mullard ultrasonic drill (50W, 20 kcyc./sec.). The homogenate was finally centrifuged at 35,000g. for

2 hours at 0° and the clear supernatant was used for electrophoresis. It was found necessary to centrifuge at high speeds to ensure that the supernatant was completely free of small particles. If a 'cloudy' supernatant was used small particles usually infiltrated the gel, resulting in an unsuitable electrophoresis pattern where the position of the bands were obscured by excessive 'streakiness'.

Extracts from cultured cells: Cells were usually grown as monolayers on glass by techniques described in a later section. They were harvested by scraping from the glass with a rubber 'policeman' or by treatment with 0.5% (W/V) trypsin (in balanced salt solution). Preliminary homogenisation was not found necessary when preparing extracts from cultured cells. The cells were suspended in 0.5 vol. of balanced salt solution and subjected to ultrasonic vibrations for 2-3 mins. An alternative method of disrupting the cells was by rapidly freezing the cell suspension to -70° (in alcohol-carbon dioxide) followed by rapid thawing at 37°. The suspension was finally centrifuged at 15,000g for 2 hrs. at 0° and the clear supernatant was used for electrophoresis. No differences in electrophoresis patterns were detected between cell extracts prepared by the 'freezing and thawing' method or by ultrasonic treatment.

Outline of tissue culture methods:

Among the established cell strains used were the mouse subcutaneous fibroblast, strain L, clone 929 (Sanford, Earle & Likely, 1948), the mouse lymphosarcoma, strain L-5178Y, (Fisher, 1958), the human cervical carcinoma cell, strain HeLa (Gey, Coffman & Kubicek, 1952), the human epithelioma cell, strain H.Ep.#1 (Toolan, 1954), and the human foetal liver (parenchymal) cell, strain HLM, (Leslie, Fulton & Sinclair, 1956). The cells were usually grown as monolayers on glass with the exception of strain L-5178Y which was grown in suspension. A variant of strain L which grows in suspension has recently been isolated in this laboratory and was used in some of the later studies. Suspension cultures have usually been found easier to maintain than monolayer cultures and also for experimental purposes replicate cell samples can be obtained with comparative ease.

Cells from disaggregated tissues: Cells were also derived from recently disaggregated tissues such as human kidney, heart and skin. The technique (Paul, 1960) was as follows. The tissues were removed aseptically. Kidneys were decapsulated and the pelvis and medulla dissected out leaving only the cortex. After the tissue was cut into very fine pieces (approx. 3-5 mm) it was washed several times with balanced salt solution.

The chopped tissue was transferred to a sterile 'fluted' Ehrlenmeyer flask (Bellco Glass Inc., Vineland, New Jersey) containing 100 ml. of 0.25% (W/V) trypsin in Eagle's medium (preheated to 37°). A sterile silicone-covered magnet was inserted and the contents of the flask stirred slowly over a magnetic stirrer for 20 mins. The supernatant was removed and discarded. Another 100 ml. of warm trypsin solution was added and after 20 mins. the supernatant was decanted into sterile centrifuge tubes. Three further harvests were similarly obtained and all the samples were pooled. Cells were separated by centrifuging at 1000g for five minutes and inoculated into flasks at 300,000 cells/ml. in Eagle's medium supplemented with 10% calf serum.

<u>Maintenance of cell cultures</u>: Cell cultures were maintained by standard techniques (Paul, 1960). The cells were fed approximately twice weekly and transferred (approximately once per week) by treatment with 0.5% trypsin. For experimental purposes cells were used at standard time intervals after transfer and feeding. The composition of the various media used for routine cell maintenance and for experimental purposes is outlined in the Appendix. Except where otherwise stated the medium was supplemented with dialysed calf or human serum. Serum was dialysed against running tap water. After

48 hrs. it was diluted (1:1) with glass distilled water, rendered iso-osmotic with balanced salt solution and filtered through a sterile Seitz filter into sterile containers.

Assay procedure for determining enzyme activity levels of extracts of cultured cells.

Preparation of extracts: The cells were harvested at standard time intervals by scraping from the glass or by treatment with 0.5% (W/V) trypsin solution. After two washings with iso-osmotic saline, extracts were prepared by freezing and thawing in 0.14M sodium chloride. Finally the extract was centrifuged at 15,000g for 1 hr. at 0° and the supernatant was used for enzyme assays.

Units of enzyme activity: Unless otherwise stated a unit of enzyme activity is defined as the amount causing the removal of 1 umole of substrate or yielding one 1 umole of product per minute. Specific activity was expressed as units per mg. of protein-nitrogen or units per million cells. Protein-nitrogen was estimated by nesslerization (Paul, 1958) and the cells were countedd with the aid of an electronic counter (Ljungberg & Co., Stockholm).

Note on the assay procedures employed: Standard procedures were adopted to suit the small amounts of material available in tissue culture work. Reagent

blanks containing water instead of the substrate and enzyme blanks containing inactivated enzyme were run simultaneously with all enzyme assays. Extinction values were determined by reading coloured solutions against a water blank on the Unicam S.P.600. The Hilger U.V. spectrophotometer was used (with a 37° attachment) for enzyme determinations necessitating readings in the ultra-violet region of the spectrum.

Esterase.

The hydrolysis of α-naphthyl acetate was followed by coupling the naphthol released with tetrazotized *o*-dianisidine (Nachlas & Seligman, 1949). The red colour was extracted with ethyl acetate and the intensity measured in a spectrophotometer.

Buffered substrate: The substrate (0.03M) was prepared by adding, slowly and with stirring, 0.005g naphthyl acetate in 1 ml. of acetone to 100 ml. of 0.06M phosphate buffer, pH 6.3. The substrate mixture was freshly prepared for each series of determinations.

Tetrazotized o-dianisidine: An aquous solution (4 mg/ml.) was freshly prepared and placed on an ice bath until required.

Trichloracetic acid: 2N.

Procedure: To 3 ml. of buffered substrate, 0.1 ml. of cell extract was added and the enzyme reaction was

allowed to proceed for 30 mins. at 22°. Tetrazotized o-dianisidine (0.5 ml.) was then added. After 10 mins. the reaction was stopped by the addition of 0.5 ml. of trichloracetic acid. The red colour was then extracted by shaking with 5 ml. of ethyl acetate and centrifuged for 10 mins. at 5000g. to remove any emulsions that may have formed. The amount of naphthol released was measured colorimetrically at 540 mu and calculated from a calibration curve.

Acid and Alkaline Phosphatase.

The method (~~[illegible]~~; Fujita, 1939) involved a colorimetric determination of the amount of ρ-nitrophenol liberated from ρ-nitrophenyl phosphate.

Substrate (0.010M): 0.1g ρ-nitrophenylphosphate (Sigma) was dissolved in 25 ml. of water and stored at -10° until required. The solution was stable for several weeks at this temperature.

Buffers: 0.1M-Acetate buffer, pH 5.2, was used for acid phosphatase determinations and 0.1M-glycine-sodium hydroxide buffer, pH 10.4 when assaying for alkaline phosphatase.

Magnesium chloride: 0.009g% of magnesium chloride (anhydrous) was dissolved in each buffer solution.

Buffered substrate: The stock substrate solution was diluted (1:1) with the appropriate buffer mixture

immediately before required.

Standard solution: An aquous solution (o.1391g%) of ρ-nitrophenol was stored in a dark bottle at -10° until required. A 'working standard' was prepared by diluting (1:200) the stock solution. A suitable standard was prepared by adding 6 ml. of 'working standard' and 1.1 ml. of 0.2N-sodium hydroxide to 4 ml. of water.

Procedure: 0.1 ml. of cell extract was added to 1 ml. of buffered substrate and enzyme action was allowed to proceed at 37°. After 30 mins. the reaction was terminated by adding 10 ml. of 0.02N-sodium hydroxide and the yellow colour was measured at 420 mu. The amount of ρ-nitrophenol liberated was calculated from the reference standard.

β-Glucuronidase.

The hydrolysis of phenolphthalein-β-glucuronide was followed by spectrophotometric estimation in alkaline solution, of the phenolphthalein released (Talalay, Fishman & Huggins, 1946).

Substrate(0.0015M): 11.75 mg. of phenolphthalein-glucuronide was dissolved in 25 ml. of buffer, 0.1M-phthalate-sodium hydroxide, pH 4.5,

Procedure: A reaction mixture was prepared as follows.

Substrate	1 ml.
Cell extract	0.1 ml.
Water	0.4 ml.

After 30 mins. at 37° the reaction was terminated by adding 5 ml. of 0.2M glycine-sodium hydroxide buffer, pH 10.4. The amount of phenolphthalein liberated was calculated from the intensity of the red colour at 550 mu, the millimolar extinction coefficient of phenolphthalein being taken as 26.6 (Talalay, Fishman & Huggins, 1946).

Glucose-6-phosphatase.

Glucose liberated from glucose-6-phosphate was estimated spectrophotometrically using glucose oxidase, peroxidase and o-dianisidine (Keilin & Hartree, 1945; Hugget & Nixon, 1957). The hydrogen peroxide liberated during the oxidation of glucose to gluconic acid (by glucose oxidase) was utilized to oxidize o-dianisidine. The red oxidation product of o-dianisidine was estimated colorimetrically at 436 mu.

Substrate (0.06M): was prepared by adding 0.365g of glucose-6-phosphate (sodium salt) to 10 ml. of 0.1M citric acid-sodium hydroxide buffer, pH 6.5.

Magnesium chloride: An aquous solution containing 1.9g% of $MgCl_2$ $6H_2O$ was prepared.

Perchloric Acid: 0.4M

Reagents for glucose estimations were supplied by C.F. Boehringer, Mannheim, and consisted of:

(a) Glucose oxidase-peroxidase mixture: The mixture

contained glucose oxidase (250 ug/ml.) and peroxidase (40 ug/ml.) in 0.12M phosphate buffer, pH 7.0. The solution was stored at -10° until needed.

(b) <u>o-dianisidine hydrochloride</u>: An aqueous solution (10 mg/ml.).

<u>Reaction mixture (a + b)</u>: Immediately before required 1 ml. of o-dianisidine mixture was added to 150 ml. of glucose oxidase mixture.

(c) <u>Glucose Standard</u>: 91 ug. of glucose/ml.

<u>Procedure</u>: A reaction mixture consisting of 0.1 ml. of substrate (glucose-6-phosphate), 0.05 ml. of magnesium chloride solution and 0.1 ml. of cell extract was incubated at 37°. After 60 mins. the reaction was terminated with 0.25 ml. of perchloric acid and the protein precipitate removed by centrifuging at 5000g for 20 mins. The amount of glucose in the supernatant was estimated with glucose oxidase as follows. 0.2 ml. of the supernatant was added to 5 ml. of the glucose oxidase-o-dianisidine mixture and enzyme action was allowed to proceed at 37° for 20 mins. The intensity of the red colour of oxidized o-dianisidine was measured at 436 mu and compared with a reference standard (0.2 ml. of standard glucose solution instead of 0.2 ml. of 'test' substance).

Lactic Dehydrogenase.

Lactic dehydrogenase levels in cell extracts were determined colorimetrically by estimating the amount of pyruvic acid converted to lactic acid in the presence of $NADH_2$ (Cabaud & Wroblewski, 1958). The pyruvic acid remaining after enzyme action was estimated with 2:4-dinitrophenyl hydrazine.

Substrate: A solution of pyruvic acid (0.02g%) was prepared in phosphate buffer (0.1M), pH 7.8 and stored at 4°. This solution was stable for one week.

$NADH_2$: An aqueous solution (10 mg/ml.) was freshly prepared for each series of determinations.

2:4-Dinitrophenyl hydrazine: A solution (0.1g%) was prepared in 2 N-hydrochloric acid.

Procedure: A reaction mixture consisting of 1.0 ml. of substrate, 0.1 ml. $NADH_2$ and 0.1 ml. of extract was incubated at 37°. After 30 mins., 2:4-dinitrophenyl hydrazine (1.0 ml.) was added and the solutions were left at room temperature for another 20 mins. 10 ml. of 0.4M-sodium hydroxide was then added and the intensity of the red colour was read at 550 mu after 10 mins. Extinction values at 550 mu were correlated with units of lactic dehydrogenase activity by extrapolating from a standard curve (Cabaud & Wroblewski, 1958).

Arginase.

Arginase activity was determined by measuring the amount of urea formed from arginine under standard conditions (Archibald, 1945; Brown & Cohen, 1959).

Substrate (0.125M): An aquous solution of arginine mono-hydrochloride (2.628g%) was adjusted to pH 9.5 with 0.5N-sodium hydroxide.

Buffer: 0.1M-glycine-sodium hydroxide, pH9.5.

Procedure: The assay system consisted of 0.1 ml. of arginine, 0.25 ml. of buffer, 0.1 ml. of manganese chloride ($MnCl_2$), 2.5 milli-molar, and water to a final volume of 1 ml. After 30 mins. incubation at 37° the reaction was terminated with 0.5 ml. of 4N-perchloric acid. The mixture was then centrifuged at 5000g for 20 mins. and 1 ml. of the supernatant was used for the estimation of urea - by reaction with α-isoN-propiophenone (Archibald, 1945).

Arginase activity can be increased several fold by preincubation with manganous ions (Hellerman & Perkins, 1935). Extracts were therefore preincubated for 30 mins. at 50° with 0.05M-manganese chloride ($MnCl_2$) as recommended by Brown & Cohen (1958).

Glutamyl Transferase.

The enzyme that catalyses the synthesis of glutamine from glutamic acid and ammonia also catalyses a transfer

reaction in which γ-glutamylhydroxamate is formed from glutamine and hydroxylamine in the presence of catalytic amounts of adenosine diphosphate, arsenate and manganese (Stumpf, Loomis & Michelson, 1951).

$$\text{L-glutamine} + NH_2OH \longrightarrow \gamma\text{-glutamylhydroxamate} + NH_3$$

. γ-Glutamylhydroxamate was estimated colorimetrically with ferric chloride, (Lipmann & Tuttle, 1945).

Substrate (0.03M): 0.8700g of L-glutamine was dissolved in 20 ml. of water by heating to 38°. The substrate was stable for several weeks if stored at -10°.

Buffer: 0.5M-Acetate, pH 5.6.

Hydroxylamine hydrochloride: An aquous solution (0.9500g%) was freshly prepared for each series of assays and the pH was adjusted to pH 5.6 with 2N-sodium hydroxide.

Sodium arsenate ($Na_2HAsO_4 . 7H_2O$): An aquous solution (7.8g%) was adjusted to pH 5.6 with 2N-hydrochloric acid.

ADP (milli-molar): 0.0150g ADP/25 ml.

Manganese chloride ($MnCl_2 . 4H_2O$): 0.1650g/50ml.

Ferric chloride ($FeCl_3 . 6H_2O$): 10g% dissolved in a mixture of 0.7N-hydrochloric acid and 0.2N-trichloracetic acid.

Standard: An aquous solution of synthetic glutamyl hydroxamate (1 uMole/ml.) was freshly prepared for each series of determinations (glutamylhydroxamate was generously donated by Dr. L. Levintow, National Institues of Health,

Bethesda, Maryland).

Procedure: The reactants were mixed in the following proportions:

Glutamine	:	0.2 ml.
Buffer	:	0.3 ml.
Hydroxylamine	:	0.1 ml.
Arsenate	:	0.1 ml.
ADP	:	0.1 ml.
$MnCl_2$	:	0.1 ml.
Cell Extract	:	0.1 ml.

After 1 hr. at 37° the reaction was terminated by the addition of 0.3 ml. of 10% $FeCl_3 . 6H_2O$. Precipitated-protein was removed by centrifuging at 1,500g for 20 mins. Formation of γ-glutamyl hydroxamate was measured at 540 mu. Under these conditions 1.0 umoles of synthetic glutamylhydroxamate had an extinction value of 0.510 in a 1 cm. cuvette.

Adenosine Deaminase.

Adenosine deaminase was estimated spectrophotometrically by measuring the rate of decrease in the extinction value (at 265 mu) when adenosine was deaminated to inosine (Mitchell & McElroy, 1946).

The cell extract (0.1 ml.) was added to 2.7 ml. of Adenosine solution (19.8 ug/ml. dissolved in 0.1M-phosphate buffer, pH 7.0) in a 1 cm. cell. Disappearance

of adenosine was followed by measuring the decrease in optical density, at 30 sec. intervals, for 10 mins.

Xanthine Oxidase.

The spectrophotometric method (Kalkar, 1947) was used to follow the disappearance of hypoxanthine at 283 mu. The rate of increase in optical density was measured when 0.3 ml. hypoxanthine (6.6×10^{-4}M) was added to 2.3 ml. 0.1M glycine: sodium hydroxide, pH 8.3, and 0.1 ml. extract.

Glucose-6-phosphate dehydrogenase

Enzyme activity was measured by spectrophotometric estimation of the rate of increase in the extinction value at 340 mu in the presence of glucose-6-phosphate and NADP. (Glock & McLean, 1953). Enzyme determinations were carried out immediately after preparation of the cell extracts.

Substrate: 0.06M-glucose-6-phosphate (Disodium salt).

NADP: 1.5. 10^{-3}M.

Buffer: 0.15M tris: maleate; sodium hydroxide, pH 7.5, was prepared by dissolving 3.63g tris and 3.48g malic acid in 100 ml. water. The pH was adjusted with 0.6 N NaOH and water was added to a final volume of 200 ml.

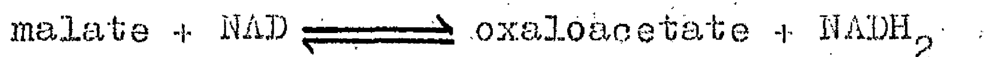
Magnesium chloride (hexahydrate): 1.9% (W/V).

Procedure: The following substances were added to a 1 cm. quartz cuvette, 2.1 ml. buffer, 0.1 ml. glucose-6-

phosphate, 0.1 ml. NADP, 0.1 ml. magnesium chloride. The reaction was initiated by adding 0.1 ml. of extract and readings were taken at 30 sec. intervals for 10 mins. A unit of enzyme activity was defined as an increase in optical density of 0.001/min/million cells.

Malic Dehydrogenase.

Malic dehydrogenase catalyses the oxidation of malate to oxaloacetate thus



$$\text{malate} + \text{NAD} \rightleftharpoons \text{oxaloacetate} + \text{NADH}_2$$

As the equilibrium favours the reverse reaction the enzyme is conveniently assayed by using oxaloacetate as substrate and $NADH_2$ as coenzyme. Because of the instability of the substrate in aquous solutions, oxaloacetate is produced in the reaction mixture by treating α-ketoglurate and aspartate with glutamic oxaloacetic transaminase.

Reagents: All solutions required for the assay are supplied in a 'test combination' supplied by C.F.Boehringer, Mannheim.

Procedure: The reaction mixture consisted of,

2.75 ml. of aspartate ($4.2.10^{-2}$M) dissolved in 0.1M phosphate buffer, pH 7.4.

0.05 ml. of α-ketoglutarate (sodium salt) (6.10^{-2}M).

0.05 ml. of $NADH_2$ (10^{-2}M).

0.05 ml. of glutamic oxaloacetic transaminase (1.0 mg. protein/ml.).

Formation of oxaloacetate was allowed to proceed at 25°. After 5 min., 0.1 ml. of extract was added and the extinction value at 340 mu was measured at 1 min. intervals for 10 min. Extracts were diluted so that ΔE did not exceed 0.030/min. A unit of enzyme activity was defined

as the amount of enzyme required to alter the extinction value by 0.001 per min.

SECTION 3. R E S U L T S

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The starch-gel electrophoretic technique introduced by Smithies (1955) and the modification described by Hunter & Markert (1957) were employed to study the distribution of homologous proteins in several tissues from various species of animals. The investigation was particularly aimed at answering the following questions (a) What is the extent of the molecular variation between homologous proteins from different species of animals and from different tissues within a species? (b) To what extent is the enzymic constitution of animal cells subject to environmental changes?

Reproducibility of the method:

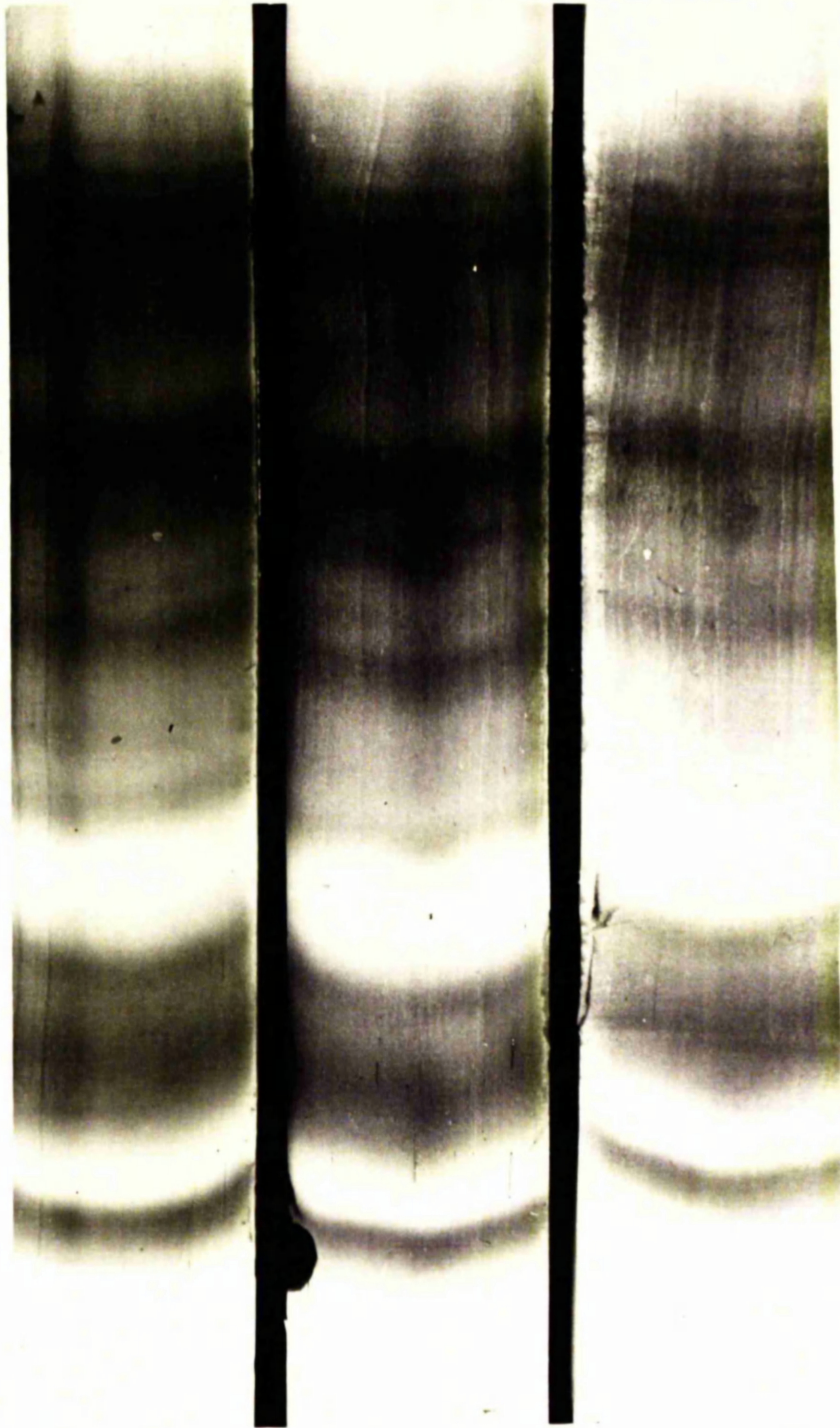
Before comparing homologous proteins from different species the degree of variation from individual to individual within a species was examined. For this purpose extracts were prepared from the livers and kidneys of over 40 mice from a stock inbred strain. Extracts were also prepared from the hearts of 12 of these mice. The extracts were subjected to electrophoresis and the gels were stained for protein and esterases. The results demonstrated that the protein and esterase patterns obtained from the three mouse tissues were reproducible from individual to individual (figures 4 & 5). There were two interesting exceptions to these general findings. In one of these animals an esterase band

was missing from the sixteen bands usually observed in mouse liver zymograms. In addition it was found that two esterase bands (including the one missing from liver) were absent from zymograms prepared from the kidney of the same animal (Figures 6 &7). In the other 'abnormal' mouse an esterase band (with an identical mobility to the one missing in the former case) was absent from the liver zymogram and an identical band from the kidney zymogram. It was interesting that in both cases no deviations in the protein patterns from the normal ones were detected. However as demonstrated in Figure 8, tissue protein patterns were in general not nearly as satisfactory as the corresponding zymograms. With the exception of serum more bands were usually visible on the esterase zymogram than on the corresponding section of the starch gel stained for protein.

It was concluded from these experiments that with one or two exceptions the esterase zymograms and protein patterns obtained from similar tissues in different individuals of the same species were identical. The abnormal esterase patterns displayed by two of the mice may reflect differences in genetic constitution between these animals and the thirty-eight others. This is not too unlikely in view of the recent fidings of Allen (1961) who correlated alterations in esterase

Figure 4.

Origin



Esterase zymograms from the livers of
different mice of the same inbred strain.

Figure 5 Protein patterns from different organs of the mouse.

