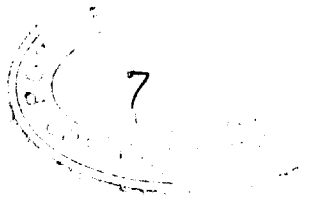

AN EXAMINATION OF THE ACTIVITIES OF SEVERAL ENZYMES AND OF THE CONCENTRATIONS OF THEIR SUBSTRATES IN NORMAL AND NEOPLASTIC TISSUE

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ABSTRACT

Three aspects of the analysis of tissues are examined critically and experiments providing the answers to some questions are described.

(1) The determination of the levels of labile substances in resting tissues. This is an essential prerequisite to the identification of the sites and mechanisms of control of reactions in normal and diseased, including neoplastic, tissue. The information presently available regarding the concentrations of labile substances is full of contradictions. This is largely because the sampling procedure has not been assessed critically enough. Minimal ether anaesthesia and guillotining were shown experimentally to be preferable to other methods of producing unconsciousness in the rat. These two procedures were then appraised by observing the effects of stresses on the concentrations of several labile intermediate metabolites of glycolysis; the response was assessed by examining both the sensitivity of the levels and the range of the concentrations. It was concluded that minimal ether anaesthesia is unlikely to disturb the resting levels of these substances greatly.

(2) Correlation between the amounts of components of the glycolytic pathway in extracts of different tissues. The demonstration of relationships between the components of a metabolic pathway has been delayed because of inadequate methods of analysis and sampling of tissues. The data

derived from (1) plus assays of activities of related enzymes were used in order to show, in a number of tissues of the rat, that there are significant correlations between (a) the concentrations of fructose diphosphate and pyruvate, (b) the activities of aldolase and lactate dehydrogenase and (c) the amounts of each substrate and the activity of the related enzyme.

(3) Comparison of the composition of normal and neoplastic cells. An attempt was made to compare the amounts of some of the constituents of the epithelial cells of human colonic mucosa, carcinomata and the polyps of multiple polyposis. These cells were isolated successfully, but during the process many of the constituents leaked out. It is concluded that the composition of a particular type of cell in a tissue can be established only by sampling the contents of single cells.

PREFACE

The work for this thesis was carried out in the Department of Chemical Pathology, Postgraduate Medical School of London.

There are many whom it is a pleasure to thank for their assistance. But first I would like to pay tribute to my supervisor, the late Professor E. J. King, who took so much pleasure in shepherding his assistants along this path.

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P A R T I

INTRODUCTION

AND

REVIEW OF LITERATURE

INTRODUCTION

One of the major pre-occupations of biochemists today is the discovery and understanding of the factors which control reactions in cells. The reactions where control occurs are known as 'pacemakers', and they may be identified by changes occurring in the concentrations of metabolites as a result of a change in the environment (KREBS and KORNBERG, 1957). The importance of clues provided in this way is exemplified in the discovery by LYNEN in 1941, that the change from respiration to fermentation in yeast is accompanied by an increase in the concentration of orthophosphate (reviewed by LYNEN and others, 1959). More recently a site of control of glycolysis in heart and diaphragm, namely, the phosphofructokinase reaction, was revealed by the observation of changes in the concentrations of the hexose phosphates during anoxia (NEWSHOLME and RANDLE, 1961). This same reaction may be concerned also with the disturbances of carbohydrate and fat metabolism which occur in diabetes (RANDLE and MORGAN, 1962).

Pacemakers presumably control the metabolism of neoplastic as well as that of normal tissues: indeed it is conceivable that neoplasia is due to a breakdown in the normal control mechanisms. Certainly the general similarity in the chemistry of tumours and normal tissues appears to rule out the effects of qualitative

differences in the causation of cancer (GREENSTEIN, 1954). The detection of the sites of control, and this may include those which produce cancer, pre-supposes that the concentrations of the relevant metabolites in the tissues can be determined accurately; small differences in the concentration could provide essential information.

The author's interest in the problem started with an investigation into some aspects of the biochemistry of multiple polyposis of the colon in man. Here is a situation where normal, potentially pre-malignant and malignant tissue can be obtained; it was believed that some of the early changes of cancer might be found in the polyps. However, it soon became evident that human tissue is unsatisfactory because it cannot be obtained in sufficiently large amounts in fresh condition. The realisation that the difficulties were due to changes occurring in the tissue before extraction focussed the writer's attention on the more general problem of measuring the amounts of labile substances in animal tissues, including tumours, without disturbing the resting state.

KREBS and KORNBERG (1957) pointed out that information concerning the concentrations of intermediate

metabolites is limited because of the absence of adequate methods of analysis. However, in spite of the recent rapid development of enzymic and chromatographic methods of analysis which appear to be highly specific and sensitive, some very divergent results have been obtained. Thus the amounts of fructose diphosphate and pyruvate in rat liver (μmM./g. fresh tissue) are given as 160 and 33, respectively, by THRELFALL and STONER (1961) and as 22 and 154, respectively by HOHORST and his colleagues (1959). There is no evidence to indicate which of these two sets of results is more likely to be correct.

It appeared to the writer that the differences could arise because insufficient attention was given to the effects of the sampling procedure by previous investigators. Thus the analyses of FROHMAN and others (1951) were carried out in tissues taken from rats which were previously starved, and then bled under anaesthesia. Likewise the analyses of LEPAGE (1948) were made on the tissues of rats which were anaesthetised and then frozen whole. HOHORST informed the author that no attempts were made to examine the effects of the sampling procedure used by him and his colleagues (1959). This doubt as to which, if any, of the analyses to accept,

was reinforced by the knowledge that homeostatic mechanisms act very quickly. The mere handling of an animal is calculated to provoke a response in its central nervous system and its adrenal medulla, and thereby in all of its tissues.

The uncertainty about the significance of analytical results is in sharp contrast to the range and sensitivity of the techniques which have become available in the past ten years. The advent of enzymic and chromatographic methods, and especially gas chromatography, have opened new vistas for the analyst. A confident attack on the accurate determination of the concentrations of the intermediates of the glycolytic pathway, the tricarboxylic acid cycle and the intermediates of fat metabolism may now be made; and small differences in concentration due to the action of the pacemakers may be revealed.

But the results of these technical advances will be meaningless unless the sampling of tissue can be achieved without disturbing the resting animal appreciably. For this reason it was regarded as essential to undertake an investigation of sampling techniques and an assessment of the probable amount of disturbance produced by them in the concentrations of labile sub-

stances. Although normal tissues were used for this investigation the results can be applied equally well to tumours. Thus it is hoped that the accurate determination of the concentrations of labile substances will reveal quantitative differences in the composition of normal and neoplastic tissues.

It is obvious that the amounts of each of the components of a tissue, as detected in extracts prepared from it, must be integrated in the cells with the amounts of the other constituents. However, when attempts are made to relate the amounts of the individual components in extracts to characteristics of the tissue as determined in whole organs, slices or homogenates, there is little or no evidence of such integration. In the case of the labile substrates the dearth of reliable information makes any attempt worthless. The information about enzymes is somewhat more reliable; yet there is no relation between the published activities of aldolase and lactate dehydrogenase in rat tissues and the respiratory or glycolytic rates as given in a recent compilation (KRATZING, 1961). The difficulties of reconciling the maximum activity of an enzyme, as determined <u>in vitro</u>, with its function in the cell were remarked by PARDEE (1959) and CHANCE

(1961). They have not been overcome as yet. Furthermore, it is not even justifiable to regard the maximum activity of a given enzyme as measured in extracts of different tissues as an index of the relative level of function of the metabolic pathway concerned in each tissue.

The reasons for these difficulties lie in the differences between the intact cell and the extract. They include:

(a) the consequence of structure, namely, the possibility of ... concentration of the reactants.

(b) the effects of the complexity of the reactions in the cell, namely, the sharing of and/or competition for enzymes, substrates and co-factors, and

(c) the presence of an open chemical system in the intact cell. The chief differences between the closed and open systems are (i) there is a flux of material through the open system, (ii) the thermodynamic equilibrium of the closed system is replaced by the steady state of the open system, and (iii) the presence of a catalyst in the open system alters the concentrations of the reactants. The steady state bears a superficial resemblance to the thermodynamic equilibrium in that the concentrations of the reactants appear to be

constant; this is because the rates of supply and removal of each reactant are equal.

One of the objects of this thesis is to produce a partial interpretation of the analytical data in terms of the function of the intact cell. This is attempted against the background of a critical examination of the information relating to the differences just outlined. The impossibility of reaching an interpretation based on the absolute values of the contents of the cell will be signified. Attention is confined to an interpretation based on the relative amounts of substances in different tissues. An important pathway common to all tissues was selected for this purpose, namely the Embden-Myerhof pathway of glycolysis. The observation of a correlation between the maximum activities of enzymes from different sites in the pathway in the different tissues would be evidence in support of a corresponding functional capacity in those tissues. Additional evidence would be provided by the demonstration of correlations between the activities of the enzymes and the amounts of their substrates or products, or between the amounts of a number of substrates in different tissues. The existence of numerous correlations of this kind in extracts of tissues would be presumptive evidence of the similarity

of the pathway in the tissues concerned and would justify making comparisons of the level of activity in the pathways in those tissues. Likewise, the failure to find such correlations would indicate that qualitative similarities are misleading: it would also provide grounds for a closer examination of the individual steps in the pathway in order to determine how the quantitative differences arise in different tissues.

The enzymes selected for investigation were aldolase and lactate dehydrogenase, and the corresponding substrates were fructose diphosphate and dihydroxyacetonephosphate, pyruvate and lactate. The choice of tissues was limited by their accessibility, by the amount available and by the desirability of excluding tissues containing cells with functions differing too widely, for example, intestine which contains muscle and gland cells.

Tissues of the rat were used exclusively for the determination of the substrate content. Although it is necessary to use human tissue ultimately when human disease is being investigated, it is often desirable to carry out the preliminary experiments on tissues which are more readily available and accessible. In the case of labile intermediates the uncertainty

surrounding the significance of the previous determinations in animal tissue was a sufficient reason to avoid the doubt which would be attached to analyses of operation material or biopsy specimens obtained from man. Having established the probable levels of any given labile substance in the tissues of the rat it is then reasonable to apply the techniques to the estimation of those substances in biopsy specimens obtained from man. The normal tissues used were liver, kidney, skeletal muscle, testis and cells of the blood; the tumours were a sarcoma and a hepatoma.

The exercise of control depends partly on the maximum activities of enzymes; differing amounts of a given enzyme in normal and neoplastic tissues might reveal differences in the control within the pathway concerned. The activities of most enzymes in neoplastic tissues are similar to their activities in normal tissues (GREENSTEIN, 1954). However, the similarities may be more apparent than real. All tissues consist of many kinds of cell including a relatively high percentage of connective tissue, blood vessels, lymphatics and nerves. Thus it must rarely be possible to compare the activity of an enzyme in normal cells of a given type with malignant cells derived from the same type. In this

thesis the results of an attempt to achieve this ideal are presented. The tissues used were the mucosa and carcinomata of human colon. One of the reasons for the choice of colon was that a potentially pre-cancerous condition, namely, multiple polyposis, occurs in it. Thus, there was the possibility of observing whether the activities of enzymes in a potentially pre-malignant condition differed from those in normal and malignant tissue. The activities of the enzymes mentioned above, namely, aldolase and lactate dehydrogenase, were assayed. In addition the activity of deoxyribonuclease was measured because it increases in the liver in the pre-malignant phase of the formation of drug-induced hepatomata in rats (SCHNEIDER and others, 1953; LAMIRANDE and others, 1954).

II

THE RELATION BETWEEN ESTIMATED AND EFFECTIVE CONCENTRATIONS OF INTRACELLULAR REACTANTS

The object of the analysis of extracts of tissues is to provide information concerning intracellular events. Such analyses are the crudest possible way of estimating intracellular function. However, the value of the results can be increased by examining the main physical and chemical differences between an extract and what is known of the intact cell. These differences will be considered from three viewpoints.

First, the effects of structure which is essential for organised function and which is destroyed in the preparation of an extract. From the time of the first microscopists, Hooke, Malpighi and Grew in the 17th century, to the present-day electron microscopists, in particular, Palade and Siekewitz, it has been evident that cells contain compartments and membranes. However, structure extends beyond compartments. Thus mitochondria contain particles, and within these are more particles; the smallest appear to be units of biochemical function.

The surfaces of cells are likewise structures. The potential importance of surfaces was first stressed by

J. W. Gibbs in 1878. Ten years later J. T. Thompson, in his book "Applications of Dynamics to Physics and Chemistry", produced a complete theory of surface forces and intimated that the subject had not received sufficient attention. PETERS (1931) stated that the exact significance of surfaces was not understood; he emphasised that 'surface effects take precedence over ordinary statistical mass action relationships'. The importance of surfaces was again underlined by DANIELLI and DAVIES in 1951. They concluded that 'the final steps in the synthesis and the initial steps in degradation of these macromolecules (polysaccharides, proteins and nucleic acids) must be regarded as occurring in the surface phase of the macromolecule itself'. Notwithstanding these statements by eminent biochemists, LIPMAN found it necessary to remind the Ciba Conference on the Regulation of Cell Metabolism (1959) that "we are dealing with a heterogeneous system'. The need for regular reminders is manifest when it is recalled that at present 'the surface chemist can do little more than indicate the possible behaviour of molecules at interfaces within the cell' (CHEESMAN and DAVIES, 1954).

The second important difference between the living cell and extracts obtained from it is that the open

chemical system of the cell is replaced by the closed system of the extract. HOPKINS (1913) recognised that biological systems exhibited the characteristics of the dynamic equilibrium, that is, the steady or stationary state, a phenomenon already familiar to physical chemists (BODENSTEIN, 1913). Little was heard of this important aspect of cell function until A. V. HILL (1930) used it to account for the difference in electromotive force between the inside and the outside of a muscle cell. The concept of the open catenary system with transient and steady states is now accepted as describing the behaviour of biological systems.

The third and last important difference between whole cells and extracts which will be discussed, is that of complexity. Thus different reactions proceed simultaneously in different compartments of the cell, the product of one compartment often becoming the reactant of another. There are branching chains and cycles of reactions linked together (KREBS, 1946-1948). Reactions are directly or indirectly under homeostatic control. Finally, the number, kind, charge, size and shape of the particles within the cell vary with respect to place and time.

The first part of this chapter comprises a general

discussion of the properties of cells based on the outline just given. In the second part an attempt is made to appraise estimates of substrate concentration and enzyme activity as found in extracts in relation to the normal cell. Particular attention is given throughout to the substrates and enzymes of interest in this thesis.

THE PROPERTIES OF CELLS

The Effects of Structure

The Whole Tissue

The Cells. Estimates of the activity of a given enzyme or the concentration of a given substrate from extracts represent weighted mean values relating to all of the types of cell present. Thus the understanding of an analysis depends on the interpretation given to mean values and will vary with tissue, enzyme and substrate.

The Extracellular Compartment. The extracellular 'space' has been estimated as occupying volumes varying between 15 and 30 per cent of the body weight. The subject was reviewed by MANERY (1954), ELKINTON and DANOWSKI (1955) and ROBINSON (1960), among others. This space comprises both blood and lymph vessels, and the interstitial fluid, both free with within connective tissue. There are in addition, the spaces occupied

by secretions or excretions which undergo functional exchange with the luminal cells. These spaces contain more or less of the constituents of the cells and therefore obscure the concentrations of reactants within the cells; the magnitude and direction of the effect varies with each tissue.

The Single Cell

The Compartments. The substances in an extract of tissue are a weighted mean, from each of the different kinds of intracellular structure. There is ample evidence of specialisation of function in the different fractions (DIXON and WEBB, 1958; de DUVE, WATTIAUX and BAUDHEIM, 1962). Some enzymes appear to be present in more than one fraction, and parts of some complex metabolic processes, for example, protein synthesis, are farmed out to almost all regions of the cell. Aldolase is present chiefly in the supernatant fraction of rat liver, and brain (KENNEDY and LEHNINGER, 1949; JOHNSON, 1960), and there is a small percentage of uncertain function in the nuclei of rat liver (ROODYN, 1957). Lactate dehydrogenase is probably wholly in the supernatant of rat liver, brain and whole chick embryo (DELBRUCK and others, 1959; JOHNSON, 1960; MAHLER and others, 1958); however, NOVIKOFF, in a

personal communications to de DUVE (1962) claims that about 50 per cent is present in the mitochondrial and post-mitochondrial fractions. Deoxyribonuclease occurs in the lysosomes and related droplets of rat liver and kidney (de DUVE, WATTIAUX and BAUDHEIM, 1962). The same authors claim that the distribution of the enzyme in mouse liver and pancreas, rat brain and azodye hepatomata and calf thymus indicates the occupation of a similar site in these tissues.

There are few observations on the distribution of labile metabolites within the cell. One of the established locations is that of citrate which occurs chiefly in the mitochondria (SCHNEIDER, STRIEBICH and HOGEBOOM, 1956).

It is probably reasonable to assume that fructose-1,6-diphosphate, one of the specific substrates and/or products of aldolase activity, is situated near the aldolase molecules. The same inference may be made regarding dihydroxyacetonephosphate and glyceraldehyde-3-phosphate because the enzymes catalysing their major transformations are chiefly in the supernatant fraction (de DUVE and others, 1962).

With pyruvate the situation is less clear. The enzyme catalysing its formation in the Embden-Myerhof pathway, namely pyruvate kinase, occurs chiefly in the

supernatant fraction of rat brain (JOHNSON, 1960). The location of the enzyme catalysing its conversion to acetyl Co-A, namely pyruvate dehydrogenase, does not appear to be recorded. Other important transformations of pyruvate include the formation of malate (catalysed by the 'malic enzyme' of OCHOA, MEHLER and KORNBERG, 1948) and the formation of oxaloacetate by pyruvate carboxylase (UTTER and KEECH, 1960). Eighty per cent of the 'malic enzyme' is in the supernatant fraction in pigeon liver (RUTTER and LARDY, 1958). Pyruvate carboxylase is present in particles in avian and beef liver. The inference is that pyruvate is present in both the supernatant fraction and the mitochondria.

A consequence of the compartition of cells is the presence of membranes. These are probably necessary for storage. They may also prevent uncontrolled access of an enzyme to its substrate; for example, the access of phosphorylase to orthophosphate, of phosphofructokinase to fructose-6-phosphate, and of glucose-6-phosphatase to glucose-6-phosphate (CORI, 1956). Membranes may impose an upper limit to the velocity of an enzyme-catalysed reaction by restricting the concentrations of substrate (see also page 56).

Fine structure. The limits of structure are not yet known. There are two main schools of thought. GREEN

(1957) believes that enzymes may occur in juxtaposition in the order in which they occur in a reaction sequence. DIXON and WEBB (1958), while not denying the proximity of enzymes concerned with particular metabolic pathways, claim that rigid structure is not necessary. Inasmuch as mitochondria are divisible into smaller particles, there is some structure at this level. Inasmuch as many enzymes can be split into a protein and a prosthetic group, there is structure at the level of aggregation of molecules.

Intraparticulate structure. Particulate fractions of mitochondria, containing the whole cytochrome system, were first prepared by KEILIN and HARTREE (1938). The cytochrome system was found to comprise two main fractions, namely succinic dehydrogenase and cytochrome b, and the cytochromes a, cytochrome oxidase and copper (EICHEL and others, 1950). Both of these systems have been obtained in soluble form (CLARKE and others, 1954; SMITH and STOTZ, 1954). GREEN and his colleagues described a different fraction obtained from heart mitochondria (1957); it appears to be similar to another isolated from liver mitochondria and catalysing oxidative phosphorylation coupled with oxidation of β-hydroxybutyrate (COOPER and LEHNINGER, 1956). In addition to

these particles there is a soluble fraction in mitochondria. This contains L-glutamate dehydrogenase, fumarase and nucleotide coenzymes (SCHNEIDER, 1955). Microsomes contain particles also (PALADE and SIEKEVITZ, 1956); the best known are those concerned with aminoacid incorporation (LITTLEFIELD and others, 1955).

The electron microscope has revealed a complicated structure in mitochondria (PALADE, 1956). Localisation of biochemical function to structures revealed by the electron microscope is described by BAKER, NORTHCOTE and PETERS (1962). They showed that exposure of mitochondria from guinea-pig kidney to dodecanoic acid reduced their capacity to oxidise pyruvate to citrate; this change coincided with the disappearance of the structure of the cristae, only the limiting membrane remaining visible.

Molecular aggregation. A number of molecular aggregates are specific; they may be regarded as the simplest manifestation of particulate structure. One of these is peroxidase in which haem is the prosthetic group (CHANCE, 1951; KEILIN and HARTREE, 1951). Another is yeast lactate dehydrogenase which contains one haem and one flavine mononucleotide group (BOERI and TOSI, 1956; APPLEBY and MORTON, 1959).

Another kind of aggregate occurs in the glutamic dehydrogenase molecule which can be divided into four sub-units by exposure to sodium dodecylsulphate or urea; the activity is reduced coincidentally. The fragments join together and become active again in the presence of NAD or ADP (TOMKINS and YULDING, 1961); their re-union is opposed by $NADH_2$ and steroids.

GREEN, who postulated the existence of an organised mosaic of enzymes (1957), produced evidence from electrophoretic and ultracentrifugal observations which may indicate the existence of complexes larger than an enzyme plus a prosthetic group(s). He and his colleagues observed binding of mitochondrial lipide to structural protein and to cytochromes. They also demonstrated the formation of complexes between and polymers of purified structural protein and each of the cytochromes a, b and c (CRIDDLE and others, 1961; GREEN and others, 1961).

If it is ever shown that the whole cell is indeed organised to this extent, and if as suggested by CHEESMAN and DAVIES (1954) 'a substrate would pass over a row of active centres under the impulse of its own osmotic gradient undergoing a change at each centre', then the absolute concentration of both enzyme and

substrate must be very high.

Surfaces. It is probable that much of the theoretical and experimental information about inanimate surfaces can be applied to the cell. However, there are some major difficulties which have received little attention. One was indicated earlier, namely, that the law of mass action can be applied only to homogeneous systems (FREUNDLICH, 1930; PETERS, 1931). CALDWELL (1956) pointed out that the law of mass action cannot be used to determine pH in a heterogeneous system. The other difficulty is that the inanimate systems in which a great deal of the work was done, are closed systems, whereas the cell is an open system.

Despite these unsolved problems it appears to be worth examining qualitatively the possible effects of surfaces on concentrations and molecular configuration and orientation of reactants.

Effects on distribution of reactants between surface and bulk phases. THOMSON pointed out that the changed concentration of the solute at surfaces alters the energy associated with the system, and therefore the equilibrium constant (1888). DONNAN and HARRIS (1911) extended the theory of surface effects to include those associated with the separation by a membrane of a non-

diffusible ion from diffusible ions. LOEB (1921) used Donnan's theory in order to account for the effects of immersing solid gelatin hydrochloride in water, namely, the diffusion of hydrochloric acid into water, the presence of a potential difference between the protein and the water and the swelling of the protein. In 1930 FREUNDLICH suggested that surface tension effects should include solid surfaces because 'substances which are absorbed strongly are formed in large amount and the chemical equilibrium is therefore shifted in this direction'.

PETERS (1931) observed that the interfacial tension between palmitic acid or cetylamine in benzene and an aqueous buffer varied with the pH. He postulated that the changes in interfacial tension were due to changes in the degree of ionisation. He appreciated that if this were true, then the pH at the interface would be different from that in the bulk phase.

DANIELLI (1937) confirmed Peter's observations. He then showed, by using Donnan's equation and making several assumptions, that the pH of the interface between oleic acid and bromobenzene is approximately two units less than that in the bulk phase. HARTLEY and ROE (1940) obtained the same result by using a

different theoretical approach. They derived and used an equation which related the difference in pH between the surface and bulk phases to the zeta potential. In 1941 DANIELLI extended the use of the Donnan equation and showed that the interfacial pH varies with the ionic strength of the bulk phase. He also demonstrated that the effects of changing ionic strength on the titration curve of ovalbumin are probably due to the effects of concomitant changes in the pH on the acid and base binding powers of the protein.

The difference in pH between surface and bulk phases is a manifestation of the more general change in the ratio of the concentrations of all diffusible ions at interfaces. The consequences include changes in the ratio, surface to bulk, of many variables affecting substrate concentration and enzyme activity; for example, ionic strength, metal ion concentration, $\frac{SH}{SS}$, redox potential, ionisation constants, and the concentrations of activators and inhibitors. The main variables are the pH and ionic strength of the bulk phase and ions which can form unionised complexes with ions on the surface (DANIELLI and DAVIES, 1951). A number of metallic cations and certain anions, for example ortho-phosphate, may be associated with exchanges of organic

ions at surfaces (BEST, 1960).

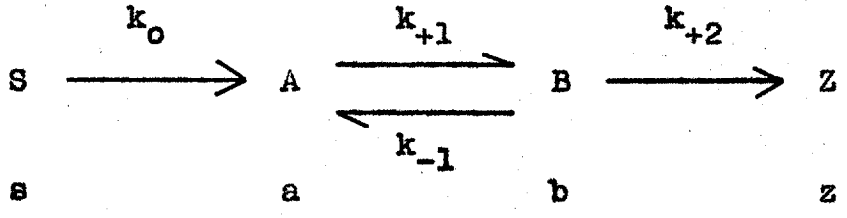
Effects of configuration and orientation of molecules. Interfaces are often monolayers and often comprise polar molecules which are packed tightly. The packing is associated with an increased surface energy. The flexibility of the molecules is reduced especially when the film contains straight chain trans isomers. The pH may affect the packing also: ionised groups may attract or repel each other (DANIELLI and DAVIES, 1951). The only available information of this kind relates to artificial systems. Indeed, in 1954, there was not a single case where the physical state of an intracellular enzyme was known (CHEESMAN and DAVIES). The potential importance of these physical factors in enzyme ractions is obvious; that they cannot as yet be appraised is equally obvious.

The Open Chemical System

The kinetics of open systems are complex and incomplete. BURTON (1939) made the first attempt to apply these concepts to biological systems. HINSHELWOOD, in his monograph "The Chemical Kinetics of the Bacterial Cell" (1946), applied principles of open systems to cell growth. DENBIGH, HICKS and PAGE (1948) carried the analysis further and HEARON has produced the most

complete mathematical treatment yet attempted (HEARON and others, 1959).

An open system is one in which there is an exchange of material and energy across its boundaries. Thus an intact tissue or cell is an open system, whereas a homogenate or an extract is a closed system. The simplest form of the open system can be expressed symbolically as follows:



where S,A,B and Z are the source, reactants (A and B) and the sink, respectively. The corresponding concentrations are s,a and b, and z. The rate equations are:

$$da/dt = k_o(s-a) + k_{-1}b - k_{+1}a$$

$$db/dt = k_{+1}a - k_{-1}b - k_{+2}(b-z)$$

There is by definition a flux of material through an open system. The size of the flux is determined by the rate of the slowest enzyme - substrate reaction in the chain of reactions. This rate, the velocity, v, of the enzyme, determines the amount of the substrate reaching all subsequent enzymes in the sequence.

The Steady State

The flux may vary as in transient states, or it may be constant at any one level as in a steady state. Transient state equations are often difficult to solve or even insoluble, and it is difficult to reproduce a particular transient state (ALBERTY, 1956a). Therefore many experiments are carried out when the metabolism is in a steady state, that is, 'when the mass and composition of the system are constant..... The stationary state is thus described or determined by the set of algebraic equations obtained by equating to zero all the time derivatives of the concentrations within the system' (HEARON and others, 1959).

Thus in the equations given, $da/dt == 0 == db/dt$. The steady state concentrations of A and B, namely α and β, respectively, may now be obtained by solving the two simultaneous equations.

$$\alpha == \frac{k_o k_{-1} s + k_o k_{+2} s + k_{-1} k_{+2} z}{k_o k_{-1} + k_o k_{+2} + k_{+1} k_{+2}}$$

$$\beta == \frac{k_o k_{+1} s + k_o k_{+2} z + k_{+1} k_{+2} z}{k_o k_{-1} + k_o k_{+2} + k_{+1} k_{+2}}$$

These equations are taken from BRAY and WHITE (1957) who modified those given by BURTON (1939). They show that the steady state concentration of a reactant is

determined by the flux from the source S to the sink Z, and by a combination of rate and equilibrium constants associated with its transformation. Thus in an open system a catalyst can change the stationary concentrations of the reactants and the rate of flux of the system (BURTON, 1939).

The steady state thus differs fundamentally from the corresponding state of a closed system, namely, thermodynamic equilibrium. At equilibrium the presence of a catalyst has no effect on the relative concentrations of reactants and products. This difference must therefore be taken into account when any equations which utilise equilibria are considered. One of these is the Donnan equation. When applied to an open system such as a cell membrane or surface it represents only an approximation. This is one of the reasons advanced by CALDWELL (1956) to account for its failure to describe accurately numerous phenomena in cells, in particular the relationship between intra- and extracellular pH.

There are several other properties of the steady state which are fundamental to the functioning of biological systems (DENBIGH and others, 1948). One of the more important is the buffering capacity. A change

in the concentration of the molecules of the source produces a change of comparable magnitude in the sum of the concentrations of the reactant molecules; this is distributed over all of the 'n' reactants in varying degrees. Another property is stability. This can only occur where there are first order reactions, that is, where 'the rates of the various elementary processes shall increase or decrease according to whether there is a rise or a fall in the concentration of the species which undergoes change'. A third property is likened to the charging and discharging of an accumulator. Thus there can be sudden changes in the output or input from a reaction sequence in the steady state without much change in the concentrations of the species present.

The term 'concentration' when used with reference to substances in an open system is applied to the molecular species. Thus in a steady state the concentration of each molecular species is constant, but the individual molecules pass through the system continuously; it is more appropriate to refer to their half life. In the tricarboxylic acid cycle this is probably no more than a few seconds (KREBS, 1954a).

The Complexity of Intracellular Reactions

Effects on Substrate Concentration

The multiplicity of reactions alone might be expected to produce identical metabolites in different pathways. The occurrence of linked cycles and alternative pathways and the funnelling of the products of progressive degradation of complex molecules (or their synthesis from 'building blocks') depend on the existence of substances common to the various pathways. For example, oxaloacetate is formed or transformed by at least six different reactions; these include formation from malate, aspartate, α-ketoglutarate and pyruvate (ATP linked), and transformation to phosphoenolpyruvate, pyruvate and citrate (KREBS and KORNBERG, 1957; UTTER and KEECH, 1960). The contribution of each pathway to the concentration of a given substrate depends on the flux from that pathway.

The factors determining which of several pathways will be taken by a substrate are numerous. In general a high affinity, and a large maximum velocity of an enzyme, optimal amounts of co-factors, absence of inhibitors and rapid removal of products will favour a given pathway (RACKER, 1954; DIXON and WEBB, 1958; CHANCE, 1959; PARDEE, 1959). This information is not

available for most enzymes and substrates at present; thus accurate estimates are not available of either the contribution of different pathways to the concentration of a substrate or of its transformations.

Metabolic Pathways of Substrates to be Examined. The substrates to be examined are components of the Embden-Myerhof pathway. They are also integral parts of other pathways. The chief of these relationships are shown in figures 1 and 2. The contribution from the different pathways to the total flux of a given substrate depends, among other factors, on the metabolic balance of the animal, the state of physical and mental rest (or exertion), the temperature and the humidity. In order to simplify the discussion, the animal can be considered to be in metabolic balance, on an optimum diet and in optimal physical surroundings.

Degradative phase. Carbohydrate provides about sixty per cent of the calories of the diet of a laboratory rat. It is degraded chiefly in the Embden-Myerhof pathway, starting from glycogen or dietary hexose.

The pentose phosphate pathway involves both D-glucose-6-phosphate and D-fructose-6-phosphate, but it does not seem to account for an appreciable amount

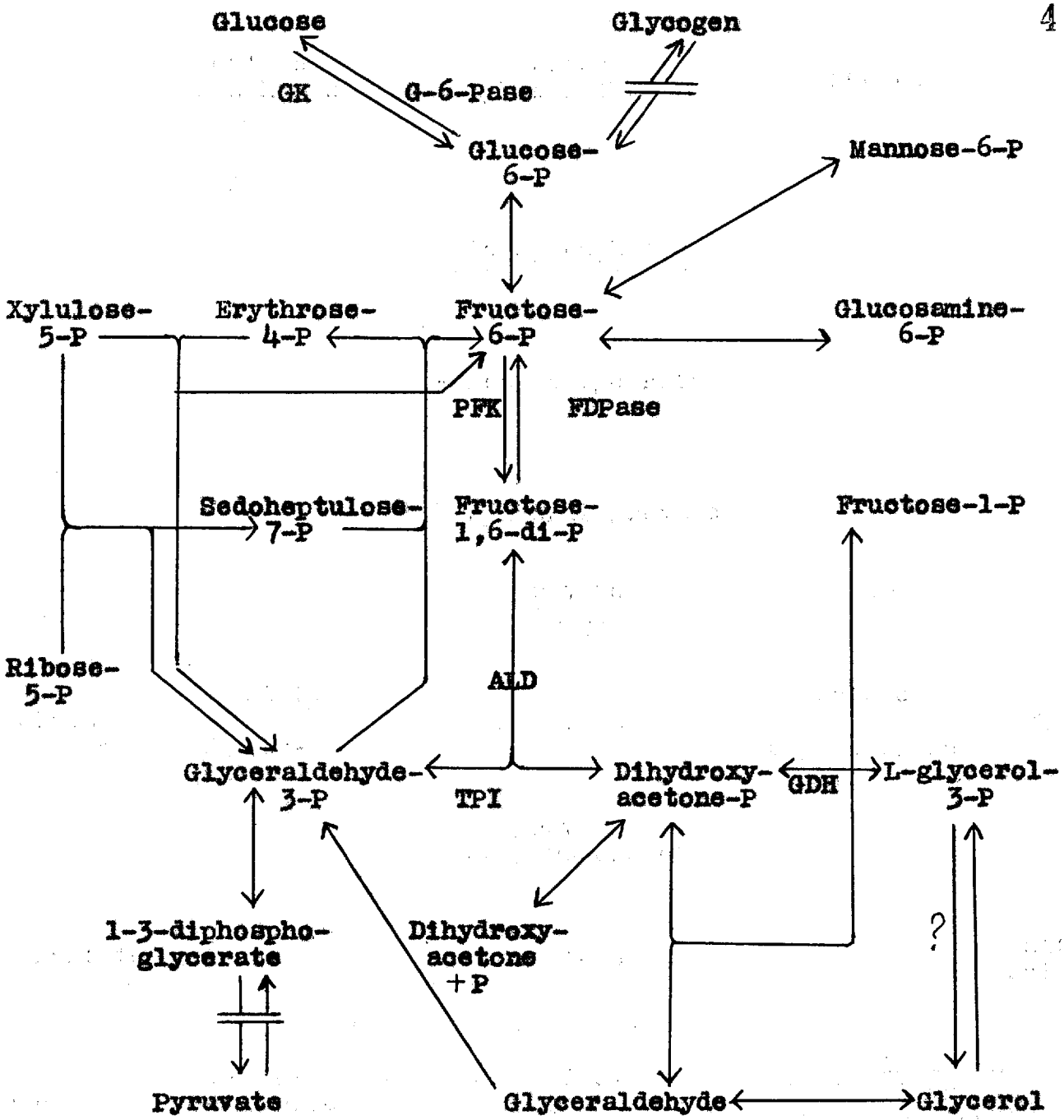


Fig. 1. Metabolic pathways of fructose diphosphate and the triosephosphates. Only the relevant enzymes are included in the chart. They are abbreviated as follows: ALD, aldolase; GDH, glycerolphosphate dehydrogenase; GK, glucokinase; G-6-Pase, glucose-6-phosphatase; FDPase, fructose-1-,6-diphosphatase; PFK, phosphofructokinase; TPI, triosephosphate isomerase. Phosphate is represented by P.

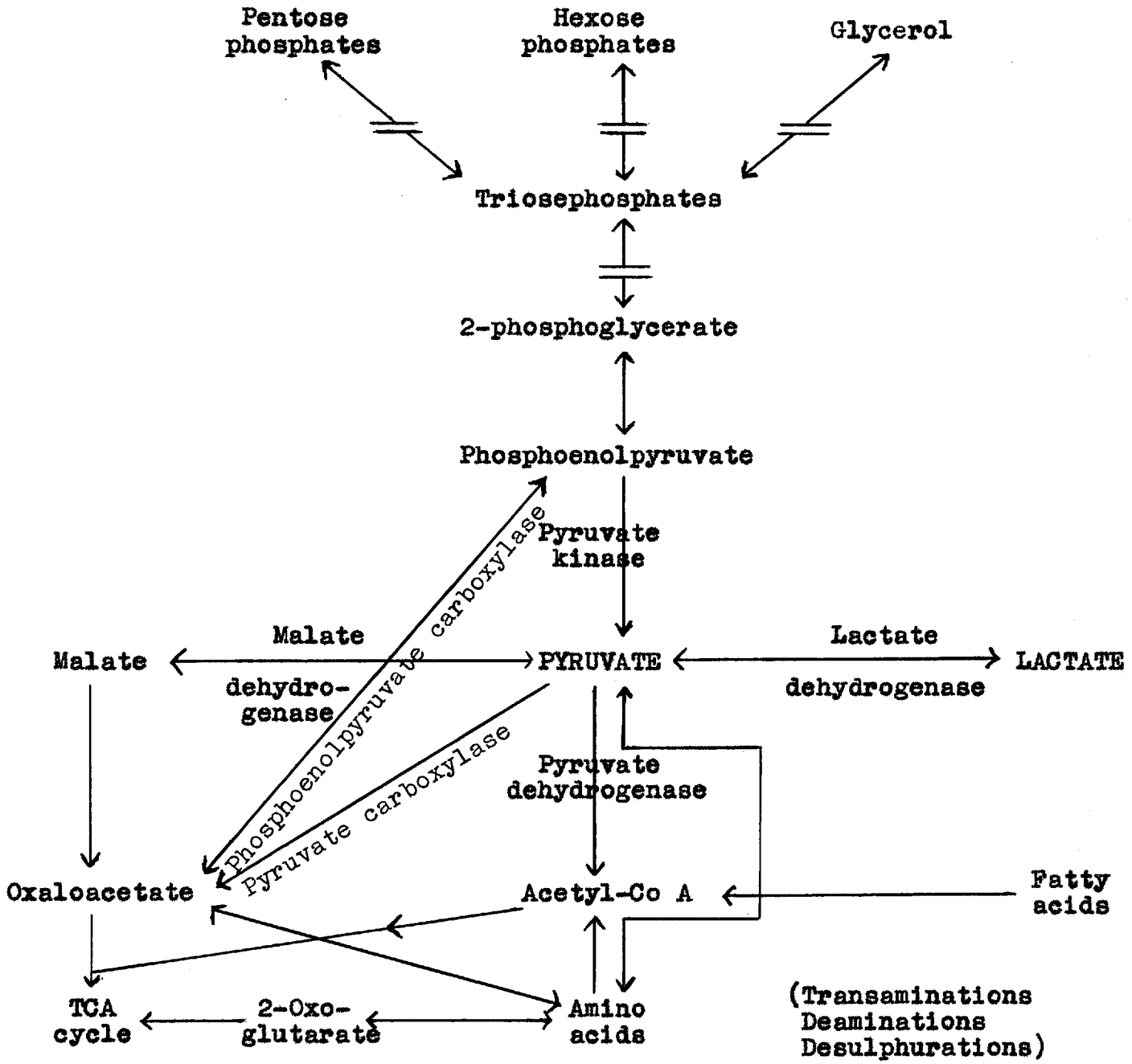


Fig. 2. Metabolic pathways of pyruvate and lactate.

of their oxidation in liver or possibly in muscle (BLOOM and others, 1953; KATZ and others, 1955; ASHMORE and others, 1956). It was suggested recently that the shunt in muscle is of greater importance than was indicated by earlier investigations (ROSSI and others, 1963). About ten per cent of the glucose in human leucocytes is oxidised in this pathway (BECK, 1958). Various other hexose phosphatas and amino sugars are converted to glucose- or fructose-6-phosphate and therby enter the glycolytic pathway. The triosephosphate pool receives a small contribution from the pentose phosphate pathway, glycerol arising from the hydrolysis of glycerol esters, and α-glycerophosphate arising from phospholipids.

Synthetic phase. Synthesis of the constituents of the Embden-Myerhof pathway occurs especially after ingestion of food and in the recovery phase after exercise when lactate leaves muscle and enters the blood. Molecules enter the pathway at various stages from the tricarboxylic acid cycle upwards. It is probable that any molecule entering the cycle can leave it via oxaloacetate, as was postulated by KREBS (1954b) for pyruvate. Thus acetate in the form of acetyl-COA arising from the degradation of fats and proteins, and α-ketoglutarate,

oxaloacetate and succinate arising from protein degradation could enter the synthetic pathway of carbohydrate. Pyruvate is provided by the deamination of alanine, serine and the sulphur containing aminoacids and also by the transaminations between alanine and hydroxypyruvate or α-ketoglutarate (FRUTON and SIMMONDS, 1957).

Other substances enter the triose and hexose phosphate pools as already indicated. One of the more important is fructose-1-phosphate in the liver.

Summary. The principle sources of fructose-1,6-diphosphate are glycogen and dietary glucose via fructose-6-phosphate, and dietary fructose, glycerol residues and lactate via the triosephosphates. The triosephosphates arise from fructose diphosphate in addition to the sources mentioned. Pyruvate derives chiefly from glycogen, dietary hexoses and lactate, and to a small extent from amino-acid residues. The contribution of the different pathways to the pools of different substrates varies not only with the tissue, but with the phase of metabolism.

Effects on Maximum Activity

The factors affecting the activity of an enzyme are related in the Michaelis-Menton equation

$$v = \frac{V.S.}{K_s + S}$$

where v == the velocity of the reaction under stated conditions

V == the maximum velocity, and is obtained only when the enzyme is saturated with respect to substrate.

== k.e,

where e == the concentration of enzyme

k == the rate of breakdown of the enzyme-substrate complex into product plus enzyme.

S == the total concentration of substrate, both free and bound. The percentage bound is regarded as negligible in classical enzyme kinetics.

K_s == the dissociation constant of the enzyme-substrate complex

$$== \frac{k_{-1}}{k+_1}$$

This equation is still acceptable in form, if not in detail. The significance of the dissociation constant and the constants of V are still in doubt, although as suggested by DIXON and WEBB (1958), 'it may well be

found that when more data are available that in the majority of cases K_m is a close approximation to K_s'. (K_m is a steady state constant involving dissociation constants plus rate constants).

Factors Affecting Concentration of Reactants. (a) Concentration of substrate. 'Such maximal rates are exceptional under physiological conditions, the limiting factor being usually, but not always, the amount of substrate' (KREBS, 1937). Similar conclusions are stated by RACKER (1954) and by WEBB (1960).

(i) The activity of other enzymes in the sequence (DIXON and WEBB, 1958; WEBB, 1960). The hexokinase reaction may restrict the flux because of limiting amounts of enzyme (LePAGE, 1950a; KREBS and KORNBERG, 1957). This observation was confirmed by BECK (1958). He showed in glycolysis in human leucocytes that hexokinase is the only enzyme whose addition will increase the rate. However, it may not be the absolute amount of hexokinase which is insufficient. McILWAIN (1959) indicated that in brain there is approximately ten times the amount of hexokinase needed to provide the recorded rates of the initial stages of the tricarboxylic acid cycle. There may be other factors involved such as the rate that glucose reaches hexokinase molecules

(KREBS and KORNBERG, 1957). The possibility of a by-pass in the formation of glucose-6-phosphate reduces the importance of the hexokinase reaction in rate limitation in tissues containing glycogen. NEIFAKH and MEL'NIKOVA (1958) suggested that phosphofructo-kinase is rate-limiting in glycolysis in muscle, after observing that this was the only enzyme whose addition increased the rate of lactate formation in preparations of rabbit muscle. This view is supported by the failure to detect appreciable amounts of fructose-1,6-diphos-phatase in rat heart or diaphragm (NEWSHOLME and RANDLE, 1962). Further evidence indicating that the activity of phosphofructokinase controls glycolysis in these two tissues was reviewed by RANDLE (1963). The regu-lation of the activity of fructose diphosphatase in the liver is not yet understood.

Another reaction which may limit the flux is that catalysed by triosephate dehydrogenase (KREBS and KORNBERG, 1957). The reaction requires ADP and ortho-phosphate. These substances are required also for respiration and oxidative phosphorylation; hence respiration inhibits glycolysis.

(ii) The competition by other enzymes for the substrates. Many enzymes have a broad specificity.

One of the consequences is that the path to be taken by a substrate molecule may be controlled in this way (see pages 60-61). However, the presence of a number of enzymes which can catalyse a given reaction in a homogenate is not necessarily evidence of competition *in vivo*. Thus the relative situations of the enzymes in the cell may be remote; there may be differentials in the supply of essential co-factors; and finally the energy barriers for different enzymes may be different, that is, the reaction mechanisms may differ. A reaction may be favoured because of coupling to an exergonic process (KREBS and KORNBERG, 1957).

The only competitor with aldolase for fructose diphosphate is fructose diphosphatase, the enzyme concerned with hydrolytic formation of fructose-6-phosphate from the diphosphate (GOMORI, 1943; POGELL and McGILVERY, 1954; KREBS and KORNBERG, 1957). The degradative activity of aldolase is favoured as long as pyruvate is oxidised and there is obviously no competition during synthesis. The phosphofructokinase reaction is irreversible because of an energy barrier: therefore it offers no competition to aldolase.

The formation of fructose diphosphate from and its degradation to the triosephosphates are catalysed

by the same enzymes as far as is known; presumably they act in unison, the direction being determined other factors. The only important enzyme competing with aldolase for either of the triosephosphates during synthesis of fructose diphosphate appears to be α-glycerophosphate dehydrogenase; this enzyme could be important in the control of carbohydrate metabolism (RANDLE, 1963).

The enzymes of the pentose phosphate pathway may compete for dihydroxyacetonephosphate or glyceraldehyde-3-phosphate; however, as the glycolytic pathway is dominant, such competition fails to affect greatly the maximum velocity of the aldolase reaction in either direction.

The best known mammalian enzymes affecting the concentration of pyruvate, apart from lactate dehydrogenase, are pyruvate dehydrogenase, kinase and carboxylase, the malic enzyme and various enzymes concerned with amino acid metabolism. The amount of competition offered to lactate dehydrogenase presumably depends on the function of the tissue. Thus in skeletal muscle and in other tissues in which the rate of formation of lactate is high the competition cannot be effective. In liver, on the contrary, the competition

is such as to support the formation of lactate from only ten per cent of labelled pyruvate added to a homogenate; up to seventy-five per cent of labelled pyruvate is removed via oxidation to acetyl-CoA, up to 40 per cent is carboxylated by an undetermined reaction(s) to dicarboxylic acids and up to 15 per cent is converted to alanine (HASLAM and KREBS, 1963). The transaminase reaction is unlikely to offer intense competition; nor is the pyruvate kinase reaction because it is effectively irreversible (KREBS and KORNBERG, 1957). The other three enzymes may offer in life just that amount of competition witnessed in the homogenate. If the function of lactate dehydrogenase in liver and other tissues is to initiate synthesis of glycogen from lactate it obviously does not enter into competition in the reactions discussed. Evidence in support of this view comes from the demonstration that in fasting rats labelled pyruvate and lactate is rapidly synthesised to glycogen via the tricarboxylic acid cycle (TOPPER and HASTINGS, 1949; LORBER and others, 1950)

(iii) The rate of diffusion of substrate between the sites of formation and transformation. The rate of diffusion of a substrate from its source could be a limiting factor in determining the potential maximum

velocity. An extreme form of such limitation is the presence of stores of glycogen. It would be possible to be misled by steady state concentrations in such cases (CORI, 1956).

DIXON and WEBB (1958) refer to the time taken for diffusion as the 'transit time', and they point out that after dilution this becomes more important. It could impose a limit on the velocity attainable. There is some support for this view but it comes from unnatural conditions. Thus SLATER (1949) observed that cytochrome c within mitochondrial particles is about 100 times more active than a similar amount added to a solution of the same volume as that containing the particles. MAZIA and HAYASHI (1953) found that the rate of degradation of albumin in fibres of pepsin and albumin formed at pH 4.4 and exposed to pH 1.5 was about 3 times greater than it was when albumin was exposed to larger amounts of pepsin in simple solution. These differences may not be due to limiting speeds of diffusion. PARDEE (1959) gives the diffusion time of many molecules as 10^{-2} seconds per micron and points out that this is negligible compared with the time required for further reaction.

BEST (1960) and BEST and HEARON (1960) discussed

the possibility of more subtle diffusion effects, namely diffusion coupling of substrates and products. They suggested that this could occur through electrostatic interactions, ion-exchange interactions or the formation of complexes between the diffusing species. It is conceivable that diffusion of pyruvate or a derivative, for example acetyl-CoA, into the mitochondria could be rate limiting for the tricarboxylic acid cycle.

(b) Concentration of Cofactors. The absolute concentrations of cofactors at sites of enzyme activity are unknown: they are the objects of competition by enzymes as are substrates and they are represented by a concentration factor in the Michaelis-Menton equation. They differ from substrates in that they are subject to cyclic changes rather than to the progressive changes of degradation or synthesis. The availability of these co-factors in the form appropriate to the occasion is one of the most important variables which determine the maximum activity of an enzyme. The direction of the flux in a reaction sequence illustrates this: the predominance of synthesis or breakdown of fatty acids depends on the relative values of the ratios $\frac{NADH_2}{NAD}$, $\frac{\text{Acetyl-CoA}}{\text{CoA-SH}}$, and $\frac{\text{reduced flavoprotein}}{\text{oxidised flavoprotein}}$ (KREBS and

KORNBERG, 1957). A less obvious example is the observation that oxidations of lactate and of alcohol are faster in the presence of free NAD and reductions of pyruvate and acetaldehyde are faster in the presence of $NADH_2$ bound to glyceraldehyde-phosphate dehydrogenase (NYGAARD and RUTTER, 1956). In this connection, it is noteworthy that in yeast alcohol dehydrogenase the $NADH_2$ bound to protein forms a high percentage of the total $NADH_2$ (DUYSENS and KRONENBERG, 1957).

Aldolase in mammalian tissues requires no co-factors (RUTTER, 1960). Lactate dehydrogenase requires $NADH_2$ as a proton donor for the reduction of pyruvate. In vitro this factor is provided in optimal amounts and in the absence of NAD. In the liver cell both forms of the coenzyme coexist; the ratio $\frac{NADH_2}{NAD}$ in resting tissue is debated, values quoted varying from 1 : 4 (CHANCE, 1954) to 1 : 1000 (HOLZER, 1959a). It varies with the state of oxidation, $NADH_2$ being produced as in the early stages of oxidation of carbohydrate. It is conceivable that the concentration of $NADH_2$ required for the maximum activity of lactate dehydrogenase in the direction of lactate may not be reached in the liver.

(c) Concentration of Enzyme. The number of active centres of an enzyme available for the catalysis

of the reactions of a given substrate may be only a fraction of the total number. This could be due to (i) competition by other substrates, (ii) effects of inhibitors or activators, and (iii) changes in molecular aggregation.

(i) Competition. A second substrate acts as a competitive inhibitor and reduces the maximum velocity with respect to the first substrate (DIXON and WEBB, 1958). The favoured substrate may vary with the prevailing conditions (PARDEE, 1959).

Many substances serve as substrates for aldolase (RUTTER, 1960) and for lactate dehydrogenase (MEISTER, 1950; CZOK and BUCHER, 1960). However, the rates obtained with most of them do not compare with those obtained with the natural substrate. Furthermore, there appears to be no evidence that many of the possible substrates occur in sufficient concentrations in cells to provide appreciable competition.

Aldolase is highly specific towards dihydroxyacetonephosphate, but numerous aldehydes may replace D-glyceraldehyde-3-phosphate (RUTTER, 1961). Thus L-sorbose-1,6-diphosphate, which is formed by the condensation of L-glyceraldehyde-3-phosphate and dihydroxyacetonephosphate, is split by muscle aldolase at about

80 per cent of the rate of splitting of the D isomer. D-fructose-1-phosphate is split by muscle aldolase at about 20 per cent and by liver aldolase at about 40 per cent of the rate of splitting of D-fructose-1,6-diphosphate by muscle aldolase (RUTTER, 1960). The affinity of muscle aldolase for fructose diphosphate is 100 times that for fructose-1-phosphate (TUNG and others, 1954). Neither of these hexose phosphates (sorbose-1,6-di- or fructose-1-phosphate) appears to form part of pathways with a high flux in mammals. This factor together with the lower affinity and maximum velocity of aldolase towards substrates other than fructose diphosphate make it unlikely that they offer appreciable competition.

Various aldehydes from the pentose pathway are theoretical competitors, but animal tissues contain sufficiently large amounts of the specific transaldolase or transketolase (HORECKER, 1961) to make competition unlikely.

Of all of the substrates for lactate dehydrogenase, only α-ketobutyrate, hydroxypyruvate and glyoxylate are reduced at rates comparable to pyruvate (MEISTER, 1950; CZOK and BUCHER, 1960). None of these substances occurs in pathways of high flux. Therefore it is unlikely that their presence would reduce the maximum

velocity of lactate dehydrogenase even if it could be shown that they were in the same vicinity in the cell. However, there is another variety of substrate binding which could reduce the amount of free enzyme; this is the binding of $NADH_2$ (CHANCE and NIELANDS, 1952).

(ii) Inhibitors and/or activators. The full activity of an enzyme might not be reached *in vivo* because of the presence of inhibitors, competitive or non-competitive. Accumulation of products may have the same effect. The importance of adrenalin in the action of phosphorylase is an example of an activator.

(iii) Molecular aggregation. The changes in the degree of aggregation and activity of glutamic dehydrogenase which can be produced *in vitro* may be of importance in controlling rates *in vivo* (see page 36).

Factors Affecting the Rate Constants. CHANCE (1961) states that 'the reaction velocity constants appear invariant in enzyme systems'. This is doubtless true, but because of the complexity of the protein molecule 'complicated effects may be encountered because of increased probability of aggregation, dissociation, changes in shape and ion-binding' (ALBERTY, 1956b). For these reasons, quoting ALBERTY again, 'an equation expressing reaction rate in terms of substrate concen-

tration at constant pH, metal ion concentration etc., is simply a specific case of the complete rate equation and ..... the values of the constants in such an equation will depend upon the pH, metal ion concentration etc.'. Furthermore, the ionisation and equilibrium constants used in enzyme kinetics are not the same as 'the thermodynamic equilibrium constants obtained for simple reactions.' In fact, they are not constant, but change with the net charge on the protein molecule. They are also affected by the activity of each of the reacting species.

There are insufficient data available to assess quantitatively possible differences between rate constants *in vivo* and *in vitro*. However, some information is available and this facilitates an attempt to put the problem into perspective.

Hydrogen Ion Concentration. The complexity of the cell interior includes the factors affecting hydrogen ion concentration. Thus apart from the effects of surfaces on the distribution of ions, protons are exchanged between molecules in the same or in different compartments of the cell in many reactions. The existence of the cell depends on their removal as water molecules. In fact, the complexity is such that the pH

conditions in the vicinity of the sites of reaction are unknown. The concentration of hydrogen ions in the cell was estimated to be 60 per cubic micron by NETTER (1934) and 30 by McLAREN (1960). The latter author is of the opinion that the number of hydrogen ions in the cell is so small that there is considerable point to point variation in their concentration.