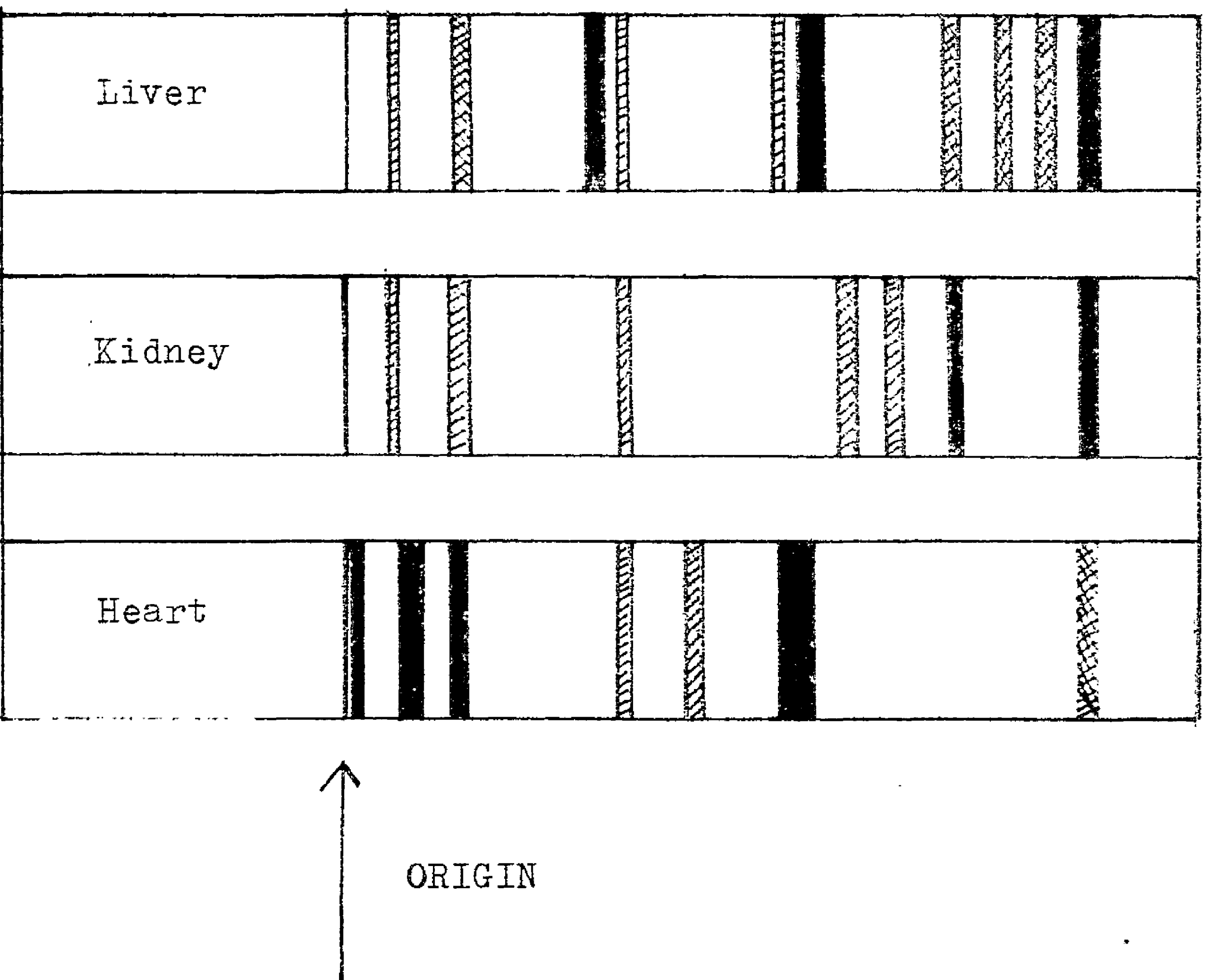
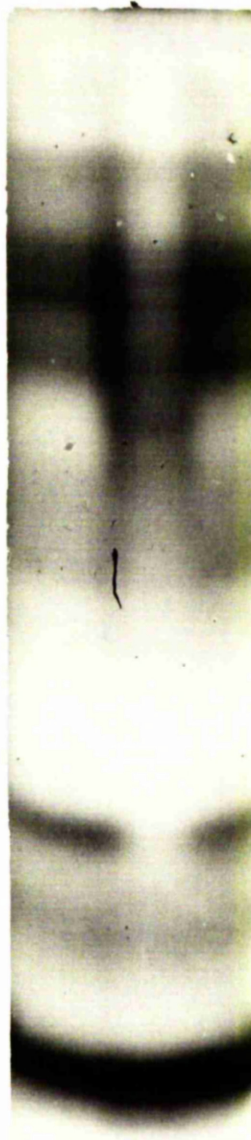


Figure 6.

Fig. 6. Comparison of liver and kidney extracts from two normal mice and one in which esterases were absent. The three preparations were inserted side by side in the gels.

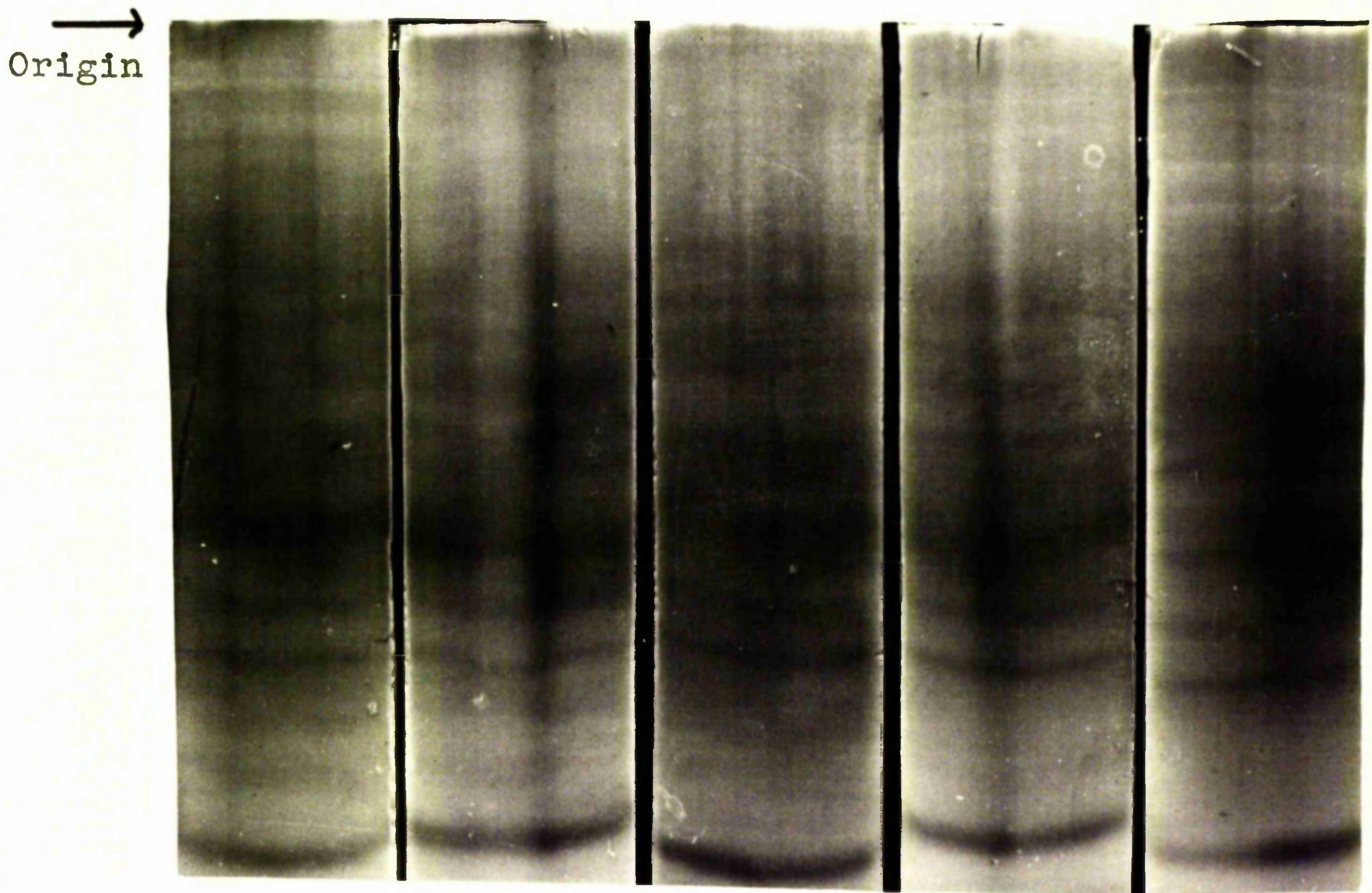
Figure 7.

Origin



Comparison of esterase zymograms from the kidneys of two normal mice with an abnormal one where an esterase band was missing. The three preparations were inserted side by side in the gel.

Figure 8.



Protein patterns from the livers of different mice of the same inbred strain.

zymograms prepared from Tetrahymena pyriformis with single gene changes in these organisms.

Classification of the various Proteins displaying esterase activity on the zymograms.

Esterases represent a large group of enzymes with distinct but overlapping substrate specificities. Although three main types with reasonably defined substrate specificities have been isolated from animal tissues, a large number of esterases which hydrolyse a variety of substrates have also been identified (Gomori, 1952). Many hydrolytic enzymes such as trypsin and chymotrypsin also display secondary esterase activity (Kaufman, Schwert & Neurath, 1948; Tinoco, 1958). In order to obtain a better understanding of the nature of the various esterases revealed by the zymogram technique a classification according to the method described by Augustinsson (1958) was undertaken. In this classification the esterases were separated into three groups depending on the degree of inhibition by specific inhibitors such as physostigmine and tetraisopropyl pyrophosphoramide. This method was adopted because of its simplicity and because of its ease of application to the zymograms.

The three main families of esterases described by Augustinsson were as follows -

(1) Cholinesterases (or C-esterases) which were specifically

Table 1. The effect of physostigmine and tetraisopropyl pyrophosphoramide on esterase zymograms from mouse and human liver.

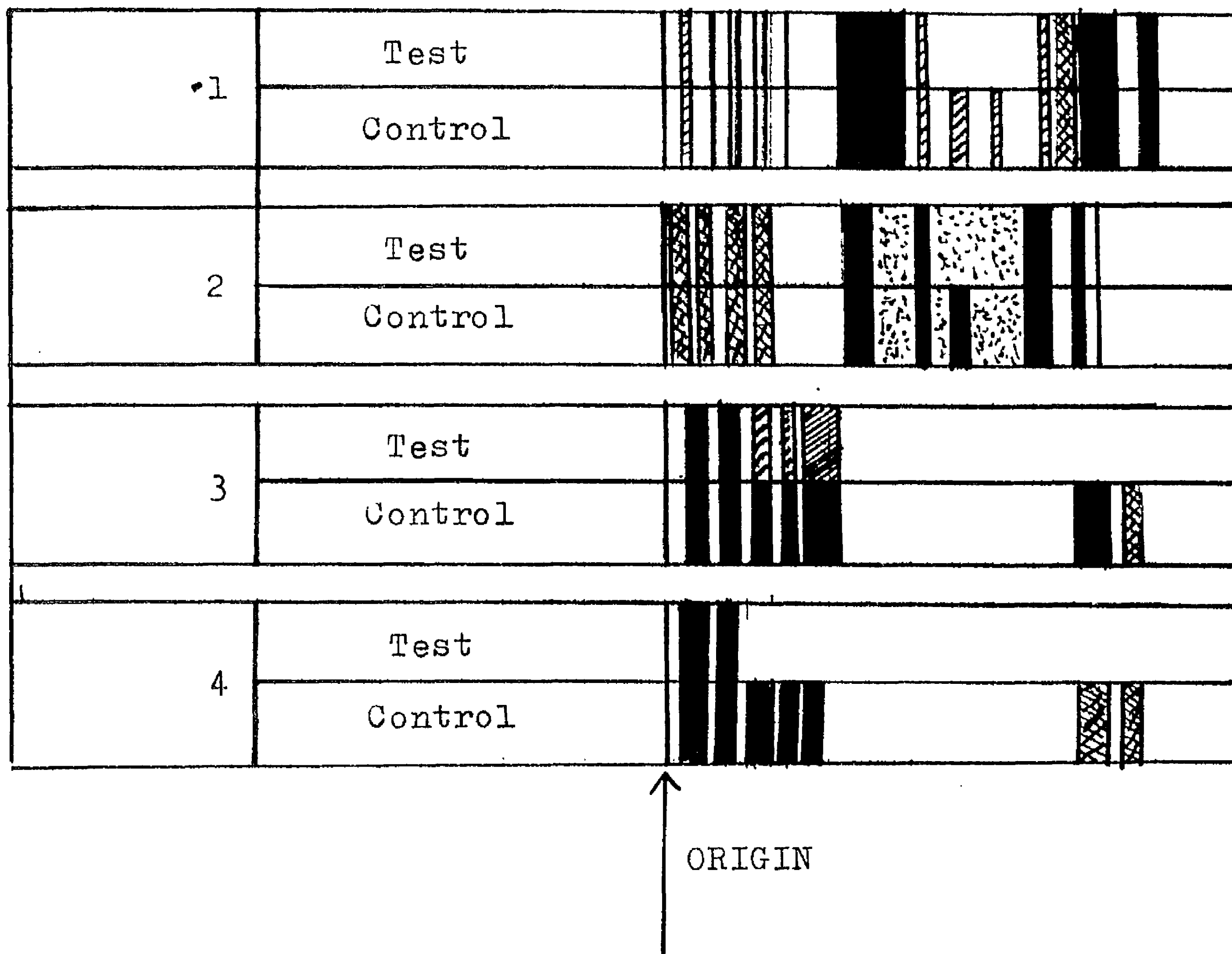
Degrees of inhibition are signified by +, ++, +++, -, represents no inhibition.

Inhibitor	Concentration	Mouse Liver	Human Liver
Physostigmine Salicylate	10^{-5}M	-	-
	10^{-4}M	+	+
	10^{-3}M	+	+++
Tetraisopropyl pyrophosphoramide	10^{-4}M	-	-
	10^{-3}M	-	++

<u>Fig. 9.</u> The effect of physostigmine salicylate (10^{-4}M) on esterase zymograms prepared from mouse liver, human liver, strains L and HeLa.

1. Mouse liver.
2. Strain L.
3. Human liver.
4. Strain HeLa.

Figure 9 Inhibitor Studies.



inhibited by physostigmine at a concentration of 10^{-5}M. Esterases which were inhibited by higher concentrations of physostigmine were not considered as cholinesterases.

(2) Aliphatic esterases (or B-esterases) which were not affected by physostigmine at this concentration (10^{-5}M) but were inhibited by tetraisopropyl pyrophosphoramide.

(3) Aromatic esterases (or A-esterases) which were not inhibited by either physostigmine or tetraisopropyl pyrophosphoramide.

The inhibitors were tested (as described in Methods section, Page No. 68) on zymograms prepared from human and mouse liver and two cultured cell lines HeLa and strain L. The results showed (Table No. 1) that there was no apparent inhibition of any esterases by physostigmine at 10^{-5}M. At higher physostigmine concentrations (10^{-4}M and 10^{-3}M) inhibition was observed in all cases (Figure 9), particularly with the esterases from human liver and HeLa which were completely inhibited at 10^{-3}M. With tetra-isopropyl pyrophosphoramide no inhibitory effect was observed on any of the esterases at a concentration of 10^{-4}M. At ten times the latter concentration there was extensive inhibition of the human esterases while mouse esterase were not affected at all.

Esterases of human origin were therefore more

sensitive to the inhibitors than mouse esterases suggesting that differences may exist in the 'active sites' of esterases from different sources. There was good agreement in the degree of inhibition of esterases from human liver and from HeLa and a similar situation was found to exist for esterases from mouse liver and strain L. As regards the nature of the various esterases on the zymogram it was concluded that the majority were non-specific aromatic (A) esterases.

Serum Protein and Esterase patterns from different species of animals.

An initial comparison of the serum protein and esterase patterns from nine different species of animals was undertaken to obtain some idea about the extent of the differences that could be expected when tissues were examined. As shown in figure 10 each animal species displayed a characteristic protein and esterase pattern which was easily distinguishable from that of other species. Augustinsson (1958) has similarly demonstrated marked differences in the number and electrophoretic mobility of serum esterases from a variety of animal species by quantitative electrophoresis on cellulose columns.

The possibility that the multiple esterase bands on the zymograms might be due to adsorption of esterases on

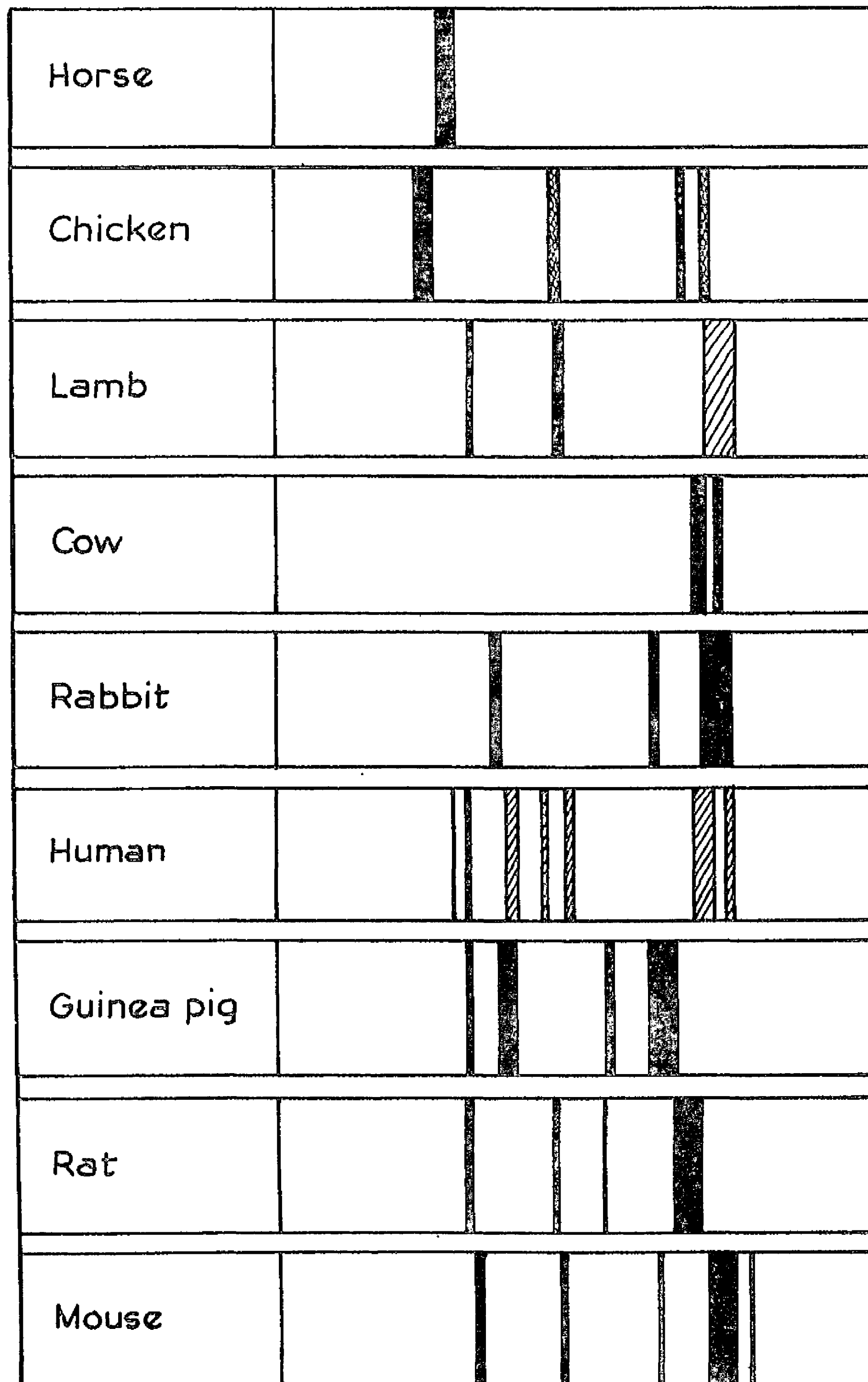


FIGURE 10. Esterase zymograms of serum from different species.

↑ Origin

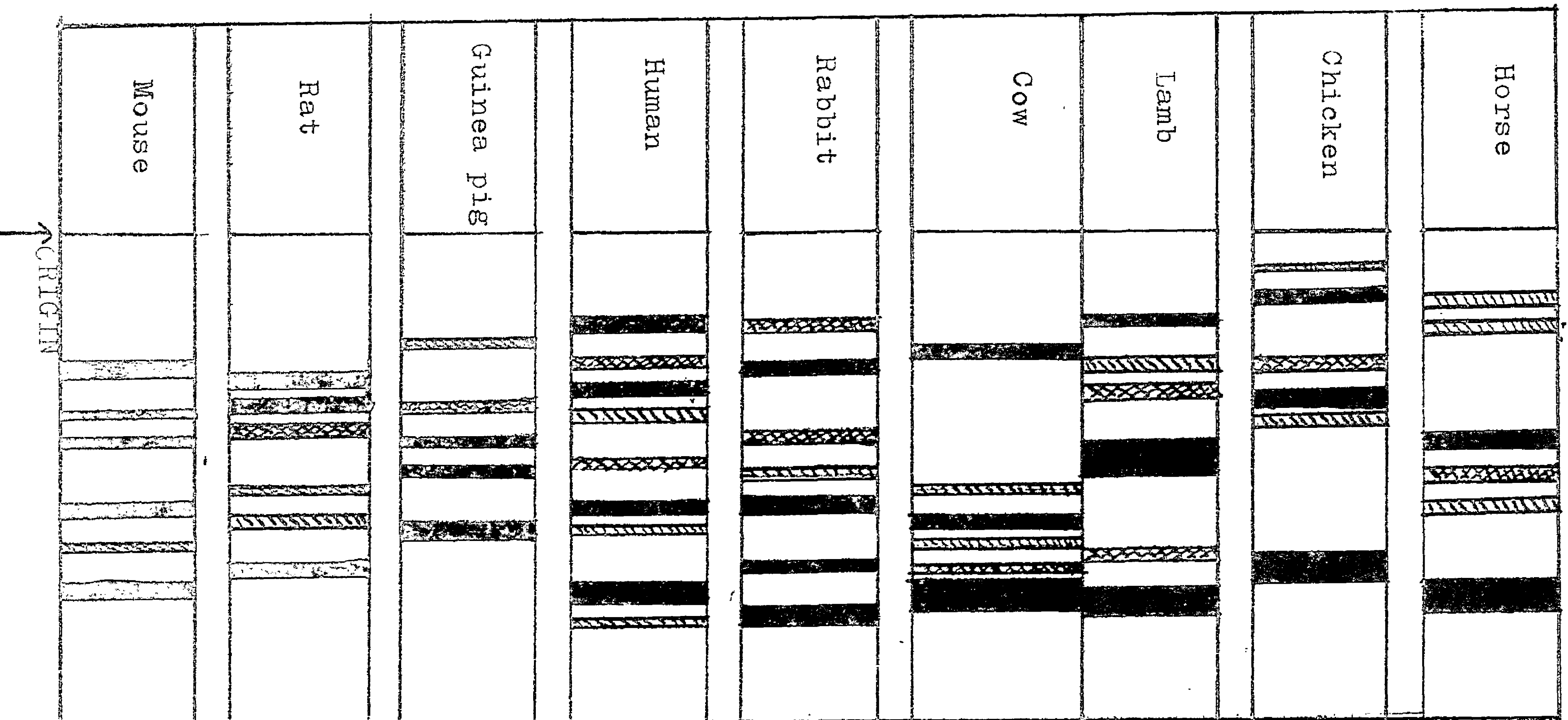
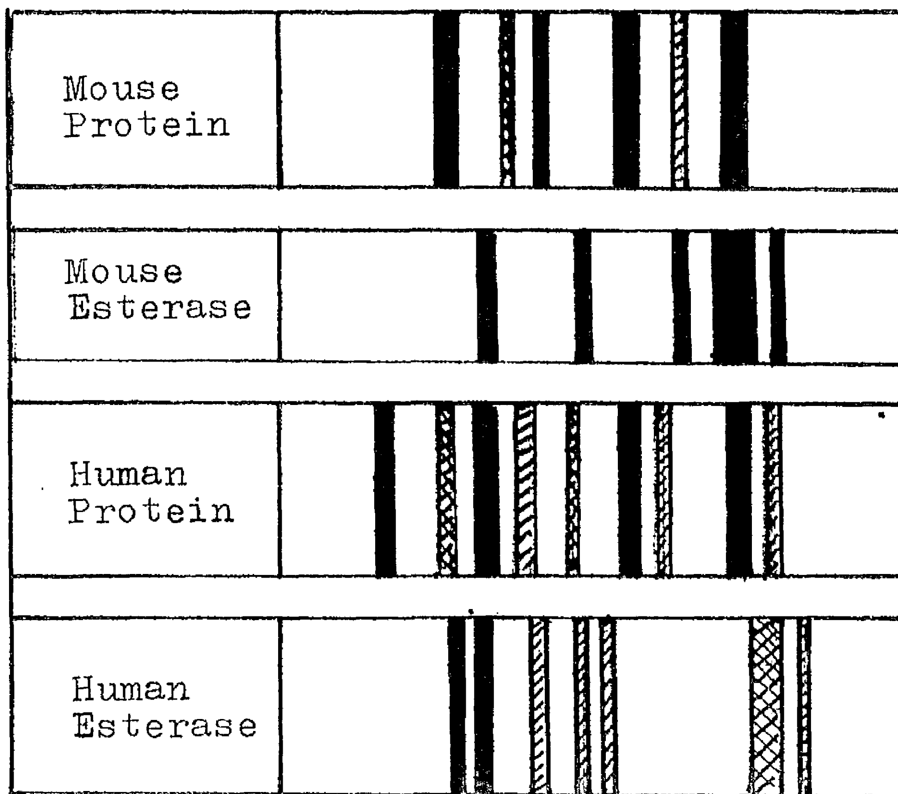


Figure 11 Serum proteins.

Figure 12 Serum - proteins and esterases from mouse and human.



to non-specific protein molecules was examined. The serum esterase zymograms were therefore compared with the corresponding serum protein patterns (figures 11 & 12) and it was found that areas of high esterase activity did not correspond with regions of high protein concentration. The possibility that the large number of esterase bands was due to adsorbtion was therefore considered unlikely.

Esterase and Protein patterns from similar organs in different species.

Esterase zymograms and protein patterns were prepared from the livers and kidneys of several species, including mouse, rat, guinea pig, rabbit, hen and human. When these patterns were compared (figures 13 & 14) a remarkable dissimilarity was observed in both the number and distribution of the bands. The differences were particularly pronounced when the esterase zymograms were examined. It was found that esterase zymograms from different species were so distinct that the species of origin could be identified from the type of zymogram produced.

These observations confirmed the previous results obtained when sera from various species were examined. They are also in agreement with the findings of Markert & Møller (1959) who demonstrated a similar type of variation in lactic dehydrogenases isolated from different species.