Ionic Strength. One of the more complex parameters of chemical reactions is the ionic strength, a quantity defined by LEWIS and RANDALL (1921) as

$$I = 0.5 \sum c_i z_i^2$$

where I represents the ionic strength, and c_i and z_i the concentration and valence of each of the ionic species in the system. The effect of ionic strength on the rates of reactions catalysed by enzymes is well known; KISTIAKOWSKY and SHAW (1953) showed by means of the changes in urease activity, that the effects of ionic strength can be described by a modification of the Debye-Huckel equation.

The importance of ionic strength in reactions catalysed by enzymes is thus established. The actual value in the whole cell and particularly at sites of reaction remains uncertain, as with hydrogen ion concentration. It is conceivable that the large numbers of ions of varying concentrations, valencies and flux

result in a fairly constant average ionic strength in cells. However, it is difficult to avoid the conclusion that point to point differences are probable, particularly when there is a change of flux. These could have considerable effects on (i) the thermodynamic activity of the reactants and the activated complex, and (ii) the degree of ionisation of the acidic and basic groups of protein which in turn affect the activity of the enzyme (ALBERTY, 1956b).

APPRAISAL OF EFFECTS OF DIFFERENCES BETWEEN INTACT CELLS AND EXTRACTS OF TISSUES

The existence of compartments and of units of structure as small as molecular aggregates with specific functions indicate that the weighted mean value of the concentration of a reactant as obtained by analysis of an extract fails to provide even an approximation to its absolute concentration. HOLZER (1959a) expressed the difficulty as follows: 'in no case is the size of the "dissolving space" in which metabolites are contained within the cell known exactly'.

Concentration of Substrate

The inability to measure the absolute concentrations of substrates does not signify that the results of tissue analyses are meaningless. The evidence indicates that

the substrates being examined are confined to particular sites and sequences in the cell; there is reason to expect, therefore, that the ratios of the concentrations of substrates in a given sequence in different tissues may be similar.

Stability of the Resting Concentrations of Substrate

One of the chief doubts concerning the estimates of labile substances in animal tissues is the possible disturbance caused by obtaining the samples. The properties of the steady state, namely, stability, buffering capacity and accumulator function, all decrease the probability and extent of the effects of disturbances likely to accompany sampling. Thus it appears possible that the total amount of a substrate as determined in an extract is the same as that present in the tissue of origin in the resting animal.

Sources and Sinks of Substrates

These were shown to be chiefly in the Embden-Myerhof pathway for fructose diphosphate, dihydroxyacetonephosphate, pyruvate and lactate. Aldolase was shown to be the enzyme most commonly catalysing the reactions of both sugar phosphates. Pyruvate kinase is the enzyme most frequently associated with formation of pyruvate; however, the contribution of lactate dehydro-

genase from lactate returning from the blood-stream may be considerable in tissues other than muscle and especially in liver. Pyruvate is converted chiefly to lactate in muscle, but in other tissues the greater percentage probably enters the tricarboxylic acid cycle. Thus in spite of the complexity of the metabolism of the cell it is probable that the substrates discussed are each derived chiefly from one or two reactions. This information will simplify the interpretation of the analyses.

Maximum Activity of Enzymes

The difficulties of reconciling the maximum activity of an enzyme as determined *in vitro* with the maximum rates, actual or potential, *in vivo*, are well known (PARDEE, 1959; CHANCE, 1961). Very few data are available regarding the activities of enzymes and more important, the concentration of most reactants, in the cell. Various attempts have been made to relate the velocities obtained from whole cells to those obtained from homogenates or purified enzymes. Thus BECK (1958) observed that the rates of lactate and carbon dioxide production from [^{14}C] glucose were similar in whole leucocytes and homogenates. The enzymes of aromatic biosynthesis have the activities *in vitro* expected from

the synthetic rates *in vivo* (PARDEE, 1959). CHANCE (1961) obtained the maximum *in vivo* activities in mitochondria for both respiration and glycolysis by uncoupling oxidative phosphorylation. As he remarks, 'these plateau values may be greater or less than those that would be obtained in the enzyme system *in vitro*, but they must be the ones that apply to the operation of the enzyme system *in vivo*'. The only attempt to relate directly the *in vitro* and *in vivo* velocities of a single enzyme is that of HOLZER (1961). He observed that the maximum rate of decarboxylation of pyruvate in intact yeast is approximately the same as that obtained from purified decarboxylase derived from a comparable amount of yeast when it is allowed to react in a system containing the amounts of pyruvate, hydrogen ion and acetaldehyde present in the intact yeast.

Thus the experimental information available indicates that activities *in vivo* may reach those *in vitro*. However, it has been suggested by RACKER (1954), KREBS and KORNBERG (1957) and WEBB (1960) that the maximum rates may be attained rarely *in vitro*. This is because the conditions used *in vitro* are optimal with respect to the substrate, co-factors, pH, ionic strength and any other factor known to affect the rate. Contrariwise, in the cell the amount of substrate may not reach saturating levels and there is competition for both substrate and co-factors by other

enzymes. In addition, the rate constants may be affected by the local hydrogen ion concentration, ionic strength, the presence of surfaces, the oxygen tension and the thermodynamics of the open system. The evidence available presently is insufficient to reach any conclusion except that the rates *in vivo* are not likely to exceed those observed *in vitro*.

As with substrates, the absolute maximum velocity of enzymes in the cell cannot be determined because the actual concentrations of all reactants within the compartments or particles are unknown. But where a particular metabolic sequence common to a number of tissues is concerned, it is conceivable that the activities of the enzymes in the sequence in each tissue will have approximately the same ratios; if this were true, then the relative maximum activities of a single enzyme in different tissues would be a useful guide as to the relative maximum flux through the given pathway in those tissues. The extent of any correlation between the ratios of the activities of serial enzymes in the same pathway in different tissues depends on whether the corresponding enzymes in each tissue are identical. It is often taken for granted that because two enzymes isolated from different tissues are specific

for the same substrate, all of their properties including the K_m and the maximum velocity are identical. There is increasing evidence suggesting that certain enzymes occur in more than one form, each having specific catalytic properties. One of these is lactate dehydrogenase for which five molecular forms were demonstrated by immunological techniques (CAHN and others, 1962). Another is aldolase which has a different specificity towards fructose-1-phosphate and fructose-1,6-diphosphate, according to whether it occurs in muscle or liver (RUTTER, 1961). These differences will be discussed more fully later.

III

THE DISTURBANCE ASSOCIATED WITH ANALYSIS OF ANIMAL TISSUE

The estimation of the amounts of substrates and the activities of enzymes involves removal of samples of tissue and preparation of extracts. The problems relating to substrates and enzymes are sufficiently different to be treated separately.

SUBSTRATES

The concentrations of many substances in animal tissues are easily altered. The procedures used to obtain and to process samples of its tissue inevitably disturb first the animal and then the samples of its tissue. The objects of this section are to inquire into the means of obtaining data regarding the amounts of labile substrates in the tissues of the resting rat and of assessing the amount of disturbance produced by the sampling procedure.

Means of Obtaining Data

Information regarding substrates is of value only if it can be related to a definite state of metabolic activity. The ideal level of activity is the resting state. This is because (a) it is a state in which each tissue is at a comparable level of its range of activity, (b) it is a steady state, (c) it is reproduced more

readily than any other given state of activity and (d) it is probably the standard state in relation to which homeostatic mechanisms function.

Removal of Tissue

It is necessary to avoid disturbing the animal from the time that it is removed from its cage to the time when the sample is taken. This is the most difficult aspect of the whole analytical procedure. No systematic attack on the problem appears to have been made. The technique of obtaining a sample of tissue is described usually without reference to the effect of any disturbance produced. All methods involve the production of more or less rapid loss of consciousness as the first step, followed by removal of the sample. The methods of producing unconsciousness which will be described are anaesthesia, guillotining and freezing the whole animal.

Anaesthesia. The advantages of anaesthesia are twofold. First, there is time to obtain access to all tissues and to carry out various experimental procedures. Secondly, there is no major physical assault on the animal; this eliminates bleeding, respiratory embarrassment and intense nervous stimulation. The disadvantages of anaesthesia are also of two kinds. There may be a

direct effect on the enzyme systems in the tissue to be sampled; also, either the anaesthetic itself or fear associated with its administration may result in a larger output of catechol amines from the adrenal medulla.

The account which follows will be limited to diethyl ether and the barbiturates because they were the anaesthetics used in the investigation.

General effects on carbohydrate metabolism. The foundation to the understanding of the action of anaesthetics was laid in the nineteen twenties and thirties. RONZONI, KONCHIG and EATON described the rise in blood lactate during ether anaesthesia in 1924. EVANS, TSAI and YOUNG (1931) and MURPHY and YOUNG (1932) remarked the fall in the glycogen content in the liver of the cat after prolonged anaesthesia produced by means of various barbiturates and ether; the effects of ether are manifest in the first 6 minutes of anaesthesia. Muscle glycogen decreases up to 55 per cent after two to three hours of ether anaesthesia, but little change is found after one hour of amytal anaesthesia (MAJOR and BOLLMAN, 1932).

Specific effects on actions of enzymes concerned with carbohydrate metabolism. The effect of drugs on

enzymes is nicely stated by HUNTER and LOWRY (1956): 'All drugs have at least indirect effects on enzyme systems, and few systems are not affected ultimately by some drug'. QUASTEL and his colleagues have done a great deal of work on the effects of narcotics on the enzymes concerned with glycolysis and respiration. They used brain slices chiefly, but there is no reason to believe that in other tissues the effect of drugs on a given enzyme sequence are different.

(a) Nature of effect. JOWETT and QUASTEL (1937a) were the first to demonstrate the inhibition of respiration produced by anaesthetic doses of hexobarbitone and phenobarbitone in slices of brain, kidney and diaphragm. Ether was not found to have an effect at concentrations sufficient to produce surgical anaesthesia (JOWETT and QUASTEL, 1937b).

High concentrations of pentobarbitone inhibit some of the dehydrogenases of the Embden-Myerhof pathway and the tricarboxylic acid cycle (PERSKY and others, 1950). At anaesthetic concentrations, however, a wide range of barbiturates including pentobarbitone, was shown to have little effect on anaerobic glycolysis <u>in vitro</u> (WEBB and ELLIOTT, 1951). This view is shared by QUASTEL (1953) and HUNTER and LOWRY (1956). The state

of activity of the tissue affects the sensitivity to depressants. McILWAIN (1953a) observed that electrical stimulation of brain slices increases both the oxygen consumption and the sensitivity of respiration and glycolysis to depression by phenobarbitone. This observation renders the absence of any effect of a drug on tissue in the extreme state of rest of the tissue slice of doubtful value.

(b) Site of action of depressants of the central nervous system. HUNTER and LOWRY (1956) reviewed the evidence indicating that the site of action is in the electron transfer system at the site of transfer from NAD or NADP to cytochrome b. HULME and KRANTZ (1954) have evidence which indicates that ether produces some uncoupling of oxidative phosphorylation.

(c) Time factor. (i) Barbiturate anaesthesia. The routes for administration of barbiturates, namely, the rectal or the intraperitoneal, result in slow absorption. This allows the drug to act for a relatively long time, that is, at least 5 - 10 minutes. (ii) Ether anaesthesia. The time required for anaesthesia in the rat is 60 - 90 seconds. Depression of oxidation of glucose in slices of rat brain by 0.031 molar ether occurs only after 40 minutes (JOWETT and QUASTEL, 1937b).

The concentration required for anaesthesia is 0.02 molar. Thus depression of oxidation is unlikely to affect the concentrations of carbohydrate intermediates within the time required for sampling of tissues.

Effects of anaesthesia mediated via the adrenal medulla. (a) Evidence indicating that the medulla is stimulated by anaesthetics. (1) Emotional and/or physical stimulation. CANNON and de la PAZ (1911) demonstrated that fear produces an increase in the output of catechol amines from the adrenal medulla. CANNON and BRITTON (1927) showed that fright, rage, pain, asphyxia, anaesthesia, exercise, exposure to cold and stimulation of sensory nerves produce an increase in the rate of the denervated heart of the cat; adrenalectomy prevented these effects. More recently EULER and his colleagues observed increased amounts of both adrenaline and noradrenaline in the urine of men one to two hours after exercise and during aeroplane flights (1952, 1954). CRAWFORD and LAW (1958) made similar observations on 24-hour urine samples of rats given a single injection of sodium chloride subcutaneously. It is concluded that even if great care is taken in handling animals and administering anaesthetics, the probability of some stimulation of the adrenal

medulla is high.

(ii) Specific effects of ether. CANNON and de la PAZ (1911) showed that the blood in the inferior vena cava of a cat under ether anaesthesia contained increased amounts of adrenaline. ELLIOTT (1912) observed that there is a decreased output of adrenaline from the adrenal gland after prolonged ether anaesthesia. He remarked also that splanchnic section abolished the effect of ether. WATTS (1955) noticed an increase in the content of the catechol amines in the blood of rabbits given sufficient ether to produce surgical anaesthesia. CRAWFORD and LAW (1958) showed that exposure of rats to ether for 10 minutes is sufficient to produce a significant increase in the 24-hour output of catechol amines in the urine; this increase is prevented by previous adrenalectomy. The need for an intact sympathetic system, as first intimated by Elliott, has been confirmed by several investigators (BHATIA and BURN, 1933; JOHNSON, 1949; BREWSTER, BUNKER AND BEECHER, 1952). It is concluded that ether stimulates sympathetic nerves, which transmit the effects to the adrenal medulla.

(b) Action of catechol amines on carbohydrate metabolism. (i) Nature and site of action. The glycolytic action of adrenaline on liver was observed by

VOSBURGH and RICHARDS in 1903, and in muscle by TOLSTOI and others in 1923-4, and CORI and CORI in 1928. It was shown in liver slices, to be due to the activation of phosphorylase, the enzyme which catalyses the degradation of glycogen to glucose-1-phosphate (SUTHERLAND and CORI, 1951). The same action was demonstrated subsequently in diaphragm, and in slices of heart, uterus, bladder, cerebral cortex and kidney (ELLIS and others, 1957). In liver the degradation proceeds as far as glucose which passes into the blood; in muscle the reaction proceeds as far as lactate which also enters the blood (CORI, 1931). Glucose-6-phosphate was observed in increased amounts, following the action of adrenaline in both muscle (CORI and CORI, 1931; ELLIS and others, 1957) and liver (SUTHERLAND and CORI, 1951). The blood is found to contain increased amounts of pyruvate, α-keto-glutaric acid and citrate (PANSINI and CAIRELLA, 1953; HENNEMAN and others, 1955) as well as the increases in glucose and lactate already described.

(ii) Time factor. CORI, CORI and BUCHWALD (1930) showed that liver glycogen starts to decrease within 15 minutes of subcutaneous injection in a conscious animal. They also showed that the blood sugar level in the anaesthetised rabbit does not increase until 4

minutes after giving a physiological dose of adrenaline intravenously. In man it was remarked that the blood lactate fails to rise until 10 minutes after the infusion of adrenaline (BEARN and others, 1951). In contrast to these observations, ELLIS (1956) states that he observed that adrenaline injected intravenously activates hepatic phosphorylase maximally within one minute. Furthermore, he points out that the sharpest part of the hyperglycaemia curve is during the first minute after intravenous infusion.

(iii) Conclusions. The action of adrenaline appears to be limited to the activation of phosphorylase. The purpose of this reaction in the liver appears to be the production of increased amounts of glucose for metabolism by peripheral tissues; however, it is possible that some of the glucose-6-phosphate formed enters the main glycolytic pathway. The concentrations of these intermediates in the liver and other tissues could be affected also as a result of an increased amount of lactate returning via the blood-stream from muscle. This would obviously take some time, and there is no evidence indicating that it occurs to a significant extent in the first one to two minutes of anaesthesia. In muscle the formation of lactate is preceded by the

formation of fructose diphosphate, dihydroxyacetone-phosphate and pyruvate, among other substances. If large amounts of adrenaline reach the muscles before they are sampled, increases in the concentrations of all of these intermediates might occur.

Guillotining. The use of the guillotine offers several definite, but limited advantages over anaesthesia. It is possible to decapitate a rat without any apparent disturbance before the decapitation, to open the animal's belly, remove a sample of liver and immerse the sample in liquid nitrogen in 7 to 10 seconds (THRELFALL and STONER, 1962). This technique is suitable for a tissue which is easily accessible, for example, liver. It cannot be used in order to obtain resting samples of muscle because the violent clonic spasms resulting from the stimulation of the central nervous system are associated with rapid and extensive glycolysis. Decapitation with reception of the head directly into liquid nitrogen is suitable for obtaining samples of brain (MANDEL and HARTH, 1961).

There are several reasons why guillotining might affect the amounts of labile substances in the liver. Decapitation produces exsanguination, cessation of respiration and stimulation of the central and autonomic

nervous systems. Exsanguination and cessation of respiration both produce anoxia of all tissues. The result is that electron transport together with respiratory chain phosphorylation of adenosine diphosphate ultimately cease. Likewise the reactions of the tricarboxylic acid cycle stop. The small reserves of oxygen dissolved in the liver may delay these effects for sufficient time to enable the enzymes to be inactivated by freezing.

Exsanguination also prevents the normal exchange of the small molecules concerned with practically every aspect of the function of the cell. This will ultimately become a limiting factor in all reactions, but its effect in the interval between decapitation and freezing cannot be assessed.

Stimulation of the whole nervous system has widespread effects of many kinds. (a) Direct effects. Stimulation of the hepatic plexus after adrenalectomy, or of nerves in the cut hepatic pedicle produces hyperglycaemia by inducing glycogenolysis in the liver (BEST and TAYLOR, 1945). (b) Indirect effects. Stimulation of the adrenal medulla via the sympathetic system produces the well known glycogenolytic effects of adrenaline in both liver and muscle. This is unlikely to occur in the decerebrate animal because of circulatory

impairment. Stimulation of the central nervous system results in strong clonic contractions of all muscles in the animal. The products of glycogenolysis from muscles, whatever the source of stimulation, are carried to the liver where glycogen is reformed; again, this is unlikely to occur in the decerebrate animal.

Freezing the whole animal. This method of producing unconsciousness was started by STONE (1938) in order to investigate the effects of anaesthetics on the lactate content of brain. Freezing the whole conscious animal is used in order to avoid anaesthesia and to fix the concentrations of metabolites. It can hardly be doubted that the freezing of a conscious animal produces intense nervous and adrenal medullary stimulation. Therefore, in order to minimise these effects it must be carried out rapidly; this means that only small animals are suitable. When a rat of weight 250 gram is frozen in liquid air, the time taken for its stomach to reach 0° is approximately 40 seconds (LePAGE, 1946). When a rat of 40 gram is frozen in liquid air, the time taken for the deep parts of the brain to reach 0° is 9 to 20 seconds (DAWSON and RICHTER, 1948). The time required for loss of consciousness is only 2 - 3 seconds. The remaining time is taken up by the cooling process. It

seems probable that the advantages obtained by avoiding anaesthesia may be lost because of the inevitably slow cooling of an object the shape and size of a 40 gram rat.

Fixation of Tissue

The fixation of tissue for biochemical purposes has as its object the preservation of the concentrations of metabolites as they occur in life. This may be carried out by exposing the tissue to extremes of temperature or pH, to enzyme poisons, or by combining these procedures. The method selected will depend on the substances to be estimated. The most effective method for heat-labile, acid-stable substances, and this includes those of interest in this project, is rapid freezing followed by extraction in acid (HOHORST and others, 1959).

Early attempts to fix the content of phosphocreatine, glycogen and lactate in brain by immersing samples in liquid air were unsatisfactory (McGINTY and GESELL, 1925; KINNERSLEY and PETERS, 1929). KERR (1935) improved on this technique by pouring liquid air on to the exposed brain *in situ*. STONE (1938), as indicated already, observed that immersion of the whole animal in liquid air gave similar results. However, the results obtained by means of these devices remained suspect and LePAGE

(1946) stated that freezing animals larger than mice should be preceded by anaesthesia, thus defeating the original object.

Rapid freezing by means of pre-cooled metal tongs. The use of rapid freezing of tissue for the fixation of the concentrations of the metabolites was obviously an advance in technique. However, it was not until much later that a fundamental approach was made. ERANKO (1954) pointed out that the immersion of a piece of tissue in liquid air or oxygen was not an efficient way of cooling. The rate of cooling of the centre of an object depends on its dimensions, the temperature gradient, and density, specific heat and heat conductivity of both the tissue and the coolant. The values for the intensity factors for water, copper, aluminium and oxygen are presented in table I (Handbookof Chemistry and Physics, 42nd edition, 1961). There is relatively little difference in the amount of heat required to raise the temperature of $1cm^3$ of any of these substances by 1°. (This quantity is measured by the product, specific heat x density). However, the heat conductivity of copper and aluminium, respectively, is about 20,000 and 10,000 times that of liquid oxygen. It is clear that the transfer of heat from tissue to liquid oxygen is slower than it would be to copper or aluminium. The rate

Table 1

Physical properties of substances relevant to cooling of animal tissues by means of pre-cooled metal tongs

Substance	Density g./cm^3		Specific heat cals./g./1°		Approximate no. cals. required to heat 1 cm^3 1°	Conductivity cal./(sec.)(cm.^{2})(1°/cm.)	
Water	1.00	4°	1.000	14°	1.00	0.00135	0°
			0.329	-100°	0.33		
			0.162	-200°	0.16		
Copper	8.50	20°	0.092	20°	0.78		
			0.078	-100°	0.66		
			0.050	-189°	0.43	1.09700	-160°
Aluminium	2.70	20°	0.214	20°	0.58		
			0.168	-100°	0.45		
			0.076	-200	0.20	0.51400	-160°
Oxygen	1.14	-184°	0.229	-181°	0.26		
			0.394	-200°	0.45	0.00005	-200°

of heat transfer from a solid to a volatile coolant such as liquid oxygen is reduced even further because there is usually a layer of ice and always a layer of gaseous oxygen separating them.

Eranko overcame all of these disadvantages by designing a pair of tongs fitted with a copper block at the ends of each blade. The tongs were pre-cooled in liquid air and then used to grasp and to compress the tissue. The sample of tissue obtained measures approximately 10 x 10 x 1 mm. and loses about 20 calories as the temperature falls from 37° to -150°. This is associated with a rise of about 40° in the temperature of the copper blocks. Compression of the tissue to about 1 mm. is important because, as already indicated, the rate of cooling of the centre of an object depends on its distance from the coolant.

The rate of cooling of tissue obtained in this way was investigated by WOLLENBERGER and others (1960). The metal blocks were made from aluminium and the tongs were pre-cooled in liquid air. Thermocouples were placed in the tissue which was then compressed to about 0.7 mm. by means of the pre-cooled tongs. The time required for the temperature of the tissue to fall from 38° to 0° was 0.1 seconds, and from 0° to -80°, a further 0.1 seconds.

Comparable times of cooling for tissue immersed in liquid air at approximately -190° and in isopentane at -150° were 16 and 4 seconds, respectively, to reach 0° and a further 3 and 10 seconds, respectively, to reach -80°.

It is necessary to inquire whether there are any disadvantages associated with fixation of substrate levels by rapid freezing. When freezing is slow the intracellular water passes progressively into the extracellular space as the extracellular water freezes (MERYMAN, 1957). Thus the intracellular water becomes hypertonic and reactions could theoretically be speeded up because of the increased concentration of the reactants. However, when freezing is rapid there is very little time for reaction. For example, when tongs, precooled to -190° are used for cooling, the time taken for the temperature to decrease from 0° to -80° is 100 milliseconds. In fact, the evidence available indicates that reactions proceed more rapidly in unfrozen tissue. Thus the pyruvate content is lower and the lactate content higher in liver extracted directly into acid than it is in liver which is frozen before being extracted (HOHORST and others, 1959).

Assessment of Value of Data Obtained by Analysis

There is no way of knowing with certainty that the results of an analysis of animal tissues represents the resting *in vivo* state. However, a number of potential sources of variation of substrate can be eliminated readily, for example, differences due to strain, sex, age and diet. In the absence of markers of the resting levels of metabolites it is necessary to use indirect methods of assessing the significance of analyses.

The Time Factor: The Times of Chemical and Physical Reaction in Relation to the Time Required to Obtain and Fix a Sample of Tissue

CHANCE (1943) measured the reaction times of a number of reconstituted enzyme systems. He found that the formation of a peroxidase-hydrogen peroxide complex in the presence of ascorbate, an oxygen acceptor, occupies 50 to 100 milliseconds; the decomposition of the complex takes a few seconds, depending on the relative amounts of substrate and acceptor. The times in whole cells are much longer. The time for 50 per cent reduction of the pyridine nucleotides in starved yeast, for example, is about 5 seconds after the addition of glucose (CHANCE, 1953-4). In a similar experiment, the amount of fructose diphosphate formed by starved yeast was found to be

only about 30 per cent of the maximum after 20 seconds (HOLZER, 1959b).

These observations are pertinent, but they are derived from simple systems and were obtained under special conditions. In an animal the total reaction times are affected by the nervous and circulatory systems. The reaction time of the nervous system, including humoral transmission at nerve endings, is measurable in milliseconds. On the other hand, the circulation time from the adrenal medulla to an effector organ in the rat is approximately 5 to 10 seconds. The time required for reaction to adrenaline injected intravenously is uncertain, but ELLIS (1956) believes it to be less than one minute.

The time required to guillotine a rat and to obtain a sample of liver is 7 to 10 seconds and the time taken to freeze tissue in Eranko's tongs is about 200 milliseconds. Thus the release of adrenaline from the adrenal medulla is not likely to affect tissue obtained by decapitation. There is still time for anoxic effects and/or release of adrenaline at sympathetic nerve endings in the liver.

The time required to anaesthetise a rat with ether is a minimum of one to two minutes. No data are

available regarding the early release of adrenaline from the medulla after inhalation of ether vapour. Therefore, the potential effects of ether cannot be assessed.

Comparison of Results Obtained by Different Methods of Sampling and Fixation

The probability that the results obtained by a given method of sampling tissue are close to the *in vivo* values would be increased if another method which disturbed the animal in a different way gave the same results. For example, it is possible that ether might affect the yields of intermediates via increased adrenaline secretion and thence increased muscle glycogenolysis. Decapitation would be more likely to affect primarily respiration, electron transport and the concentrations of NAD and ATP; glycolysis would not be affected until later.

The lactate content of the brain of mice frozen without anaesthesia (STONE, 1938) is similar to that of the brains of cats and dogs frozen *in situ* under anaesthesia (AVERY and others, 1935). The percentage of adenosine phosphates present as the diphosphate was found to be approximately the same in the brains of mice frozen without anaesthesia and in the brains of rats

whose heads were cut off with or without chloral anaesthesia and allowed to fall into liquid nitrogen (MANDEL and HARTH, 1961). In these two instances the results obtained after different methods of producing unconsciousness are confirmatory.

In other tissues, for example in liver and muscle, comparisons fail to provide confirmation. Thus the pyruvate content of rat liver obtained by direct fixation in acid is about two thirds of that fixed first by freezing the tissue by means of pre-cooled tongs (HOHORST and others, 1959). The fructose diphosphate, pyruvate and lactate contents of rat liver obtained after guillotining are less than those obtained after pentobarbitone anaesthesia (THRELFALL and STONER, 1961).

Attempts to compare the results obtained by different investigators are not helpful; the problem is complicated because of different techniques being used for every stage of the sampling analysis. Even when the results appear to be consistent for one tissue, for example, pyruvate and lactate in rat muscle, they diverge disconcertingly in rat liver (LePAGE, 1948; BUCHER and KLINGENBERG, 1958; THRELFALL and STONER, 1961).