Figure I3

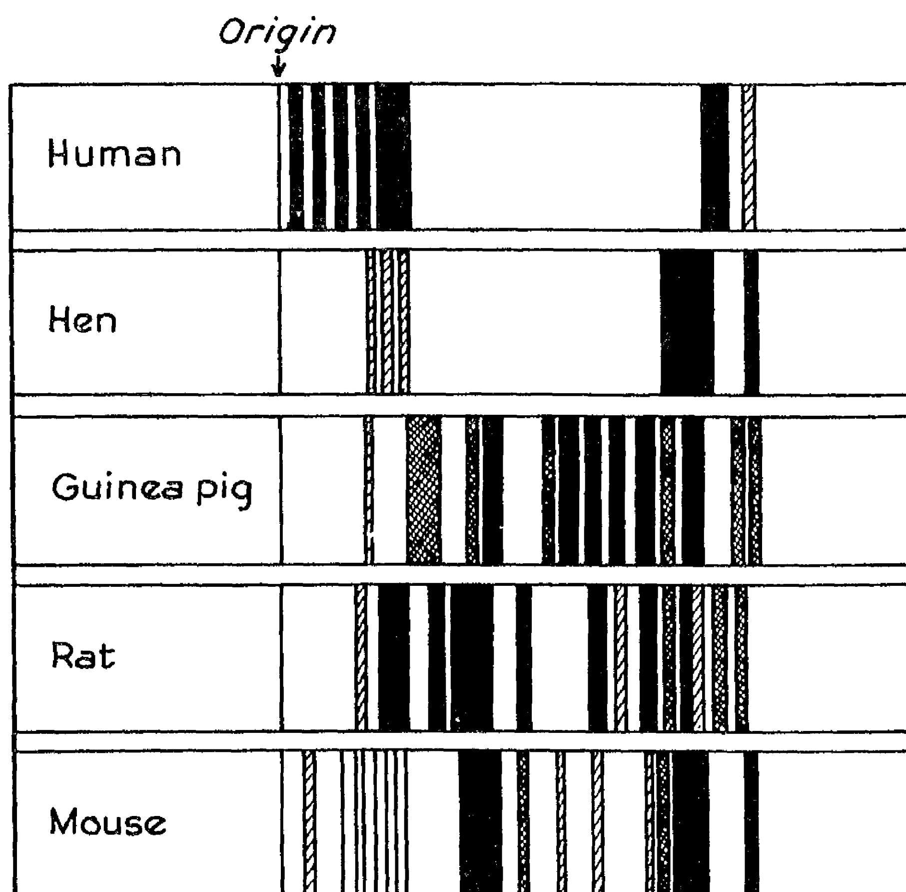


FIGURE 13. Esterase zymograms of liver preparations from different species.

Figure I4 Protein patterns from the livers of different species.

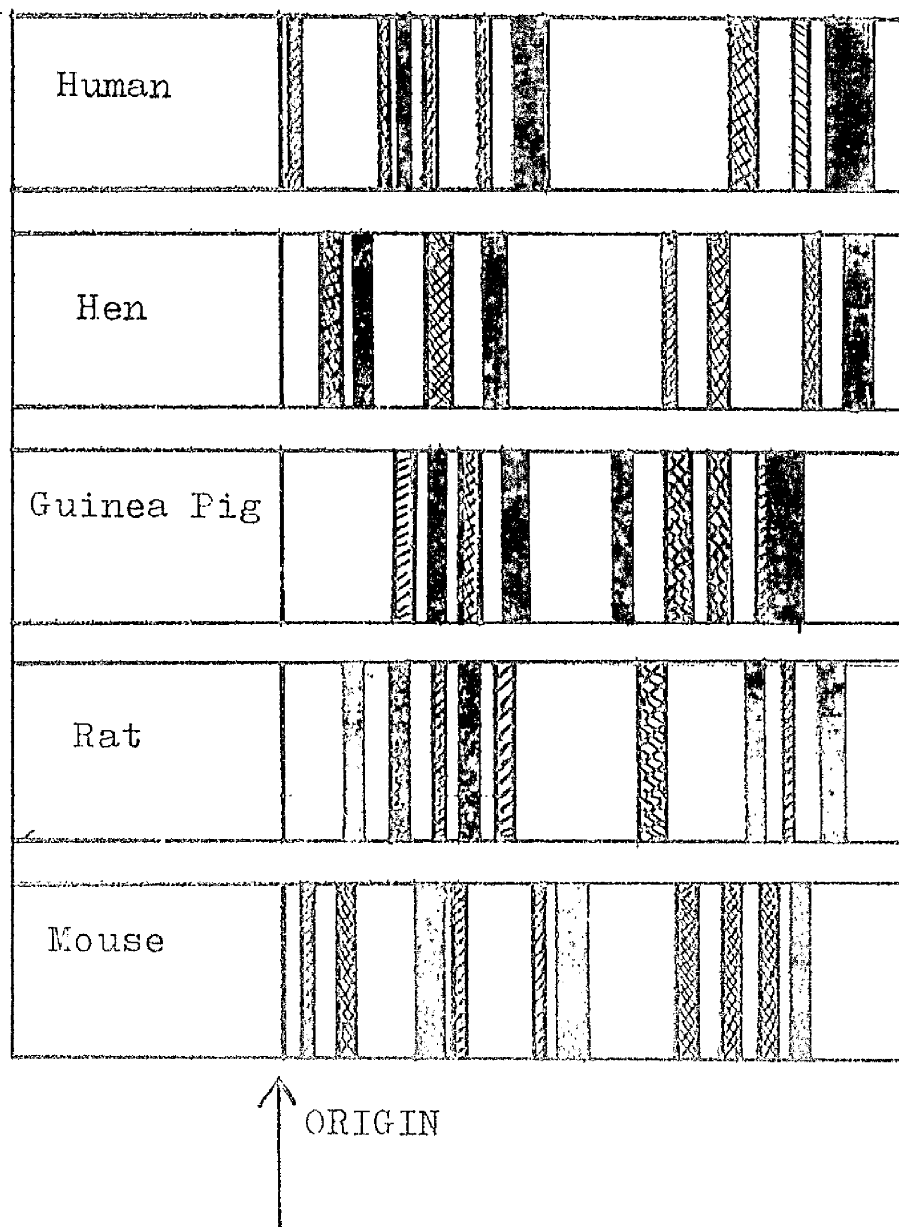


Figure I5

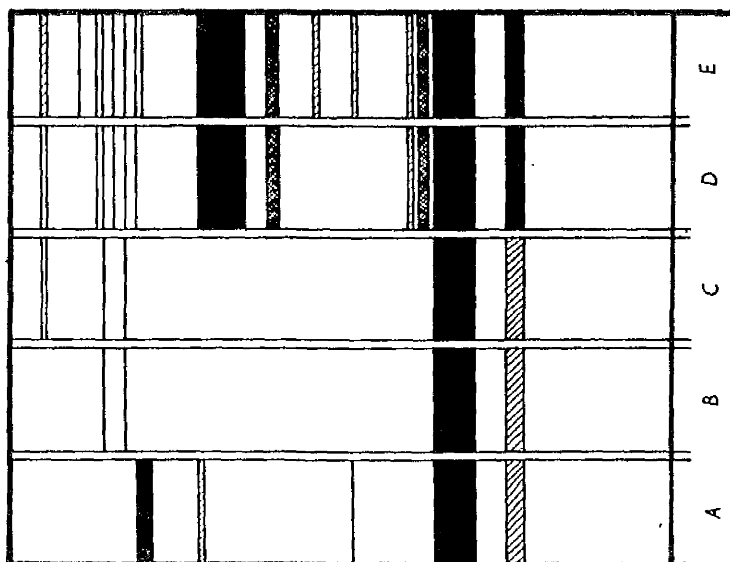
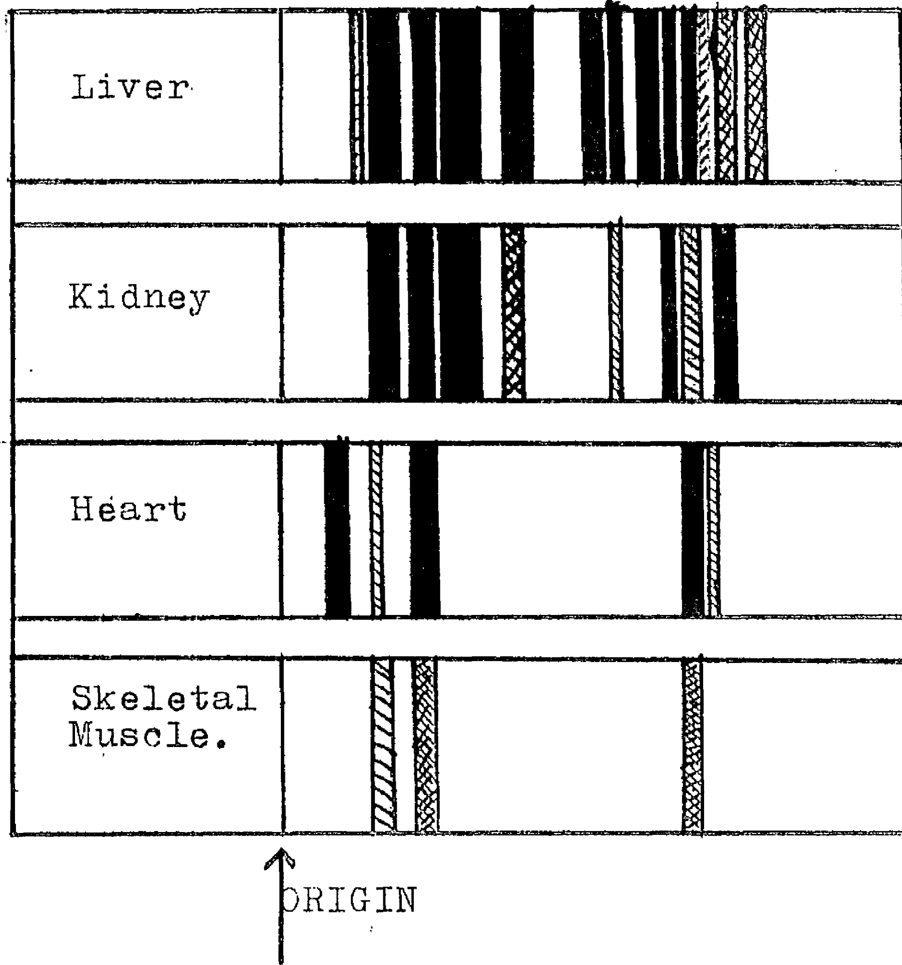


Fig. 15. Zymograms from extracts of different organs of the mouse. Relative intensities of bands are indicated by shading. *A*, Serum; *B*, skeletal muscle; *C*, heart; *D* kidney; *E*, liver.

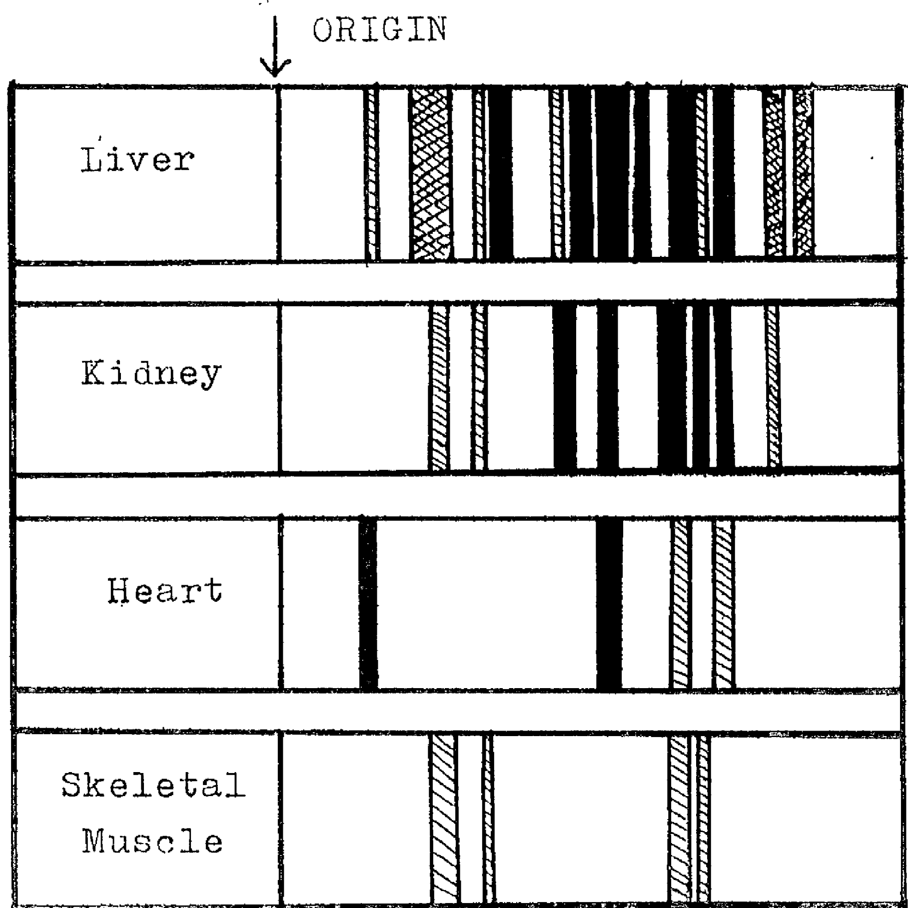
Origin

Figure 16



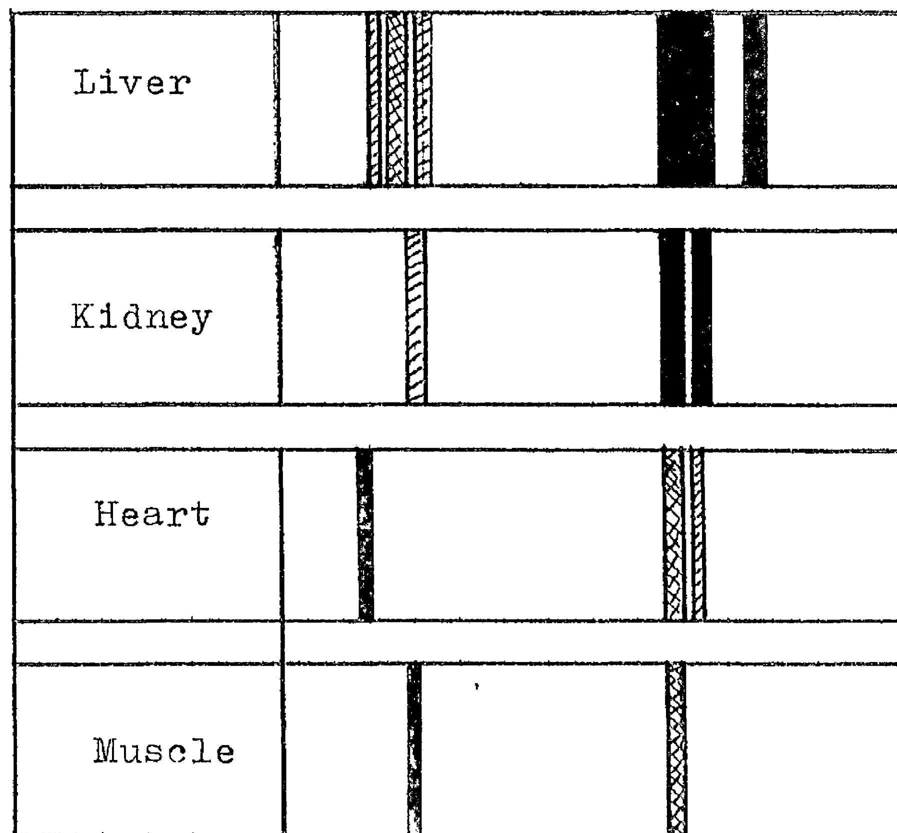
Esterase zymograms from different organs of the rat.

Figure 17



Esterase zymograms from different organs of the guinea pig.

Figure I 8



Esterase zymograms from different organs of the hen.

Figure I9

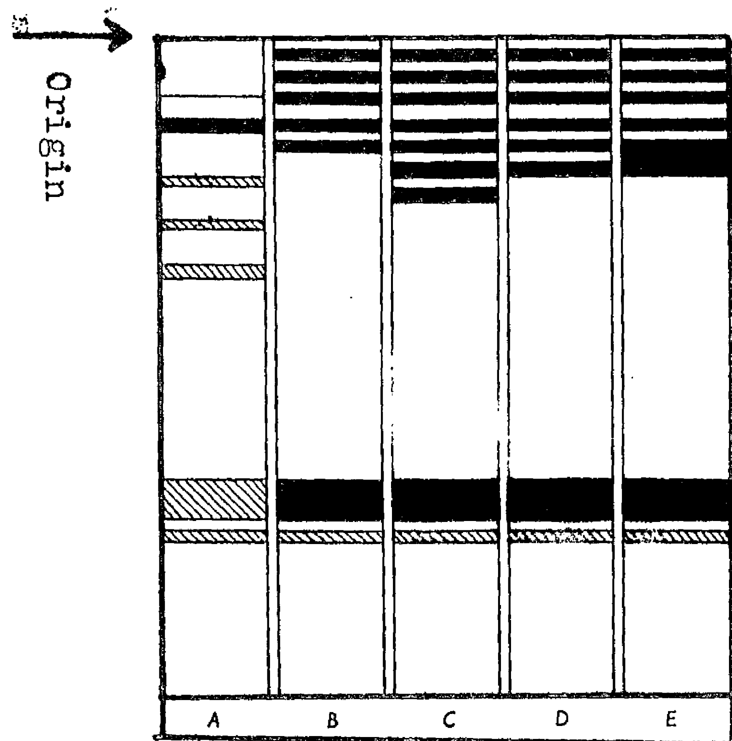


Fig. 19. Zymogram patterns obtained from different human organs. Relative intensities of bands are indicated by shading. *A*, Serum; *B*, stomach, small intestine, lung, thymus, adrenal, testis, spleen, heart; *C*, thyroid; *D*, kidney; *E*, liver.

Esterase and Protein patterns from different Organs of the same animal.

Variations in electrophoretic mobility between homologous proteins from different species of animals are almost certainly due to differences in genotype. Considering the evidence now available suggesting that protein structure is genetically controlled (Allison, 1959) it would be expected that homologous proteins from different tissues within an individual would be structurally similar. However, Henion & Sutherland (1957) demonstrated that phosphorylases prepared from various tissues of the dog were not immunologically similar. In these studies organ specific esterase zymograms were found to exist in the mouse, rat and guinea pig. In all cases it was possible to recognise the tissue of origin from the type of zymogram produced (figures 15-18). Zymograms from liver usually displayed the largest number of esterases which were distributed among the other tissues to a lesser degree. In marked contrast to the situation observed in the mouse, rat and guinea pig, esterase zymograms prepared from several human tissues showed a striking similarity to each other (figure 19). Of the eleven human tissues examined only liver, kidney and thyroid displayed tissue specific esterase zymograms. In the latter instances the differences were usually less pronounced and merely involved an addition of one or two

extra bands to seven bands found on zymograms prepared from the other tissues.

Another interesting feature which these studies revealed, was the large number of proteins, displaying esterase activity, in the various tissues examined. Extracts prepared from mouse, rat and guinea pig liver contained sixteen, thirteen and twelve different esterases respectively. Whether these various esterases should be designated as isozymes according to the classification of Markert & Møller (1959) will be discussed in a later section. Whatever the nature of the numerous esterases these investigations demonstrated that, with the exception of the human being, organ specific esterases could be identified in several species of animals. These findings are in good agreement with some recent observations on immunological differences between lactic dehydrogenases in different tissues of the rabbit (Niesselbaum & Bodansky, 1959). By separating enzymes with the aid of starch gel electrophoresis Tsao (1960) demonstrated that organ specific glucose-6-phosphate dehydrogenases and malic dehydrogenases could be identified in certain tissues of the rat.

Esterase zymograms from Cancer Tissues.

In view of the extreme sensitivity of the zymogram technique in detecting differences in enzyme content of

Figure 20.

Comparison of esterase zymograms prepared from normal human kidney (a) and a hypernephroma (b). The two preparations were inserted side by side in the gel.

the various tissues examined, it was decided to prepare zymograms from tumour tissues to determine if these displayed different patterns from normal tissues. The 'deletion' hypothesis of Van Potter (1944) proposes that some tumour tissues are lacking (or have lower activity levels) in many hydrolytic enzymes and are therefore better equipped for performing anabolic reactions than normal tissues. Esterase zymograms prepared from two human tumours, a hypernephroma and a carcinoma of the rectum were compared with the tissue of origin of the tumour. The patterns obtained from the tumour tissues were identical with the zymograms normally obtained from human tissues. Studinzska (unpublished) found that a difference existed in the esterase zymogram prepared from another hypernephroma when compared with the normal pattern obtained from human kidney (figure 20).

Esterase zymograms and Protein patterns from mammalian cells maintained in culture.

Previous results demonstrated that species and organ specific esterase zymograms could be prepared from several animals. Esterase zymograms were then prepared from extracts of several cultured cell lines to determine if species characteristics persisted during cultivation in artificial media. The majority of the cell types were of human origin and in all cases were found to retain the

characteristic human esterase pattern (figure 21). The period during which these cells had been in culture varied from a few weeks for kidney cells to several years for HeLa cells. The other cell lines investigated were strain L and L-5178Y, (a lymphoma), both of murine origin. The esterase zymograms prepared from these cells did not resemble any of the zymograms prepared from cell lines of human origin thereby further demonstrating that species characteristics were retained under tissue culture conditions.

In the course of these investigations it was demonstrated that the ERK cell line (originally claimed to have been derived from a rabbit by Westwood et al. in 1957) displayed an esterase zymogram which closely resembled that of the HeLa cell line. Recent immunological evidence (Coombs et al. 1961) has indicated that these two cell lines are antigenically similar. It is generally accepted now that the ERK cell line was probably contaminated at some stage by HeLa cells which have since outgrown the original cells.

The remarkable retention of species characteristic esterases by animal cells maintained in culture, even after several years in heterologous media in some cases, indicates that the zymogram technique can be employed for the purpose of establishing the cellular identity of cultured cells.

Figure 21

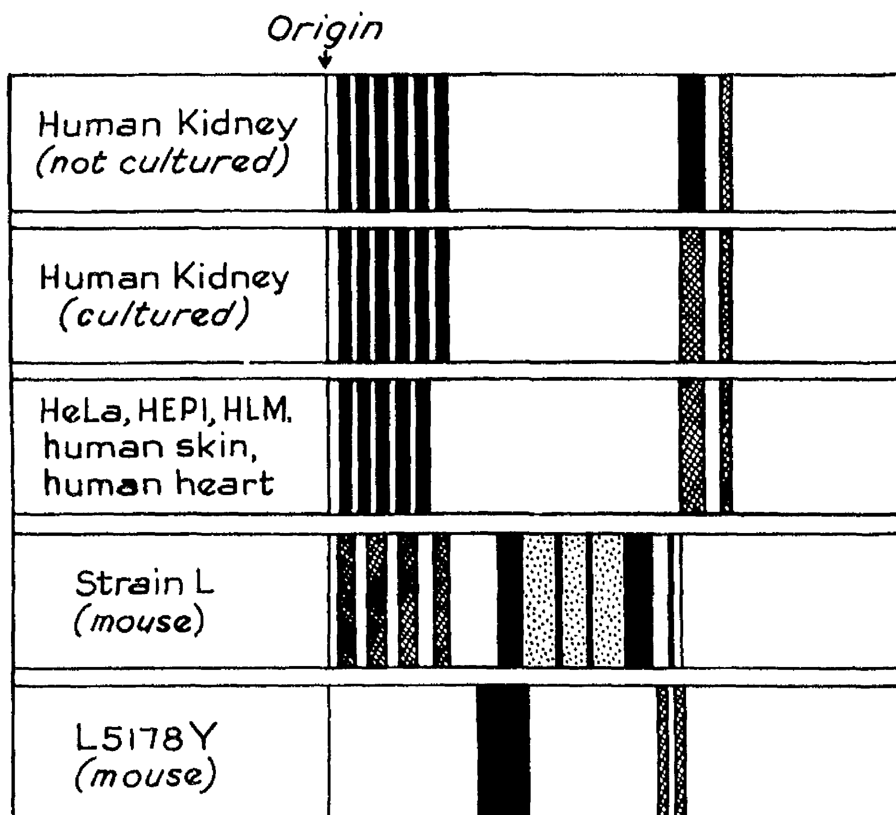
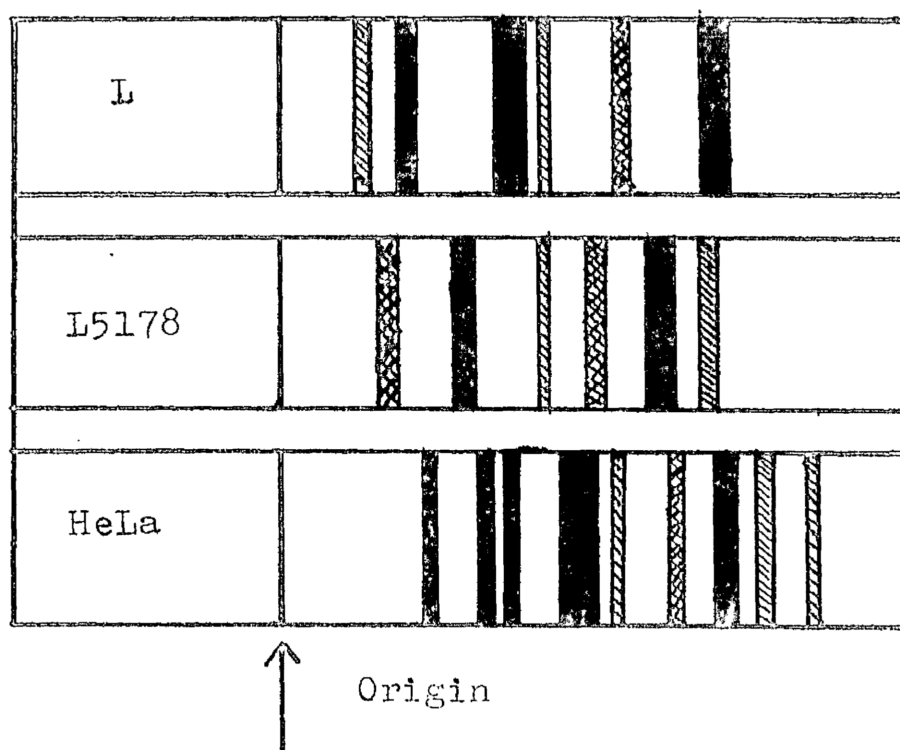


FIGURE 21. Esterase zymograms from cultured cell strains compared with human kidney. HeLa, HEP1, HLM are all of human origin.

Figure 22



Protein patterns from cultured cell lines.

Esterase patterns of cultured cells grown in media containing organic esters.

Further proof of the stability of the esterase patterns of cultured cell lines was obtained when direct attempts were made to alter the characteristic pattern. It was previously reported (Vandelli & Scaltriti, 1943) that increased esterase activity occurred in rats which were fed on diets containing high concentrations of aromatic and aliphatic esters. In this study human kidney and strain L cells were grown for eight days in Waymouth's medium supplemented with 5% dialysed calf serum and containing acetylsalicylic acid (1mM) or triacetin (0.2mM). Duplicate cultures were set up and control cultures were grown in a similar medium without esters. The medium was renewed after three and six days and the cells were harvested on the eighth day. Zymograms prepared from control and test cultures displayed identical esterase patterns (figure 21). This experiment suggested that the distribution of esterases on zymograms prepared from cultured cells was not influenced by the presence of esters in the growth medium. It is possible however that the esters may have been hydrolysed, before entering the cells, by the esterase activity of the serum present in the culture medium. In addition, the zymogram technique would not detect slight

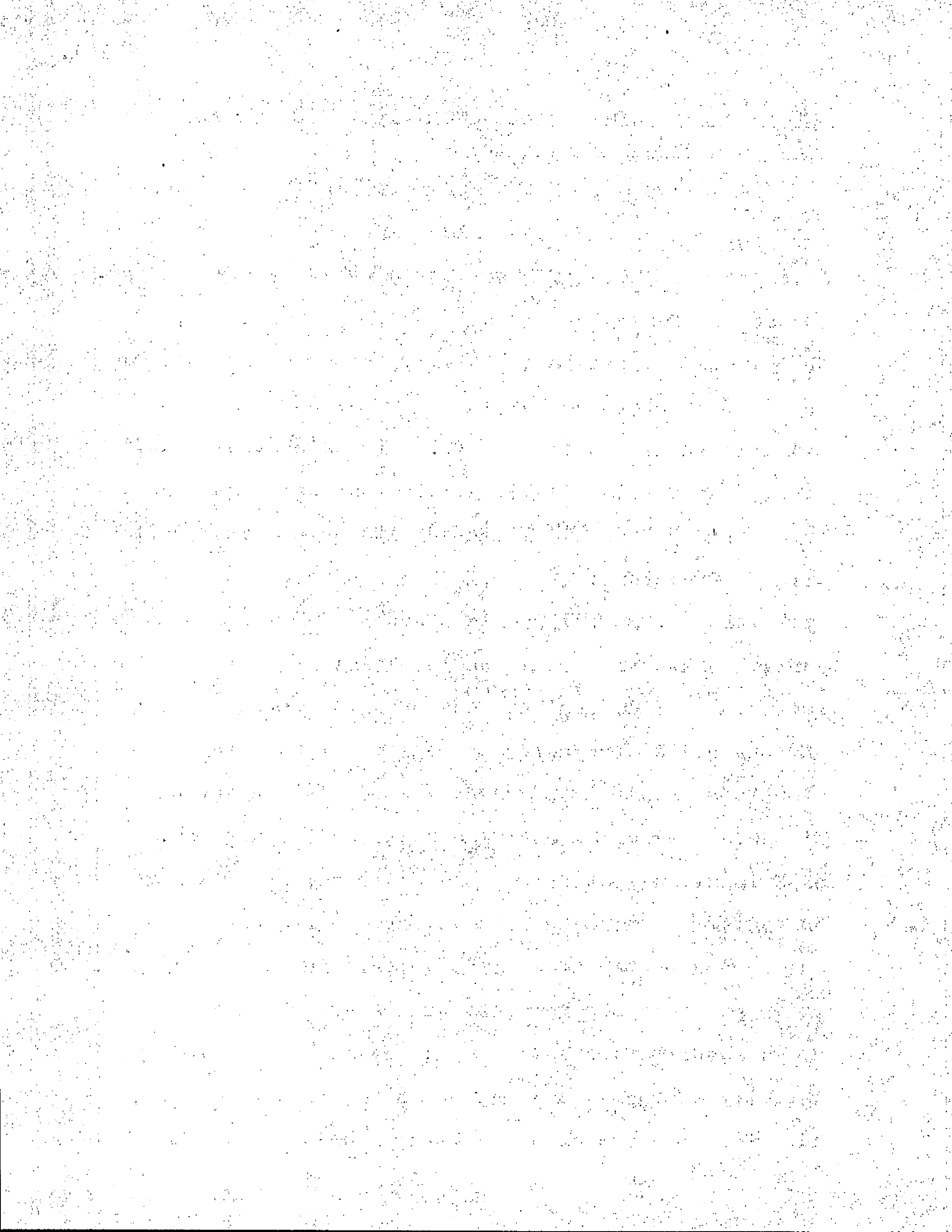


Figure 23

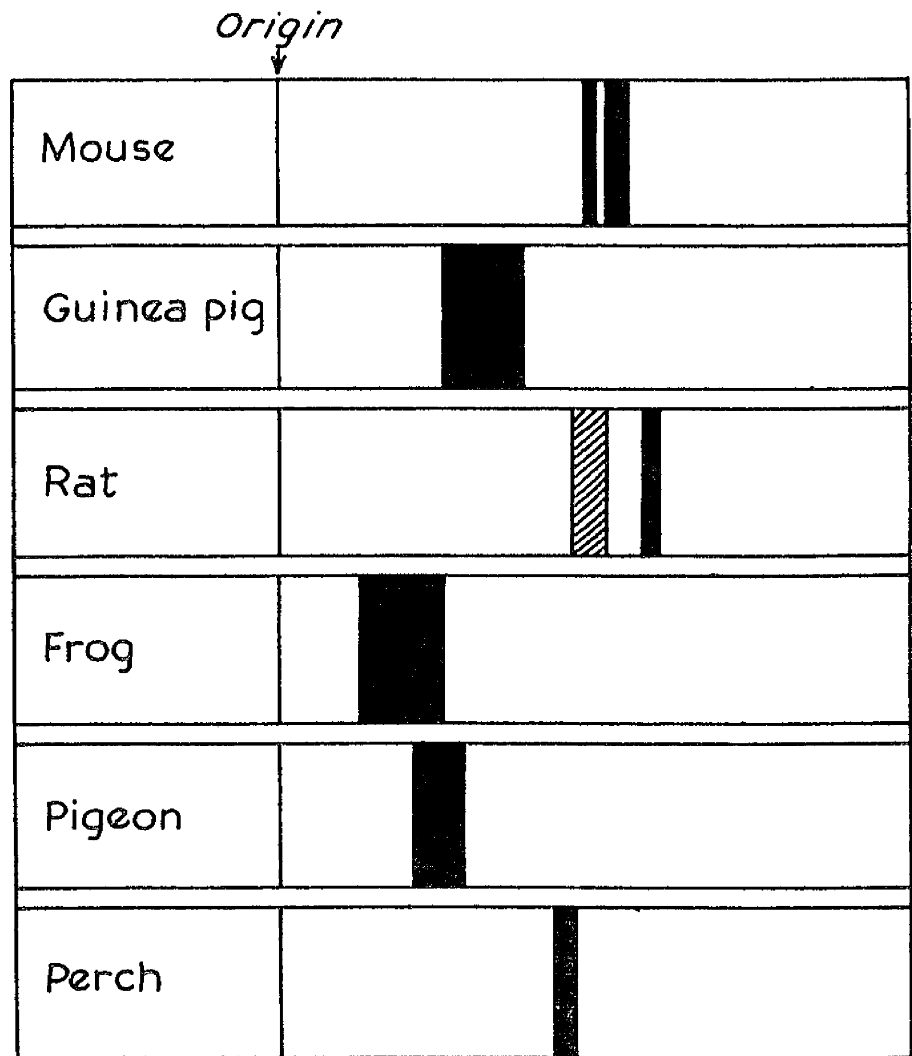


FIGURE 23 Alkaline phosphatase zymograms of liver preparations from different species.

Figure 24

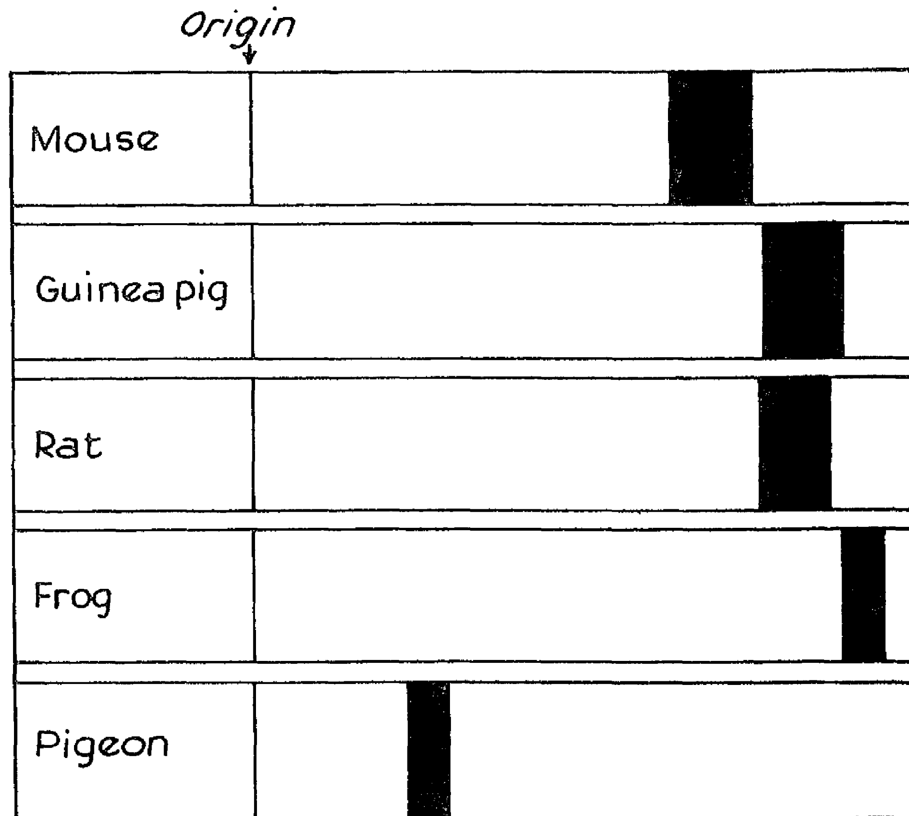


FIGURE 24: Peroxidase zymograms of liver preparations from different species.

Figure 25

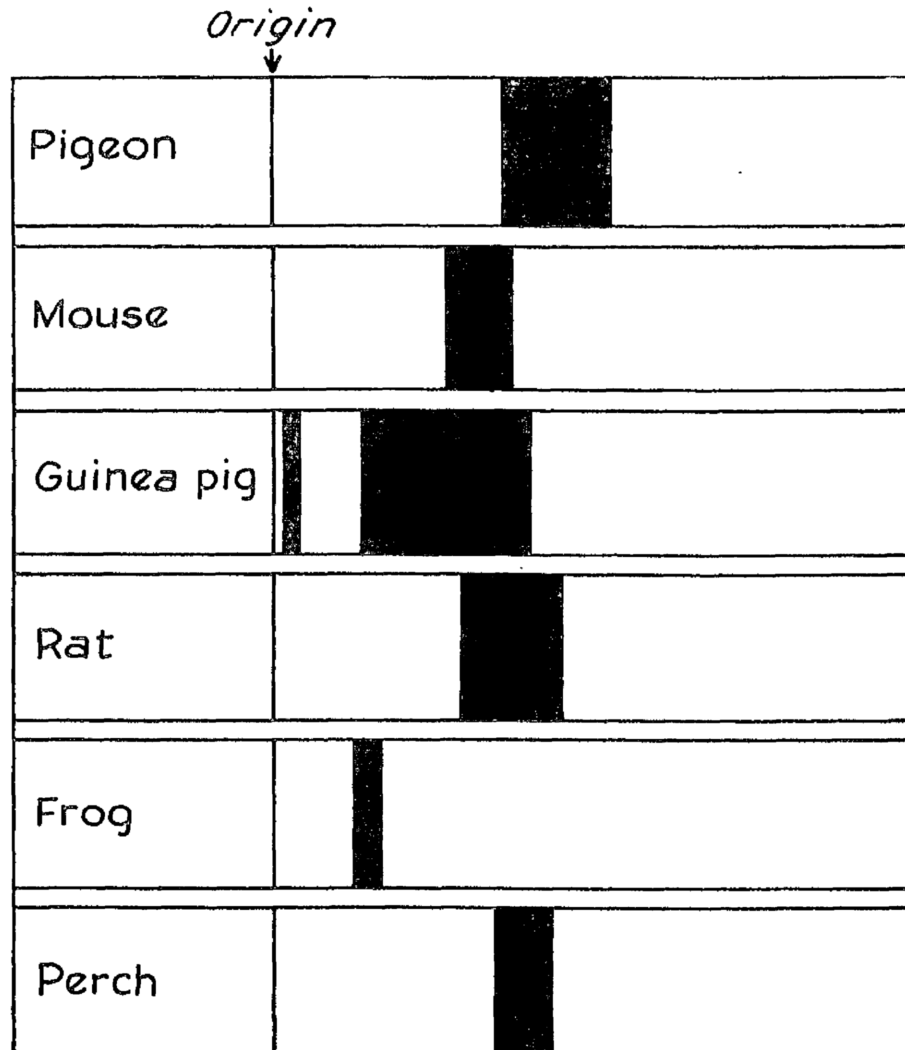
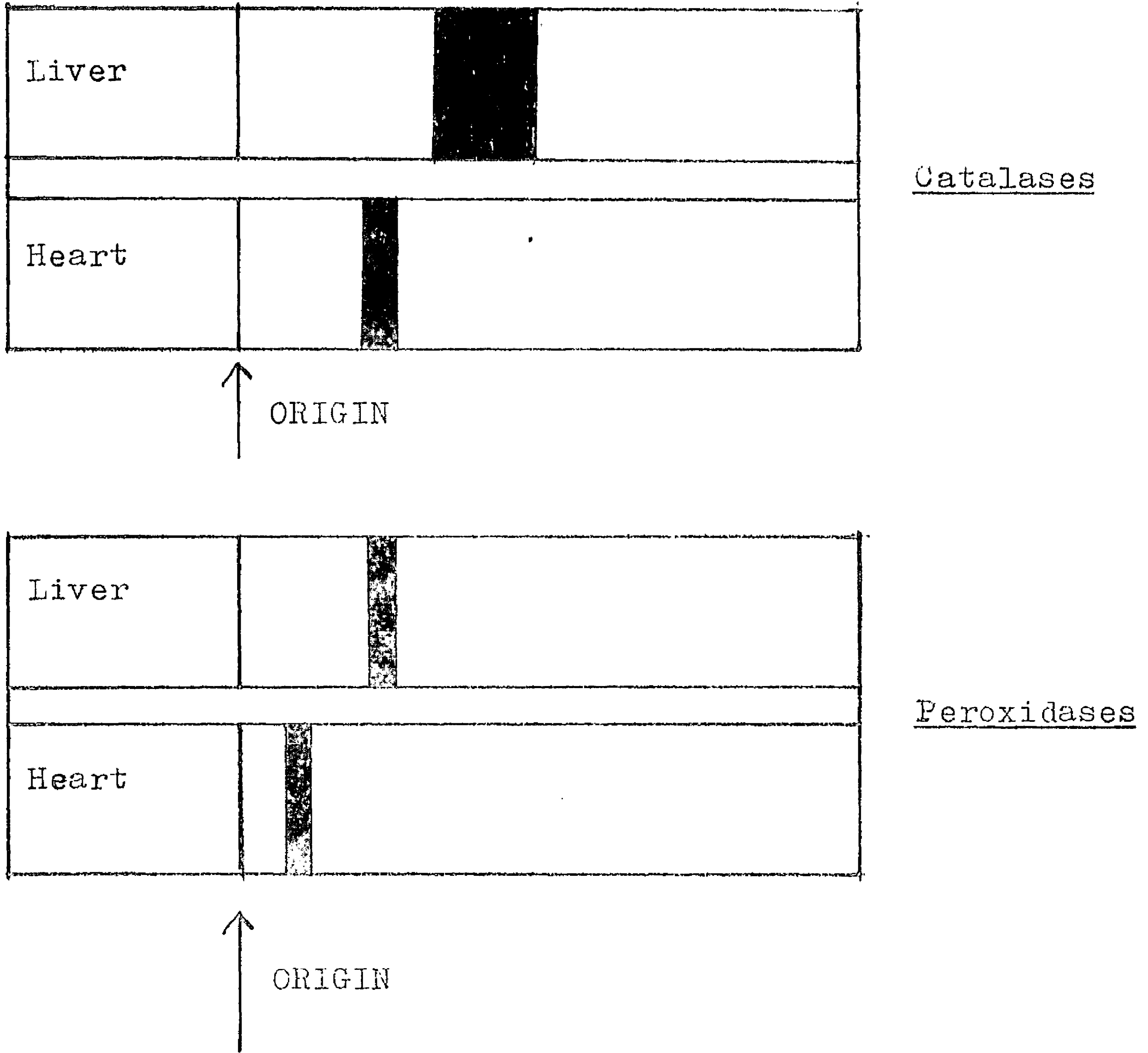


FIGURE 25. Catalase zymograms of liver preparations from different species.

Figure 26



Catalase and peroxidase zymograms from different organs of the pigeon.

quantitative alterations in esterase activity which may have occurred. The limitations of this experiment were eliminated in a later series where quantitative esterase determinations were carried out and a serum-free medium was used (see Page No.117).

Species differences in other enzymes as revealed by the zymogram technique.

Since the esterases represented a family of enzymes with a broad spectrum of substrate specificity it was decided to investigate enzymes with more limited substrate specificities to determine if species variations could also be demonstrated. As in the case of the esterases the enzymes were chosen because the assay systems could be applied to the zymogram technique. Alkaline phosphatase peroxidase and catalase zymograms were prepared from liver extracts of several different species including frog, rat, guinea pig, mouse, perch and pigeon. Species differences were again apparent (Figures 23-25 but in this instance enzyme activity of the extracts was located kn one or two bands on the zymogram. Organ specificity was demonstrated when the catalases and peroxidases from pigeon liver and heart were compared (figure 26).

Quantitative Enzyme Studies on Mammalian Cells maintained in culture.

In the previous section it was demonstrated that functionally similar proteins from different species of animals and from different tissues within an individual animal displayed molecular heterogeneity when examined by the zymogram technique. In all cases investigated the species of origin could be recognised by the type of zymogram produced. In addition it was found that species specific esterase patterns persisted in cultured animal cells.

In this section the results of a quantitative enzyme survey on cultured animal cells are described. Large variations have been encountered in the enzyme content of different tissues and organs in the intact animal (Greenstein, 1947). It was considered of interest to determine if these differences persisted in cultured animal cells. The cell lines investigated were HeLa, HLM, skin fibroblasts, Hep I (all of human origin) and Strain L and L-5178Y.

In general, a basic similarity was found in the activity levels of several enzymes from different cell lines (Table No. 2), thus confirming previous observations by Lieberman & Ove (1958). Interesting exceptions were observed however when the levels of alkaline phosphatase,

Table 2. Activity levels of various enzymes in the cell lines used in these investigations.

Each figure represents the mean value of at least three determinations. Enzyme activity is expressed as enzyme units/mg. of protein - nitrogen.

	HeLa	HLM	L
Acid phosphatase	4.0	130	12.3
Alkaline phosphatase	18.7	1740	2.5
Glucose-6-phosphatase	2.1	8.6	0.3
Glucose-6-phosphate dehydrogenase	39	29	24
Esterase	2.2	2.8	2.3
β-Glucuronidase	21.3	17.2	14.5
Glutamyl transferase	2.0	1.4	0.48
Lactic dehydrogenase	77	42	98
Tryptophan pyrrolase	0	0	0
Adenosine deaminase	0	0	0
Xanthine oxidase	0	0	0

glucose-6-phosphatase and the ratio of acid to alkaline phosphatase of different cell strains were compared. The relatively high levels of alkaline phosphatase and glucose-6-phosphatase in HLM cells (derived from liver) compared for instance with HeLa cells is similar to the situation that exists in the whole animal.

In contrast to these observations was the failure to detect any tryptophan pyrrolase activity (which is confined exclusively to the liver in intact animals) in HLM cells. Auerbach & Walker (1959) reported that tryptophan pyrrolase was also absent from the Chang liver cells. In addition these workers were unable to detect, in the Chang liver cells, many other enzymes which were present in the livers of whole animals. These enzymes included tyrosine transaminase and histidase, while glucose-6-phosphatase, fructose diphosphatase and fructokinase were also reported (Perske, Parks & Wallace, 1957) absent from this cell line.

The extremely low levels of arginase detected in these cells was in contrast to the findings of Westfall et al. (1958) who found that mouse liver cells in culture displayed arginase activity comparable to the tissue of origin. Very low levels of arginase compared to the original tissue were detected in some cell strains, e.g. human skin, by these latter workers. A finding

(by Freshney - unpublished) in this laboratory, demonstrated that although the L-5178Y cells in vivo contained considerable arginase activity none was detected in vitro. In view of this observation L5178 cells which had been grown for several months in vitro were reinoculated into the species of origin (DBA2 mice - F1 crosses) and assayed for arginase after two weeks. Considerable arginase activity was once again detectable in these cells.

These results and those of other workers indicated that the use of enzymic parameters for comparing cultured cells with the tissue of origin was subject to certain limitations. In some cases it was found that enzymic characteristics were retained by cultured cells, e.g. high activity levels for glucose-6-phosphatase and alkaline phosphatase in HIM cells as compared with other cell lines which did not originate from liver tissue. There was however a remarkable similarity in the activity levels of many enzymes in cell lines which originated from different tissues, in marked contrast to the situation that exists in the whole animal.

Another point of interest which emerged from these investigations was that although the activity levels of the various enzymes were reproducible from preparation to preparation there was however one exception. This involved the level of acid phosphatase which was found

to vary considerably during the growth cycle of strain L. After the cells were trypsinized and transferred to fresh medium a seven fold decrease in acid phosphatase activity occurred. During logarithmic growth the phosphatase activity of the cells gradually increased again and finally reverted to its original level when the cells entered stationary phase. This variation in enzyme activity appeared to be peculiar to acid phosphatase as the levels of other enzymes such as alkaline phosphatase and β-glucuronidase remained constant during the growth cycle of strain L (figure 27). A similar variation in the alkaline phosphatase activity of Chang liver cells has recently been reported (Nitowsky & Herz, 1961). Different activity levels for the thymidine kinases during the growth cycle of strain L have also been demonstrated by Weissman, Smellie & Paul (1960).

Alterations in enzyme content during growth has been demonstrated in bacteria (Needham, 1950; Gale, 1943; Sheinin, 1958) and in animal tissues (Van Potter, 1944). That similar variations can occur in animal cells in culture demonstrates that great care must be taken when comparing enzyme activity levels in cultured cells. This would apply when different experimental conditions which may affect the growth rate are employed.

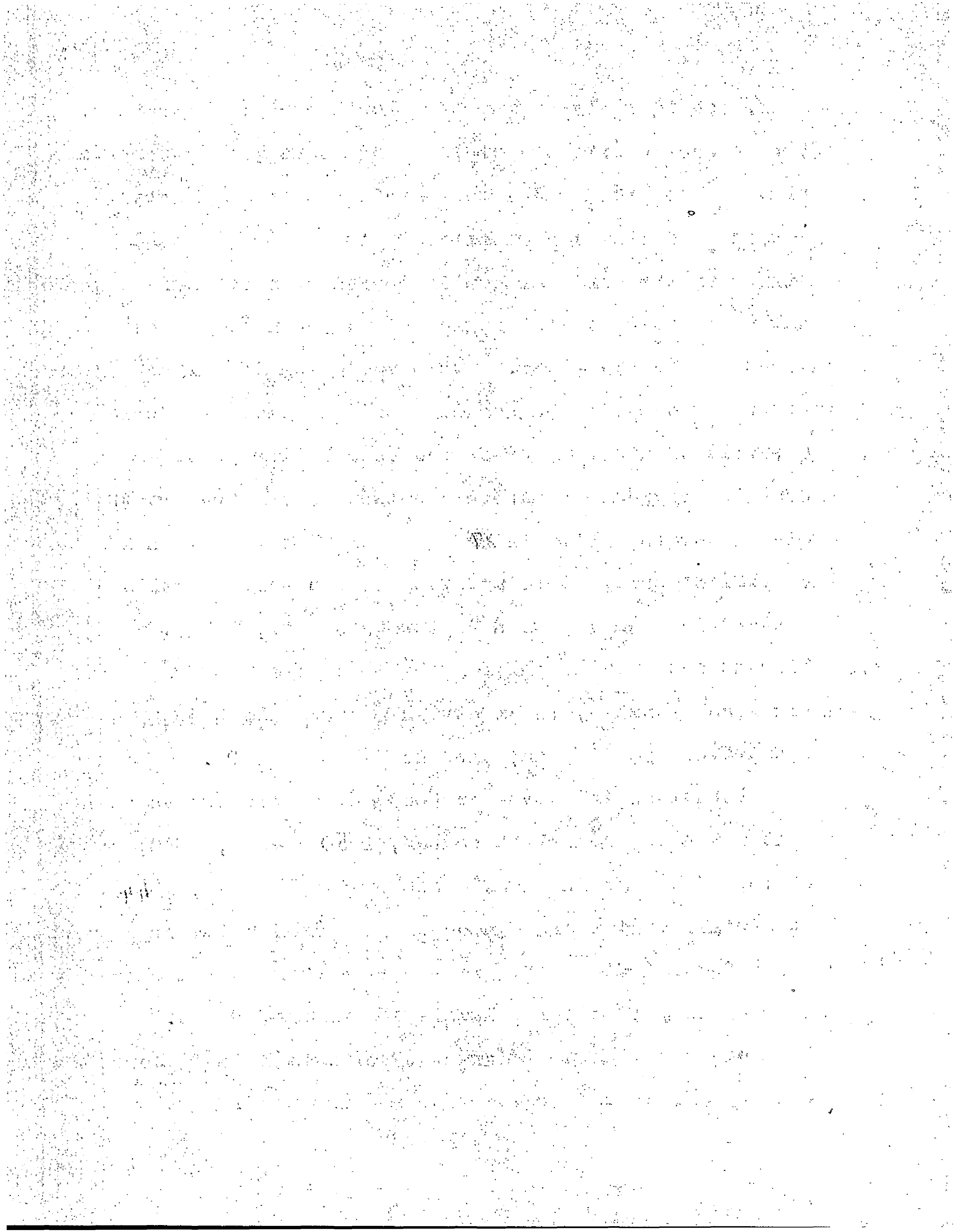


Fig. 27. Variations in the levels of acid phosphatase during the growth cycle of strain L. Triplicate samples were collected at daily intervals (approx.) after the cells were trypsinized and transferred to fresh medium.