Correlation of Results Obtained With Those Expected on Theoretical or Experimental Grounds

The lactate concentration has been used as an indicator of the amount of departure from the normal resting state since it was shown to accumulate in muscle after exertion (FLETCHER and HOPKINS, 1907) and in many tissues during anaerobic glycolysis (WARBURG, 1926). However, the lactate level is, at best, a crude index of normality. This is because it is the 'sink' of an open system: any increase in the rate of glycolysis is manifest as an increase in the concentration of lactate but there is not necessarily a corresponding increase in the concentrations of the intermediates preceding lactate. Furthermore, lactate is produced chiefly by skeletal muscle from which it enters the blood and the extracellular fluid and ultimately the cells of many tissues. Thus the lactate content of a tissue is not only a crude index of normality, but it may be misleading.

The use of a relative index rather than an absolute one has more to offer. It is possible that this exists for assessment of estimates of the content of intermediates in brain. Thus it appears that adenine should be present chiefly as the triphosphate, and to a limited extent as the monophosphate (MANDEL and HARTH, 1961).

ENZYMES

The term 'activity' as used in relation to enzymes is applied to the maximum activity obtainable from a homogenate and referred to the weight of fresh tissue or to the total nitrogen content. The usefulness of estimates of activity in crude extracts of tissues depends on (a) the completeness of extraction of the enzyme in the native state, and (b) the possibility of measurement of the activity of a single enzyme in the presence of other enzymes and of potential inhibitors.

Completeness of Extraction

There is no way of testing the completeness of extraction of an enzyme. However, as a result of the intensive research into the preparation and purification of enzymes over the past 40 years, it is possible to prepare and manipulate homogenates of tissues with reasonable assurance that excessive losses of enzyme activity have been avoided.

Measurement of Activity of a Single Enzyme in a Homogenate

The possibility of measuring the activity of a single enzyme in the complex mixture of enzymes present in a homogenate was converted into a reality by POTTER

and ELVEHJEM (1936) and POTTER (1946a & b).

Dilution of the Homogenate

This procedure eliminates many reactions and facilitates the observation of the reaction of interest under zero order conditions. These are conditions where the rate of the reaction is directly proportional to the concentration of the enzyme, that is, to the amount of homogenate added to the assay mixture. The validity of the method depends also on the rate being linear and being controlled by one specific enzyme, that is, the one whose activity is being assayed (POTTER, 1946b). The method can be criticised on two grounds. One is that non-competitive inhibitors cannot be diluted out and they would, therefore, result in an underestimate of the maximum activity. The other is that another enzyme acting on the same substrate cannot be diluted out and it could result in over-estimation of the maximum activity. The possibility of the latter artefact is reduced by the specificity of the conditions of assay.

Addition of Essential Reactants

The co-factors and conditions essential for the measurement of the maximum activity must be known (POTTER, 1955). A recent example which illustrates the importance of the full knowledge of the essential

conditions is the discovery of some of the factors required for the activation of phsophorylase by adrenaline. These are an activating enzyme, ATP and particles sedimentable at low speed from a homogenate (RALL and SUTHERLAND, 1959).

It is just as important to avoid the addition of reagents which might alter the activity in a way not occurring in the cell. For example, phosphate ions have been shown to affect both the maximum initial velocity and the K_m of the hydration of fumarate and the dehydration of L-malate as catalysed by fumarase (ALBERTY and others, 1954). The activity of triose-phosphate isomerase is reduced to 25 per cent in the presence of 0.05 molar phosphate (BEISENHERZ and others, 1953). Ions of the reaction medium can affect velocities either by (a) reacting with other reactants and so producing a new term in the rate equation, or (b) affecting the rate constants via alterations in the activity coefficients of the reactants (ALBERTY, 1959).

The reaction of phosphate with protein is an example of the more general reactivity of proteins with anions. Therefore the maximum activity, as determined <u>in vitro</u> could be misleading, unless the effects of buffer ions are known.

PART 2

EXPERIMENTAL DETERMINATION OF THE CONCENTRATIONS OF SUBSTRATES AND THE ACTIVITIES OF ENZYMES

IV

HUMAN COLON AND RECTUM

The investigation of human colon is considered first, and separately, because the problems associated with the extraction of enzymes were experienced and overcome in this tissue. In addition the substances determined in the colon differed from those estimated in the tissues of the rat. Finally, a method of separating the epithelial cells from the supporting connective tissue was developed in the colonic mucosa and carcinoma.

MATERIALS AND METHODS

The Tissue

Carcinoma of the Large Intestine

Samples of carcinoma and the apparently normal mucosa removed with it were obtained chiefly from St. Mark's Hospital, London E.C.1. Of the 27 samples described in the "Results", 19 were situated in the rectum, 6 in the pelvic and sigmoid colons, one in the transverse colon and one in the caecum. In 22 specimens the tumour was in the form of an ulcer with a scirrhous base and everted edges; the remainder were fungating masses. The length of apparently normal bowel resected in addition to the tumour varied from 10 to 30 cm.

Multiple Polyposis

Nine specimens were obtained from St. Mark's Hospital where Dr. Cuthbert Dukes and Dr. Basil Morson are carrying out an investigation into many aspects of this disease. The whole colon was excised in all cases. Polyps were present throughout the length of every specimen. They varied in size from 1 to 2 cm. to 1 mm. or less in diameter; those less than 3 to 4 mm. in diameter were usually sessile and those greater were usually pedunculated. The number of polyps varied greatly. In one specimen the appearance resembled a dense bed of sea-weed whereas in another there were only 3 to 4 polyps visible on casual inspection. Both number and size of the polyps were greater in the rectum.

The condition of the specimens differed greatly according to the delay between the time of excision and the time of reception in the laboratory; this varied from 5 to 45 minutes. Correspondingly the specimens were warm and contractile or cool and inert.

All specimens were treated as follows. The mesentery and/or sub-peritoneal fat was removed, the antimesenteric taenium was incised longitudinally and the mucosa was wiped clean by means of filter paper. The specimen was pinned out on a bark board. The mucosa was separated from the wall of the colon at the level of the muscularis mucosae by means of a tooth brush, the bristles of which were cut to 0.5 cm. in length. Samples of carcinoma were taken from the least

necrotic and most cellular parts of the tumour; they were trimmed free of necrotic and haemorrhagic areas. Whole polyps were removed in the cases of multiple polyposis. All samples were placed in test tubes and stored in ice.

Extraction of Enzymes

There is no way of ascertaining when complete extraction of the enzymes from a tissue is achieved. It is assumed that the point is reached when the yield cannot be increased further. Extraction of a tissue involves physical destruction of its fabric always, and chemical destruction sometimes. The latter, known as denaturation in the case of proteins, must be avoided; again, the only guide is the highest attainable yield.

It is clear that the efficacy of the extraction process must be balanced against the probability of denaturation. A homogeniser which functions by cutting the tissue with blades is unsuitable because it causes frothing; this increases the extent of the surface and denatures the enzymes at the same time. The homogeniser designed by POTTER and ELVEHJEM (1936) is suited to the production of disruption without denaturation. It produces its effects by the generation of shearing forces between the pestle and the glass tube, Frothing is avoided by preventing the pestle from leaving the suspension during homogenisation.

A standard procedure was adopted after numerous preliminary experiments were carried out. Approximately 1 g.

tissue was weighed accurately into a heavy glass homogeniser tube. A volume of 0.25 M mannitol, equal to nine times the weight of the tissue, was added by means of a pipette. The tissue was then disrupted by the action of a plastic pestle which was rotated at high speed by means of a 1/4 horse-power motor. Forty runs of the pestle against the tube were sufficient; the amount of enzyme present in the supernatant was not increased by more homogenisation. During this manipulation the glass tube was held in a plastic bottle containing melting ice; this prevented the contents of the homogeniser becoming warm and also protected the hands of the operator. Many of the tumours proved very difficult to homogenise because of the content of hard fibrous tissue; this necessitated cutting them into pieces 1 to 2 mm. in length with scissors before homogenisation.

After the homogenisation was completed separate aliquots were removed for the estimation of deoxyribonucleate phosphorus, total nitrogen, and the activities of the enzymes. The aliquot destined for the estimation of aldolase was spun in a refrigerated centrifuge at approximately 500 x g at 2° for 5 minutes and the supernatant was removed and used immediately or stored up to 3 days before use. The time lapsing between the removal of the tissue and the estimation of aldolase activity was about 6 hours. The aliquot reserved for the estimation of the activity of deoxyribonuclease was

frozen and thawed twice in order to release the fraction of the enzyme which remains in the lysosomes (de DUVE and others, 1955); it was then spun as indicated for aldolase and the supernatant was removed. The activity of lactate dehydrogenase was determined on this supernatant also.

Analytical Methods

All the methods used are well known and do not require to be described in detail. Any alterations in technique or any difficulties encountered are indicated.

Aldolase

The method used was that of SIBLEY and LEHNINGER (1949). It depends on trapping the products of the cleavage of fructose diphosphate, namely dihydroxyacetonephosphate and glyceraldehydephosphate, as hydrazones and their subsequent estimation as dinitrophenylhydrazones. The reaction is particularly suitable for assays in crude extracts of tissue because it is free from the side effects due to the action of other enzymes to which reactions linked to $NADH_2$ are susceptible. The original method was followed with the exception that 20 per cent trichloracetic acid was used to stop the reaction after incubation. The colour of the chromagen fades rapidly; therefore the readings were always made at a known time, namely 10 minutes, after the addition of the last reagent. The substrate calibration curve was verified and found to agree with that of Sibley and Lehninger. The rate was linear up to extinctions of at least 1.0. The

amount of triosephosphate formed was calibrated by plotting the extinction of the triose-2,4-dinitrophenylhydrazone against that of the alkali-labile phosphate as suggested by the authors.

The result is expressed as micromoles of fructose diphosphate split per minute per gram of fresh tissue (or per mg. N) at 37° and pH 8.6. It is obtained from the solution of the following equation. Velocity == activity ==

$$\frac{\text{Extinction observed}}{\text{Extinction equivalent to 1 µg. P}} \times \frac{\text{Dilution factors}}{\text{2 x time of incubation (min.) x molec. weight P}}$$

The factor, 2, appears in the denominator because 2 moles of triose-phosphate are formed from each mole of hexose diphosphate.

Reagents and equipment. Fructose-1,6-diphosphate, calcium dihydrogen salt (British Drug Houses Ltd., Poole, Dorset), 0.05 M, pH adjusted to 8.6 by addition of 2.0 M sodium hydroxide. The precipitate of calcium phosphate was removed by centrifuging. The solution keeps for 4 weeks at 2°. Hydrazine, 0.56 M, prepared by dissolving hydrazine sulphate in 2.0 M, sodium hydroxide until the pH is 8.6 and then making the volume up to the mark. Tris-HCl buffer, 0.1 M, pH 8.6. Sodium hydroxide (AR), 0.75 M. Trichloracetic acid, 20 per cent w/v. 2,4-dinitrophenylhydrazine, 1 g. dissolved in 1 litre of 2 M HCl. Water bath, 37°. Glass cuvettes, 10 ml. Photoelectric colorimeter with Ilford 625 green filter.

Lactate Dehydrogenase

The method of KUBOWTIZ and OTT (1943) as described by KORNBERG (1955) was used. It depends on the oxidation of $NADH_2$ which occurs when pyruvate is reduced to lactate in the presence of lactate dehydrogenase, and it is suitable for use in crude extracts of tissue. All reactants except the enzyme are provided in relative excess; the rate then depends on the amount of the enzyme. The original method was followed exactly. Calibration curves for pyruvate and $NADH_2$ were made; they agreed with those prepared by Kubowitz and Ott. The temperature coefficient was measured by observing the rates in the presence of purified reagents between 17° and 29°; this was used to construct a calibration curve from which all rates were corrected to 25°. The reaction was followed at 340 mμ for 3 minutes, readings being taken every 30 seconds. Rates of change of extinction of 0.02 to 0.05 per minute were used. The change in extinction with time was plotted and a line fitting the points was drawn. A tangent to the curve between 30 and 90 seconds was then drawn and the rate of change of extinction for this period was measured. The solution of the following equation gives the activity in micromoles of pyruvate transformed per minute per gram of fresh tissue at 25° and pH 7.4.

Velocity == activity ==

$$\frac{\text{Change in extinction during 1 minute}}{6.22 \times 10^3} \times \frac{\text{Volume in cuvette}}{1000} \times \frac{10}{\text{Vol. 1:9 brei used}}$$

where 6.22×10^3 cm^2. == molecular extinction coefficient of $NADH_2$ at 340 mμ.

Reagents and equipment. Sodium pyruvate (L. Light and Co. Ltd., Colnbrook, Bucks.) was found to contain 95 per cent pyruvate by enzymic analysis (p.174); this compares with 97.9 per cent found by the retailer's analyst who used a perchloric acid titration. The commercial product was purified by recrystallising twice. 5 g. were dissolved in a minimum amount of water and cold ethanol was added to 80 per cent of the volume. The crop of crystals was filtered with aid of a Buchner funnel plus suction. The crystals were washed first in ethanol and then in di-ethyl ether (AR quality). Finally they were dried in a vacuum dessicator and stored over calcium chloride at -20°. $NADH_2$ (Sigma Chemical Co., St. Louis, Mo., U.S.A.) was obtained in batches of 100 mg. It was stored in lots of 2.5 - 3.5 mg. in sealed glass ampoules at -20°. Phosphate buffer, 0.1 M, pH 7.4. The solutions of sodium pyruvate and $NADH_2$ were made up in phosphate buffer just before use. The reference cell used in the estimation contained sodium dichromate solution diluted so that the test solution gave a reading of approximately 0.3 on the spectrophotometer (Zeiss PMQ II). A plastic stirrer of the type described on page 165 was used to intro-

duce and mix the substrate with the contents of the test cuvette.

Deoxyribonuclease

The method used was based on the modification made by SCHNEIDER and HOGEBOOM (1952) to the procedure described by KUNITZ (1949) for the estimation of the activity of the enzyme with purified reagents. It depends on the increase in the extinction of a solution of deoxyribonucleate when it is incubated with deoxyribonuclease. 100 µg. purified deoxyribonucleate were incubated in acetate buffer (pH 5.0) in the presence of tissue extract at 37° for 30 minutes. The total volume was 1.0 ml. The reaction was stopped by the addition of 1.0–2.0 M perchloric acid. The precipitate of protein and undigested deoxyribonucleate was allowed to age at 0° for 45 minutes and then it was spun at 18,000 x g at 2° for 5 minutes in the high-speed head of the MSE refrigerated centrifuge. The crystal-clear supernatant was removed and the extinction was read at 259 mµ in the Zeiss PMQ II spectrophotometer. The blank was treated in the same way with the exception that the substrate was added after the acid. The units are arbitrary and are expressed as the change in extinction ($\log \frac{I_o}{I}$) occurring at 259 mµ per minute per gram of tissue at pH 5.0 and 37°. The pH optimum was checked and was found to be sharply defined at pH 4.9 - 5.0. The optimal concentration of substrate was 100 µg. per ml. reaction mixture. The enzyme concentration curve was linear. Mg^{2+} were inhibitory at a

concentration of 10^{-2}M. The ionic strength of the buffer was 0.1; ionic strengths greater than 0.2 and less than 0.05 were inhibitory. There was no evidence of a deoxyribonuclease acting at pH 7.4 in the presence of Mg^{2+}.

Reagents. Deoxyribonucleate. The rapid method for the large scale preparation of calf thymus nucleate as described by HURST (1958) was used. This involves homogenisation and repeated washing of thymus in 0.1 M ethylenediaminetetra-acetate. The thymonucleoprotein which is present in the residue is dissolved in 2 M sodium chloride and the protein-nucleate bond is broken by means of potassium thiocyanate. The protein is adsorbed on to celite and the deoxyribonucleate in the filtrate is precipitated in alcohol.

230 g. thymus were used and 3.5 g. deoxyribonucleate were obtained, a yield of 1.5 per cent. The whole procedure took 4½ days. The final product was white, fibrous, matted and tough, having the appearance of asbestos. It was stored *in vacuo* over $CaCl_2$ at -20°. It contained 22.2 per cent water, 0.33 per cent ribonucleate, and the biuret test was negative. The maximum extinction was at 259 mμ and the minimum at 230 mμ at pH 7.0; these values agree with those given in the literature. The degree of purity and the lack of degradation were assessed by estimating the nitrogen and phosphorus content and the extinction coefficient per mole of phosphorus. The results of these determinations and the values regarded as criteria of satisfactory purity

(CHARGAFF, 1955) are summarised below.

	N	P	N/P	ϵ_P in 0.15 M NaCl, pH 7.0
	%	%		
DALE, 1963	14.4	9.2	1.57	6660
CHARGAFF, 1955	15.3	9.2	1.66	6650 ± 50

The deoxyribonucleate was made up as a solution of 1.0 mg./ml. in 0.15 NaCl solution and stored at 2°.

Acetate buffer, 0.1 M, pH 5.0 was prepared every 2 weeks and stored at 2°. Perchloric acid, 2 M was made up as required and stored at 2°. Glass distilled water was used.

Total Nitrogen

Homogenates of the colonic tissues in mannitol were used. The samples (in duplicate) were first subjected to a Kjeldahl digestion (KING, 1946) and then the nitrogen was estimated by nesslerisation (KING and WOOTTON, 1956). The extinction was read at 480 mμ in a Zeiss spectrophotometer, immediately following the addition of the Nessler reagent. There was a linear relation between concentration and extinction up to extinction values of 0.5; all digests were diluted with water in order to obtain a reading of 0.3 to 0.4. The amount of nitrogen (mg. per g. fresh tissue) is obtained from the following equation.

$$\text{Total N} = \frac{\mu\text{g. N in standard}}{1000} \times \frac{E_{test}}{E_{standard}} \times \frac{10}{\text{volume of 1:9 homogenate digested}} \times \text{dilution factor, if any required to reduce extinction of test solution}$$

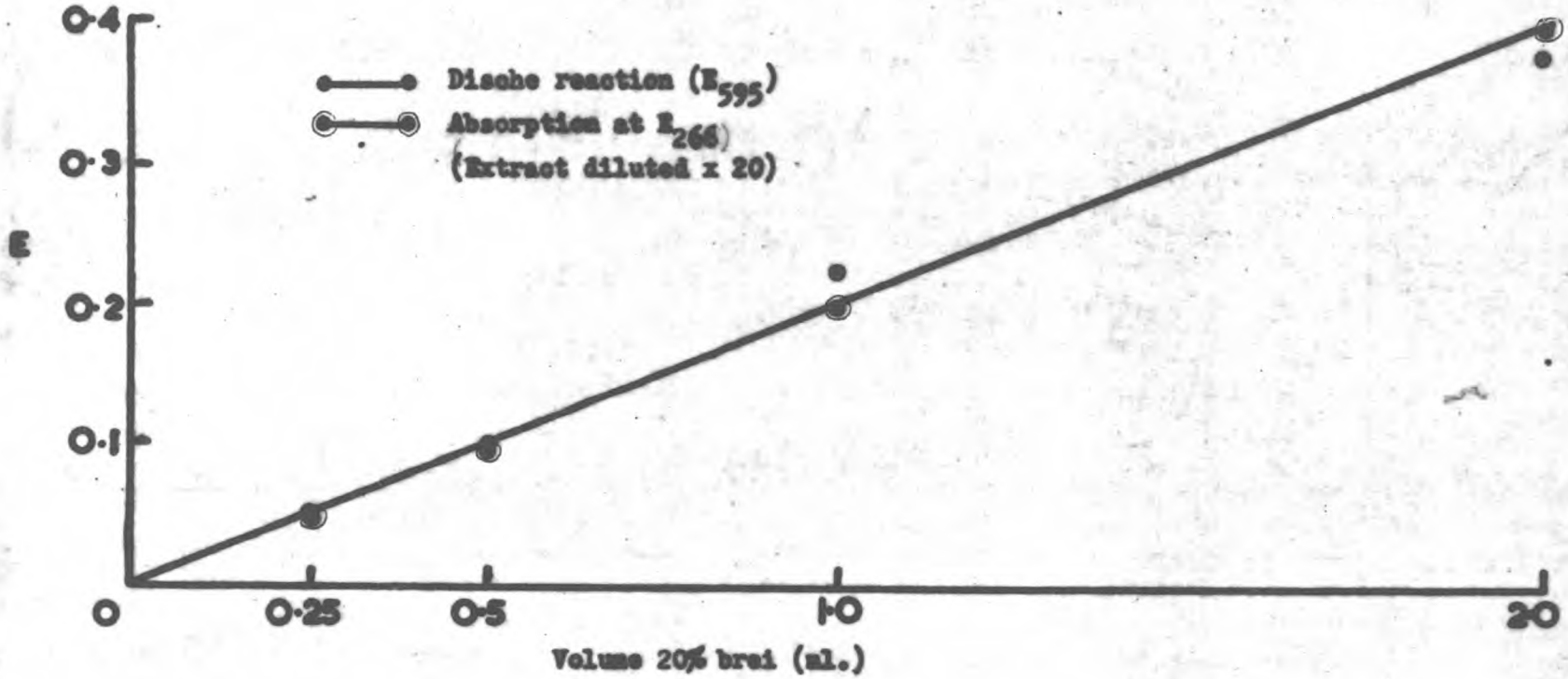


Fig. 3. Calibration curve of deoxyribonucleate (DNA) extracted from mucosa of human colon.

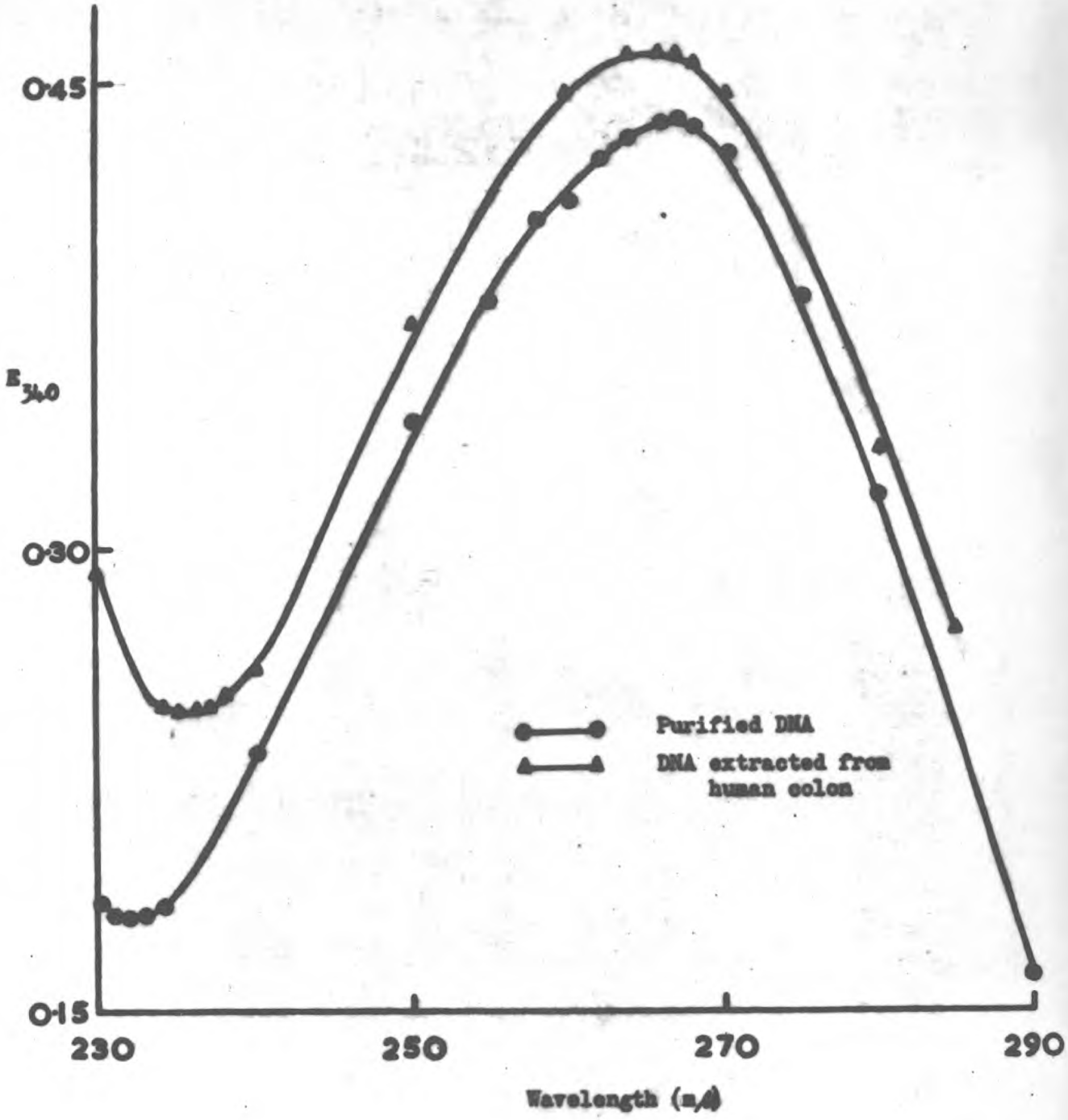


Fig. 4. Absorption spectra of deoxyribonucleate. (measured in 0.5 M perchloric acid).

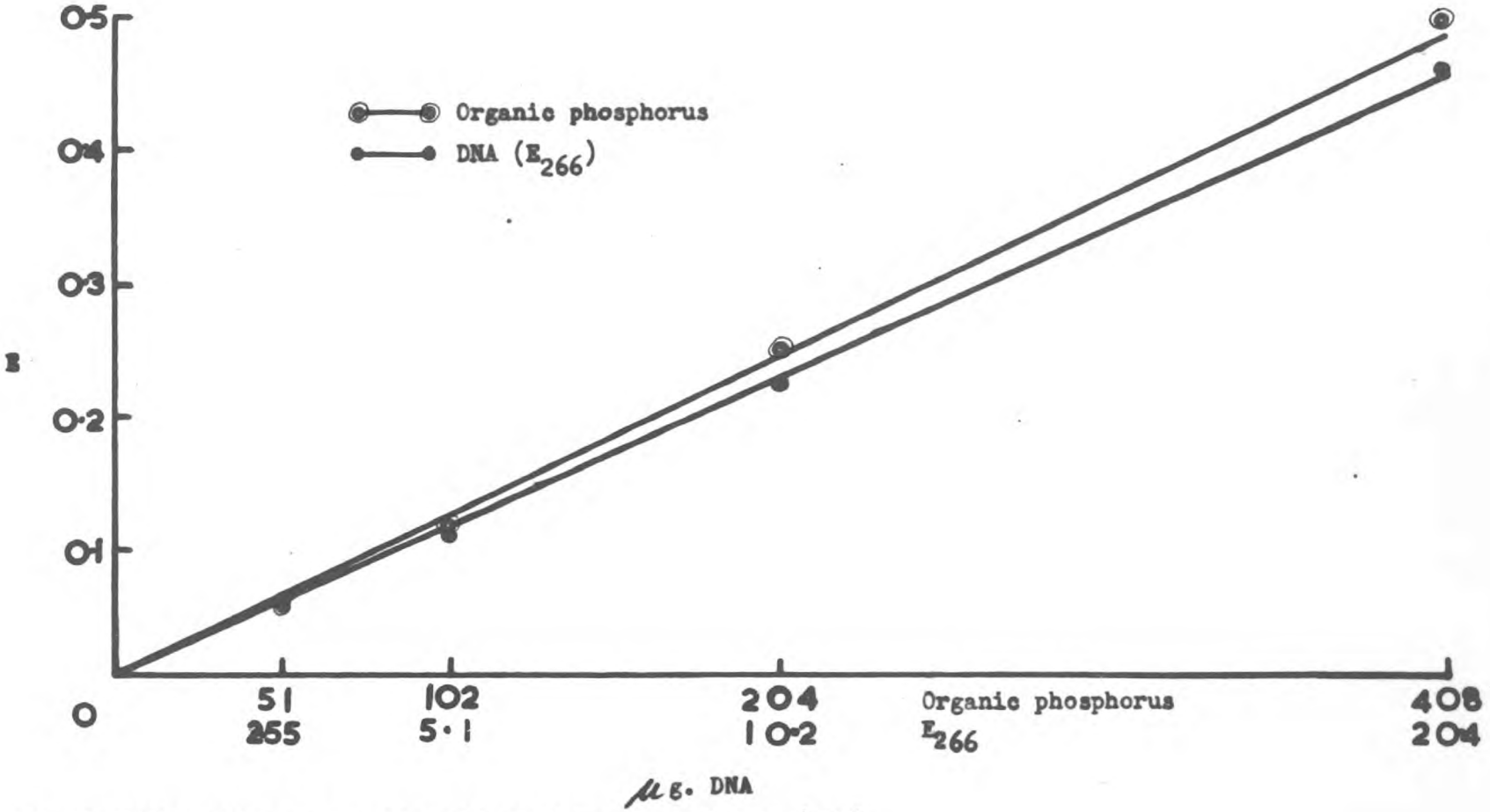


Fig.5. Calibration curve of purified deoxyribonucleate (DNA).