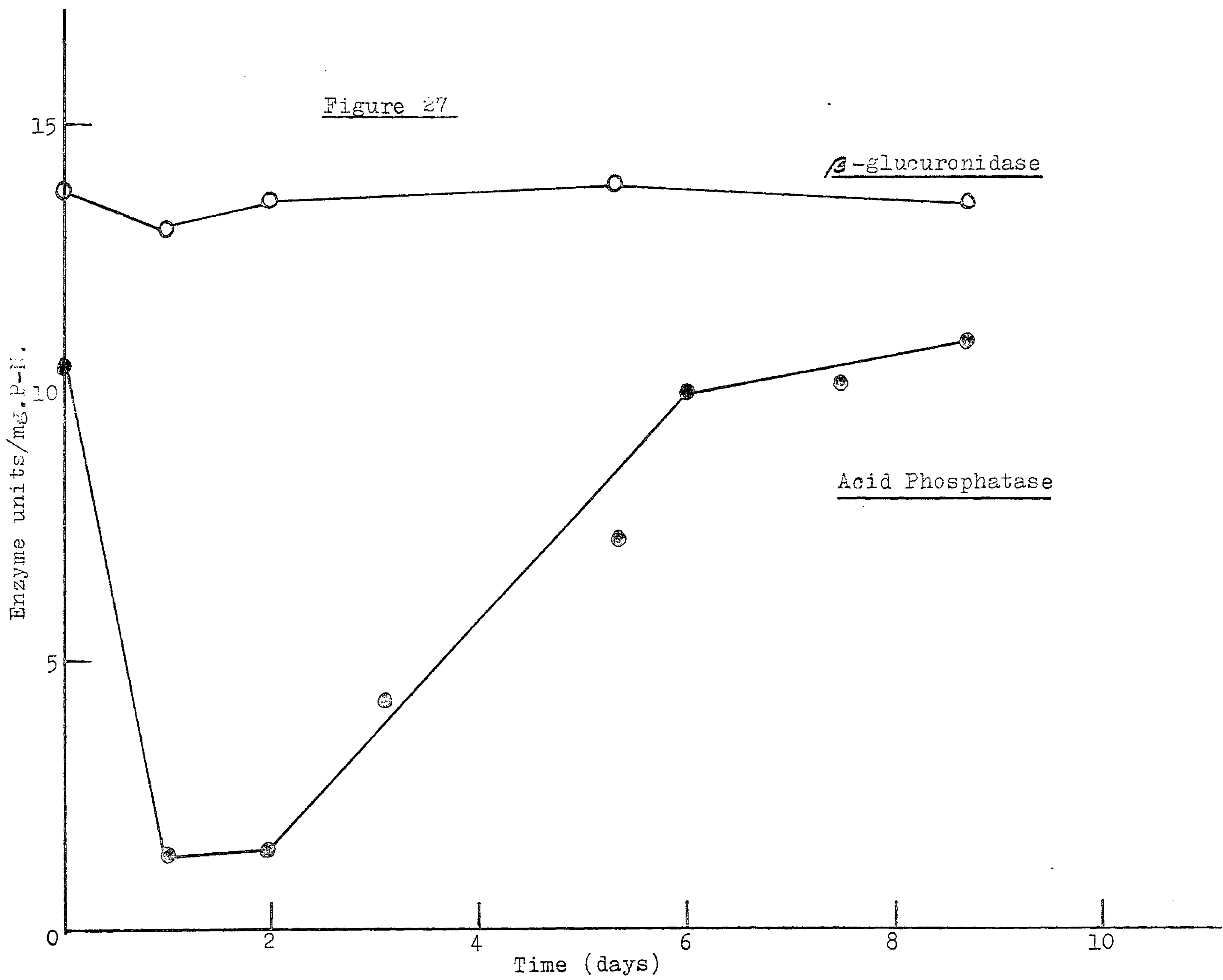


Figure 27
β-glucuronidase
Acid Phosphatase
Enzyme units/mg.P-N.
15
10
5
0
2
4
6
8
10
Time (days)

Attempts to Alter the Enzymic Constitution of Cultured Cells.

The experiments outlined in this section describe some attempts to alter the levels of several enzymes in cultured cells. Many of the enzyme systems investigated had previously been reported to be of an adaptive nature in whole animals (see Knox, Auerbach & Lin, 1956). Cells were generally grown in the presence of specific enzyme substrates, products, or related compounds and the enzyme activity levels compared with control cultures.

Malic Dehydrogenase

Paul & Danes (1961) have demonstrated that inhibition of the respiratory rate of cultured cells occurred after growth at low oxygen tensions. The possibility that decreased respiration could be correlated with a reduction in the level of some of the Krebs cycle enzymes was investigated here. For this purpose HLM cells were placed in an atmosphere of nitrogen for 48 hr. and the malic dehydrogenase levels were compared with control cultures. The effect of another inhibitor of the Krebs cycle, fluoroacetate, (which competitively inhibits aconitase through the formation of fluoro-acetyl-CoA) was also investigated. It was thought that inhibition of respiration might be reflected in a decrease in the level of malic dehydrogenase.

The experiment was set up by inoculating 12 flasks

with 10M cells each, in Waymouth's medium supplemented with 10% dialysed calf serum. Three flasks served as 3 groups (3 in each group) and treated as follows:

Group (a) glucose was added to the medium - final concentration 800 mg%.

Group (b) fluoroacetic acid was added to the medium final concentration 1.6×10^{-5}M.

final concentration 1.6×10^{-5}M.

Group (c) the gas phase in the flask consisted of nitrogen: carbon dioxide (95%:5%) and 500 mg% ribose (final concentration) was added to the medium as a source of pentose for nucleotide synthesis.

After 60 hr. the cells were harvested and the malic dehydrogenase activity of the extracts was determined. The levels of malic dehydrogenase in extracts of cells treated with fluoroacetic acid or high glucose concentrations were not significantly different from the values obtained from control cultures (Table 3). On the other hand a marked reduction in the levels of malic dehydrogenase in extracts prepared from cells grown under anaerobic conditions was demonstrated. When enzyme activity was expressed as enzyme units per million cells a 40 to 50-fold difference between malic dehydrogenase levels in the latter cells and the others was found to exist. Cells grown in an atmosphere of nitrogen were found to have a diminished

Table 3. The malic dehydrogenase activity of HLM cells after growth in the presence of nitrogen, fluoroacetate and high glucose concentration.

Fluoroacetic acid, nitrogen or excess glucose were added to the test cultures while control cultures contained basal medium (Waymouth's) only. The cells were collected after 60 hr. for malic dehydrogenase assay.

Additions to the medium.	Cell No. (M)	Malic Dehydrogenase (units/million cells).	Esterase (units/million cells).
Control	27	113	0.21
Fluoroacetic Acid ($1.65.10^{-5}$M)	24	88	0.18
Glucose (800 mg.%)	33	102	0.25
Nitrogen (95%): CO_2 (5%) + Ribose(500 mg%)	16	2	0.09

growth rate and a lower protein nitrogen content than usual. It was possible therefore that the reduced level of malic dehydrogenase in these cells was part of a general reduction in protein synthesis. To determine if this was true the experiment was repeated and in addition to malic dehydrogenase activity, esterase levels were also estimated in the various extracts. Variations in the esterase levels were much less pronounced (Table 3) indicating that some specific mechanism may be responsible for the decrease in malic dehydrogenase activity. The nature of this mechanism has, so far, not been defined.

In view of the recent findings of Gregolin et al.(1961) who demonstrated that separate forms of lactic dehydrogenase, with different kinetic properties, existed in yeast when the cells were placed in an aerobic and anaerobic environment; it is tempting to postulate that some similar mechanism may be responsible for the 'malic dehydrogenase effect'. Two forms of malic dehydrogenase with distinct kinetic properties, have been isolated from rat liver (Thorne, 1960) and one of these forms (located in the mitochondria) was inhibited by high concentrations of oxaloacetate (Delbruck et al.1959).

Arginase.

Arginase activity has been induced in intact animals (Lightbody & Kleinman, 1939) and in bacteria (Gorini & Maas, 1958). Klein (1961) found that addition of arginine to the growth medium of HeLa cells had little effect on the resultant arginase activity. If however the cells were grown in the presence of yeast RNA (250 ug/ml.) for fourteen days a seven fold increase in arginase activity was observed. The arginase activity was further increased to approximately ten times the original level when arginine was added to these cells.

Arginase was found to be either completely absent or present in very small amounts in all cell strains maintained in this laboratory (Freshney, unpublished observation.). This was particularly striking with the L5178Y strain which displayed high arginase activity in 'vivo'. When L5178 cells which had been maintained in culture for several months were reinoculated into the species of origin (DBA-2 mice, F1 crosses) the arginase activity reverted to its original high level. This finding suggested that the disappearance of arginase activity from cultured cells might be due to adaptive phenomena. This was later confirmed when arginase activity (approximately 12 times greater than normal) was induced in HeLa cells after growth in the presence of arginine (10^{-2}M), uridine (10^{-3}M) and

Table 4. Induction of arginase in HeLa cells.

Arginine (10^{-2}M), uridine (10^{-3}M) and glutamic acid (4.10^{-3}M) was added to test cultures while control cultures contained basal medium only.

	Cell No. (Millions)	Arginase
Control	11	0.08
Test	13	1.12

glutamic acid (4.10^{-3}M) for seven days (Table 4) inoculating six flasks with 1 million HeLa cells in Eagle's medium supplemented with 5% calf and 2% human serum.

Attempts to induce arginase activity in strain L cells have so far been unsuccessful but have resulted in an interesting finding. After three days growth in a medium containing arginine (10^{-2}M), uridine (10^{-3}M), ornithine (10^{-2}M), citrulline (10^{-2}M) and glutamic acid (4.10^{-3}M), giant cells, approximately three times larger than control cells, have been observed. The giant cells reverted to their normal size four days after control medium was substituted for the test medium. Giant-cell formation has previously been observed in cultured cells treated with X-rays (Puck & Marcus, 1956; Dickson, Paul & Davidson, 1958) and 5-bromodeoxyuridine (Hakala, 1959). X-irradiation of cultured cells caused suppression of cell division but the synthesis of all components (including DNA and protein) continued and resulted in giant-cell formation (Dickson & Paul, 1961). Since no satisfactory explanation of the mechanism of giant-cell formation has been evolved it is difficult to interpret the results obtained here with strain L. It remains to be seen whether a possible connection exists between the absence of arginase activity in these cells (under conditions which

induced the enzyme in HeLa cells) and the formation of giant cells.

β-Glucuronidase.

Several unsuccessful attempts were made to induce β-glucuronidase in strains L and HeLa by growing the cells in the presence of phenolphthalein glucuronide (1mM). The possibility was considered that the level of β-glucuronidase was controlled by a repressor which originated from glucose present in the medium, as occurred with the inducible β-galactosidase in E.coli (Cohn, 1956; Mandelstam, 1961). An experiment to investigate this possibility was set up as follows - Nine flasks were each inoculated with one million HeLa cells in Eagle's medium. In three flasks which served as controls the glucose concentration in the medium was 1 mg/ml. In the six remaining flasks lactic acid (1 mg/ml.) and ribose (5 mg/ml.) substituted for glucose in the medium. A possible repressor of β-glucuronidase potassium glucuronate (2mM) was added to three of the latter flasks. The medium was renewed in all flasks on the third and sixth day after commencement of the experiment. The cells were harvested after eight days.

The results (Table 5) showed that while the levels of β-glucuronidase were identical in cells grown in media

Table 5. Suppression of β-Glucuronidase Activity by Potassium Glucuronate in HeLa Cells.

Group I served as controls and in groups II and III glucose was omitted from the medium. Potassium glucuronate (2mM) was added to group III.

Group	Cell No.	β-glucuronidase
I	12	0.92
II	10	0.87
III	8	0.19

with or without glucose a five fold suppression of β-glucuronidase activity resulted after growth in the presence of potassium glucuronate. This finding demonstrated that the levels of β-glucuronidase may be regulated by a repressor and suggested that suitable conditions for inducing the enzyme might be a glucose-free medium containing a glucuronide.

Glutamyl Transferase

In contrast to the situation that exists in vivo, most mammalian cell strains have an absolute requirement for glutamine in the growth medium. Some cell lines can be adapted to grow in the absence of glutamine providing that it is replaced by a high concentration (20mM) of glutamic acid (Eagle, 1959). When HeLa cells were propagated in a glutamine-free medium the specific activity of glutamyl transferase was found to increase several fold (De Mars, 1958). It was decided here to determine (a) if this phenomenon could be demonstrated in other cell lines and (b) the nature of the mechanism involved. Glutamyl transferase is almost certainly identical with glutamyl synthetase (Krisenaschwamy & Meister, 1960) which is responsible for the biosynthesis of glutamine from glutamic acid and ammonia.

Strain L cells were initially adapted to growth in a glutamine-free medium as follows. Five million cells were inoculated into flasks containing Waymouth's medium (-glutamine) supplemented with 10% (V/V) dialysed calf serum and 20mM glutamic acid. The medium was renewed twice weekly for several weeks. Periodic examination of the cultures revealed that the majority of the cells were in suspension and were not in a healthy condition. After five weeks approx., a small colony of cells with typical

Table 6. Induction of glutamyl transferase in strains L and HLM.

The specific activity of glutamyl transferase was compared in cells grown, for 48 hr., in the presence and absence of glutamine. Glutamic acid (20mM) was substituted for glutamine in the medium of test cultures.

		Glutamyl Transferase (units/million cells)
HLM	Control	0.053
	Test	0.438
L	Control	0.020
	Test	0.187

fibroblastic morphology and attached to the glass was observed one of the flasks. These cells were eventually transferred to other flasks and could be propagated without difficulty in a glutamine-free medium even when the glutamic acid concentration was reduced to 1mM.

Strain HIM was found to adapt to a glutamine-free medium far more readily than strain L. The glutamyl transferase activity of cells grown in the glutamine-free medium was found to be eight to ten times higher than that of control cultures which were grown in the presence of glutamine. The initial level of glutamyl transferase in HIM cells was approx. 2.5 times higher than strain L but the magnitude of the increase was of the same order in the two cell lines (Table 6). The suppression of glutamyl transferase activity in cells grown in the presence of glutamine appeared to be analogous to the feed back control systems described in bacteria (Yates & Pardee, 1957). Further studies into the nature of this phenomenon were performed and will be described in a later section.

Acid and Alkaline Phosphatases

Attempts were made to induce phosphomonoesterase activity in strains HIM and L by growth in the presence of phosphate esters. Six flasks were inoculated with 1 million HIM cells in Waymouth's medium supplemented with 10% dialysed calf serum. Three flasks served as controls

and sodium-β-glycerophosphate (0.2mM) was added to the three remaining flasks. Nine flasks containing 1 million L cells in Eagle's medium supplemented with 0.5% (W/V) bactopeptone were set up at the same time. Glucose-6-phosphate, sodium salt (0.8mM) was added to three flasks, sodium-β-glycerophosphate (0.2mM) was added to three of the remaining flasks and as before three flasks served as control cultures.

Samples of the medium were taken every 48 hr. when fresh medium was added, to determine if any increase in the phosphatase activity was detectable. After two weeks the cells were harvested and assayed for phosphatase activity. As demonstrated in Table No. 7 there was no evidence for any increase in phosphatase activity with cells grown in the presence of phosphate esters. With the HLM cells it was possible that the slight phosphatase activity of the serum present in the medium hydrolysed the phosphate esters. This was not true with the L cells which were grown in a serum-free medium. In view of these results and those of other workers (Klein, 1961) it would appear that the phosphatases of strain L are constitutive in nature.

Induction of alkaline phosphatase in HeLa cells by means of prednisolone has been reported (Cox & Pontecorvo, 1961). These findings were confirmed in this investigation

(Table 8) by growing HeLa cells, at an initial inoculum of 1 million in Eagle's medium + 5% calf serum and 2% human serum, for 10 days in the presence of prednisolone (1 ug/ml). As previously mentioned alterations in the acid phosphatase levels of strain L occurred during the growth cycle. The nature of this variation has, so far, not been defined.

Esterases

Fifteen flasks containing an initial inoculum of 1 million HIM cells, in Waymouth's medium supplemented with 10% dialysed calf serum, were divided into 5 groups of triplicate cultures and the following additions were made:

Group I. No addition - controls.

Group 2. acetylsalicylic acid (0.2mM).

Group 3. acetylsalicylic acid (1.0mM).

Group 4. triacetin (0.2mM).

Group 5. triacetin (1.0mM).

Nineteen flasks of L cells with 1 million cells each, in Eagle's medium + 0.5% (W/V) bactopeptone were also set up. Three flasks served as controls and the following substances were added to eight pairs of the remaining flasks:

Group 6. acetylsalicylic acid (0.2mM).

Group 7. acetylsalicylic acid (1.0mM).

Group 8. triacetin (0.2mM).

Group 9. triacetin (1.0mM).

Table 7. Attempts to induce alkaline phosphatase in strains L and HLM.

The cells were grown in the presence of various phosphate esters for two weeks and the levels of alkaline phosphatase and glucose-6-phosphatase were compared with control cultures. Samples of the medium were collected at various times and examined for any increase in alkaline phosphatase activity.

	Substance added to the medium.	Alkaline Phosphatase (units/mg. protein-nitrogen).	Glucose-6-Phosphatase (units/mg. protein-nitrogen).
Strain L	-	2.4	0
	Sodium-β-glycero-phosphate.	2.1	0
	Glucose-6-phosphate	1.9	0
Strain HLM	-	1735	8.4
	Sodium-β-glycero-phosphate	1687	7.8
	Glucose-6-phosphate	1811	8.8

No difference was detected in the alkaline phosphatase levels in the medium of control and test cultures.

Table 38. Effect of prednisolone on the alkaline phosphatase activity of HeLa cells.

Prenisolone (1 ug/ml.) was added to the test cultures and the alkaline phosphatase levels were compared with control cultures after five and ten days.

Culture	Days	Cell No.	Alkaline Phosphatase
Control	5	7	0.47
	10	13	0.54
Test	5	4	2.94
	10	9	4.80

Table 9. Attempts to induce esterase in strains L and HLM.

Organic esters were added to the medium of L and HLM cells and the esterase levels were compared with control cultures after two weeks. Samples of the medium were also collected and examined for possible differences in esterase activity.

	Substance added to the medium.	Esterase (units/mg. protein nitrogen).
Strain L	-	2.6
	Acetylsalicylic Acid	2.9
	Triacetin	2.6
	Acetylthiocholine Iodide	2.2
	Tween 20	2.5
Strain HLM	-	3.1
	Acetylsalicylic Acid	2.7
	Triacetin	2.6

No differences were detected in the esterase levels in the medium of control and test cultures.

Group 10. acetylthiocholine iodide (1.0mM).

Group 11. Tween-20 (0.8 ug/ml).

Samples (1.0 ml.) of the medium were taken daily (for esterase determinations) and the entire medium was renewed every 48 hr. After two weeks the cells were assayed for esterase activity but no differences were detected in the activity levels of control and test cultures (Table 9). Esterase activity was not detected in the growth medium of strain L. The zymogram studies indicated the possible stability of the esterase levels in cultured cells; these results substantiate this.

Xanthine Oxidase, Adenosine Deaminase and Aspartatete-α-ketoglutarate Transaminase

Ten flasks of HIM cells were set up with 2 million cells each in the following media (in duplicate).

(1) Eagle's medium.

(2) Medium 199.

(3) Waymouth's medium.

(4) Waymouth's medium + hypoxanthine (0.2mM).

(5) Waymouth's medium + adenosine (0.2mM).

All media were supplemented with 10% dialysed calf serum and the entire medium was renewed every three days. After 12 days the cells were collected and assayed for xanthine oxidase, adenosine deaminase and aspartate-α-ketoglutarate transaminase. Since medium 199 contains many non essential

Table 19. Attempts to induce adenosine deaminase, xanthine oxidase and aspartate-α-ketoglutarate transaminase in HLM cells.

Medium	Aspartate-α-ketoglutarate transaminase (units/mg. protein nitrogen)
199	4.7
Waymouth's	5.2
Eagle's	4.3

No adenosine deaminase or xanthine oxidase activity was detected in control cultures or with cells grown in the presence of hypoxanthine or adenosine.

amino acids it was though that cells grown in this medium might have lower transaminase levels than those grown in Eagle's medium, which contains essential amino acids only. However, the results (Table 10) demonstrated that the aspartate-α-ketoglutarate transaminase activity was similar in HLM cells grown in both types of medium. Adenosine deaminase or xanthine oxidase activity was not detected in any of the cultures, suggesting that either the enzymes are not present in the cells or that the concentrations are so low as to escape detection by the assay systems employed.

Tryptophan Pyrrolase

The previous results suggested that many of the enzymes investigated, in the search for an adaptive enzyme system, were probably constitutive in nature, especially in strain L. Since the induction of tryptophan pyrrolase in the intact animal represents the best example so far of enzyme induction in whole animal it was decided to investigate it here. Tryptophan pyrrolase has been induced in rats (Civen & Knox, 1959) and in Rana pipens (Stearns & Kostellow, 1958). Although the enzyme is exclusively confined to liver it was not detected in Chang liver cells which were grown in the presence of tryptophan (Auerbach & Walker, 1959). The HLM cell was also devoid of tryptophan pyrrolase activity but resembled liver

tissue in that it contained relatively high levels of glucose-6-phosphatase and alkaline phosphatase. In this experiment an attempt was made to induce tryptophan pyrrolase in HLM cells. Six cultures each containing 10 million cells were set up in Waymouth's medium supplemented with 10% dialysed calf serum. The tryptophan concentration in three of the cultures was just sufficient (10 uM) to maintain a maximum growth rate while in the three other cultures the tryptophan concentration was increased to 1mM. The cells were collected after 3 days and assayed for tryptophan pyrrolase. No tryptophan pyrrolase activity was detected in any of the cultures. The absence of tryptophan pyrrolase from these cells even after growth in a medium containing a high concentration of tryptophan suggests that either (a) the enzyme system is not inducible (at least by the method employed here) or (b) that the enzyme forming system is absent.

Glucose-6-phosphatase, Glucose-6-phosphate dehydrogenase and Lactic-dehydrogenase

Several workers have recently reported (Freedland & Harper, 1958; Fitch & Chaikoff, 1959) significant increases in the levels of rat liver glucose-6-phosphate dehydrogenase following the administration of diets rich in glucose or fructose. Appreciable amounts of these enzymes were already present in HLM cells and it was

Table 11. Glucose-6-phosphatase, glucose-6-phosphate dehydrogenase and lactic dehydrogenase activity of HLM cells, after growth in the presence of high concentrations of glucose or fructose.

Glucose (800 mg.%), fructose (800 mg.%) or lactic acid (100 mg.%) were added to the medium of HLM cells. After 48 hr. the levels of the three above enzymes were compared with control cultures (glucose concentration 100 mg.%).

Additions to the medium.	Cell No. (M)	Glucose-6-Dehydrogenase (units/million cells).	Glucose-6-Phosphatase (units/million cells).	Lactic Dehydrogenase (units/million cells).
-	19	3.8	0.87	5.4
Glucose	22	3.2	0.65	5.7
Fructose	17	4.7	0.71	5.8
Lactic Acid	18	4.2	0.67	5.8

decided to investigate if these levels could be increased by growing the cells in a medium with a high concentration of glucose or fructose.

Sixteen flasks were inoculated with 10 million HLM cells each, in Waymouth's medium (+ 5% dialysed calf serum + 2% human serum) as follows:

(a) 4 flasks contained Waymouth's medium.

(b) 4 flasks contained Waymouth's medium + 800 mg% glucose.

(c) 4 flasks contained Waymouth's medium (-glucose) + 800 mg% fructose.

(d) 4 flasks contained Waymouth's medium (-glucose) + 100 mg% lactic acid.

After 48 hr. the cells from two flasks in each group were harvested. The medium was renewed in the eight remaining flasks after 3 days and the cells were finally harvested on the sixth day. Glucose-6-phosphatase, glucose-6-phosphate dehydrogenase and lactic dehydrogenase determinations were performed on the various cell extracts but as demonstrated (Table 11) no significant differences in enzyme activity levels were observed. The enzyme levels were remarkably constant in the different cultures and from these findings it would appear that some other factors (probably hormones) were responsible for the increased activity in intact animals.

Studies on the Mechanism of Adaptive Enzyme Formation in Cultured Cells.

The results in the previous section illustrate that while many enzyme systems in animal cells appear to be constitutive in nature some adaptive enzymes are also present. In this section some investigations carried out to determine the mechanism involved in adaptive enzyme formation are described. These studies were mainly confined to the glutamyl transferase system in strain L and some preliminary findings on the arginase system in HeLa cells will also be described.

Some kinetic studies on mouse liver glutamyl transferase were carried out to determine the optimum conditions for the assay system. The results of these investigations are given in the accompanying figures (Nos. 27,28 & 29) and table (No.12).

Time-course of the induction of glutamyl transferase.

Ten flasks were each inoculated with 10 million cells and Waymouth's medium (-glutamine) supplemented with 20mM glutamic acid and 10% dialysed calf serum. The cells were previously grown for several days in a similar medium containing glutamine. Duplicate flasks were removed at zero time and at daily intervals thereafter for 4 days. When glutamyl transferase estimations were performed on extracts prepared from these cells an eight to tenfold increase in activity was demonstrated during the first

Fig. 28. pH - activity curves of mouse liver glutamyl transferase in three different buffer systems, acetate (0.1M), phosphate:citrate (0.1M) and citrate (0.1M).

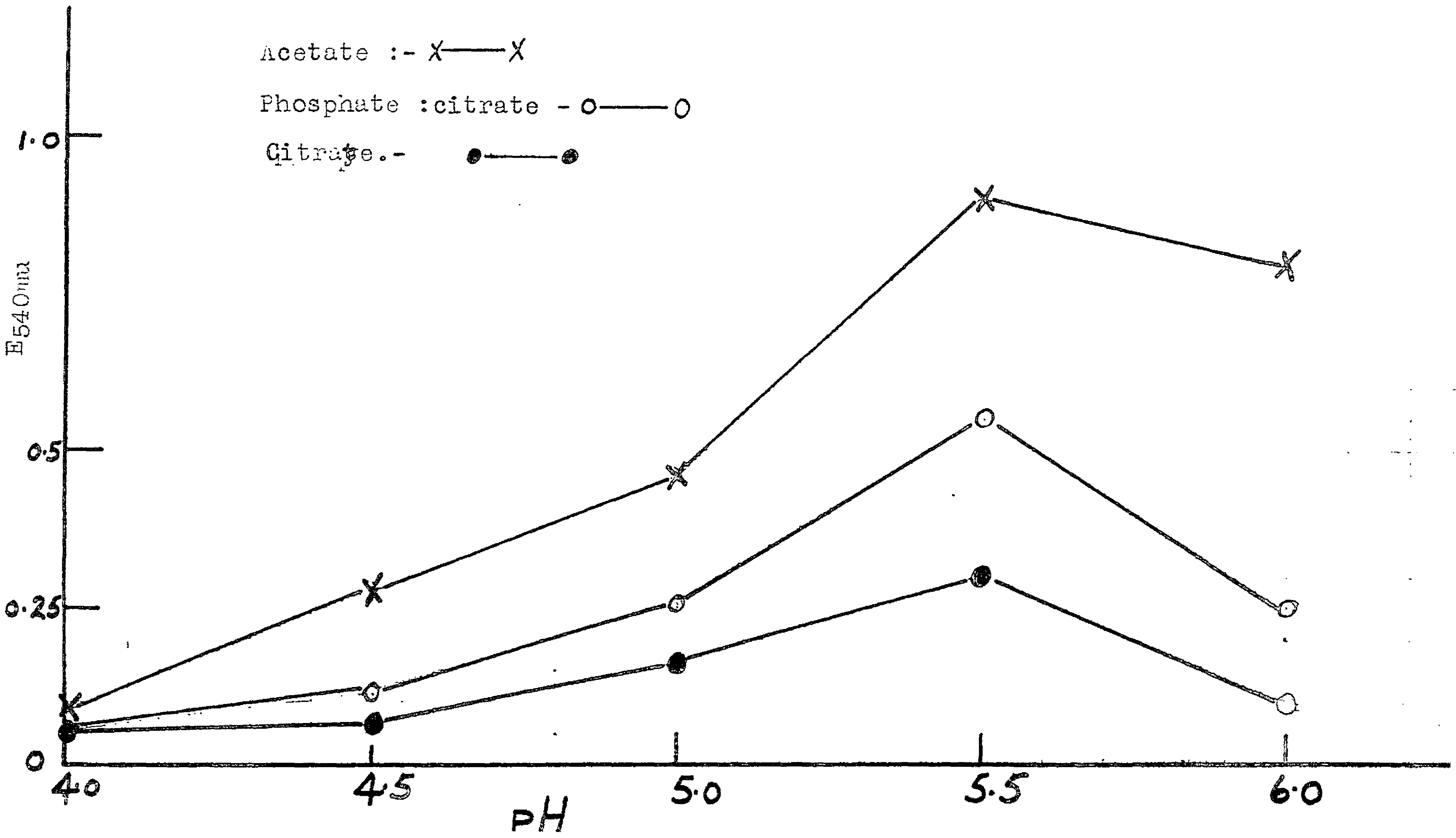


Figure 28
Acetate :- X——X
Phosphate :citrate - o——o
Citrate.- ●——●
1·0
0·5
0·25
0
E540mu
4·0
4·5
5·0
5·5
6·0
pH

Fig. 29. Activity of mouse liver glutamyl transferase plotted against time (min.).

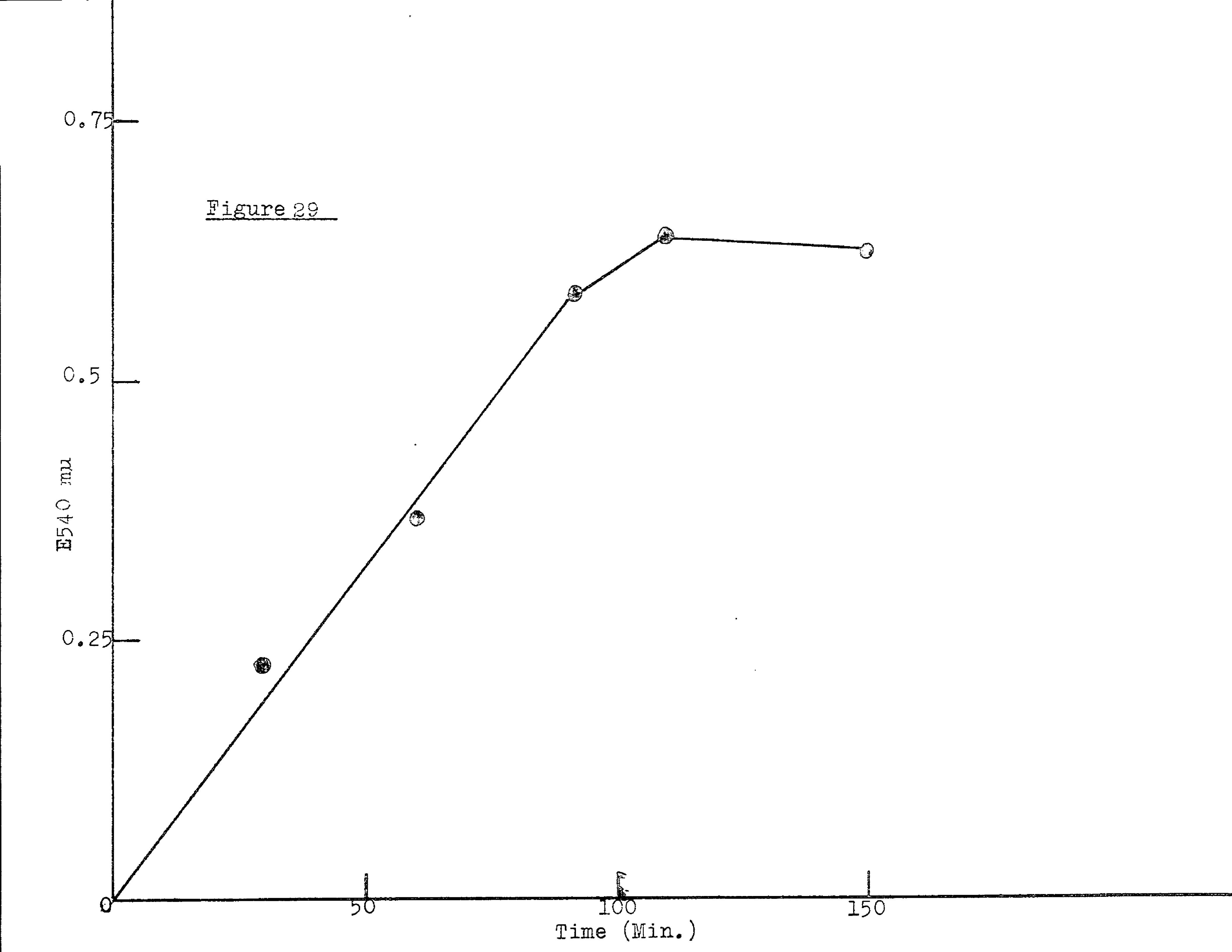


Figure 29
0.75
0.5
0.25
0
E540 mμ
50
100
150
Time (Min.)

Table 12. The effect of varying the concentrations of manganese chloride and sodium arsenate on mouse liver glutamyl transferase.

The concentrations of manganese chloride and sodium arsenate represent final concentrations in the reaction mixture. The mean-value of three determinations was used in each case.

$MnCl_2$ uMoles/ml.	$Na_3A_sO_4$ uMoles/ml.	E540 Mu
5	25	0.870
2.5	25	0.970
0.5	25	0.763
5.0	12.5	0.860
5.0	1.25	0.630
0	25.0	0.069
5.0	0	0.148

Table 13. Time-course of induction of glutamyl transferase in strain L.

Time (hr)	Cell Number (million)	Glutamyl Transferase (units/mg. P.- N.)
0	10	0.41
24	14	1.28
48	25	2.42
68	39	2.33
92	48	2.40

Fig. 30. Time-course of the induction of glutamyl transferase in strain L. The cells were inoculated at a density of 2.10^5 ml. in Waymouth's medium (- glutamine) + 20mM glutamic acid + 10% dialysed calf serum. Duplicate samples were removed at zero time and at intervals of 24 hr.

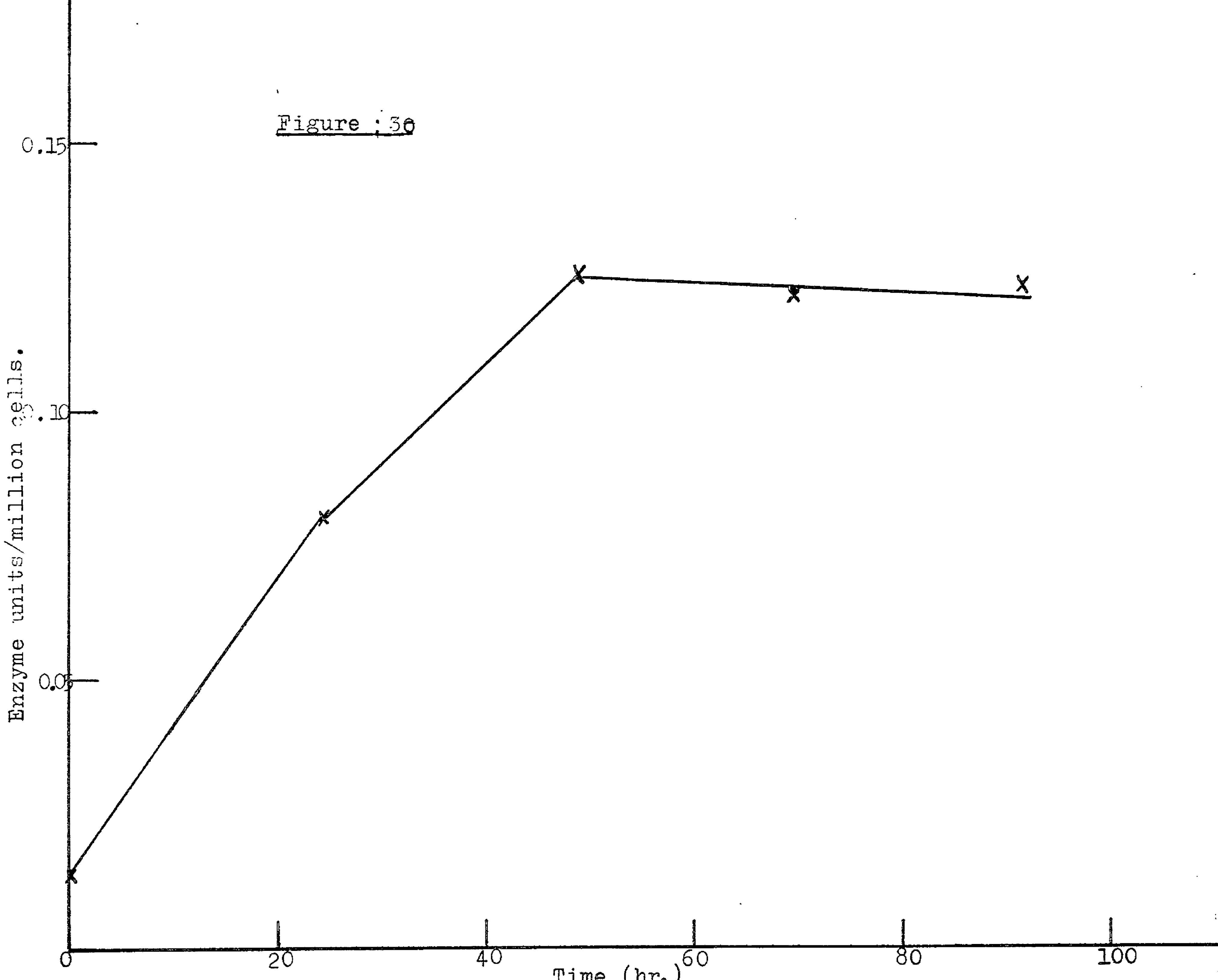


Figure : 3e
0.15
0.10
0.05
Enzyme units/million cells.
0
20
40
60
80
100
Time (hr.)

48 hr. (Figure 30). During this period cell number and protein nitrogen content increased approx. 3-fold (Table 13). Maximum glutamyl transferase activity was obtained after 48 hr. and thereafter the level remained constant for a further 44 hr.

When this experiment was repeated the pattern of induction was exactly the same and on no occasion could the level of glutamyl transferase be increased by more than tenfold. The extent and the rate of glutamyl transferase induction in animal cells was obviously very different from similar phenomena in bacteria where in some instances enzyme activity levels have been increased by several thousand following the addition of a suitable inducer.

Time-course of the suppression of glutamyl transferase by glutamine.

This experiment was undertaken to determine the time required for glutamine to suppress glutamyl transferase formation in 'induced' cells. Ten flasks were therefore inoculated with 10 million 'induced' cells in Waymouth's medium (-glutamine) plus 10% dialysed calf serum and 20mM glutamic acid. After 24 hr. cells from two flasks were collected as initial samples. Glutamine (final concentration 2.4mM) was then added to four flasks and an equivalent amount of glutamic acid to the four flasks which served as control cultures. One flask from the

Table 14. Time-course of the repression of glutamyl transferase in strain L.

An initial sample of induced cells was removed. Further samples were taken after glutamine was added to the test cultures and glutamic acid to control cultures.

	Time (hr) after addition of glutamine or glutamic acid	Cell Number (M)	Glutamyl Transferase (units/mg. P - N).
Initial	0	16	2.35
Control	4	15	2.41
	12	24	2.38
	16	23	2.49
	24	27	2.25
Test	4	16	1.62
	12	22	0.45
	16	25	0.51
	24	30	0.38

Fig. 31. Time course of the repression of glutamyl transferase in strain L. Glutamine (2.4mM) was added to the medium of 'induced' cells (density 2.10^5/ml.). Glutamic acid (2.4mM) was added to control cultures. Duplicate samples were removed from control and test cultures at 4 hr., 12 hr., 16 hr. and 20 hr. after addition of glutamine and glutamic acid.

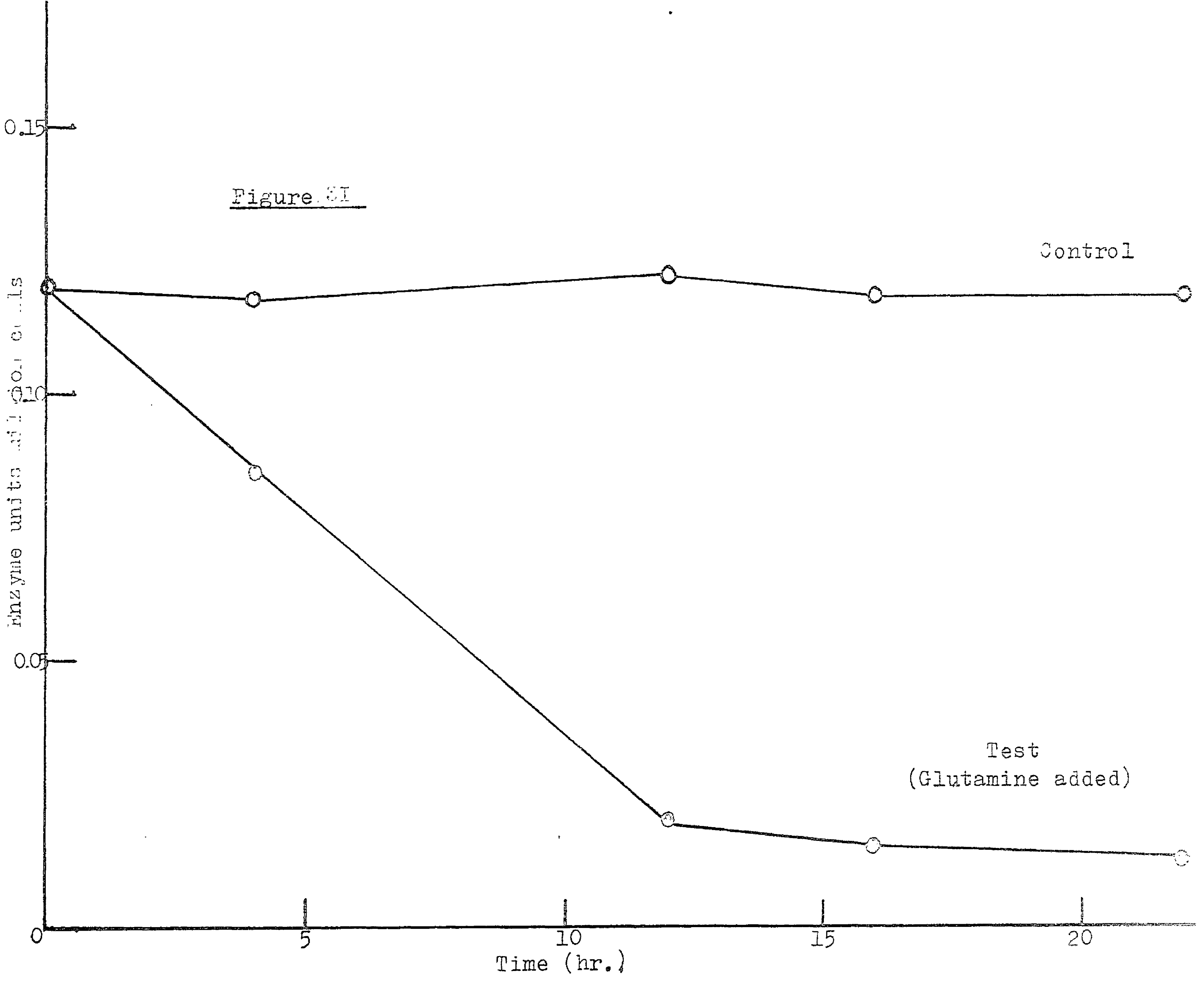


Figure 31

glutamine and glutamic acid treated cultures was harvested at 4, 12, 16 and 24 hr. intervals and the cells were assayed for glutamyl transferase activity.

Glutamyl transferase activity (in enzyme units/million cells) was plotted against time (Fig.31 and table 14).

In the light of these results the glutamyl transferase system was considered as an example of enzyme repression where enzyme formation was controlled in some way by glutamine. This would explain the low levels of glutamyl transferase in cells which were grown in the presence of glutamine. Some other mechanism which rapidly inactivates glutamyl transferase activity must also operate, to account for the rapid disappearance of glutamyl transferase activity following the introduction of glutamine into the growth medium of 'induced' cells. Glutamyl transferase activity diminished far too quickly to be explained by dilution of existing enzyme during subsequent cell growth.

Studies on the glutamyl transferase system in cell-free extracts.

In an effort to obtain information about a possible mechanism that would explain the rapid disappearance of glutamyl transferase, in the previous experiment, some investigations were performed on the stability of the enzyme in cell free homogenates. Because of the technical difficulties involved in obtaining sufficient quantities of material from cultured cells, an extract prepared from

Table 15. The effect of incubating glutamyl transferase with various substances at 37°C.

Substance	% Protection	% Inhibition
Nitrogen	82	
Nitrogen + Glutamic Acid	184	
Nitrogen + Glutamine	118	
Glutamic Acid	47	
Glutamine	28	
$NADH_2$	56	
$NADH_2$ + Glutamic Acid	172	
$NADH_2$ + Glutamine	53	
Iodoacetic Acid	-	56

$$\% \text{ Protection} = \frac{\text{Activity of Test} - \text{Activity of Control}}{\text{Activity of Initial} - \text{Activity of Control}} \times 100$$

$$\% \text{ Inhibition} = \frac{\text{Activity of Control} - \text{Activity of Test}}{\text{Activity of Control}} \times 100$$

mouse liver was used in these studies. A suitable extract was prepared by homogenising the liver with 30 volumes of ice-cold, 0.85% (W/V) sodium chloride in a Potter-Elvehjem homogeniser. Cell debris (nuclei and mitochondria) were removed by centrifuging at 15,000 g. and 0° for 1 hr.

When portions of this extract were incubated with an equal volume of 0.1M phosphate buffer, pH 7.4, at $37^{o}C$ a progressive decrease in glutamyl transferase activity was observed. Transferase activity completely disappeared after 36 hr. under these conditions. Inactivation of the enzyme could be prevented, however, if the extract was incubated in an atmosphere of nitrogen or if glutamine (2.4mM) or glutamic acid (2.4mM) were added to the incubation mixture. Reducing agents such as $NADH_2$ ($5.10^{-3}M$) and ascorbic acid were also found to have a protective effect on the transferase activity. Iodoacetic acid ($1.5.10^{-3}M$) on the other hand inhibited transferase activity. Activation of transferase activity was observed when the extract was incubated with either nitrogen + glutamic acid or $NADH_2$ + glutamic acid (Table 15). Although glutamine was found less efficient than glutamic acid as a 'protective agent' for glutamyl transfer there was no evidence of inhibition of enzyme activity by glutamine. It is not unlikely however that the protective

effect of glutamine may be due to some of the amide being converted into glutamic acid under the influence of glutaminase.

These findings demonstrated that glutamyl transferase was an unstable enzyme, inactivated by iodoacetic acid but stabilized by anaerobioses and several substances including reducing agents. Since these properties are peculiar to many sulphydryl enzymes it seems probable that sulphydryl groups are in some way necessary for maximum function of glutamyl transferase. While considering that some degree of caution must be exercised when extrapoleting from in vitro to in vivo conditions it is possible that the intracellular activity of glutamyl transferase can be altered by substances which would bring about oxidation of thiol linkages on the enzyme molecule.

The nature of the increased glutamyl transferase activity in cells grown in the absence of glutamine.

This series of experiments were designed to determine whether the increase in glutamyl transferase activity was due to activation of an inactive precursor or if de novo protein synthesis was involved. The low specific activities obtained with the glutamyl transferase system indicated that the amount of enzyme formed was only a small fraction of the total cellular protein. This prevented any studies being performed on the incorporation of radioactive amino

acids, purine and pyrimidines. In some inducible bacterial enzyme systems the amount of induced enzyme formed, represented a considerable proportion of the total protein concentration of the cell, e.g. 16% for the β-galactosidase system in E.coli. Also because of the low levels of glutamyl transferase it was not possible to demonstrate directly that protein synthesis had occurred. Experiments such as those of Halvorson (1960), Hogness, Cohen & Monod (1955) and Pollock (1959) who isolated induced enzymes from bacteria and with the aid of radio-active amino acids demonstrated that nett protein synthesis had occurred, were not feasible in the present system. A more indirect approach involving the use of metabolic inhibitors of protein, RNA and DNA biosynthesis, was therefore adopted in these investigations.

<u>The Effect of ρ-fluorophenylalanine on the induction of Glutamyl Transferase.</u>

Evidence from bacterial studies suggested that in some cases protein synthesis was inhibited by the presence of an amino acid analogue in the growth medium (Chantrenne, 1961). Some amino acid analogues, on the other hand, were incorporated into bacterial proteins resulting in the formation of catalytically active (Vaughan & Steinberg, 1959) or inactive (Richmond, 1960) protein molecules. Investigations on the incorporation of ρ-fluorophenyl-

3 - ^{14}C -alanine into the proteins of rabbit muscle, blood and liver have revealed that while incorporation was extensive the catalytic activity of aldolase and glyceraldehyde-3-phosphate dehydrogenase was not altered (Boyer & Westhead, 1961). Rabinowitz & McGrath (1959) however demonstrated that protein synthesis in reticulocytes was inhibited in the presence of o-fluorophenylalanine.

To determine the effect of p-fluorophenylalanine on the induction of glutamyl transferase, 10 flasks were each inoculated with 10 million cells in Waymouth's medium (+ glutamine) supplemented with 10% dialysed calf serum. After 24 hr. two flasks were taken as initial samples and the remaining flasks treated as follows. The medium was removed and the cells washed in situ with sterile balanced salt solution to remove all traces of glutamine. Four flasks served as controls and fresh medium, consisting of Waymouth's medium (- glutamine) + 20mM glutamic acid + 10% dialysed calf serum, was added. The medium added to the four remaining flasks was composed of balanced salt solution + 10% dialysed calf serum + 20mM glutamic acid + 2.10^{-4}M p-fluorophenylalanine.

Duplicate samples were removed from each group at 24 and 48 hr. intervals after changing the medium. When the glutamyl transferase activity of the two groups was compared (table 16) it was found that no induction had

Table 16. The effect of p-fluorophenylalanine on the induction of glutamyl transferase in strain L.

Test cultures contained p-fluorophenylalanine (2.10^{-4}M) which was omitted from the controls. Samples were taken at 24 hr. and 48 hr. after the addition of p-fluorophenylalanine.

	Time (hr)	Cell Number (M)	Glutamyl Transferase units/million cells
Initial	0	9	0.017
Control	24	14	0.070
	48	19	0.123
Test	24	10	0.022
	48	13	0.019

occurred in the medium containing ρ-fluorophenylalanine while the usual 8-fold increase occurred in control cultures. The protein-nitrogen content of the cells grown in the depleted medium, containing ρ-fluorophenylalanine, was appreciably lower than that of control cultures (~~Table 16~~).

It was concluded from this experiment that protein synthesis was necessary for the induction of glutamyl transferase. It was not possible to say whether ρ-fluorophenylalanine (a) inhibited enzyme synthesis or (b) was incorporated into the enzyme molecule and inhibited the catalytic activity of the protein.

The effect of 8-azaguanine on the induction of glutamyl transferase.

8-Azaguanine, a potent inhibitor of RNA synthesis, has been found to inhibit the formation of inducible enzyme systems in bacteria (Creaser, 1956). Paul & Hagiwara (1961) have demonstrated inhibition of RNA synthesis in strain L cells when 8-azaguanine was included in the culture medium. To study the effect of 8-azaguanine on the induction of glutamyl transferase, 10 Roux flasks were inoculated with 10 million cells in Waymouth's medium (+ glutamine) + 10% dialysed calf serum. As in the previous experiment the contents of two flasks were harvested as initial samples and the medium changed

Table 17. The effect of 8-azaguanine on the induction of glutamyl transferase in strain L.

8-Azaguanine (2.10^{-4}M) was added to the test cultures and samples were collected after 24 hr. and 48 hr.

	Time (hr)	Cell Number (M)	Glutamyl Transferase (units/million cells)	Esterase (units/million cells)
Initial	0	10	0.014	0.25
Control	24	15	0.051	0.23
	48	21	0.119	0.28
Test	24	12	0.027	0.14
	48	14	0.020	0.12

in the eight remaining flasks. All flasks contained Waymouth's medium (- glutamine) + 10% dialysed calf serum + 20mM glutamic acid. Four flasks served as controls and 8-azaguanine (2.10^{-4}M) was added to the remaining cultures.

Duplicate samples from control and test cultures were collected at 24 hr. and 48 hr. intervals thereafter and assayed for glutamyl transferase activity. The results demonstrated that induction of glutamyl transferase was inhibited in the cultures treated with 8-azaguanine (table 17). The protein-nitrogen content and the cell number were considerably diminished by the presence of 8-azaguanine in the growth medium. However the levels of a constitutive enzyme such as esterase were not appreciably altered by 8-azaguanine. Creaser (1956) described a similar phenomenon when investigating the effect of 8-azaguanine on constitutive and adaptive enzymes in bacteria.