Organic Phosphorus

The phosphorus content of deoxyribonucleate was determined by the method of BEVERIDGE and JOHNSON (1949).

Deoxyribonucleate

Separation from non-nucleate phosphorus-containing compounds and from ribonucleate in the homogenate was carried out by the procedure described by SCHNEIDER (1945). The amount of deoxyribonucleate in the extract was estimated at first by the determination of both the pentose content (SCHNEIDER, 1955b) and the extinction at 268 mμ. (The maximum extinction shifts from 259 to 268 mμ and the minimum shifts from 230 to 232 mμ as a result of the heating of deoxyribonucleate in perchloric acid during the extraction procedure). The results obtained by the two methods agree. The calibration curves were almost identical (fig. 3). Also the spectrum of the extract was similar in shape to that of purified deoxyribonucleate, with a peak at 268 and a trough at 232 mμ (Fig. 4). Therefore the simpler method of measuring the extinction at 268 mμ was adopted. This was calibrated against the extinction at 268 mμ and the phosphorus content of purified deoxyribonucleate, the curves for which appear in fig. 5. The amount of deoxyribonucleate phosphorus per gram of tissue was calculated as follows:

$$\text{Concentration of standard solution} \times \frac{E_{extract}}{E_{standard}} \times \text{dilution factors required to convert volume of extract used to g. tissue} \times \frac{7.16}{100}$$

where 7.16 = percentage of phosphorus in deoxyribonucleate.

Table 2

Effects of repeated freezing and thawing on activities of aldolase, lactate dehydrogenase and deoxyribonuclease in colonic mucosa

No. times tissue frozen and thawed	Aldolase μM/min./g. fresh tissue				Lactate dehydrogenase μM/min./g. fresh tissue				DNA ase units/min./g. fresh tissue			
	Experiment				Experiment				Experiment			
	1	2	3	4	1	2	3	4	1	2	3	4
1	1.13	1.05	1.44	1.8	3.5	9.2	6.9	—	1.12	3.6	0.71	2.24
2	1.36	1.04	1.26	---	3.1	9.2	---	---	1.58	—	0.86	2.44
3	1.45	1.28	1.20	1.7	3.5	8.8	6.5	15.2	1.93	3.8	0.78	2.32
4	---	0.84	---	---	---	---	---	16.1	1.98	---	---	---

Experiments Designed to Establish Optimal Conditions of Extraction and Storage of Colon

The procedure finally adopted was reached by trial and error. This was necessary because little information was discovered regarding the optimal conditions of extraction and storage and because it was impossible to carry out the analyses on one sample in less than 3 days.

Release of Deoxyribonuclease from Lysosomes

Enzymes residing in cell sap, such as aldolase and lactate dehydrogenase, are readily extracted from tissues by simple homogenisation. Deoxyribonuclease occurs largely in the lysosomes and is released by disrupting these particles. The method used was repeated freezing and thawing of the tissue (de DUVE and others, 1955). Some enzymes are denatured by this procedure, therefore its effects on the activities of aldolase and lactate dehydrogenase were checked. The results of these experiments appear in table 2. With deoxyribonuclease there was a suggestion that the yield was greater after 2 lots of freezing and thawing. With the other 2 enzymes there was no evidence that two or three lots of freezing and thawing affected the yield. The relatively small increase in the activity of deoxyribonuclease is probably due to the previous release of a large percentage from the lysosomes following prolonged anoxia (de DUVE and BEAUFAY, 1959).

Effect of Simple Freezing and Thawing

At this stage all homogenates were frozen and thawed

Table 3

Effect of a single freezing and thawing on mean activities of aldolase and lactate dehydrogenase

Tissue (6 samples)	Aldolase µM/min./g. fresh tissue		Lactate dehydrogenase µM/min./g. fresh tissue	
	Frozen and thawed	Unfrozen	Frozen and thawed	Unfrozen
Mucosa	4.7	5.6	42	41
Carcinoma	3.4	3.9	42	42

twice in order to ensure the complete release of deoxyribonuclease from the lysosomes. Some time later it was considered worth determining whether the contents of aldolase and lactate dehydrogenase were adversely affected by the first lot of freezing and thawing. The results are presented in table 3. There was no evidence indicating that the activity of lactate dehydrogenase was affected, but the values obtained for aldolase were lower after the single freezing and thawing. The difference was not significant; nevertheless the activity of aldolase was estimated without freezing and thawing from then on. This precaution proved to be justified because when a larger number of unfrozen samples was analysed the activity was found to be significantly greater than in the frozen specimens ($p < 0.01$). Additional evidence which indicates that the activity of aldolase is reduced by a single freezing and thawing was obtained in rat liver (table 35). The only way to store the enzyme overnight appeared to be to keep the extract at 2°. This was attempted, but the activity decreased by 20 to 30 per cent. Therefore the activity of aldolase was always estimated as soon as possible after preparation of the extracts. The activities of lactate dehydrogenase and deoxyribonuclease were estimated after storage overnight at -20° plus two lots of freezing and thawing.

Effect of Storage at -20°

It is an advantage to assay substances in tissues in batches; this is especially true when it is not possible to

Table 4

Effects of storage at -20°

These results were obtained from tissues which were washed three times with 0.25 M mannitol and frozen and thawed twice before the estimations were made. The number of specimens analysed appear inside the brackets at the head of each column.

	Mucosa		Carcinoma	
	2 days or less* (10)	Up to 3 weeks (7)	2 days or less* (9)	Up to 3 weeks (7)
Aldolase µM/min./g.	1.7	1.6	2.8	1.5
LDH µM/min./g.	12.4	6.4	30.2	15.5
DNA P µg./g.	294	230	392	278

* Aldolase and LDH were estimated after storage for 13 hours. DNA P was determined after storage for 2 days.

Table 5

Effects of repeated freezing and thawing on the content of DNA P in colonic mucosa

No. times frozen and thawed	DNA P µg./g. fresh tissue	
	Sample 1	Sample 2
1	235	175
2	205	190
3	208	175
4	215	---

obtain unlimited supplies of specimens. Therefore a number of samples of mucosa and carcinomata were stored at -20° for one to three weeks before any analyses were carried out. The results of assays for the activities of aldolase and lactate dehydrogenase and the content of deoxyribonucleate in these tissues and in some others stored for the minimal time are given in table 4. Storage resulted in decreases in the content of all substances in the carcinomata and in the activity of lactate dehydrogenase and the content of deoxyribonucleate in mucosa.

Storage for 1 to 2 days of the homogenates destined for the determination of deoxyribonucleate was sometimes necessary; this involves freezing and thawing. Therefore the effects of this procedure on the content of deoxyribonucleate in a homogenate of mucosa were assessed (table 5). There was no evidence of a decrease. It was concluded that storage at -20° for 2 days plus freezing and thawing would not result in appreciable degradation of the deoxyribonucleate.

Effect of Washing the Colon in 0.25 M Mannitol

The mucosa and the tumours in the colon are inevitably associated with more or less faecal matter. Attempts were made to remove this by washing the tissues in 0.25 M mannitol solution in order to exclude any substances which might interfere with the analyses. The effects of this procedure on the content of several substances in both mucosa and carcinomata of the colon are presented in table 6. With the exceptions

Table 6

Effects of washing tissue in 0.25 M mannitol

Constituent	Mucosa		Carcinoma	
	Washed	Unwashed	Washed	Unwashed
Aldolase μM/min./g.	1.7 (10)	4.7 (19)	2.8 (10)	3.1 (19)
LDH μM/min./g.	12.4 (9)	47 (27)	30.2 (9)	49 (27)
Lactate μM/g.	4.2 (8)	21.4 (27)	12.4 (7)	25.9 (27)
Total N mg./g.	11.3 (10)	24.6 (27)	16.6 (9)	24.4 (27)
DNA P μg./g.	294 (10)	510 (27)	392 (10)	390 (27)

Washing was carried out by inverting the tissue in a test tube containing 20 - 30 ml. mannitol. The pieces of mucosa swelled considerably as a result. The pieces of tumour (~0.3 mm. in thickness) did not alter noticeably.

of aldolase and deoxyribonucleate in the tumours, the amounts of all of the constituents examined were diminished. The effects in the mucosa were more evident than in the tumours. This is presumably because the tissue of the mucosa was in the form of thin filmy sheets whereas that of the tumours was in solid chunks; the access of the mannitol solution to the mucosa would be freer. In both tissues the amount of lactate was affected most, the value being reduced to approximately 20 and 50 per cent in the mucosa and the carcinomata, respectively. This is doubtless related to the smallness of the lactate molecule. The substance least affected in both tissues was deoxyribonucleate; this is attributed to its intranuclear site and to the binding to the histone molecules. The losses of aldolase and lactate dehydrogenase in the mucosa were approximately 35 and 25 per cent, respectively. The reduction in the total nitrogen in the mucosa was 50 to 55 per cent and in the tumours, 30 to 35 per cent. Thus it is clear that the losses varied according to the type of tissue and the molecular species. Clearly washing must be avoided if an accurate assessment of the amounts of the normal constituents of the tissue is to be made. The losses of these substances was not expected because mitochondria are prepared in isosmotic sucrose or mannitol solutions, apparently with retention of their content. The period of deprivation of blood supply before excision plus the delay before washing may have resulted in damage to the cell membranes with a

consequent increase in their permeability. This interpretation is supported by observations indicating that lysosomal membranes are disrupted progressively by anoxia; the activities of acid phosphatase, acid ribonuclease, acid deoxyribonuclease, cathepsin and β-glucuronidase are released into the cell sap of a homogenate of liver to the extent of 40 per cent within a few hours of ligation of the blood supply (de DUVE and BEAUFAY, 1959).

Isolation of Epithelial Cells of Mucosa and Carcinomata

It was pointed out in Chapter I that the amounts of a given constituent of a tissue are a weighted mean of the content in all of the types of cell present. It would revolutionise the interpretation of the results if the type of cell of interest could be isolated. This was found to be possible in both the mucosa and the carcinomata as the result of an observation made after gentle disruption carried out in a Potter-Elvehjem homogeniser; the acini were seen to be lying free in the medium. As a result of this observation a technique was developed for the isolation of the epithelial cells of both the mucosa and the carcinomata. This involves gentle disruption of the tissue by means of the homogeniser followed by repeated differential centrifuging of the resultant suspension of tissue fragments. The histological appearance of the mucosa and tumour before disruption is shown in fig. 6 a, b and c. In fig. 6a a section of the mucosa superficial to the muscularis mucosae is seen, and in fig. 6b there is a

Key to Fig. 6.

a. Section of the mucosa of the colon prepared from the sheets of mucosa stripped off the submucosa with the aid of a stiff brush. x 60

b. Section of the growing edge of a carcinoma of the colon showing the tissue from which the epithelial cells of the carcinoma are isolated. x 60

c. Surface view of the mucosa showing the acini in plan (unfixed). x 60

d. Expression of the acini from the lamina propria at an early stage (unfixed). x 60

e. Extruded acinus lying among the intact nuclei of epithelial cells (unfixed). x 80

f. Surface view of the lamina propria from which the acini were expressed (unfixed). x 60

g. Clumps of tumour cells and connective tissue as seen during disruption of a carcinoma (unfixed). x 60

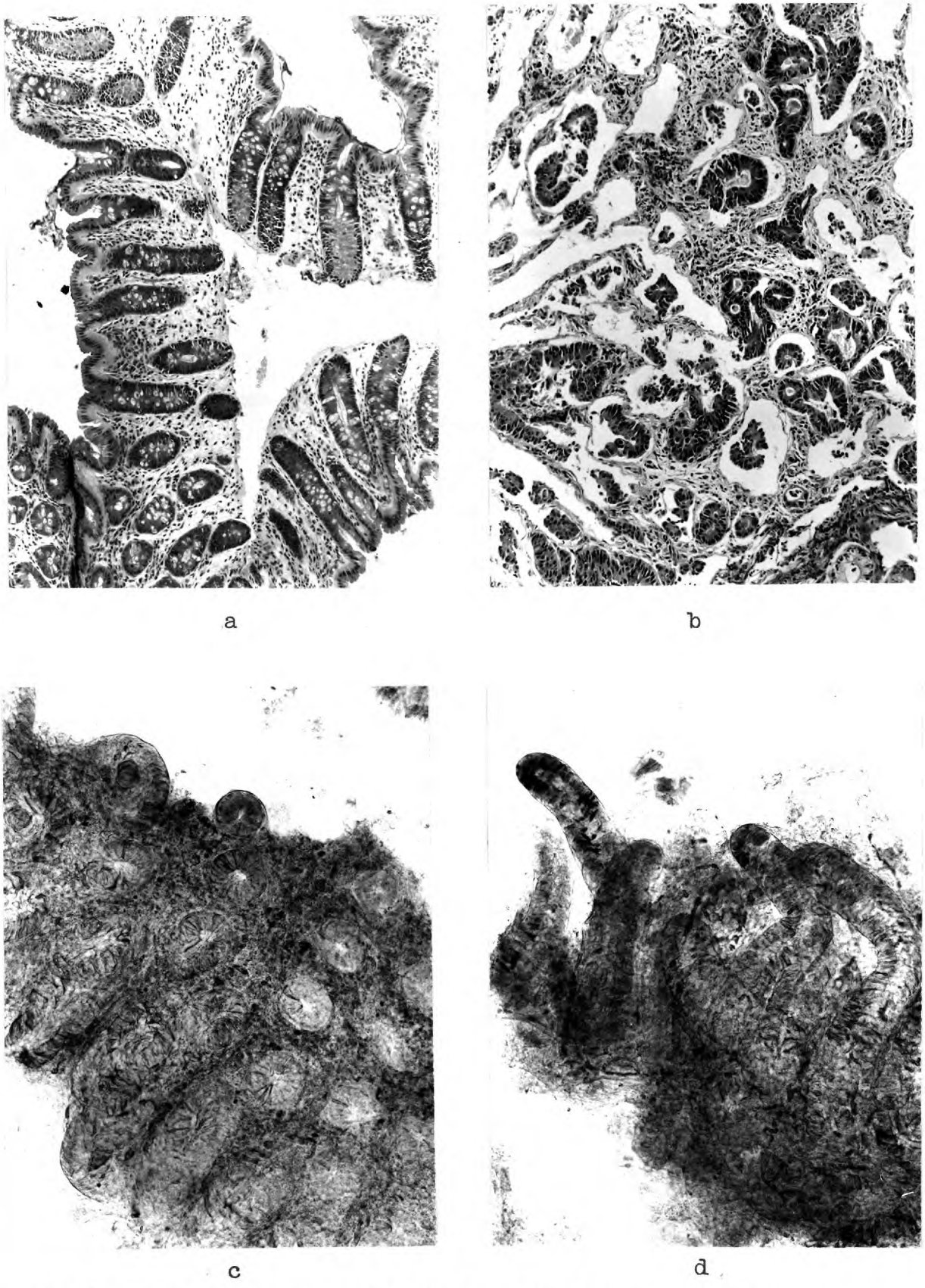
h. Section of the epithelial cells and nuclei isolated from the lamina propria. Note that many of the acini are intact. x 60

i. High-power view of Fig. h. showing that the cells are apparently intact. x 600

j. Section of the lamina propria showing that the connective tissue cells are still <u>in situ</u>. x 60

k. Section of some epithelial cells of a carcinoma isolated from the stroma. x 180

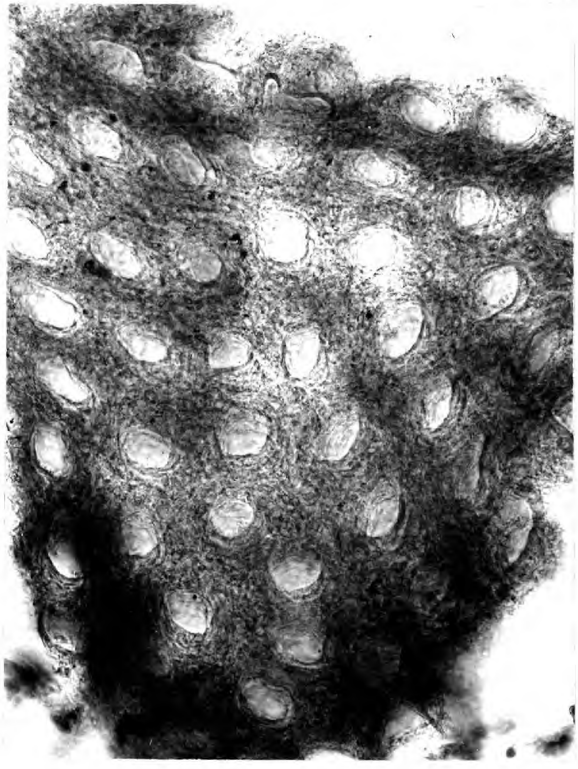
l. Stroma of a carcinoma after isolation of the epithelial cells showing that the connective tissue cells are still <u>in situ</u>. x 60



a b c d

Fig. 6. Isolation of epithelial cells from human colon.

e



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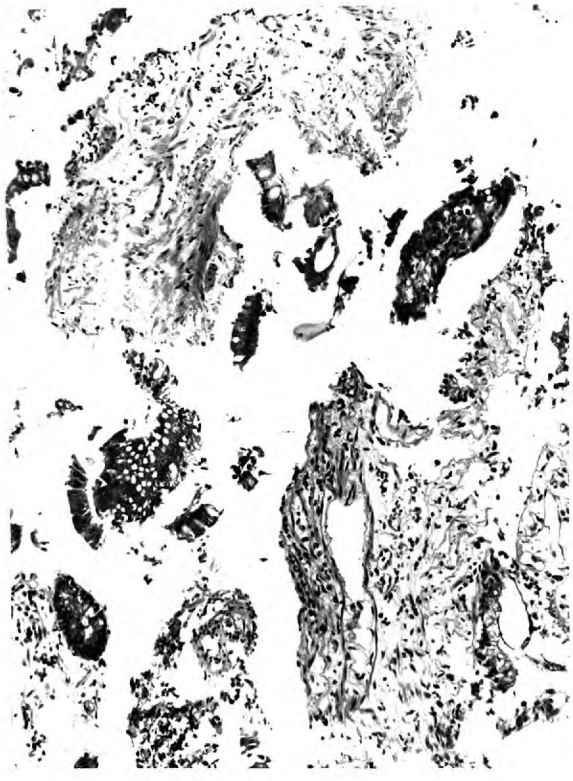

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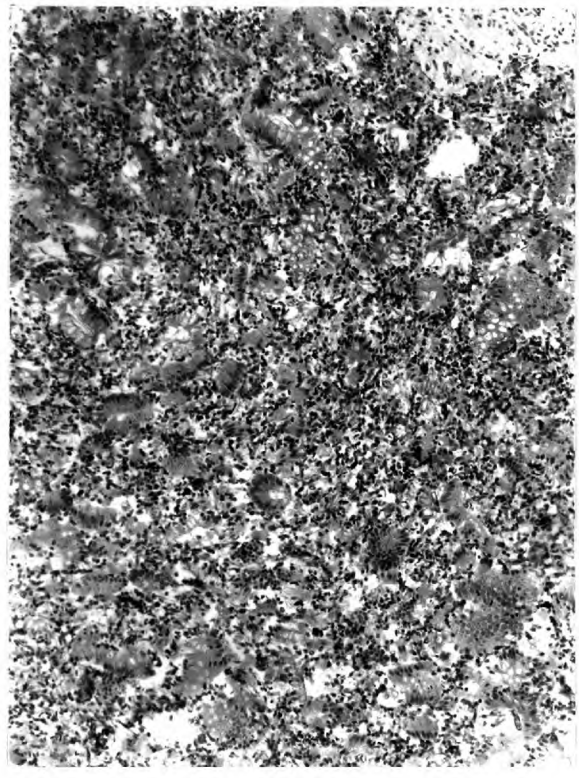
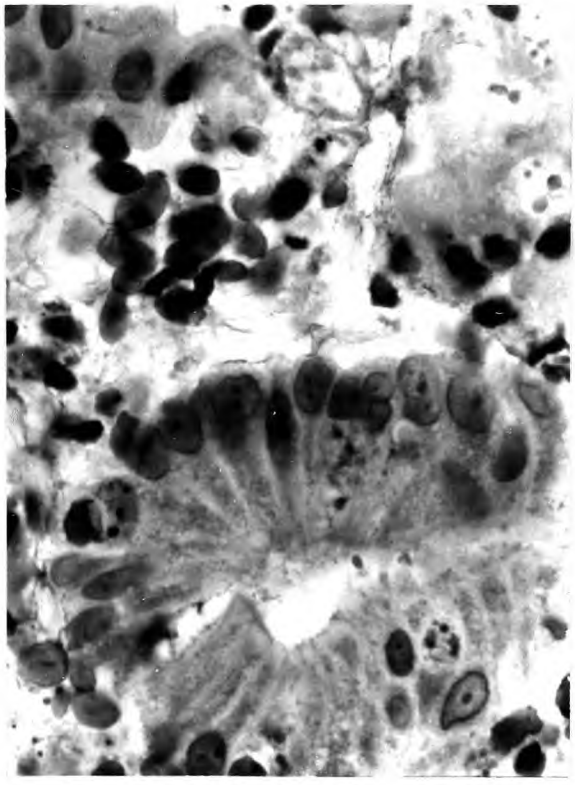


h

Fig. 6. Isolation of epithelial cells from human colon.



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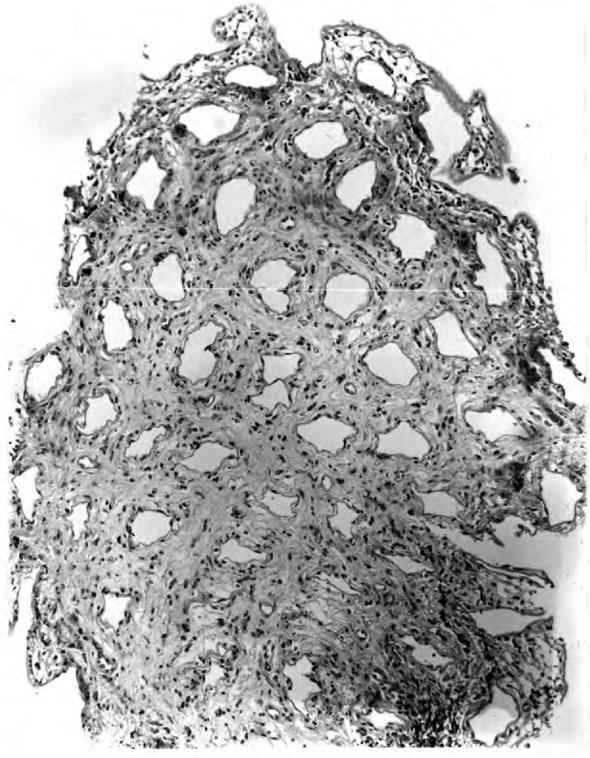

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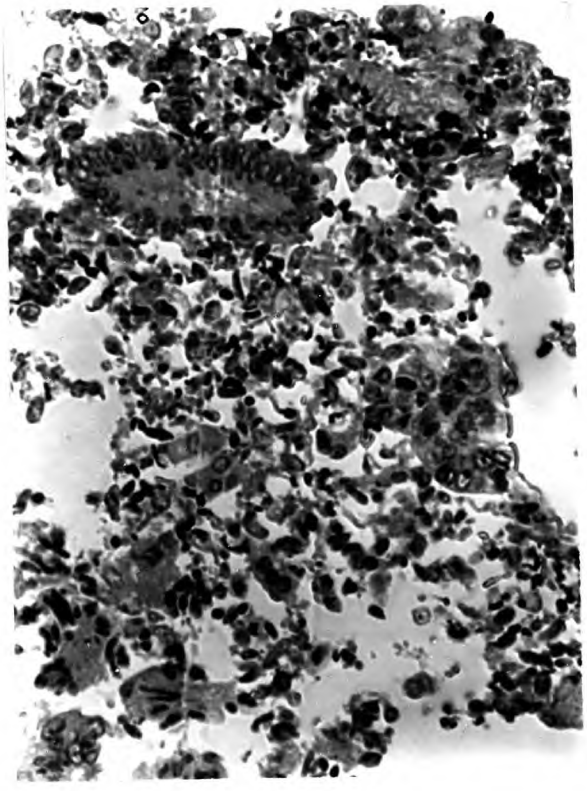

k

l

Fig. 6. Isolation of epithelial cells from the human colon.

section of the growing edge of a carcinoma of the colon. In both of these sections the epithelial cells and the stroma are clearly defined from each other. In fig. 6c there is a view of the surface of a piece of fresh mucosa mounted under a coverslip in 0.25 M sucrose. The acini and the individual cells of which they are comprised are seen in plan in situ in the lamina propria. When the microscope is focussed up and down on such a preparation the lumina of the acini have the appearances of tunnels.

Disruption of the Tissue

The movement of the pestle against the homogeniser tube compresses the tissue in such a way that the epithelial cells are expressed from, and/or stripped off, the stromal tissue. The appearance in the fresh state of the suspension so produced from the mucosa is seen in fig. 6 d, e and f. In fig. 6d the expression of the acini from the lamina propria is shown at an early stage and in fig. 6e an acinus is shown lying free surrounded by the nuclei of disrupted cells. The appearance of the lamina propria is seen in fig. 6f in which the complete removal of the acini is demonstrated. A section of the tumour in the same stage of preparation appears in fig. 6g in which clumps of tumour cells and fragments of connective tissue are visible.

Differential Centrifugation

As a result of centrifugation the suspension is resolved into the following layers from below upwards: pieces of

connective tissue containing a few acini, clumps of cells, single cells, nuclei, mitochondria and microsomes. The connective tissue, cells and nuclei are usually held together as a pink fluffy layer by the mucus which is released from the goblet cells. This makes it difficult to separate the various components of the suspension. However, separation is achieved, as outlined below, by a system of centrifugations followed by washing of the deposit with the top layers of the supernatant liquid. The end-result is apparently a complete resolution of most of the epithelial cells from the stroma.

The appearance of sections of the epithelial cells and nuclei prepared in this way from the mucosa are seen in fig. 6 h and i. Many of the acini and separate cells appear to be intact and even where the cell membrane was broken the nuclei do not seem to have been damaged. Most of the cells and nuclei are clearly epithelial, but there is a small percentage of nuclei whose origin it is impossible to ascertain. It is unlikely that many of these nuclei arise from the connective tissue because, as shown in fig. 6j, the lamina propria does not appear to have been disturbed by the procedure used to express the acini.

The same general comments apply to the sections prepared from the epithelial cells and connective tissue residue of the carcinoma. In fig. 6k both the malignant cells and the free nuclei appear to be intact. In fig. 6l the connective

tissue of the tumour is still cellular but owing to the lack of regular architecture it is difficult to be certain that none of the cells from the connective tissue was shorn off in the homogeniser.