The Effect of Fluorodeoxyuridine on the Induction of Glutamyl Transferase.

While DNA is now regarded as the ultimate carrier of genetic information for the structural configuration of proteins, results from bacterial systems have indicated that protein synthesis can occur when DNA synthesis is inhibited (Spiegelman, 1956). The synthesis of DNA in

Table 18. The effect of fluorodeoxyuridine on the induction of glutamyl transferase in strain L.

Test cultures were treated with fluorodeoxyuridine (2.10^{-6}M) which was omitted from control cultures. Samples were harvested after 24 hr. and 48 hr.

	Time (hr)	Cell Number (M)	Glutamyl Transferase (units/million cells)
Initial	0	11	0.013
Control	24	16	0.068
	48	23	0.103
Test	24	10	0.054
	48	12	0.082

animals was inhibited by fluorodeoxyuridine (FUdR) and the inhibition was reversed by thymidine (Heidelberger, 1957). Inhibition of DNA synthesis by FUdR has also been demonstrated in strain L cells (Paul & Hagiwara, 1961). To investigate the effect of FUdR on the induction of glutamyl transferase an experiment, similar to the two preceding ones, was set up. One group of cultures contained FUdR (2.10^{-6}M) and duplicate samples from control and test cultures were harvested after 24 hr. and 48 hr. as before. In marked contrast to the results obtained in the previous experiments the induction of glutamyl transferase was found to be only slightly effected by FUdR (table 18). DNA synthesis was therefore not necessary during the induction of glutamyl transferase.

It was concluded from these experiments that active protein and RNA synthesis were essential for the induction of glutamyl transferase. The mechanism responsible for the rapid disappearance of glutamyl transferase on the addition of glutamine has not been defined. The evidence from these investigations strongly suggests that this phenomenon is an example of enzyme repression whereby the formation of glutamyl transferase is regulated in some way by glutamine.

Arginase.

Previous findings had demonstrated that marked increases in arginase activity of HeLa cells resulted after growth in the presence of arginine, uridine and glutamic acid. Glutamic acid and uridine were included in the medium for the purpose of priming the ornithine cycle with carbamyl glutamate. If uridine functioned by repressing aspartyl transcarbamylase, as described in bacterial systems (Yates & Pardee, 1957), an accumulation of carbamyl phosphate might be expected. In the presence of excess glutamic acid, surplus carbamyl phosphate might be metabolised through the ornithine cycle. An increased need for the ornithine cycle might therefore result in an elevation in the levels of some of the enzymes in the cycle. This experiment describes some preliminary investigations into the nature of the increased arginase activity. To test the ability of other components of the ornithine cycle to induce arginase activity, thirty eight flasks were inoculated with one million HeLa cells each, in Eagle's medium supplemented with 5% calf and 2% human serum. Two flasks served as controls and various substances were added (in duplicate) to the remaining flasks (see table 19). The medium was renewed in all flasks four days after setting up the experiment, with the exception of three flasks from group 17 where the medium

Table 19. Induction of arginase in HeLa cells - additions to the medium.

Group No.	Arginine (10^{-2}M)	Ornithine (10^{-2}M)	Citrulline (10^{-2}M)	Glutamic Acid (4.10^{-3}M)	Uridine (10^{-3}M)
1	-	-	-	-	-
2	+				
3		+			
4			+		
5				+	
6					+
7	+	+			
8	+		+		
9	+			+	
10	+				+
11		+	+		
12		+		+	
13		+			+
14			+	+	
15			+		+
16				+	+
17	+			+	+

Table 20. The effect of various compounds on the arginase activity of HeLa cells.

The various substances added to the cultures are listed in the accompanying table (No. 19).

Group No.	Cell No. (10^6)	Arginase
1	13	0.09
2	12	0.34
3	12	0.25
4	11	0.27
5	14	0.09
6	14	0.08
7	11	1.41
8	12	1.67
9	12	0.40
10	11	0.31
11	14	0.35
12	12	0.13
13	10	0.20
14	12	0.22
15	13	0.42
16	13	0.08
17(a)	14	1.10*
17(b)	15	0.280

*The medium was not renewed in group 17(a) during the experiment.

was not altered. After eight days the cells were collected and assayed for arginase.

Several compounds were found to be capable of inducing arginase activity (Table 20). Arginine, citrulline or ornithine caused a 2-3 fold increase in enzyme activity when included alone in the medium. The highest arginase activity was displayed by groups 6 and 7 (arginine + ornithine and arginine + citrulline). It was interesting that uridine and glutamic acid either alone or in combination with each other had no effect on the arginase levels. Similarly arginine + uridine and arginine + glutamic were no more effective than arginine alone. However arginine + uridine + glutamic increased the level of arginase by approximately 10 fold. The reason for this is not clear at present but it might indicate that some other enzyme(s) associated with or present in the ornithine cycle may also be of an adaptive nature.

An interesting finding emerged when the arginase activity of group 17 cultures were compared (Table 20). Renewal of the medium caused a three fold diminution in the arginase activity compared with those cultures where the medium was not changed. This result suggested that the medium was 'conditioned' possibly by the accumulation of some inducing substance.

SECTION 4. D I S C U S S I O N

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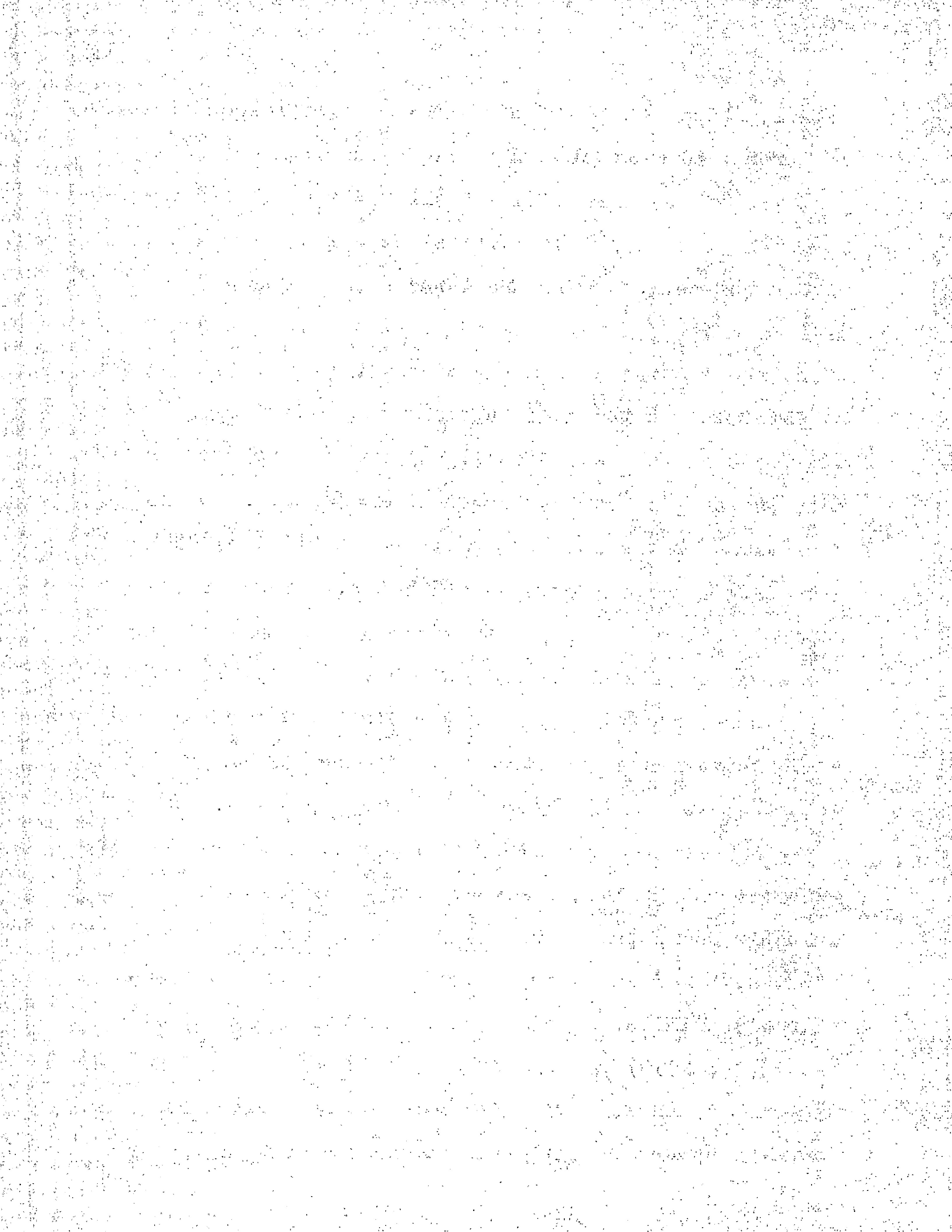
An interesting finding emerging from the zymogram studies was the occurrence of a large number of proteins possessing esterase activity. Several enzymes have recently been resolved into more than one molecular form. Lactic dehydrogenase, for instance, was once considered to exist as one molecular species but has now been separated into five different molecular forms in several mammalian tissues (Markert & Møller, 1959; Wieland & Pfleiderer, 1961). Similarly multiple forms of other enzymes such as trypsin (Timasheff, Sturtevant & Bier, 1956) and malic dehydrogenase (Hess, 1958; Tsao, 1960) have been isolated. Molecular heterogeneity of functionally similar proteins in different species, as originally demonstrated by Desreux & Herriot (1939) and Taylor, Green & Cori (1948), was attributed to differences in genotype. In recent years, interest in this subject has been stimulated by the discovery that multiple forms of homologous proteins exist within a single organism and probably within a single cell (Markert & Møller, 1959). Two aspects, of this phenomenon, which have aroused considerable interest are (a) the structural and genetic relationship between the various molecular forms and (b) the advantages which multiple molecular enzyme formation afford to an organism.

However before considering structural and genetic

relationships and the physiological significance of the occurrence of multiple molecular forms of homologous proteins it must first be established that differences observed in tissue extracts reflect actual variations within the cell. When the structural organisation of the cell is destroyed the release of proteolytic enzymes may bring about structural alterations in many native proteins. To determine whether the multiple esterase patterns were artefacts of the isolation procedure, several different methods for preparing extracts were compared. Some variations in the esterase patterns obtained by different preparative methods were demonstrated. However the majority of the esterases appeared on the same position on the zymogram no matter what extraction procedure was used.

When chromatographic or electrophoretic parameters are employed to express molecular heterogeneity the possibility of artefacts arising by adsorbtion on to non specific protein molecules should be considered. There is some evidence that this can account for a number of the multiple forms that have been shown to exist for some enzymes. For instance 8 forms of sheep ribonuclease each with similar specific activities have been isolated (Anfinsen, 1959) by chromatography on cellulose ion-exchange columns. When the 8 forms were put through a Dowex-50 column only 4 peaks of ribonuclease activity



emerged. This was explained as being the result of 4 of the ribonucleases having some non-specific protein attached to the molecule and when this was removed they reverted in chromatographic behaviour to the four original forms. In the present study it was found that areas of high protein concentration did not usually correspond to regions of high esterase activity on the starch gel and therefore the possibility of adsorbtion was considerably reduced.

The possibility that some of the esterase bands on the starch gel may have arisen by depolymerization of a larger molecule was also considered. A recent example of this involving the enzyme urease has been reported (Creech & Nichols, 1960) which demonstrated that although a single electrophoretic peak was obtained for the crystalline enzyme, three or four peaks were found after ultracentrifugation. This was shown to be the result of depolymerization brought about by rupture of disulphide bridges in the native enzyme. Frieden (1959) has also demonstrated various degrees of polymerization of glutamic dehydrogenase in the ultracentrifuge and that the number of monomers obtained was decidedly influenced by NAD. Various degrees of polymerization have been shown to exist for other proteins such as haemocyanine (Pedersen, 1949) and insulin (Rees & Singer, 1955). Some of the

differences that were obtained in the esterase zymograms by different extraction procedures usually involved a disappearance of a few bands and an increase in the intensity of one or two others. This might indicate that the missing bands had polymerized into a larger molecular species. Some of the esterases may therefore represent monomeric forms of larger molecules which had become depolymerized during extraction. It seems unlikely however that depolymerization was responsible for the majority of the esterase bands that appeared on the zymograms because of the large variety of combinations of the esterases that occurred in the different animal tissues investigated.

It would appear therefore that the majority of the esterases revealed by the zymogram technique are not due to artefacts resulting from (a) the extraction procedure or (b) adsorbtion on to non specific proteins. The multiple esterase patterns probably reflect structural differences between various proteins displaying esterase activity. In the absence of any detailed information about the structural configuration of the various esterases it is not possible to explain how molecular variation is compatible with similarity in function. Some suggestions, however, can be put forward when the findings of other workers on structural variations in homologous proteins

is considered.

Molecular Variation in Homologous Proteins from Different Species.

There is now a great deal of evidence available demonstrating that homologous proteins from different species of animals differ widely in their physico-chemical properties. Many of these differences have been detected by such techniques as chromatography, electrophoresis and immunology and in some cases have been correlated with variations in chemical configuration between the molecules. Thus molecular heterogeneity in ribonucleases isolated from various species of animals has been demonstrated (Anfinsen, 1959) to be due to differences in amino acid sequences. Similarly species variations have been detected in Cytochrome C (Tuppy, Paleus & Bodo, 1958) and in the protein hormone insulin (Harris, Sanger & Naughton, 1956; Isihara et al. 1958). These observed species variations have been shown to occur at specific sites on the polypeptide chain. For instance the species differences in insulins isolated from various sources were all restricted to one portion of the β-chain and similarly in Cytochrome C the amino acid sequence of that part of the polypeptide chain associated with the haem prosthetic group was relatively constant in all the species examined.

It therefore seems possible that the differences in physico-chemical properties which are due to changes in molecular configuration are confined to specific regions of homologous protein molecules. In other words wide structural variations can occur in some parts of homologous proteins without altering the specific function of the molecule. Other parts of the molecule on the other hand display practically no molecular variation and it is not too unreasonable to assume that these 'parts' are directly involved in the functional activity of the proteins. There is evidence available which indicates that this may indeed be the case for some proteins at least. It is now known that certain parts of functional protein molecules can be removed without interfering with function to any extent. The best known example of this is papain which has been degraded by leucine aminopeptidase to 40 per cent of its original length without loss in function.(Hill & Smith, 1960). Similarly adrenocorticotrophin was found to retain maximum activity after a third of its amino-acid residues had been removed from the carboxyl terminal end of the chain (Bell et al. 1956). It will be interesting to see if species variations in papain and adrenocorticotrophin are confined to those parts of the molecule which are apparently unnecessary for function.

Further evidence demonstrating that certain areas of protein molecules have more rigid structural requirements than others has been derived from studies on the amino acid sequences around the active centres of several hydrolytic enzymes. Jantz et al. (1960) have demonstrated that a common amino acid sequence exists around the 'active centre' of several esterase and proteases. With only one exception all these enzymes had a similar amino acid sequence around their di-iso-propylfluorophosphate-sensitive active centres as follows:

Glycine	Aspartic Acid or Glutamic Acid	Serine	Glycine or Alanine

To explain the occurrence of the large number of proteins displaying esterase activity, distributed among various species, it is proposed that species variations are confined to the 'non-essential' parts of the molecule. During the course of evolution the amino acid sequence around the active centre of the esterase molecule must remain intact or the protein is non-functional and is eventually discarded (Anfinsen, 1959). Mutations involving alterations in the amino acid sequence in the remainder of the molecule would not interfere with function to any extent. During the course of evolution there is thus ample opportunity for the manufacture of

species characteristic modifications of homologous proteins without altering the function of the molecule.

The similarity in chemical structure between human haemoglobins and those of other vertebrates (Zuckerkandl et al. 1960; Gratzer & Allison, 1960; Braunitzer et al. 1960) has led Ingram (1961) to a hypothesis as to how the various forms of vertebrate haemoglobins were derived. With a few interesting exceptions all known vertebrate haemoglobins consist of four polypeptide chains of equal molecular weight (17,000). Among the exceptions are (1) lamprey haemoglobin which consists of one polypeptide chain (molecular weight 17,000) and (2) hogfish haemoglobin which contains two polypeptide chains. On the assumption that these two examples represent possible stages in the evolution of vertebrate haemoglobins, Ingram polstulated that the four types of peptide chains found in human and vertebrate haemoglobins were derived from a common primitive myoglobin-like molecule. During the course of evolution gene mutations gave rise to alterations in the structural configuration of the various haemoglobin molecules which were then either favoured or discarded through the process of natural selection. The present day haemoglobin molecules are pictured by Ingram as being the result of 'non-lethal' mutations and that the genes responsible for their structural configuration may

represent mutant forms of the gene controlling a primitive lamprey type of haemoglobin. Much more evidence on the structural and genetic relationship between other vertebrate and human haemoglobins is required before Ingram's hypothesis can be tested. The scheme however gives some insight into the origin of the enormous number of homologous proteins which are distributed between so many different living organisms.

Molecular Heterogeneity in Homologous Proteins from Different Organs of the Same Species.

Variations in physico-chemical properties between homologous proteins from different species are almost certainly reflections of differences in genetic constitution. It is now generally agreed that, with a few exceptions, the basic genetic constitution of all the cells in a single organism is the same. The reasons for this general agreement stem from the similarity in morphology and mechanism of replication of the chromosomes from different organs in any one organism (Sonneborn, 1960). Homologous proteins from different tissues of the same individual would be expected to be controlled by the same structural genes and therefore to have identical physico-chemical properties. However when the esterase zymograms from various organs of the same individual were compared marked differences in the overall distribution

of esterases on the zymograms were observed. Markert & Møller (1959) have similarly demonstrated that tissue specific lactic dehydrogenase zymograms could be obtained from extracts of various pig organs. The lactic dehydrogenases from various rabbit and human organs were also found to be immunologically dissimilar (Nisselbaum & Bodansky, 1959).

Electrophoretic and immunological differences between functionally similar proteins from different organs have sometimes been found to be greater than variations between species. This was true for instance when the alkaline phosphatases from various tissues of the dog were immunologically compared both with each other and with several other animals (Schlamowitz, 1957). The problem of explaining the nature of tissue variations in homologous proteins is made more difficult because it is not simply a matter of a difference between one esterase or lactic dehydrogenase from heart, for example, and one esterase or lactic dehydrogenase from liver. Multiple forms of these and many other enzymes such as alkaline phosphatase and peroxidase have now been located within a single organ and even within a single cell. The term isozyme has been proposed by Markert Moller (1959) "to describe the different molecular forms in which proteins may exist with the same enzymatic specificity".

The large number of functionally similar enzymes that can be isolated from a single organ, e.g. sixteen esterases from mouse liver, five lactic dehydrogenases from rat heart raises the question as to why so many modifications of the same protein are manufactured in a single organ. Many electrophoretically homologous esterases from different tissues have been identified during this investigation and this was also demonstrated with lactic dehydrogenases from various tissues (Markert & Møller, 1959). It is not known yet whether electrophoretically homologous enzymes are identical in other properties as well.

The possibility that the multiple enzyme patterns obtained by starch gel electrophoresis might be due to artefacts produced during the extraction procedure has already been discussed. Several other possibilities which are not directly concerned with the extraction procedure were also considered. The multiple forms of esterases, for instance, that were revealed on the zymogram may simply represent a mixture of enzymes with distinct but overlapping substrate specificities. Considering also that many proteins possess secondary esterase activity the multiple forms of esterases revealed on the zymogram could well be attributed to action of different genes.

Physiological Significance of Isozymes.

A few recent examples of differences in kinetic properties between functionally similar enzymes isolated from different cellular organelles may explain the occurrence of some multiple forms of enzymes in cell extracts. The mitochondrial and supernatant forms of rat liver L-glutamate-oxaloacetate transaminase, for instance, have been shown (Boyd, 1961) to have different electrophoretic mobilities and to differ in their substrate affinities and optimum pH's. Similarly differences have been observed in the kinetic properties of malic dehydrogenase isolated from the supernatant and the mitochondrial fractions of the cell (Delbruck et al. 1959). It will be interesting to see if similar differences exist for other enzymes which occur in particulate and supernatant cell fractions. In addition it would contribute to our understanding of the physiological significance of the occurence of multiple enzymes to know if these forms were interconvertible. For instance Pollock (1959) has demonstrated that two forms of penicillinase, an α and β form could be isolated from B. cereus. The α form was more stable and was resistant to iodine and urea whereas the β form was not. It was also demonstrated that the α form was converted to the β form by adsorbtion onto glass or cell surfaces.

Considering the existence of isozymes as part of the general control mechanism in living cells would certainly underline their biological significance. The influence of environmental factors on the isozymic constitution of a tissue has hardly been investigated at all, although in these studies it was found that the pattern of the esterase zymograms was not affected by alterations in the environment. Recent studies such as those of Slonimski & TYSAROWSKI. (1958) and Gregolin, Kearney & Singer (1961) on the D and L forms of lactic dehydrogenase in yeast illustrates how the environment can affect the isozymic composition of a cell. These workers found that when yeast cells were grown under anaerobic conditions the highest lactic dehydrogenase activity was shown by a D(–) lactic dehydrogenase which reacts with various dyes but not with cytochrome C. In aerobic cells the highest lactic dehydrogenase activity was shown by two L(+) lactic dehydrogenases one of which reacted with cytochrome b_2 and the other with cytochrome C. In addition to demonstrating that different forms of lactic dehydrogenase appeared in the cells under aerobic and anaerobic conditions these studies also show that the oxidation of lactate can proceed by two pathways under aerobic conditions. Thus, the cells can regulate the oxidation of lactate by two separate means. As mentioned previously the decrease in

the level of malic dehydrogenase in strain L cells following growth in the absence of oxygen may involve a similar mechanism.

A clear cut and extremely interesting explanation for the occurrence of two isozymes of aspartokinase in E.coli has recently been provided (Stadtman, Cohen & Le Bras, 1961). This has already been mentioned in connection with feedback inhibition and repression. The finding that one aspartokinase was inhibited by L-lysine and the other by L-threonine demonstrates that isozymes may play an important part in regulating metabolic pathways. The formation of different forms of an enzyme whose substrate or product is a common intermediate for a number of metabolic pathways guarantees that independent regulation can be exercised over each biosynthetic pathway. There are a few examples of isozymes cited in the literature which would be interesting to study from the point of view of metabolic control. For instance two forms of hexokinase, A and B, have been separated from yeast extracts (Kaji, Trayser & Colowick, 1961). Hexokinase is known to be inhibited by glucose-6-phosphate and it has not been stated whether hexokinase A and B showed any difference in the degree of inhibition by various hexose phosphates.

Genetic Control of Isozyme Formation.

The existence of isozymes within a single organ or

cell raises the question as to how these individual enzymes are genetically controlled. If the structure of each individual isozyme was controlled by a separate gene then a situation analogous to the production of adult and foetal haemoglobin would exist. Genes for the production of adult and foetal haemoglobin are present in the same cell but are controlled so that they function at different periods during development (Allison, 1959). Heterozygous individuals have also been identified (Ingram, 1961) possessing genes responsible for the synthesis of normal and abnormal varieties of haemoglobin. Markert & Møller (1959) have demonstrated an increase in the number of isozymes of lactic dehydrogenase during the development of embryonic pig heart. This finding may also indicate that a process of gene activation occurred giving rise to an increase in the number of active genes structurally controlling the various forms of lactic dehydrogenase.

It will be difficult to assess the genetic basis of isozyme formation until the various isozymes are subjected to genetic analysis. Horowitz (1953) for example investigated the two forms of tyrosinase found in two strains of Neurospora of different genotype. It was found that the two types of tyrosinase had identical optimum pH values and Michaelis constants etc. but that one form was more heat stable than the other. The two forms of

tyrosinase were controlled by different genes and if a heterokaryon was formed in which the two nuclei were present in the same organism both types of tyrosinase were clearly controlled by two distinct structural genes. The recent findings of Allen (1961) suggest that some of the esterases revealed by the zymogram technique may possibly represent modifications of the product of a single gene. It was shown (Allen, 1961) that a single gene change in the protozoan, Tetrahymena pyriformis, altered the electrophoretic mobility of three esterases, revealed by the zymogram technique. This experiment provides the best evidence so far that multiple forms of functionally similar enzymes are not controlled by separate genes.

If all the multiple forms are controlled by the same structural gene the problem of explaining the origin of the various modifications then arises. Tissue specific isozyme patterns may be one of the results of the process of differentiation which in higher animals produces an adult with various types of organs differing morphologically and functionally from each other. The present studies on the esterase zymograms from various organs revealed that with one or two exceptions the full complement of esterases was usually contained in the liver. The esterases in other organs were usually electrophoretically

similar to those of liver but were not as numerous. Although similarity in electrophoretic mobility does not necessarily imply similarity in structure these findings indicate that the means of producing isozymes may be similar in the different organs. Much more information is obviously required about the molecular configuration of isozymes and the genetic mechanism controlling this configuration before any general conclusions concerning the physiological significance of their occurrence can be arrived at.

Enzyme Activity Levels of Cultured Cells.

Some interesting differences in enzyme levels of cell lines which originated from different organs were obtained. This resembled the situation encountered in the intact animal (Greenstein, 1947) and demonstrated that some characteristics of the parent tissue were retained by cultured cells. The specific activities of most of the enzymes were reproducible from preparation to preparation. An interesting exception to this was the acid phosphatase (of strain L) which varied several fold during the growth cycle. Similar variations in micro-organisms (Gale, 1943), of alkaline phosphatase in the Chang line (Nitowsky & Herz, 1961) and of thymidine kinases (Weissman, Smellie & Paul, 1961) in strain L have been reported.

Due allowance for such variations must therefore be made when comparing enzyme activity levels in cultured cell strains. Variations in enzyme activity levels during growth are attributable in some instances to alterations in metabolic behaviour. Saltzman (1959) demonstrated that the amount of essential constituents such as protein, RNA and DNA synthesized by cultured animal cells varied with the growth cycle. The increased levels of the thymidine kinase in strain L cells during growth phase compared favourably with similar increases in rat liver, regenerating (Weissman, Smellie & Paul, 1961). Increases in the rate of cell division are therefore compensated for by increases in the levels of the enzymes responsible for DNA synthesis.

A similar line of argument can be adopted to explain the variations encountered in the levels of hydrolytic enzymes such as acid and alkaline phosphatases during the growth cycle of cultured cells. If phosphatase activity of hydrolytic enzymes was lowest during lag and growth phase essential phosphorylated compounds could be shunted into the many synthetic reactions that take place during these phases. Thus the levels of acid and alkaline phosphatases were found to decrease during the lag and the growth phase whereas enzymes involved in synthetic reactions such as the thymidine kinases increased during

growth phase. An interesting example of this sort of phenomenon has been shown to occur in rat liver regenerating by Umbarger (1958). It was demonstrated that while the activity of aspartyl transcarbamylase increased several fold the activity of arginase was markedly decreased. These findings suggest that during a period when the main metabolic pathways of an organism are anabolic in nature the activity of hydrolytic enzymes is suppressed.

An alternative explanation of the variations in enzyme activity during the growth cycle is that these changes are caused by leakage of enzymes into the medium. Danes & Paul (1961) have demonstrated that the decreased respiratory rate of cultured cells following trypsinization was due to leakage of Krebs cycle intermediates into the medium. The decrease could be prevented if the cells were placed in a medium containing Krebs cycle intermediates. The increased 'leakiness' of some cells after trypsinizing might result in the loss of larger molecules such as enzymes. This would be particularly applicable to acid phosphatase which is present, within the cell, in small cellular organelles called lysosomes (de Duve, 1959). Trypsinization might result in the loss of lysosomes from the cells and their eventual regeneration by some mechanism such as pinocytosis. It is now thought (Novikoff, 1961)

that some of the hydrolytic enzymes incorporated into the cells in the pinocytotic vacuoles remain enclosed in these vacuoles which eventually give rise to the cellular lysosomes. This hypothesis would not explain however why other hydrolytic enzymes which are also thought to exist in the lysosomes are not affected by treatment with trypsin. Thus in this investigation, no alterations were detected in the levels of β-glucuronidase during the growth cycle of HeLa cells and similarly Nitowsky & Herz (1961) found that the level of leucine-amino-peptidase was constant during the growth cycle of the Chang cell. It is not yet certain if the leakiness of cultured cells is increased by treatment with trypsin any more than other treatments. For instance, no differences in the hexokinase activities of HEp 1 cells were detected when the cells were collected by treatment with trypsin, versene or by scraping from glass (Etingoff, & Gabrielyan 1959). This experiment might possibly have been more informative in this respect if the cells had been suspended in medium for several hours after the various treatments in order to allow ample time for any leakage to occur.

Although leakage of enzymes after trypsinizing may not be responsible for the decreased enzyme activity that was observed, the loss of activators, stabilizers or

endogenous inducers of these enzymes is a distinct possibility. All these substances would presumably be small molecules which could easily escape from the cells. The gradual increase in enzyme activity which reached a maximum during stationary phase could be attributed to the reaccumulation of these factors within the cell.

The quantitative enzyme survey also revealed that in contrast to the situation in the intact animal marked similarities existed in the levels of other enzymes in cultured cells. One of the most striking divergencies from the situation in vivo was the failure to demonstrate any tryptophan pyrrolase activity in the HLM line which has been derived from human foetal liver. There are numerous reports in the literature of the 'loss' of specific enzymes from cultured cell lines derived from liver tissue. Auerbach & Walker (1959) demonstrated that several enzymes known to be present in liver were absent from the Chang liver cells. The loss of specific enzyme systems and specialized functions such as the production of hormones by cultured cells may be attributed to several causes.

One possible explanation to account for the loss of specific characteristics by cultured cells might be attributed to the fact that only a small percentage of the different cell types in an organ survive under tissue

culture conditions. If the production of specific substances by a tissue is due to the conversion of precursor substances which are manufactured in one cell type before being converted into the active form by another type of cell, then failure of any one of these cell types to survive in culture would obviously result in the loss of some specialized function.

The possibility also exists that some specialized functions were originally present in cultured cells, but were lost during rapid growth in vitro for long periods. These losses may be nongenetic in nature and may arise as a result of the metabolic processes of the cell being diverted to maintaining rapid cell growth rather than elaborating any specialized characteristics. On the other hand gene mutations may give rise to alterations in the metabolic behaviour of the cells. The ability of cultured cell lines derived from normal tissue to produce tumours when reinoculated into the host animal may be the result of gene mutations. Finally, alterations in the metabolic behaviour of a cell line may be due to adaptive phenomena, reflecting alterations in the environment or the lack of some essential substances such as hromones.

Attempts to Alter the Enzymic Constitution of Cultured Cells

Several enzyme systems which had been reported to be of an adaptive nature in the intact animal were not found

to be adaptive in cultured cells. In many instances the nature of the increases in enzyme activity in intact animals following the administration of a specific substrate or a related compound, has not been sufficiently investigated. The induction of tryptophan pyrrolase for instance, which has been the subject of considerable study in recent years is still not clearly understood. The tryptophan pyrrolase system has clearly demonstrated that hormones can influence the results obtained. The inability in these studies using cultured cells to demonstrate increased enzyme activity following administration of several specific substrates may therefore be due to the absence of hormones or ancillary substances which would be essential to bring about a response. Specific enzymes may be lost from cultured cells because of the absence of these latter substances from the growth medium. These compounds which would be unnecessary for cell survival would nevertheless be capable of elaborating some particular cellular characteristics such as the possession of specific enzyme-forming systems. Thus, Fell & Melanby (1955) have demonstrated in organ cultures that although Vitamin A was not necessary for growth it induced metaplasia of embryonic skin to ciliated mucous-secreting epithelium. Trowell (~~1959~~ UNPUBLISHED.) found that glycogen accumulated in organ cultures of liver only when insulin

was included in the culture medium. The induction of alkaline phosphatase in HeLa cells (Cox & McLeod, 1961; Cox & Pontecorvo, 1961) by prednisolone is a recent example of the acquisition of a specific characteristic by cultured cells under hormonal influence. In these experiments several clones of various HeLa lines were tested for their response to prednisolone and not all the clones were found to react to the same extent. Although increased alkaline phosphatase activity was obtained by using high concentrations of organic monophosphates as inducers the response was approximately eight times less than that obtained with prednisolone. The findings of these workers with prednisolone were confirmed in the present investigation. The mechanism of the induction of alkaline phosphatase in HeLa cells by prednisolone has not yet been investigated. One of the disadvantages of this system was the long period (about 10 days) required for maximum induction of alkaline phosphatase. More important however, was the finding that treatment with prednisolone resulted in a marked diminution of cell growth (about 40% of the normal rate) (Cox & McLeod, 1961). In view of possible alterations in the phosphatase levels during the growth rate, this is undesirable when comparing enzyme levels in control and test cultures.

Tryptophan pyrrolase was not detected in HLM cells

after growth in the presence of tryptophan. Auerbach & Walker (1958) similarly failed to induce this enzyme in the Chang liver cells. Civen & Knox (1957), however, have reported increased levels of tryptophan pyrrolase after incubating rat liver slices with tryptophan. Liver slices incubated in the absence of tryptophan served as controls. The possibility of stabilization of the enzyme by tryptophan rather than induction must be considered in this case, since the tryptophan pyrrolase activity of the control slices diminished several fold during incubation.

A number of examples of increased activity of some glycolytic and Krebs cycle enzymes have been reported (Freedland & Harper, 1958; Fitch & Charkoff, 1961) following the administration to intact animals of diets high in glucose or fructose. When similar experiments were attempted on cultured cells in this investigation, no increases in the specific activities of glucose-6-phosphatase, glucose-6-phosphate dehydrogenase, lactic dehydrogenase or malic dehydrogenase were detected. Freedland & Harper (1958) for instance reported increases in the specific activities of glucose-6-phosphatase and glucose-6-phosphate dehydrogenase of rat liver when these animals were fed diets high in glucose or fructose. In view of the negative results obtained with cultured cells, it is possible that increased enzyme levels recorded in

similar experiments with intact animals were due to hormonal influence.

Alterations in respiration with changes in the glucose concentration (Paul & Danes, 1961) were not correlated in this investigation with alterations in the amounts of glucose-6-phosphate dehydrogenase, malic dehydrogenase or lactic dehydrogenase. It is quite probable that differences in the levels of some other glycolytic or Krebs cycle enzymes or enzymes involved in electron transport could be correlated with alterations in the respiratory rate.

Adaptive Enzyme Systems in Animal Cells maintained in Culture.

The existence of several adaptive enzyme systems in cultured animal cells has been demonstrated during the course of these studies. The suppression of malic dehydrogenase and β-glucuronidase in strains HIM and L respectively require further investigation before the mechanism of these phenomena can be underlined. The malic dehydrogenase system may be analogous to the D and L forms of lactic dehydrogenase which exist separately in yeast under aerobic and anaerobic conditions (Gregolin, Singer & Kearney, 1961). Although two isozymic forms of malic dehydrogenase have been isolated from animal cells (Delbruck et al. 1959; Thorne, 1960) there is no evidence

to suggest that one particular form predominates under aerobic and anaerobic conditions. A more likely explanation of the malic dehydrogenase effect would be that reduced malic dehydrogenase activity reflects an overall diminution in the activity of Krebs cycle enzymes under anaerobic conditions. This situation has been demonstrated with yeast cells (Slonimski, 1953; Hebb et al. 1959) where reappearance of enzyme activity occurred very rapidly during subsequent adaptation to oxygen. When animal cells were placed under an anaerobic environment, the respiratory rate decreased and did not return to normal until 24-48 hr. after removal to an aerobic environment (Danes & Paul, 1961). The 24-48 hr. period required to attain the original respiratory rate may represent the time required to resynthesise some of the Krebs cycle enzymes which may have been repressed under anaerobic conditions.

Since a relatively simple assay procedure is available and considering the stability of the enzyme itself, an inducible β-glucuronidase would be ideally suitable for investigating the kinetics of adaptive enzyme formation in animal cells. The suppression of β-glucuronidase activity in strain L suggests that the enzyme may be inducible under the proper conditions. The possibility is being considered that the presence of glucose in the

growth medium may prevent induction of β-glucuronidase in a manner similar to the glucose effect on the inducible β-galactosidase in E.coli (Cohen, 1956; Mandelstam, 1961). Preliminary investigations have revealed that the inducible arginase in HeLa cells may provide a useful system for testing whether the hypothesis of Jacob & Monod is applicable to animal cells. Indirect evidence has been obtained for the accumulation of an inducer in the growth medium during induction. Results already obtained from this system and from similar systems in bacteria (Gorini & Gunderson, 1961) strongly suggest that other enzymes in the ornithine cycle (e.g. ornithine transcarbamylase and argine synthetase) are also of an adaptive nature. If the induction of these enzymes is spontaneous, investigations into the possible existence of an operon (Jacob & Monod, 1961) in animal cells can be performed.

The glutamyl transferase system was studied in some detail in order to define the mechanism involved in adaptive enzyme formation in animal cells. Because of the low levels of glutamyl transferase encountered indirect methods, employing inhibitors of protein, RNA and DNA synthesis, were adopted. The results strongly suggested that concomittant protein and RNA synthesis were necessary for the induction of glutamyl transferase. The

glutamyl transferase system was therefore considered to be an example of enzyme repression in animal cells and analogous to many similar systems in micro-organisms. However, the extent and the rate of increase in glutamyl transferase activity was decidedly less than most of the bacterial induced enzyme systems. The forty eight hours required to attain maximum glutamyl transferase activity might possibly represent the time necessary to deplete the intracellular amino acid pool of all its glutamine. Glutamine is one of the major components of the intra-cellular amino acid pool in cultured animal cells and concentrations as high as 8mM have been reported when the extracellular concentration was only 1mM (Eagle, 1959). The rapid disappearance of glutamyl transferase activity when glutamine was added to the medium of 'induced' cells suggested a possible explanation why increases in enzyme activity higher than the usual 10 fold were not obtained. Presumably after a 10-fold increase in glutamyl transferase activity, glutamine might accumulate in the amino-acid pool thus preventing (by repression) the synthesis of further glutamyl transferase molecules. Gorini & Maas (1958) observed a similar type of phenomenon in E.coli. In these organism the synthesis of ornithine trans-carbamylase and arginosuccinase was repressed when arginine was present in the growth medium. In the absence of

arginine the levels of the latter enzymes increased to about 20 times the original levels. Increases greater than 20 fold were obtained by using a mutant stein and depleting the levels of extracellular arginine by including arginase in the growth medium.

Another interesting finding emerging from the studies on the glutamyl transferase system was the ability of several substances to protect the enzyme from inactivation. Several other mammalian enzyme systems which were stabilized by substrates and related compounds have been described. For instance glutamic-oxaloacetic transaminase was stable to heat in the presence of various dicarboxylic acids (Jenkins, Yphatis & Sizer, 1959). The inhibition of glutamic dehydrogenase by urea was similarly prevented by glutamic acid (Inagaki, 1959) and as previously mentioned tryptophan has been shown to have a stabilizing effect on tryptophan pyrrolase (Dubnoff & Dimick, 1959). It will be interesting to see if similar phenomena can be demonstrated in vivo since the existence of mechanisms for stabilizing enzymes would obviously be of great importance in metabolic control. Isotope incorporation data have revealed (Schoenheimer, 1942; Velick, 1956) that in animal tissues a constant degradation and resynthesis of proteins occurs. The levels of various mammalian enzymes are maintained relatively constant when

the synthetic and degradation rates are equal. If specific mechanisms existed for disturbing the equilibrium, enzyme levels could be elevated without increasing the rate of synthesis. In bacteria and yeasts on the other hand the evidence available suggests that proteins and nucleic acids are in a nondynamic state (Cohn, 1956). The inducible β-galactosidase of E.coli for instance was not degraded to any extent once it was formed (Hogness, Cohn & Monod, 1955). This suggests that different mechanisms may exist in animal and bacterial cells for altering intracellular levels of specific proteins.

Practically all our information on the mechanism of adaptive enzyme formation has been derived from bacterial systems. Until relatively recently no conclusive evidence was available for the existence of adaptive enzymes in animal cells. The phenomenon has now been demonstrated in animal cells but the operative mechanism has not been outlined. In recent years some advances towards elucidating the mechanism of adaptive enzyme formation in bacteria have been made. The hypothesis of Jacob & Monod (1961), based on genetic evidence, suggested that inducers and repressors of enzyme formation in bacteria functioned by interfering with the flow of messenger RNA from the structural genes to the ribosomes. Because of its instability the messenger RNA in bacteria

was constantly being replenished and a continuous flow of messenger from the structural genes to the ribosomes was therefore necessitated (Brenner, Jacob & Meselson, 1961). In animal cells, on the other hand, the available evidence suggests that messenger RNA is more stable than its bacterial counterpart and remains on the ribosomes for longer periods (Dentzis, Borsook & Vinograd, 1958; Campbell, 1961). This might indicate that a different mechanism to the one described by Jacob & Monod may operate in animal cells. Some possible alternative mechanisms include (a) stabilization of enzyme molecules by substrates or related substances, (b) mechanisms for regulating the release of the fabricated enzyme molecule from the ribosomes as suggested by Szilard (1960).

These studies and similar findings by other workers (Klein, 1961; Cox & Pontecorvo, 1961) provide good evidence for the existence of adaptive enzyme systems in animal cells. It is hoped that in the next few years more information about the precise mechanism of the phenomenon will be forthcoming. An understanding of the nature of adaptive enzyme formation should increase our knowledge of homeostatic control mechanisms in higher animals. The occurrence of adaptive enzymes in animal cells offers a suggestive hypothesis to account for the

origin and regulation of enzyme activity during the course of embryonic differentiation.

S U M M A R Y

SUMMARY.

1. To determine the extent and nature of the molecular variation between homologous proteins from various sources, several functionally similar proteins from different species were examined by starch gel electrophoresis.

2. The proteins, which were selected for their ease of identification, were located on the gels by their enzymic activity. The reproducibility of the method was demonstrated when identical esterase patterns were obtained from similar tissues of 40 mice of the same inbred strain. The two exceptions to this general finding were attributed to genetic differences.

3. Marked variations were encountered when esterases, phosphatases, catalases and peroxidases from different species were compared. The patterns were so distinct that the species of origin could be recognised from the type of zymogram produced.

4. Organ specific esterase patterns were also demonstrated in several animals including mouse, rat, guinea pig and hen. Liver usually displayed the largest complement of esterases which were distributed among the other tissues to varying degrees. In contrast esterase zymograms from different human organs were remarkably

similar to each other.

5. Species characteristic esterase patterns persisted in cultured cell lines which in some cases had been growing for many years in heterologous media. Attempts to alter the esterase patterns, by growing the cells in the presence of organic esters were unsuccessful. Therefore these patterns almost certainly reflect genetic differences.

6. How molecular heterogeneity is compatible with similarity in function was discussed in the light of recent findings on structural variations in haemoglobins, insulins and ribonucleases isolated from different sources. It was suggested that species variations were confined to those areas of the protein molecule which were not essential for function.

7. Several possibilities were considered to explain the occurrence of the large number of proteins displaying esterase activity, e.g. at least 16 in mouse liver. Particular attention was given to the possibility that the physiological significance of the occurrence of isozymes lies in their importance in metabolic control systems.

8. Investigations into the existence and mechanism of adaptive enzyme formation in animal cells were also

undertaken. Animal cells maintained in culture were employed for this study because they provided a system, free from hormonal and nervous influences.

9. Attempts were made to alter the levels of several enzymes in cultured cells by including substrates, products or related compounds in the growth medium. Under these conditions the levels of several enzymes including esterases, alkaline phosphatase, glucose-6-phosphatase, aspartate-α-ketoglutarate transaminase, lactic dehydrogenase, glucose-6-phosphate dehydrogenase, etc. were stable.

10. The levels of some enzymes, particularly glucose-6-phosphatase and alkaline phosphatase, in various cell strains were so different that the tissue of origin could be recognised from the enzymic composition of the cell strain. This resembled the situation encountered in the intact animal. In contrast however, a marked similarity was observed in the levels of several enzymes in cell strains of different origins.

11. Several examples of adaptive enzyme systems, which provide more conclusive evidence for the occurrence of the phenomenon in animal cells, were encountered during the course of these investigations.

12. The glutamyl transferase system represents an

example of enzyme repression while the β-glucuronidase system may involve feedback inhibition or repression. Arginase activity was induced in HeLa cells after growth in the presence of arginine, uridine and glutamic acid.

13. Glutamyl transferase activity increased several fold when glutamine was removed from the medium. In common with similar phenomena in bacteria, active protein and RNA synthesis was necessary for induction of the enzyme. Inhibition of DNA synthesis, on the other hand, did not prevent the induction of glutamyl transferase. Addition of glutamine to the medium of 'induced' cells resulted in a rapid decrease of glutamyl transferase activity.

14. In cell free extracts glutamyl transferase activity disappeared during incubation at 37°. The enzyme was protected from inactivation by various substances including nitrogen, ascorbic acid, $NADH_2$, glutamic acid and glutamine. Iodoacetic acid inhibited glutamyl transferase activity. These findings strongly suggested that sulphydryl groups, at or near the active site of the molecule, were necessary for maximum functioning of glutamyl transferase. The rapid disappearance of glutamyl transferase from 'induced' cells after addition of glutamine may involve mechanisms for oxidizing thiol linkages.

15. Preliminary studies on the arginase system have revealed that (a) arginine, citrulline or ornithine were poor 'inducers' of arginase activity (b) combinations of arginine with citrulline or ornithine or with uridine and glutamic resulted in a 15-20 fold increase in arginase activity (c) indirect evidence for the accumulation of an 'inducing' substance of unknown composition has been obtained (d) other enzymes in the ornithine cycle may be of an adaptive nature.

16. The mechanism involved in adaptive enzyme formation in animal cells was discussed with reference to some recent hypotheses on the nature of enzyme adaptation in bacteria.

APPENDIX

HANK'S BALANCED SALT SOLUTION (1948).

	Concentration (g./litre)
$NaCl$	8.00
KCl	0.40
$CaCl_2$	0.14
$MgSO_4.7H_2O$	0.10
$MgCl_2.6H_2O$	0.10
$Na_2HPO_4.2H_2O$	0.060
KH_2PO_4	0.060
Phenol red	0.02
$NaHCO_3$	0.35

EAGLE'S MEDIUM.

	Concentration	
	Milligrams per 1000 ml.	Approx. equiv. in millimoles
L-Arginine	17.4	0.1
L-Cystine	6.0	0.05
L-Histidine	3.2	0.02
L-Isoleucine	26.2	0.2
L-Leucine	13.1	0.1
L-Lysine	18.2	0.1
L-Methionine	7.5	0.05
L-Phenylalanine	8.3	0.05
L-Threonine	11.9	0.1
L-Tryptophan	2.0	0.01
L-Tyrosine	18.0	0.1
L-Valine	11.7	0.1
L-Glutamine	146.0	1.0
Choline	1.0	
Nicotinic acid	1.0	
Pantothenic acid	1.0	
Pyridoxal	1.0	
Riboflavine	0.1	
Thiamine	1.0	
i-Inositol	1.0	
Biotin	1.0	
Folic acid	1.0	
Glucose	2000.0	
NaCl	8000.0	
KCl	400.0	
$CaCl_2$	140.0	
$MgSO_4.7H_2O$	100.0	
$MgCl_2.6H_2O$	100.0	
$Na_2HPO_4.2H_2O$	60.0	
KH_2PO_4	60.0	
$NaHCO_3$	350.0	
Phenol red	20.0	
Penicillin	0.50	

WAYMOUTH'S MEDIUM MB752/1 (1959).

	Milligrams per 1000 ml.	Equivalent in millimoles
NaCl	6000	103
KCl	150	2.0
$CaCl_2.2H_2O$	120	0.82
$MgCl_2.6H_2O$	240	1.18
$MgSO_4.7H_2O$	200	0.81
Na_2HPO_4	300	2.11
KH_2PO_4	80	0.59
$NaHCO_3$	2240	26.7
Glucose	5000	27.8
Ascorbic acid	17.5	0.1
Choline. HCl	250	1.8
Cysteine. HCl	90	0.57
Glutathione	15	0.05
Hypoxanthine	25	0.18
Glutamine	350	2.38

The above ingredients made up at double the stated concentration, in glass distilled water, constitute solution BNI. Prepared by dissolving all components except the three buffer salts first, in about 80 per cent of the water. This prevents precipitation of Ca or Mg phosphates.

Thiamine HCl	10	0.03
Ca Pantothenate	1.0	0.003
Riboflavin	1.0	0.003
Pyridoxin HCl	1.0	0.003
Folic acid	0.4	0.008
Biotin	0.02	0.0008
m-Inositol. $2H_2O$	1.0	0.005
Nicotinamide	1.0	0.008
Vitamin B_{12}	0.2	0.00015

A stock solution of the B vitamins is conveniently made at 40 times the above concentrations.

WAYMOUTH'S MEDIUM MB752/1 (1959).

(Cont'd.)

	Milligrams per 1000 ml.	Equivalent in millimoles
L-Cystine	15	0.06
Glycine	50	0.66
L-Phenylalanine	50	0.30
L-Glutamic acid	150	1.02
L-Aspartic acid	60	0.46
L-Tyrosine	40	0.22
L-Lysine. HCl	240	1.42
L-Proline	50	0.44
L-Methionine	50	0.34
L-Threonine	75	0.64
L-Valine	65	0.55
L-Isoleucine	25	0.19
L-Leucine	50	0.38
L-Tryptophan	40	0.20
L-Arginine. HCl	75	0.36
L-Histidine. HCl	150	0.80
NaOH	to pH 7.4	2.5

The amino acids are conveniently made up in a stock at ten times the above concentrations.

Medium 752/1 is made by adding to 37.5 ml water, 50.0 ml of BNI + 2.5 ml of the stock B vitamin solution + 10.0 ml of the stock amino acid solution (total 100 ml).

CULTURE MEDIUM FOR L5178 LYMPHOBLASTS (Fisher, 1958).

	Medium concentration
	(gm./l.)
NaCl	8.0
KCl	0.4
$MgCl_2.6H_2O$	0.1
Na_2HPO_4	0.06
$NaH_2PO_4H_2O$	0.067
Glucose	1.0
$NaHCO_3$	1.0
5% acid hydrolyzed casein + tryptophan, 0.5 mg/ml.	5 ml/l
	(mg/l.)
Glycine	20
Cystine	7.5
Histidine	20
Glutamine	200
Vitamins:	
Thiamin.HCl	1.0
Nicotinamide	0.5
Ca-Pantothenate	0.5
Pyridoxal.HCl	0.5
D-Ribose	0.5
Riboflavin	0.5
Choline chloride	1.5
i-Inositol	1.5
Biotin	0.01
Ascorbic acid	1.6
Glutathione (reduced)	1.5
Serum	2-10%
Peptone	0.06%
Folic acid	10 mg/l.
Penicillin	100 units/ml.
Streptomycin	0.050 mg/ml.
Phenol red	10 mg/l.
$CaCl_2.H_2O$	0.182 gm/l.

MORGAN, MORTON AND PARKER'S MEDIUM NO. 199 (1950).

	Milli-grams per 1000 ml.		Milli-grams per 1000 ml.
L-Arginine	70.0	Riboflavin	0.010
L-Histidine	20.0	Pyridoxine	0.025
L-Lysine	70.0	Pyridoxal	0.025
L-Tyrosine	40.0	Niacin	0.025
DL-Tryptophan	20.0	Niacinamide	0.025
DL-Phenylalanine	50.0	Pantothenate	0.01
L-Cystine	20.0	Biotin	0.01
DL-Methionine	30.0	Folic acid	0.01
DL-Serine	50.0	Choline	0.50
DL-Threonine	60.0	Inositol	0.05
DL-Leucine	120.0	p-Aminobenzoic acid	0.05
DL-Isoleucine	40.0	Vitamin A	0.10
DL-Valine	50.0	Calciferol(Vit.D)	0.10
DL-Glutamic acid	150.0	Menadione(Vit.K)	0.01
DL-Aspartic acid	60.0		
DL-Alanine	50.0	-Tocopherol phosphate (Vit. E)	0.01
L-Proline	40.0	Ascorbic acid	0.05
L-Hydroxyproline	10.0	Glutathione	0.05
Glycine	50.0	Cholesterol	0.2
Cysteine	0.1	Tween 80(oleic acid)	20.0
Adenine	10.0	Sodium acetate	50.0
Guanine	0.3	L-Glutamine	100.0
Xanthine	0.3	Adenosine tri-phosphate	10.0
Hypoxanthine	0.3	Adenylic acid	0.2
Thymine	0.3	Ferric nitrate	0.1
Uracil	0.3	Ribose	0.5
Thiamin	0.010	Deoxyribose	0.5

*This medium also contains a balanced salt solution.

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