Scheme for the Isolation of Epithelial Cells

(a) (i) 1 g. tissue plus 9.0 ml. 0.25 M sucrose are placed in the tube of a Potter-Elvehjem type homogeniser (tube A), and the pestle* is forced down and up 20 times.

(ii) The suspension is centrifuged (in tube A) at 2500 r.p.m. for 3 minutes and the supernatant liquid plus the upper half of the "fluffy" layer (see above) are removed to a second tube, B.

(iii) Tube B is centrifuged at 2500 r.p.m. for 3 minutes and the upper three quarters of the suspension are removed to tube C.

(iv) Tube C is centrifuged at 5000 r.p.m. for 5 minutes, and the clear supernatant liquid is transferred to tube B, mixed with the contents remaining as under (iii) and poured into the homogeniser.

(b) (i) The pestle is forced down and up a further 80 times and the contents of the homogeniser are returned to tube B.

* A plastic pestle is used. The clearance between the wall of the tube and the pestle should be such that when the pestle is held vertically with the tube in position and containing water, the tube slowly falls.

(ii) Tube B is centrifuged at 2500 r.p.m. for 3 minutes and the upper half of the suspension is transferred to tube C.

(iii) Tube C is centrifuged at 5000 r.p.m. for 5 minutes and the clear supernatant liquid is transferred to tube B which is swirled in order to mix the contents.

(c) The procedures (b) (ii) and (iii) are repeated until the number of free nuclei remaining in the deposit in tube B is negligible, that is, approximately 5 per high-power field. Usually it is necessary to repeat procedures (b) (ii) and (iii) twice more.

All materials and equipment are kept at 0° and all procedures except weighing are carried out at this temperature. A histological examination is carried out as follows on each sample of the suspension removed for high-speed centrifugation. The sample is removed from the centrifuge tube by means of a Pasteur pipette which is filled from the surface of the suspension. This ensures that the deeper layers, i.e., the last to be removed, are situated in the distal part of the pipette. At stages (a) (iii), (b) (ii) and the corresponding stage under (c) of the scheme above, one drop from the tip of the pipette is placed on a glass slide, stained with methyl violet, and examined for the presence of stroma. If any stroma is seen a suitable volume of the suspension is returned to the centrifuge tube, and again the drop in the tip of the pipette is examined. This procedure is repeated until all of the stroma is eliminated. The remainder of the contents of the

Table 7

Whole tissue and epithelial cells; comparison of enzyme activities

Enzyme	Mucosa				Carcinoma			
	Whole tissue		Epithelial cells		Whole tissue		Epithelial cells	
	Washed	Unwashed	Washed	Unwashed	Washed	Unwashed	Washed	Unwashed
Aldolase µM/min./g.	1.8 (7)	4.6 (7)	2.1 (7)	5.2 (7)	3.3 (7)	2.6 (7)	3.5 (7)	2.7 (7)
LDH µM/min./g.	13.2 (7)	44 (7)	10 (7)	45.3 (7)	35.3 (7)	50 (7)	30 (7)	50.1 (7)

All samples were frozen and thawed 3 times before assays were made. The washed and unwashed specimens were taken from different colons.

pipette is then discharged into tube C for high-speed centrifugation. After a little experience only 3 to 4 histological examinations are needed.

Comparison of the Activities of Enzymes in Whole Tissue and in Epithelial Cells

The isolation of epithelial cells having been achieved it was obviously important to discover whether the contents of enzymes differed from those present in the whole tissue. The results obtained for the activities of aldolase and lactate dehydrogenase in both washed and unwashed tissue are presented in table 7. The activities of these two enzymes were almost the same in extracts of the whole tissue and in the epithelial cells. This could be due to the amounts being the same in all of the types of cell present, to the enzymes being present chiefly in the epithelial cells or to the leaching out of the enzymes from all of the cells during the isolation of the epithelial cells. In view of the evidence presented in table 6 indicating that large losses of all of the constituents occurred as a result of washing the tissue in mannitol, there can be little doubt that even larger amounts diffused out of the cells during the isolation procedure. It was concluded, despite the demonstration that the epithelial cells can be isolated apparently intact physically, that no useful purpose is achieved by this procedure from a biochemical viewpoint. As a result of the

Table 8

Several constituents of human colonic mucosa, carcinoma and polyps (from multiple polyposis)

Constituent	Mucosa		Carcinoma		Polyps	
	per g. fresh tissue	per mg. DNA P	per g. fresh tissue	per mg. DNA P	per g. fresh tissue	per mg. DNA P
Aldolase µM/min.	5.75 ± 0.30 (14)	11.4	3.35 ± 0.28 (14)	8.7		
	4.74 ± 0.41 (8)	8.3	4.1 (2)	—	5.38 ± 0.33 (8)	9.1
LDH µM/min.	47.2 ± 2.1 (27)	91	48.6 ± 3.1 (27)	126		
	43.3 ± 3.5 (8)	76	62 (2)	—	56.6 ± 4.6 (8)	97
DNA ase units/min.	7.10 ± 5.5 (22)	13.9	7.64 ± 0.71 (22)	19.5		
	5.84 ± 0.54 (8)	10.2	6.0 (2)	—	8.75 ± 0.50 (8)	15.0
DNA P µg.	509.6 ± 13.0 (27)	—	390.4 ± 18.6 (27)	—		
	566.3 ± 25.5 (8)	—	405 (27)	—	588 ± 14.6 (9)	—
Total N mg.	24.57 ± 0.17 (27)	—	24.41 ± 0.51 (27)	—		
	24.58 ± 0.68 (7)	—	23.2 (2)	—	24.59 ± 0.75 (8)	

The numbers of samples analysed appear within the brackets. The S.E.M. is given with each mean value as determined in the fresh tissue.

experiments described it was clear that all assays should be carried out on homogenates of whole unwashed tissue.

RESULTS

The results are summarised in table 8 and are related to both g. fresh tissue and mg. deoxyribonucleate phosphorus. The total nitrogen was not used as an index because it is almost identical in all of the colonic tissues. Furthermore, in the tumours it arose largely from dense connective tissue (fig. 6b), whereas in the mucosa and the polyps a higher percentage is derived from the epithelial cells. The content of deoxyribonucleate phosphorus is used because it represents approximately the number of cells in the tissue (THOMSON and others, 1953). It is not an ideal index because some cells have more than one nucleus and others have more than the normal complement of chromosomes; this applies especially to tumours. Furthermore, it fails to reflect differing amounts of cytoplasm in different types of cell. However, it is probably the best index available for the purpose of comparing the activities of enzymes in tissues in which the content of connective tissue differs, as it does in mucosa, polyps and carcinomata.

Total Nitrogen

The similarity in the content in the mucosa and the tumours and polyps was remarked above. It is also similar

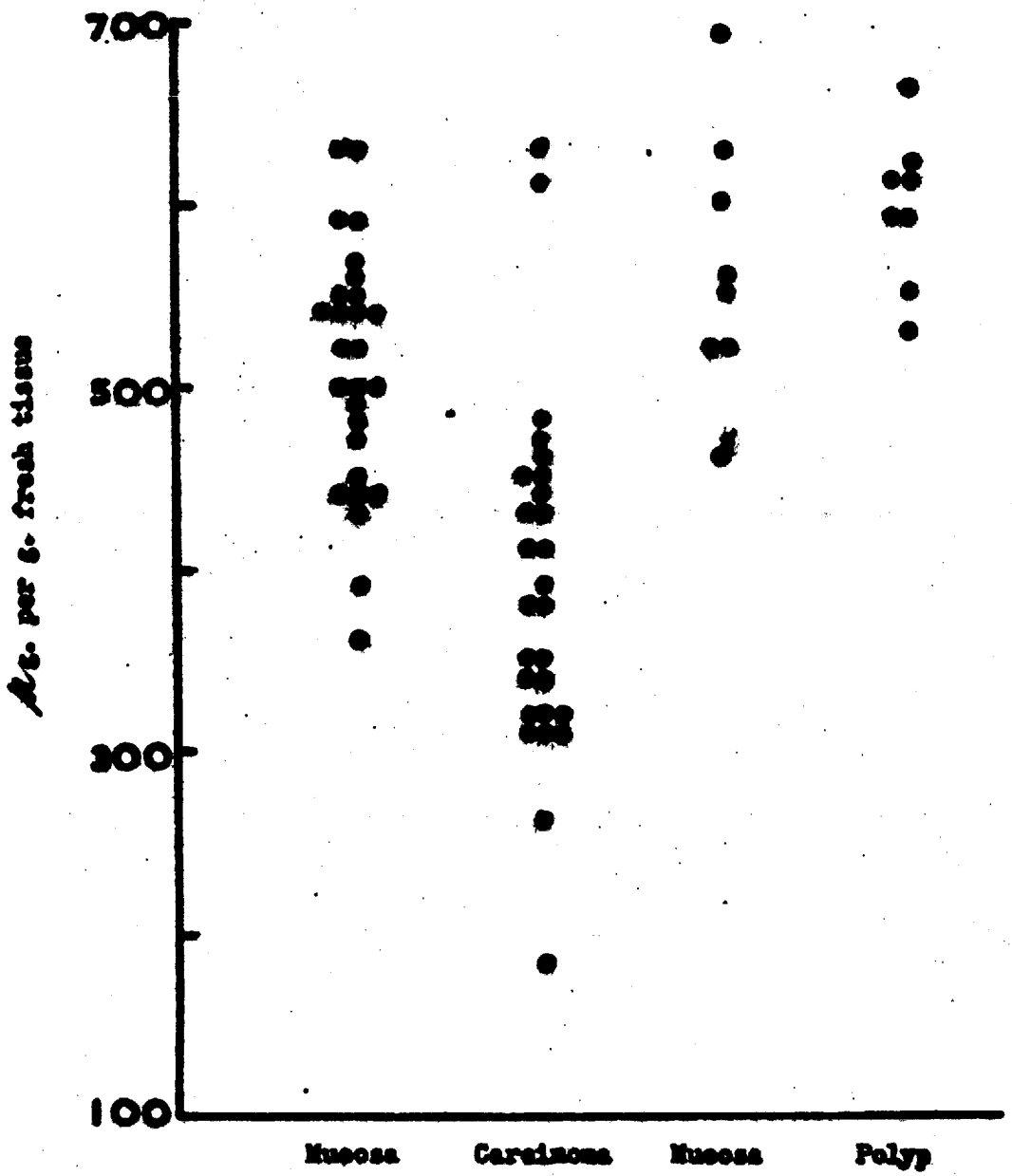

Fig. 7. Scatter diagram of content of DNA P in human colonic mucosa and tumours.

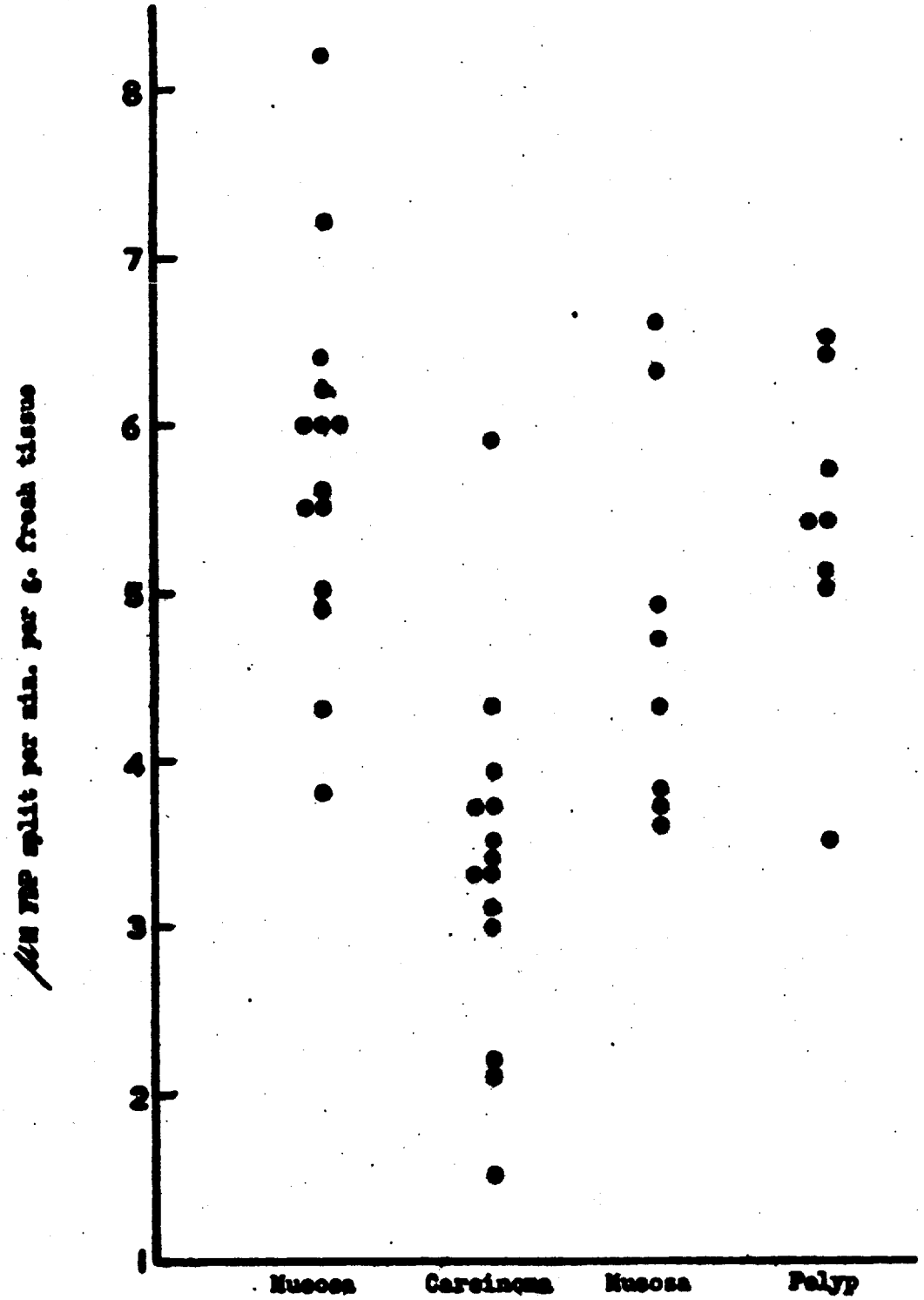

Fig. 8. Scatter diagram of activity of aldolase in human colonic mucosa and tumours.

to the content in rat hepatomata and sarcomata, but it is less than that in liver or muscle (table 30). It is slightly greater than the total nitrogen content of the intestinal mucosa of the rat, namely, 23 mg./g. (REID, 1961).

Deoxyribonucleate phosphorus (fig. 7)

The content of deoxyribonucleate phosphorus (DNA P) is significantly greater in the mucosa and the polyps than it is in carcinomata ($p < 0.001$). This is presumably due to the relatively greater content of cells and smaller content of connective tissue. The differences between the amounts present in mucosa derived from specimens excised because of carcinomata and those excised because of multiple polyposis is not significant. The content of deoxyribonucleate of human colon is similar to that of many rat tissues. For example, the content in the pancreas, liver and kidney, respectively, in the rat are 479, 264 and 418 µg./g. (SCHNEIDER, 1946), and the contents in Jensen sarcoma, Walker carcinoma and hepatomata, respectively, are 430, 450 and 546 µg./g. (ELSON, 1961).

Aldolase (fig. 8)

The activity in the mucosa of the colons excised for carcinomata is greater than that in the tumours whether referred to fresh weight ($p < 0.001$) or to deoxyribonucleate phosphorus ($p < 0.002$). No difference was observed between the activities in the mucosa and in the polyps of the colons excised for multiple polyposis. The activity in the polyps

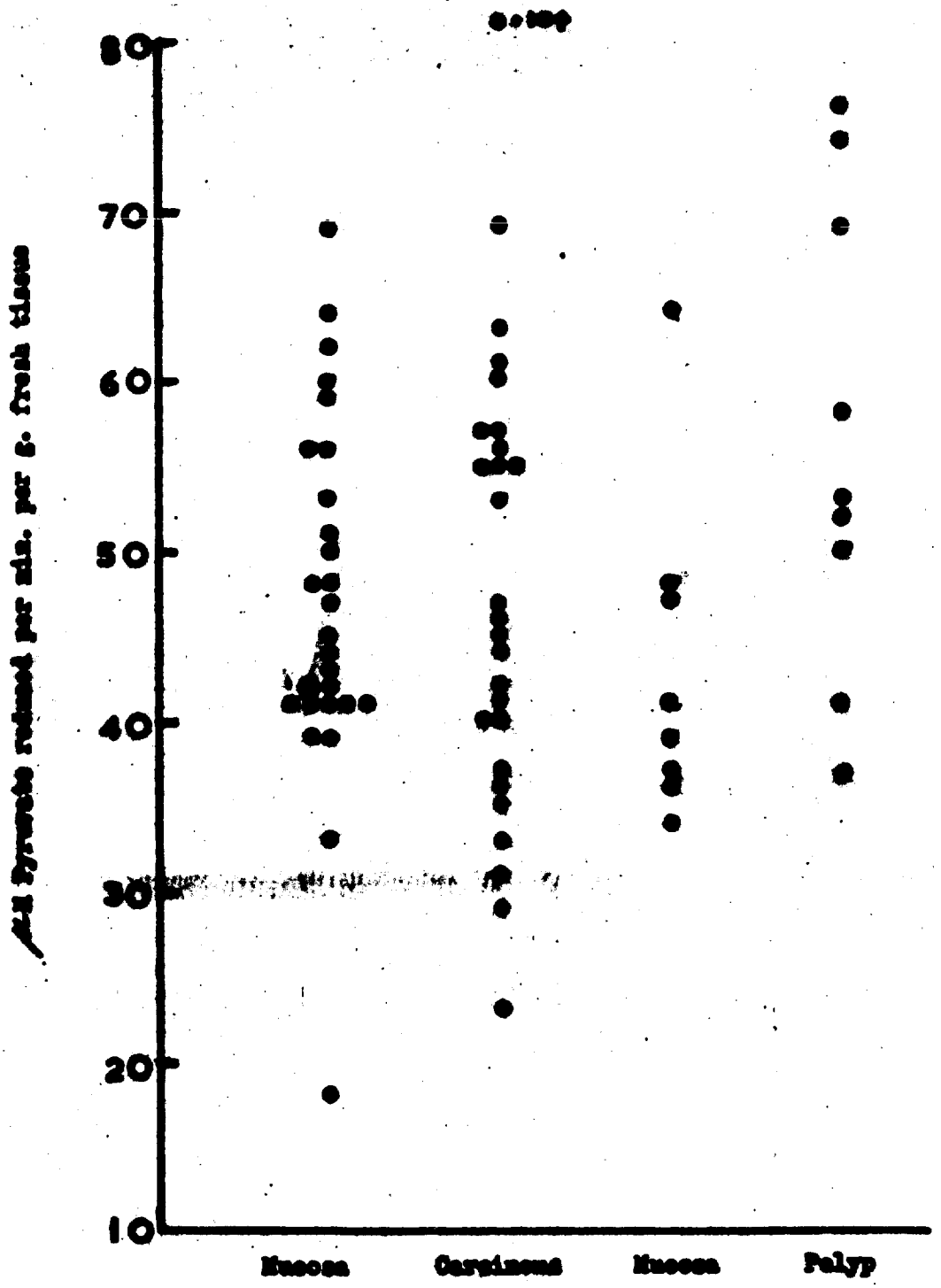

Fig.9. Scatter diagram of activity of lactate dehydrogenase in human colonic mucosa and tumours.

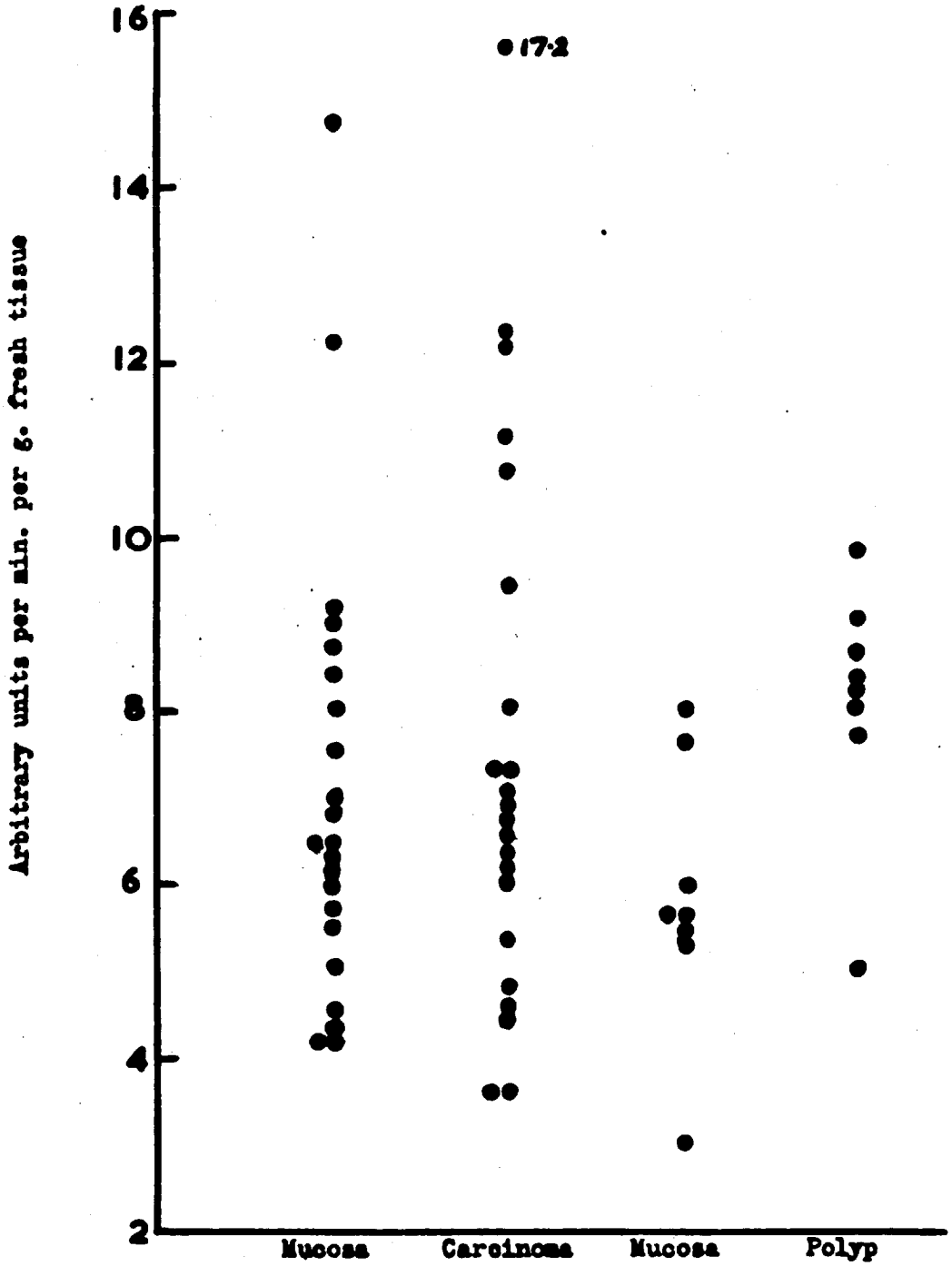

Fig. 10. Scatter diagram of activity of deoxyribonuclease in human colonic mucosa and tumours.

is greater than that in carcinomata when referred to fresh weight ($p < 0.002$) but not when referred to DNA P.

Lactate Dehydrogenase (fig. 9)

No difference was demonstrable in the activities of this enzyme in mucosa and carcinomata when referred to fresh weight, but the activity in the tumour is significantly greater when referred to the deoxyribonucleate phosphorus ($p < 0.001$). In the case of the mucosa and polyps taken from the same specimen, the activity in the polyps was greater only when referred to fresh weight ($p < 0.05$). The difference between the activities in the polyps and carcinomata is significant when referred to deoxyribonucleate phosphorus ($p < 0.05$).

Deoxyribonuclease (fig. 10)

The activities in the mucosa and carcinomata are similar when referred to fresh weight, but they are significantly greater in the tumours when related to the content of deoxyribonucleate ($p < 0.02$). The activity in the polyps is greater than that in the associated mucosa when referred to either index (fresh weight, $p < 0.002$; DNA P, $p < 0.02$). There is no difference between the activities in the polyps and carcinomata whichever index is used.

SUMMARY

(1) The difficulties associated with obtaining samples of normal and neoplastic cells preclude a comparison of the

amounts of their constituents.

(2) The relatively low activity of aldolase compared with the activities of lactate dehydrogenase and deoxyribonuclease demarcates the tissue of whole carcinoma from that of whole mucosa and polyp.

(3) The activities of lactate dehydrogenase and deoxyribonuclease, referred to gram tissue, are greater in the polyps than in the mucosa. There is no difference between the activities of these enzymes in polyps and carcinomata.

V

DETERMINATION OF CONCENTRATION OF SUBSTRATES IN EXTRACTS OF TISSUES OF THE RESTING RAT

The content of this section was adumbrated in Chapter III. Particular attention is given to the chief reasons for lack of confidence in published analytical results, namely, the failure to assess (a) the disturbance produced in the animal before and/or during removal of samples of its tissue, and (b) the effects of delay in stopping the actions of enzymes on the substrates. The disturbance produced in the intact rat was the more difficult to appraise. The method adopted included (i) the comparison of the resuls obtained by using a number of different methods of preparing the animal for sampling its tissue, that is, of producing unconsciousness, and (ii) an investigation into the range of yields of substrates resulting from, and of their sensitivity to the application of stress to the animal.

MATERIALS AND METHODS

Obtaining Samples of Tissue

The Tissues

The rats. Male animals weighing between 100 and 250 gram were used. Most of the rats were a black and white hooded strain bred at the Postgraduate Medical School of London.

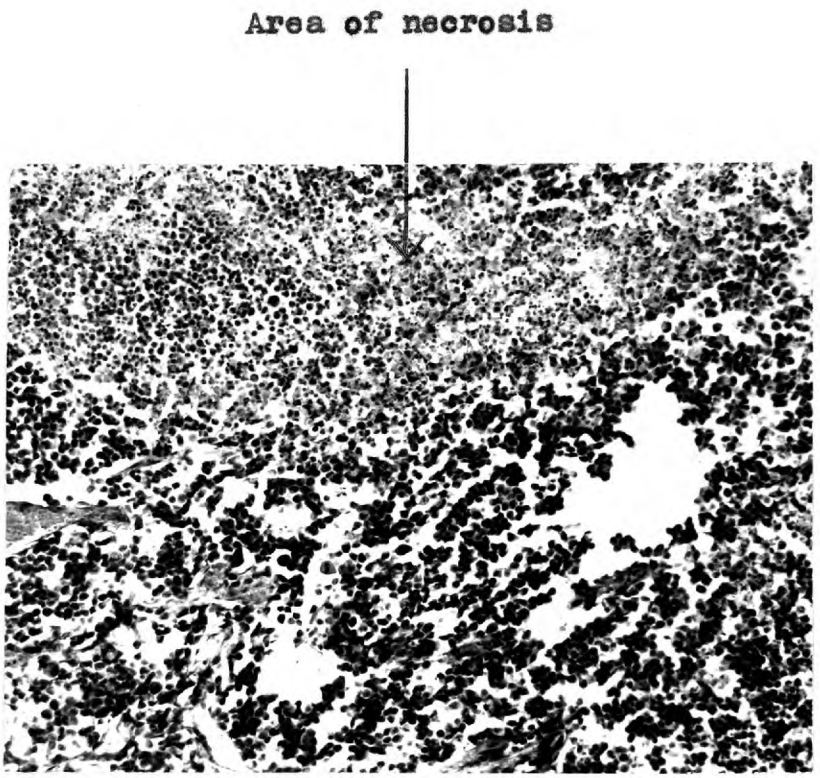

Fig. 11. Section of sarcoma (RD3). x100

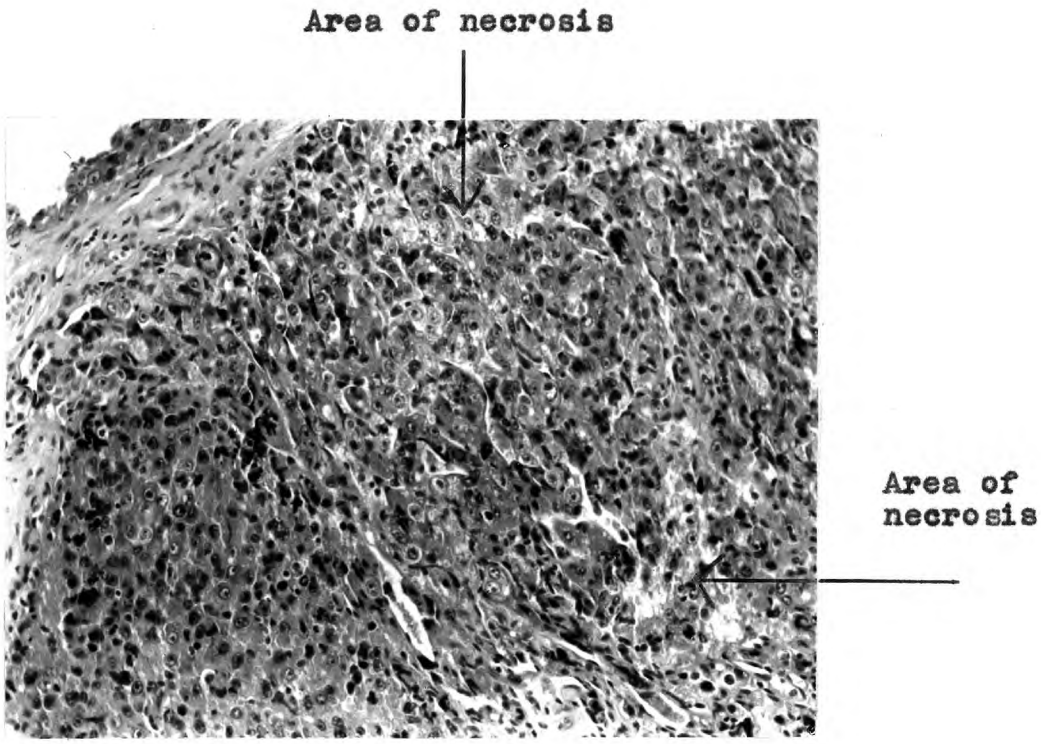

Fig. 12. Section of 3' MeDAB hepatoma. x250

Marshall and August rats were used for a few experiments and a strain of white rat, also bred at the Postgraduate Medical School, was used when 40 gram rats were required. All of these rats were used to being handled but special care was taken not to disturb them just before an experiment.

The tumours. Sarcomata (RD 3) were provided by Dr. R. H. Thomlinson of the Medical Research Council Radiobiology Unit. This tumour is grown in a highly inbred strain of white rats; it is transplanted as a mince from the donor to the flank of the host. It was analysed when it reached 2 to 3 cm. in diameter, that is, after 10 to 14 days of growth. The tumours contained areas of necrosis, but it was always possible to select samples which were relatively free from gross macroscopic necrosis. Histological examination always reveals necrosis; this may be seen in fig. 11.

Hepatomata were provided by Dr. E. Reid of the Chester Beatty Cancer Research Institute. These tumours are produced in a strain of rapidly growing white rat by feeding 3'-methyl-diaminoazobenzene at a concentration of 0.075 g.% in the food for 12 weeks. After a further period of 10 to 12 weeks on a normal diet tumours can be palpated in the liver; 3 to 7 days later the rat is killed and the tumours are removed. As with the sarcomata, ~~widespread~~ necrosis is revealed by histological examination (fig. 12).

The diet used for all rats was 41 b. This contains approximately 58% carbohydrate, 16% protein, 2.5% oil, 4.7%

ash, 11.8% water and has a calorific value of 327 cals./gram. It is supplemented with vitamins and choline (E. Dixon and Sons, Crane Mead Mills, Ware).

Means of Producing Unconsciousness

Ether anaesthesia. Ether anaesthetic, B. P. Duncan, was used throughout for anaesthesia unless stated otherwise. The ether was administered in a room at 23° to 25°. A 1.5 litre desiccator was used as the anaesthetic chamber. A layer of cotton wool about 2 cm. thick was placed on the floor of the desiccator, and a wire gauze was superimposed. The opening in the lid was stoppered with a perforated rubber bung. The perforation was needed to allow influx of air. This was essential in order to prevent anoxia. Ether was poured on to the wire gauze and cotton wool until the latter was saturated. The rat was put on to the gauze quickly and the lid was superimposed in such a way that there was about 2 mm. of overlap through which vapour could escape. The time of replacing the lid was taken. The rat was allowed to inhale the mixture of ether and air until it became unconscious; this was usually 60 to 75 seconds. A further 20 to 30 seconds of anaesthesia was allowed during which time the desiccator containing the rat was carried into a cold room maintained at 4°. The rat was then taken out of the desiccator and the appropriate tissue was removed. About 70 per cent of the rats did not struggle during the induction of anaesthesia, 25 per cent showed some movement and 5 to 10 per cent struggled

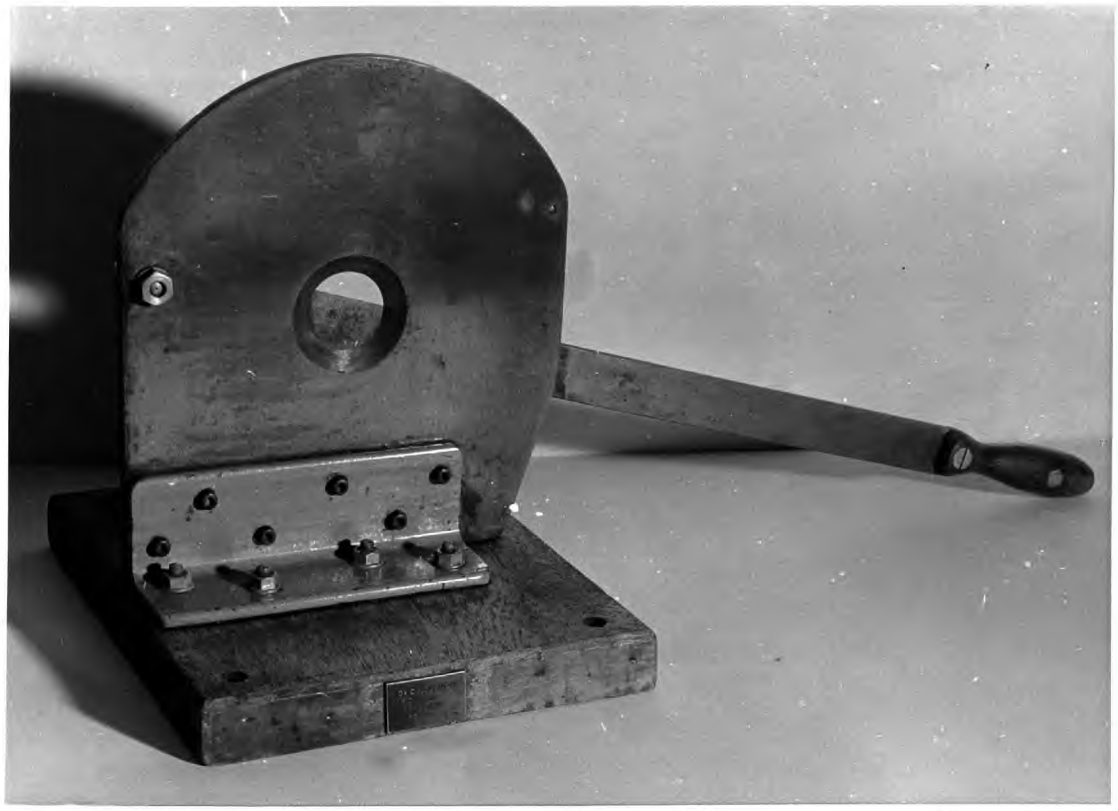

Fig. 13. Guillotine

violently. In certain experiments oxygen or nitrogen gas was admitted to the desiccator before and/or during anaesthesia. The gas entered through a thin, bent glass tube inserted through an additional perforation in the stopper, to the level of the wire gauze.

Nembutal (pentobarbitone) (Abbot) was used for a few experiments. Doses of 1 to 6 mg. were injected intraperitoneally. Anaesthesia was sufficiently deep to remove tissue after 10 to 15 minutes.

Guillotine. This was provided by H. B. Stoner and C. J. Threlfall at the Medical Research Council Toxicology Research Unit, Carshalton, Surrey. It consists of a vertical plate of stainless steel approximately 7 mm. thick, fixed to a heavy wooden block which is clamped to a bench. There is a hole with bevelled edges, approximately 3 cm. in diameter in the centre of the plate. On the side opposite the bevelling there is a stainless steel knife, 2 to 3 mm. thick, which is attached as shown in fig. 13; it is held in contact with the vertical plate by means of a steel guard. The rat is held in the left hand of the operator and it is allowed tp put its head through the hole; the right hand jerks the knife upwards in order to sever the animal's neck. The operator keeps the convulsing body flat by holding the limbs while a colleague opens the belly and removes the appropriate sample of tissue. A piece of liver can be removed with scissors within 7 seconds of guillotining. When the pre-

cooled tongs are used 2 to 3 seconds longer are required.

Freezing the whole rat. Rats weighing 40 to 60 grams were used. They were allowed to fall into and were held under the surface of liquid oxygen contained in a thermos flask. Struggling occurred for 2 to 5 seconds and then the rat became rigid. The oxygen continued bubbling vigorously for approximately 2 minutes. The carcase usually split after it froze. The tissues required were removed in the cold room with aid of chisels and forceps chilled in powdered solid carbon dioxide. They were kept floating in liquid oxygen and manipulated for 10 to 15 seconds at a time. After isolation the tissues were treated in the standard fashion (see pages 154-157).

Removal of Tissues

All samples of tissue, with the exception of blood, hepatomata and the livers of the rats which were guillotined at Carshalton, were removed in the cold room. The appropriate tissue was exposed; the sample was then removed, unless stated otherwise, with the aid of metal tongs precooled in liquid oxygen to approximately -190° (fig. 14).

Metal tongs. These were modified from the specifications of ERANKO (1954) and were made at the workshops of the Postgraduate Medical School. They consist of a pair of surgical forceps, 25 cm. long, to each blade of which is screwed a block of duralum. The blades of the forceps are bent so that the bight at closure is parallel. Each block measures

Fig. 14. Tongs used for rapid freezing of tissue

5 x 2 x 1 cm. A strip of stainless steel, 1 mm. in thickness, is screwed along the middle 3.5 cm. of each side of each block and the whole of the far end. The strips project 0.5 mm. towards the opposing block and this prevents complete closure of the tongs when pressure is applied. A leaf of frozen tissue of 1 to 1.5 mm. in thickness is obtained, the excess being squeezed through the gaps at the ends of the steel strips.

The pre-cooled tongs are used to grasp the tissue which is held as vertical as possible by means of toothed forceps. Sufficient pressure to obtain a sample 1 to 2 mm. thick is applied; the tongs together with the enclosed sample, are freed by means of scissors and then they are returned to the liquid oxygen. Should too much closing pressure be applied to the tongs, the steel shafts bend. This results in the proximal ends of the metal blocks acting as a fulcrum and the distal ends fail to close; a wedge-shaped piece of tissue is produced. This is not desirable because the distal end may be 2 to 3 mm. thick and its rate of cooling is reduced commensurately. There is no difficulty in obtaining up to 1.0 g. of tissue from liver, testis and muscle, but usually only 0.5 g. can be removed from the kidney.

Exposure. Each tissue requires a special approach in order to obtain adequate exposure.

Liver. The rat's belly was opened along the midline and along both costal margins. The lobes of the liver were

separated with the right index finger and the long central lobe was grasped very gently at the tip with toothed forceps held in the left hand. The tongs were removed from the liquid oxygen with the right hand and were used to grasp the lobe. The time lapsing from making the incision to grasping the lobe was 15 to 25 seconds.

Kidney. The belly was opened in a similar way, but the incision along the left rib margin was extended dorsally as far as the sacrospinalis muscle. The rat was then placed with its right flank across a cork ring so that the left kidney was displayed in the incision. The lower pole was freed partially from the surrounding fat by means of which the kidney was held gently in position and compressed in the tongs. The time required for the whole procedure was 20 to 30 seconds.

Testis. The belly was opened in the midline and the incision was extended laterally on both sides, about 1 cm. proximal and parallel to the inguinal ligaments. The epididymus was used to draw a testis into the abdomen. The thick capsule of the testis was snipped open with scissors; the testis was then held vertical by means of toothed forceps and the pre-cooled tongs were applied. The time required was 15 to 25 seconds.

Muscle. Abdominal muscle was used because it is the only flat sheet of muscle easily accessible and suitable for grasping with the tongs. The skin was first incised

along the midline and then at right angles laterally at both extremities of the belly on one side. It was reflected from the muscle with the aid of a new scalpel blade. The muscle was then divided along the midline by means of scissors. It was held away from the underlying viscera with the aid of a pair of toothed forceps applied at both ends of the incision. An assistant held the forceps applied to the distal end. The pre-cooled tongs were then used to grasp a section of the sheet of abdominal muscle. The whole process occupied 20 to 30 seconds.

Sarcomata. The skin between the tumour and the host was incised sufficiently to expose the tumour. Two parallel longitudinal incisions, about 1 cm. apart, were made in the tumour, thus exposing a central slab of tissue. This was readily grasped by the pre-cooled tongs. The time taken was 20 to 35 seconds.

Hepatomata. The incisions were made as for removal of a sample of liver. The tumour was treated in the same way as the sarcomata. The time required was 20 to 35 seconds.

Blood. The rat's belly was opened in the midline and the viscera were drawn aside. The aorta and inferior vena cava were cut across and the thumb was used to compress both vessels immediately. A tube containing 5.0 ml. 6 per cent perchloric acid was applied next to the severed vessels, the thumb was released and approximately one ml. blood was

collected. When the substrates in plasma (or enzymes in whole blood or plasma) were to be estimated, 3 to 4 ml. blood were collected in a tube containing 0.2 ml. heparin plus 0.3 ml. 0.2 molar sodium chloride.

Destruction of Enzymes and Extraction of Substrates

The freezing of tissues to the temperature of liquid oxygen stops the action of enzymes temporarily. They must be destroyed in order to stop their action permanently. This is conveniently carried out by means of exposure to perchloric acid into which the substrates are extracted simultaneously. The technique to be described is based on that of HOHORST and others (1959) and will be known as the standard procedure; any variations will be indicated in passing.

All of the steps up to the immersion of the tissue in ice-cold perchloric acid were carried out in the cold-room. All procedures undertaken in the laboratory were performed either in an ice-bath or in a refrigerated centrifuge at 2°. Working in a cold-room reduces the condensation of water vapour on to the frozen tissue and instruments, in addition to helping to keep the temperature of the tissue low. All instruments used to handle the frozen tissue were kept in powdered solid carbon dioxide, the temperature of which is less than -78.5°. Rubber gloves were worn. The frozen tissue projecting from the tongs was chipped off and the centre leaf was released into the liquid oxygen on which it floated

The leaf of tissue was usually 1 to 2 mm. thick, 10 mm. wide and up to 30 mm. long. It was broken into several pieces and 0.5 to 1.0 g. was transferred to a stainless steel mortar containing liquid oxygen. The cavity in the mortar was cylindrical; the pestle has a clearance of approximately 0.5 mm. If the pestle fits the mortar too tightly, the blows of the hammer used to powder the tissue may jam it and form a gas-tight seal. Any gas present expands as the mortar warms and the pestle is projected with devastating force.

The frozen tissue was powdered by 4 to 6 bouts of 5 blows on the pestle. At the end of each bout, more oxygen was poured into the mortar and the powder was stirred with a fine steel spatula. After the oxygen had evaporated the pestle was replaced and the next bout of blows was given. Four bouts were sufficient for most tissues; six were required for muscle. When pulverisation was complete more oxygen was poured on to the caked powder which was again stirred with the spatula. A large spatula was then used to transfer the powder to a test tube containing 5.0 ml. of 6 per cent perchloric acid (prepared by diluting 60 per cent acid (AR quality) with 9 volumes of water and delivered by means of a 5.0 ml. pipette reserved for this purpose). The transfer was made usually by the time the oxygen had evaporated. The tissue was stirred thoroughly in the acid with the spatula which was allowed to drain back into the test tube.

It was convenient, and economical of time to remove and to process four samples of tissue for subsequent analysis in one session. Each of the four tubes containing perchloric acid plus the sample were weighed. The four tubes were then centrifuged at 4000 x g. for 5 minutes at 2°. The supernatant was transferred by means of a pasteur pipette to 4 labelled test tubes standing in melting ice. The original tubes, containing the residue were returned to melting ice and 2.0 ml. 3 per cent perchloric acid were added to each from a 5.0 ml. graduated pipette. The residue in each tube was stirred into the acid and all four tubes were centrifuged again, as before. The supernatant from each tube was transferred by means of the same pasteur pipette to the appropriate tube containing the first extract. The original tubes were then washed with detergent, rinsed with distilled water dried with methanol and ether and re-weighed. The weight of the 5.0 ml. 6 per cent perchloric acid, always delivered from the same pipette, was known and the weight of tissue was determined by difference.

Each extract was next treated in turn as follows. It was poured into a 50 ml. beaker standing in an ice bath, the pH was brought to 3.5 $\pm$ 0.2 units by careful addition of 10 normal potassium hydroxide (AR quality, prepared in 100 ml. lots by dissolving 56 g. pellets in water and making the volume up to the mark). This was readily carried out by means of a 3.0 ml. graduated burette, the tip of which was

fitted with a capillary polythene tube which dipped into the extract. The titration was controlled by means of a glass electrode dipping into the extract and connected to an E.I.L. (model 23 A) direct reading pH meter. This pH meter is fitted with a thermostat and it is internally compensated so that readings made at 0° are automatically corrected to 20°. When the pH reached approximately 3.0, the extract was returned to the test tube in order to neutralise the extract remaining on the walls. The contents of the test tube were again emptied into the beaker and the pH was finally brought to 3.5. Approximately 0.5 ml. potassium hydroxide were needed. The standardisation of the instrument was checked against an electrometrically tested pH 5.00 buffer (B.D.H.).

The test tubes containing the extracts were allowed to stand at 0° for 30 minutes in order that the precipitate of potassium perchlorate could age. They were then centrifuged for 5 minutes at 4000 x g. as before. Each supernatant was finally transferred by means of a clean pasteur pipette to a clean test tube standing in melting ice. The samples were stoppered and held ready for the analysis of fructose diphosphate, dihydroxyacetonephosphate and pyruvate. When this was completed the remainder of the extract was stored at -20° until the lactate content was estimated.

The use of perchloric acid for extraction and potassium hydroxide for raising the pH has the advantage of removing most of the perchlorate ions. This is possible because at

0° the solubility of potassium chlorate is only 0.75gper cent (Handbook of Chemistry and Physics, 42nd edition). Ten normal potassium hydroxide is used in order to raise the pH with minimal increase in the volume of the extract. This is important because the amounts of fructose diphosphate and dihydroxyacetonephosphate in many tissues are small.

Analytical Methods

The Sugar Phosphates and Pyruvate

The methods available for the determination of sugar phosphates and pyruvate fall into 2 groups, namely, chromatographic and enzymic. The chief advantage of chromatographic methods is that many substances can be determined at once. The disadvantages are that they take a long time, the conditions are rigorous, the separations of compounds are not always satisfactory, some compounds cannot be identified and some cannot be found. Thus in a technique described by THRELFALL (1957) the time required for the whole procedure was at least 3 days; 2- and 3-phosphoglycerate and phosphopyruvate could not be identified and there was a large unknown spot present in the group of intermediates of glycolysis. Furthermore, in the absence of isotope dilution facilities, the smallest amount of sugar phosphate detectable corresponds to 17×10^{-9} mole of inorganic phosphorus.

Enzymic methods have the advantages of mild conditions, rapidity, simplicity and a sensitivity greater than many chemical methods. They sometimes suffer from lack of speci-

ficity and RACKER (1956) pointed out that confusing results will be obtained if the enzyme is not sufficiently pure, if its affinity for the substrate is not high enough and if there are inhibitory side-reactions. However, he also stated that 'Some enzymic methods used for determination of metabolic intermediates, e.g., the various hexose phosphates, have a degree of specificity rarely attained by colorimetric and other isolation procedures'. Pyruvate is another such intermediate. Although it was shown that a number of other ketoacids have a high affinity for lactate dehydrogenase (MEISTER, 1950), neither their affinity nor their quantity interferes with the determination of pyruvate in biological material (SEGAL and others, 1956). For these reasons and because a technique uniquely suitable for the present purpose was described by HOHORST, KREUTZ and BUCHER (1959), an enzymic method was used to determine fructose diphosphate, dihydroxyacetonephosphate and pyruvate.

KJELDAHL (1886) (quoted by RACKER, 1956) was probably the first to propose the use of enzymes as analytical tools. He suggested that sucrose could be determined by the action of invertase. The use of enzymes is now extensive and this is largely due to WARBURG and CHRISTIAN (1939). RACKER encountered many problems in his attempts to use a chain of enzymes to determine the activity of a given enzyme. His method for the estimation of dihydroxyacetonephosphate was one of the results (1947). The first estimation of hexose

diphosphate by an enzymic method appears to have been made by VISHNIAC and OCHOA in 1952; this was suggested, if not actually carried out, by CORI and others in 1948. SLATER used a similar method in 1953. Subsequently the method was used by RACKER and SCHROEDER (1958), WU and RACKER (1959) and HOHORST and others (1959).

The simplicity and elegance of the technique described by HOHORST and his colleagues are illustrated by the serial determinations in a crude perchloric acid extract of tissue in 30 to 45 minutes of the 3 substrates being discussed. The method depends on the specific enzyme-catalysed stoichiometric exchange of protons between $NADH_2$ and the substance to be determined or one derived from it. The reaction is followed and measured in the spectrophotometer by the decrease in the extinction ($\log \frac{I_o}{I}$) at 340 mμ. The ratio of the change in extinction during the reaction to the molecular extinction coefficient ($\epsilon 340$) is a direct measure of the initial concentration of the substance reduced.

Reactions

(a) Pyruvate.

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow[\text{dehydrogenase}]{\text{lactate}} \text{Lactate} + NAD^+$$

$K_{eq} = 3 \times 10^{-12}$ M, in the direction of $NADH_2$ (NEILANDS, 1952; GIBSON and others, 1953). The equilibrium is thus far to the right. The reduction of one mole of pyruvate is associated with the oxidation of one mole of $NADH_2$.

(b) Dihydroxyacetonephosphate

$$\text{Dihydroxyacetone-P}^{2-} + \text{NADH} + \text{H}^{+} \underset{\text{dehydrogenase}}{\rightleftharpoons} \text{glycerolphosphate} + \text{NAD}^{+}$$

$K_{eq} = 7 \times 10^{-12}$ M, in the direction of $NADH_2$ (BARANOWSKI, 1949). The equilibrium again lies far to the right; in fact, even in the presence of triosephosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase, no serious error is introduced into the estimation (RACKER, 1947). One mole of dihydroxyacetonephosphate is reduced per mole of $NADH_2$ oxidised.

(c) Fructose diphosphate

(i) $$\text{Fructose-1,6-diphosphate}^{4-} \underset{}{\overset{\text{aldolase}}{\rightleftharpoons}} \text{glyceraldehyde-3-P}^{2-} + \text{dihydroxyacetone-P}^{2-}$$

$K_{eq} = 1.2 \times 10^{-4}$ M (partially purified aldolase, MYERHOF and JUNOWICZ-KOCHOLATY, 1943). The equilibrium is in favour of fructose diphosphate which is present to the extent of 89 per cent.

(ii) $$\text{Glyceraldehyde-3-P}^{2-} \underset{\text{isomerase}}{\overset{\text{triosephosphate}}{\rightleftharpoons}} \text{dihydroxyacetone-P}^{2-}$$

$K_{eq} =$ 20 to 25 M. The equilibrium is in favour of dihydroxyacetonephosphate which comprises 96 per cent of the total triosephosphate (MYERHOF and JUNOWICZ-KOCHOLATY, 1943).

(iii) $$\text{Dihydroxyacetone-P}^{2-} + \text{NADH} + \text{H}^{+} \rightleftharpoons \text{glycerol-3-P}^{2-} + \text{NAD}^{+}$$

See (b) for further details. The estimation of one mole of fructose-1,6-diphosphate is thus associated with oxidation

of 2 moles of $NADH_2$.

Reagents. Glass distilled water was used throughout. 0.1 M-tris-HCl buffer, pH 7.6 (1 mM with respect to EDTA) was prepared every 2 weeks and stored at 4°. $NADH_2$ see p.

Aldolase, glycerolphosphate dehydrogenase (GPDH) and lactate dehydrogenase (LDH) (Boehringer und Soehne, Mannheim, Germany) were obtained as suspensions of crystals in ammonium sulphate solution. The suspensions were transferred to glass phials in lots of 0.2 ml. and stored at -20°. The manufacturers claim that the contaminating activities of each enzyme are as follows:

Contaminating enzyme	In LDH	In Aldolase	In GPDH
Dehydrogenase			
Lactate		0.05%	0.05%
Glycerolphosphate	0.005%	0.05	
Glyceraldehyde phosphate		0.01	0.05
Malate	0.03		
Pyruvate kinase	0.003	0.01	0.03
Triosephosphate isomerase		0.01	
Aldolase			0.01
Myokinase	0.01		

Triosephosphate isomerase was prepared from rabbit muscle (BEISENHERZ, 1955). 200 g. muscle was used and approximately 1.5 ml. of a suspension of needle-shaped crystals were obtained. The protein content was approximately 80 mg./ml. as determined by utilising the difference in

extinction of a solution examined at 280 and 260 mμ. The specific activity was not determined, but after dilution of the suspension 500 fold, the activity was found to be comparable to that of a mixed preparation of the isomerase and glycerolphosphate dehydrogenase of Boehringer und Soehne. No evidence of the presence of aldolase, glycerolphosphate or lactate dehydrogenases was found when the appropriate substrates were added to the reaction medium containing the enzyme plus $NADH_2$. The bulk of the stock solution was stored at -20°. An aliquot (0.1 ml.) was diluted 80 fold with a 1 per cent solution of albumin, divided into lots of 0.5 ml. and also stored at -20°. A fresh 0.5 ml. lot was used as needed.

D-fructose-1,6-diphosphate, calcium dihydrogen salt (British Drug Houses, Ltd., Poole, Dorset) (FDP) contains not less than 20 per cent water; the content of FDP is not available from the manufacturer. It was found to be 57.3 per cent after drying <u>in vacuo</u> over calcium chloride for 4 days at -18° (p.174). This value compares with 58 per cent for the Boehringer preparation (Biochemica 'Boehringer', 1958).

Dihydroxyacetonephosphate (California Corporation for Biochemical Research, California, U.S.A.) (DAP) was obtained as dihydroxyacetonephosphate (dimethylketal dicyclohexylamine salt, monohydrate). It was hydrolysed, according to instructions, by swilling for 30 seconds with moist Dowex 50 W (200 - 400 mesh, x 4) in the H^+ form), filtering through a

Buchner funnel with the aid of suction, and standing at 40° for 4 hours. The yield was just under 94 per cent compared with 95 per cent, as claimed by the supplier.

Sodium pyruvate, see p.104.

Procedure. All reagents, excluding the tris buffer, were made up daily and were kept at 0° in an ice bath. All pipettes were chilled by standing in a test tube in an ice bath. Enzyme solutions were measured by means of pipettes graduated to contain 0.1 ml.

$NADH_2$ solution was prepared by emptying the contents of an ampoule into 0.5 ml. tris buffer delivered by means of a 0.5 ml. pipette. Glycerolphosphate and lactate dehydrogenase solutions were made up separately by dissolving 0.01 ml. of the suspended crystals in 0.09 ml. tris buffer delivered by means of a 0.1 ml. graduated pipette. The concentrations of enzyme protein in the solutions were, respectively, 1 and 0.5 mg. per ml. A single solution containing both aldolase and triosephosphate isomerase was prepared; 0.02 ml. of the suspension of aldolase crystals plus 0.04 ml. of the solution of isomerase in 1 per cent albumin were added to 0.04 ml. of tris buffer delivered by means of a 0.1 ml. graduated pipette. The concentrations of enzyme protein in the mixture were, respectively, 2 and 0.4 mg. per ml.

2.5 ml. tris buffer, 0.04 ml. $NADH_2$ solution and 2.5 ml. extract were added serially from appropriate pipettes to a test tube which was placed in a water bath at room temperature for 2 minutes. The total volume of the reaction mixture

was not allowed to exceed 5.0 ml. plus the volume of the added enzyme solutions; this was partly in order to conserve extract and partly to obtain the maximum extinction. The reaction mixture was then transferred to a 4 cm. glass cuvette which was placed on a chock, 4 mm. thick, in the cell carrier of the spectrophotometer (Zeiss PMQ II). The cuvette was raised in order to ensure that the light path did not pass through the meniscus. The reference blank, set on zero, was a solution of 0.1 normal potassium dichromate in 2 N sulphuric acid diluted sufficiently to obtain an extinction of 0.4 to 0.8 at a wave length of 340 mμ when the reaction mixture was in the light path. The extinction at which the reaction was to start was chosen according to the decrease expected, that is, according to the amount of oxidation of $NADH_2$ anticipated. The slit width required to give a full scale deflection varied between 0.1 and 0.4 mm. according to the kind and amount of tissue used. Thus extracts of muscle and testis, being clear, required a slit of 0.1 to 0.2 mm., whereas extracts of kidney and liver, which were opalescent, required a slit of 0.3 to 0.4 mm. The reaction mixture was allowed to equilibrate in position in the spectrophotometer in order that any non-specific reactions could take place. The extinction was then measured every 30 seconds in order to observe the background drift. This varied with the source of the tissue. Thus with extracts of muscle and testis there was usually no drift from the time of mixing the

reactants. However, extracts of blood, liver and kidney were always associated with some drift; it usually settled down to about 0.001 per 30 seconds. It is noteworthy that the drift was present only in extracts derived from tissues with a rich blood supply.

The substrates were estimated in the following order: dihydroxyacetonephosphate, fructose diphosphate and pyruvate. This was carried out by the serial addition, at appropriate intervals of time, of 0.01 ml. of glycerolphosphate dehydrogenase (10 µg. protein), aldolase (20 µg. protein) plus triosephosphate isomerase (4 µg. protein) and lactate dehydrogenase (10 µg. protein), in that order. The addition of the enzymes to, and their mixture with the contents of the cuvette were made by means of a container cum stirrer. This consists of an 8 mm. cube of perspex from one side of which a 6 mm. cube is removed, leaving a cavity with a base of perspex 2 mm. thick. The base is perforated by 6 to 8 holes, each 1 mm. in diameter, and to it is attached a handle of stainless steel wire 9 cm. long. The central cavity holds 0.2 ml. liquid which drains through the holes in the base only if the outside of the container is wet. Thus the enzyme solution can be held in the perspex container until it is introduced into the cuvette; the holes then facilitate mixing.

The glycerolphosphate dehydrogenase was added to the cuvette and mixed with the contents after the rate of drift

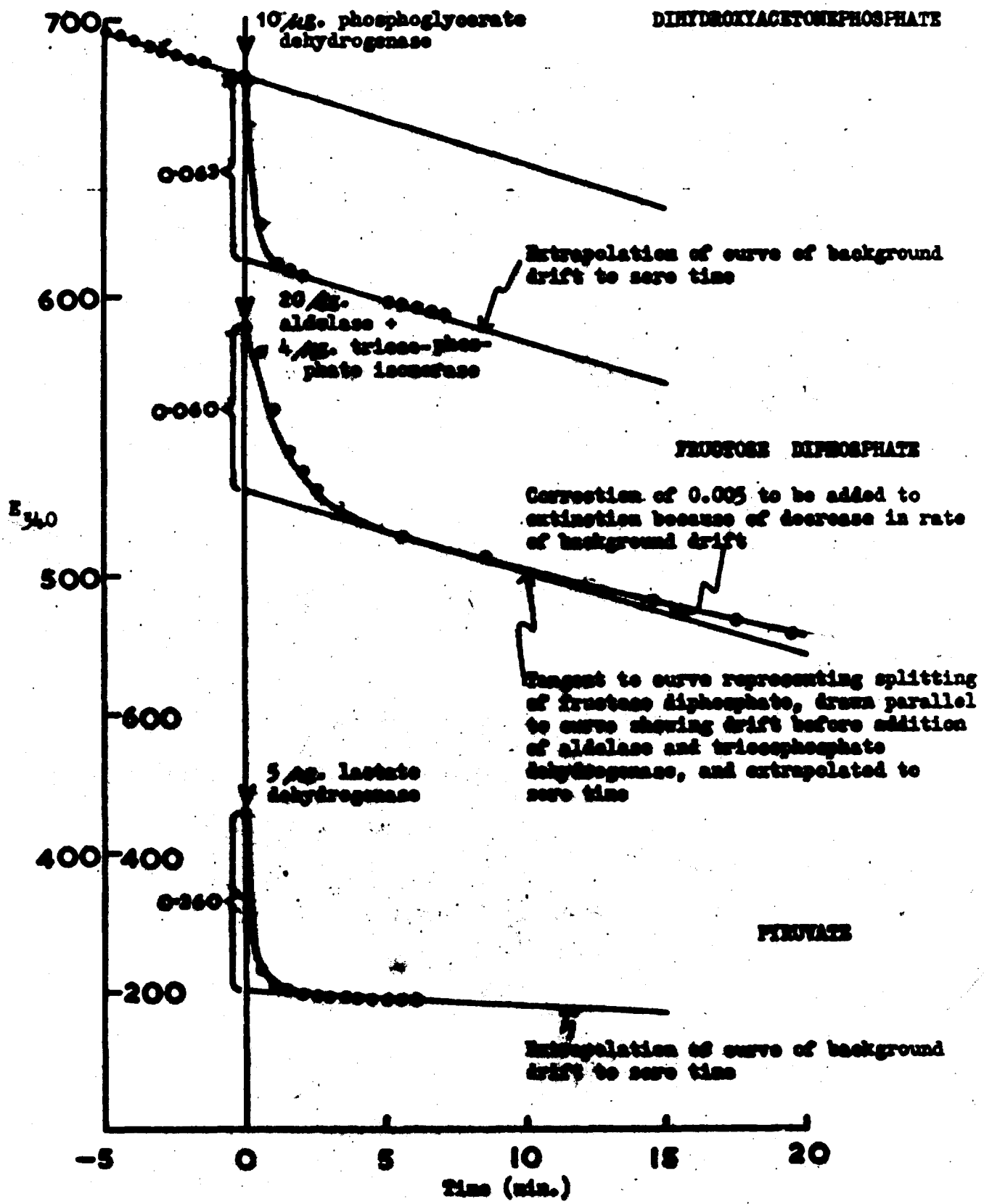

Fig. 15. Course of reactions used to determine dihydroxyacetonephosphate, fructose diphosphate and pyruvate.

was observed for 3 minutes. The time of mixing was noted and readings were then taken every 30 seconds until the rate of drift was again constant, that is, until the reaction was complete. The procedure was then repeated by adding next the mixture of aldolase and triosephosphate isomerase, and finally the lactate dehydrogenase. The stirrer was thoroughly washed with distilled water between additions.

Course of reaction. The sequence of changes in the extinction associated with the serial addition of the enzymes is shown in figure 15. The graphs of the reactions are presented in a tier rather than in series for reasons of space. The scale used for pyruvate is one quarter of that used for the sugar phosphates. The curve starts in the top left corner at an extinction value of 0.691. The rate of drift is approximately 0.003 per 30 seconds. On adding glycerolphosphate dehydrogenase there is a rapid decrease in the extinction due to the oxidation of $NADH_2$. This reaction is virtually complete in 90 seconds, but it is followed for 5 to 7 minutes in order to obtain a satisfactory estimate of the rate of background drift. The change in extinction is then obtained by the difference between the initial reading and the value obtained by extrapolating the curve of drift back to zero time. The difference is 0.063, a value often obtained when determining dihydroxyacetonephosphate.

The reaction which followed the addition of aldolase plus triosephosphate isomerase started at an extinction of

0.589. Here the oxidation of $NADH_2$ proceeds more slowly; it is the 3rd reaction in a sequence and it is complete only after 5 to 10 minutes. The end point is sometimes difficult to identify because the rate of background drift may decrease noticeably during this time. Fortunately, the potential error is not large and it can be allowed for satisfactorily as follows. It is assumed that the deceleration of drift is constant. A tangent to the curve tracing the course of the reaction is drawn parallel to the projection of the line representing the background drift after the first reaction. The distance between the ordinate and the junction of the tangent and the curve tracing the course of the reaction is measured. At a point twice this distance from the ordinate the vertical distance between the tangent and the curve is measured. It represents the decrease in the rate of background drift during the reaction and it is added to the estimate obtained by assuming a constant rate of drift. In this case the correction amounted to an extinction of 0.005, to be added to 0.060.

The reaction which occurred during the measurement of pyruvate started at 0.458 and continued until the extinction was 0.193, 2.5 minutes later. As with the first reaction, the oxidation of $NADH_2$ was followed for longer in order to obtain a better estimate of the rate of drift. The extinction value after correction for drift was 0.260.

Calculation of the amount of substrate per gram of tissue. The molar concentration of the substrate in the cuvette is obtained first. It is equal to $\frac{E}{\epsilon \cdot l}$ where

E = change in extinction due to oxidation of $NADH_2$.

ϵ = molar extinction coefficient of $NADH_2$

= 6.22×10^3 cm^2. at 340 mμ (HORECKER and KORNBERG, 1948).

l = length of light path in cm.

The total amount of substrate per gram of fresh tissue

$$= \frac{E}{\epsilon \cdot l} \times \frac{\text{Volume in cuvette}}{1000} \times \frac{\text{total volume extract}^{*}}{\text{volume used for reaction}} \times \frac{1}{\text{fresh weight of tissue}}$$

* It is assumed that (a) the extraction of the substrate is virtually complete, that is $>$ 95 per cent, and (b) the volume of solid plus liquid remaining after extraction is approximately the same as that of the sample extracted. The total volume of extract is then the combined volume of the 2 lots of perchloric acid used for extraction, namely, 5.0 plus 2.0 ml. plus the volume of potassium hydroxide used for raising the pH.

This relation applies when 1 mole of $NADH_2$ is oxidised per mole of substrate reduced, for example, when dihydroxyacetonephosphate or pyruvate are reduced. When 1 mole of fructose diphosphate is split, 2 moles of $NADH_2$ are eventually oxidised; the extinction, E, in the equation is therefore halved.

Verification of analytical procedure. No details of

any verification of the enzymic determination of the 3 substrates appear to have been published. Both for this reason and because it was obviously necessary to check that the method is reliable, and that no inhibitors were present in reagents or extracts, a series of experiments was undertaken. This included the estimation of the change in extinction per mole of substrate transformed, preparation of calibration curves and recovery of substrate added to extracts of different tissues.

(a) Extinction per mole of substrate transformed. This measurement provides an absolute check on the method if pure substrate is available (see page 160 for reactions). It was carried out during the preparation of a calibration curve for each of the substrates. Solutions in tris buffer, approximately 10^{-5} to 10^{-4} molar with respect to dihydroxyacetonephosphate, fructose diphosphate and sodium pyruvate, were accurately prepared from reagents obtained commercially (pages 162 and 163). The theoretical final molarities (assuming 100 per cent purity of the reagents) in the cuvettes were DAP 1.62×10^{-5}, FDP 1.08×10^{-5}, and pyruvate 2.04×10^{-5}. The actual molarities were determined according to the procedure already described (page 163), and the change in extinction per mole of substrate transformed was calculated. This was found to be as follows:

dihydroxyacetonephosphate 5.82×10^{3} cm^2. = 93.5 per cent of theoretical value

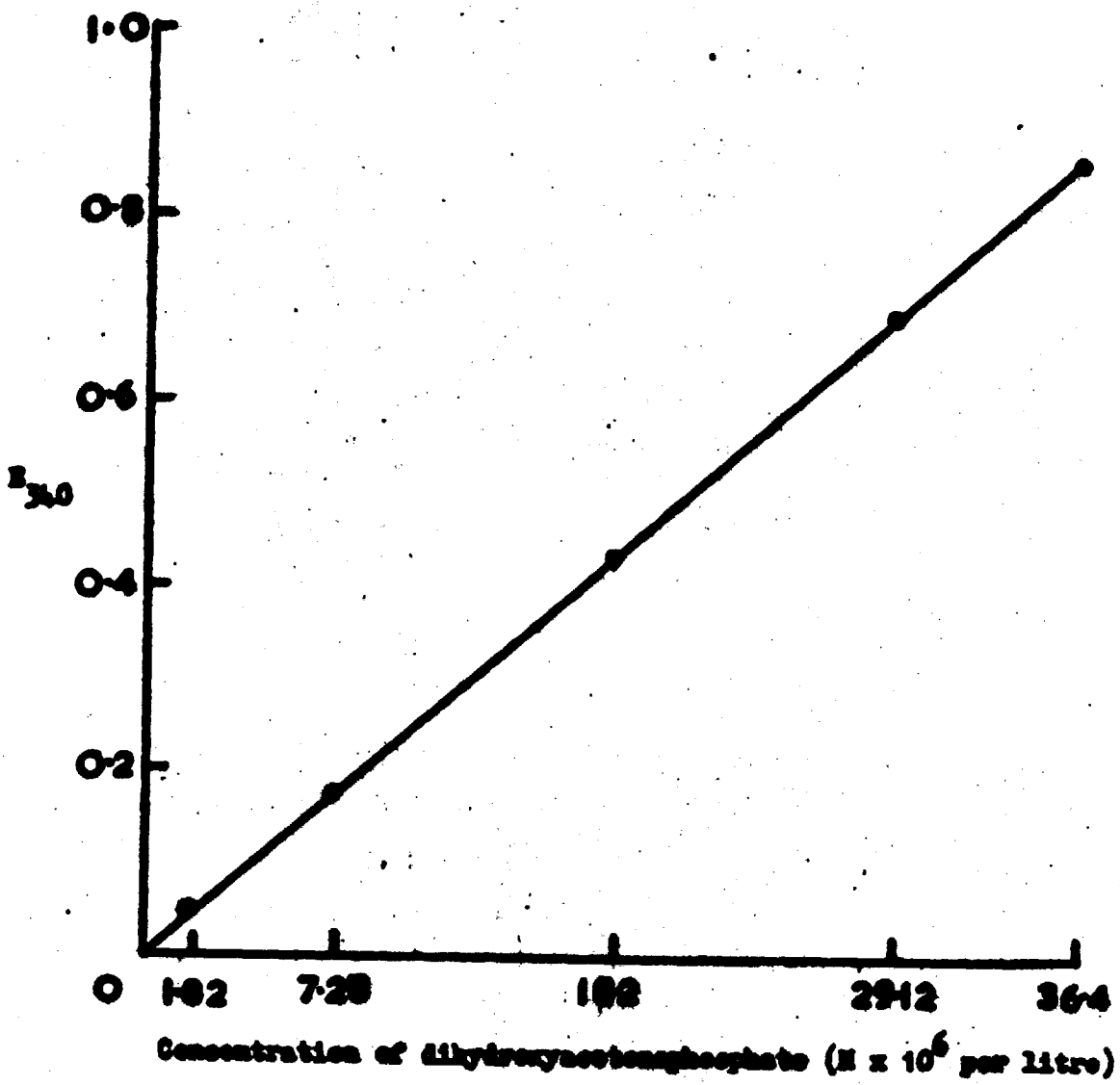

Fig. 16. Calibration curve of dihydroxyacetonephosphate.

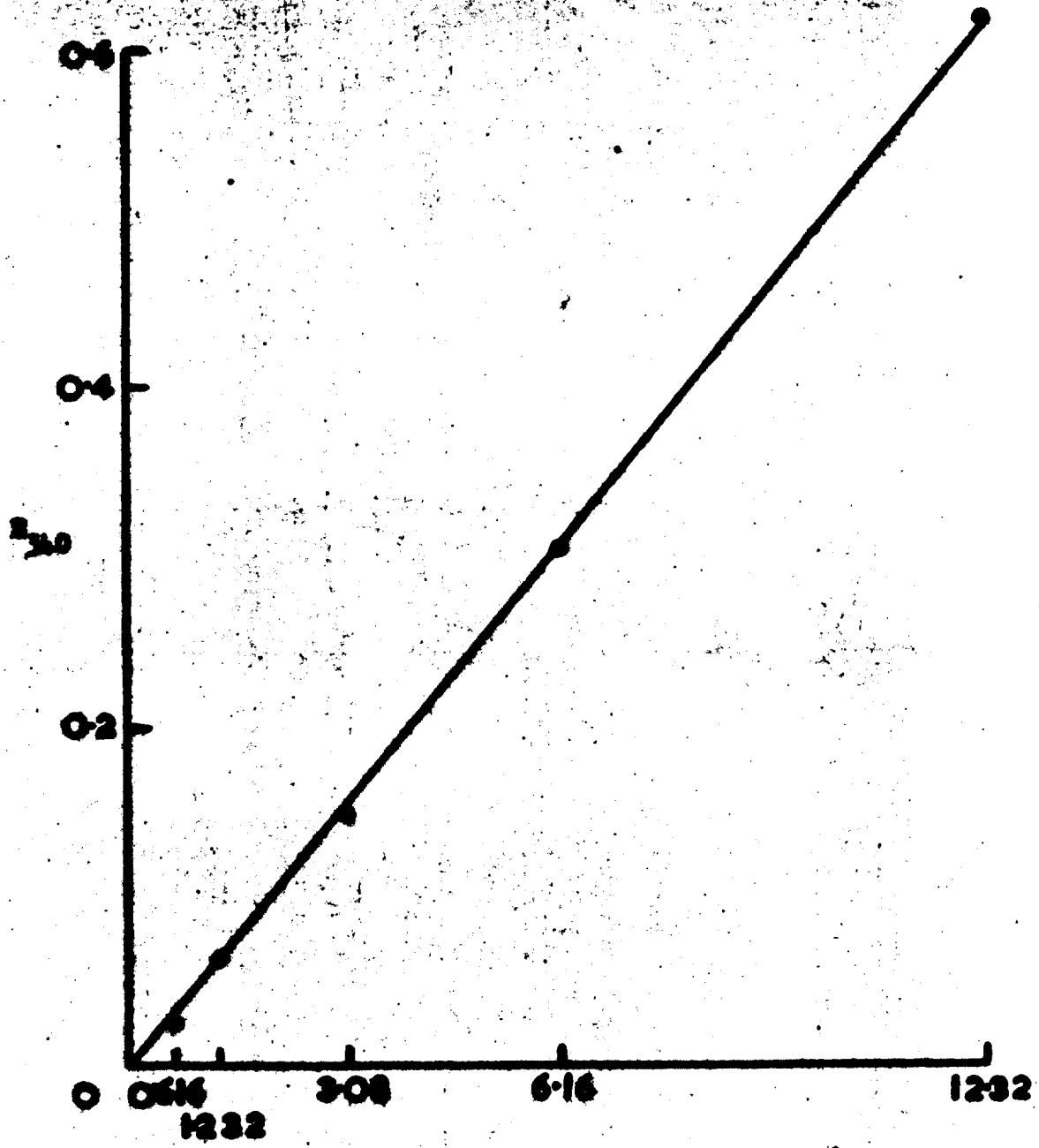

Fig. 17. Calibration curve of fructose diphosphate.

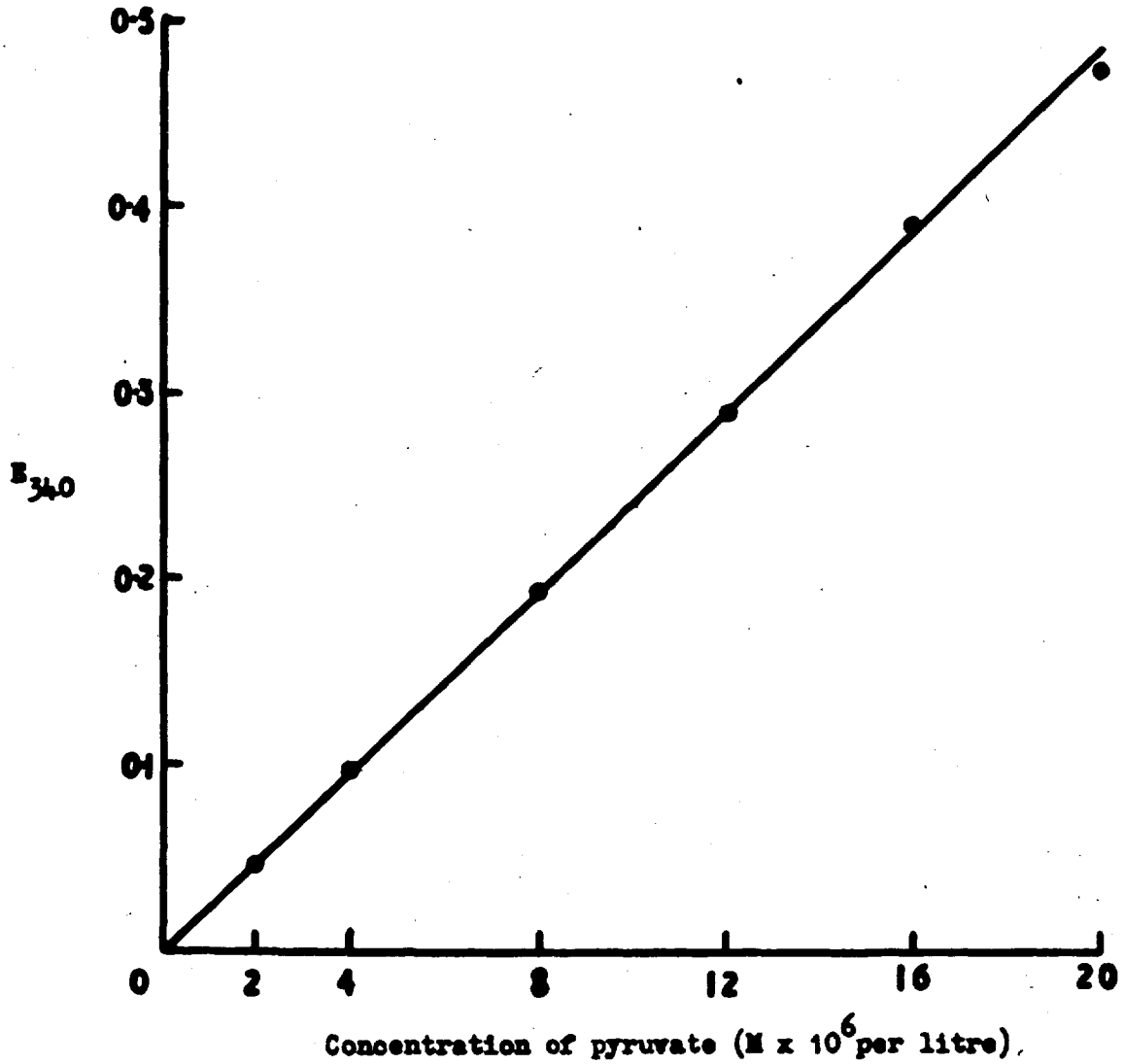

Fig. 18. Calibration curve of pyruvate.

fructose diphosphate 7.12×10^3 cm^2. ≡ 57.3 per cent of theoretical value

pyruvate 5.92×10^3 cm^2. ≡ 95.0 per cent of theoretical value

The change in extinction per mole of dihydroxyacetonephosphate transformed was actually only 1.5 per cent less than the theoretical change, 5 per cent being lost in hydrolysing the complex (see page162). Although the result of the analysis of fructose diphosphate appears low, it is in keeping with the expected purity of a commercial preparation (see page162). The result of the pyruvate analysis is satisfactory because no more than 98 per cent purity of the original salt was claimed, and because the sample used had been stored for 2 years at -20°. The experimental yields are consistent with the theoretical predictions.

(b) Calibration curves of purified substrates. These were determined for each substrate and were constructed by determining the changes in extinction due to transformation of substrate at 5 to 6 concentrations. The changes in extinction were scattered over the range of 0.02 to 0.08. The points fitted a straight line passing through the origin for all 3 substrates (figures 16, 17 and 18).

Recovery of substrates added to extracts of different tissues. This check was made by adding known amounts, both larger and smaller, of each purified substrate to extracts of tissues containing known amounts, both larger and smaller,

Table 9

Recovery of substrate added to extract

Substrate	Amount added	Amount recovered from extract	
		A (Low content of substrate)	B (High content of substrate)
DAP*	8.6	8.4	7.8
	86.0	85.0	85.0
FDP*	3.1	3.2	3.2
	30.6	31.3	31.6
Pyruvate⊕	9.7	10.5	10.1
	48.0	47.0	---
	96.5	---	90.5

All units are µmM substrate and refer to the reaction volume of 5.0 ml. in the cuvette.

* Extract A contained 3.8 µmM, extract B contained 10.1 µmM.

* Extract A contained 5.0 µmM, extract B contained 18.0 µmM.

⊕ Extract A contained 32.6µmM, extract B contained 75.0 µmM.

of the same substrate. The details appear in table 9. The volume of extract used was 2.5 ml. and to this was added 0.1 or 1.0 ml. of a solution in tris buffer of purified substrate. The final volume was made up to 5.0 ml. with tris buffer. The larger and smaller amounts of substrate already present in the extracts differed in magnitude by factors varying between 2 and 4. The recovery varied from 91 to 105 per cent over all the substrates; it did not differ especially between particular substrates or between the amounts of substrate already present or added. It is concluded that the 3 substrates can be recovered quantitatively from extracts of different tissues.

This evidence taken with the linearity of the calibration curves plus the demonstration that the changes in extinction per mole of all 3 substrates transformed are those expected, justifies the use of the enzymic method.

Investigation of some parameters of extraction procedures. No attempt was made to examine all of the parameters relating to extraction and analysis, because the method was described by HOHORST and others (1959). However, it was considered important to confirm that homogenisation is not necessary and that two extractions are sufficient. In addition, in view of the warning given by these authors that (a) pyruvate is unstable at a pH below 3.5 and triosephosphates are unstable in a more alkaline range of pH, and (b) all 3 substrates should be estimated within 12 hours of

their extraction, the effects of the final pH of the extract and of delay in estimation were examined. The authors state also that freezing and thawing of the extracts assists in the decomposition of the 3 substrates. This point was not investigated, the estimations always being carried out on unfrozen extracts as soon as possible after preparation.

(a) Effect of homogenisation. Homogenisation of tissue is carried out in order to obtain complete extraction of its constituents and where labile constituents are concerned, to allow rapid access of an agent which destroys enzymes. It appeared probable that pulverisation of frozen tissue would make homogenisation unnecessary, but it seemed important to demonstrate the point by experiment.

Homogenisation was carried out by means of the kind of apparatus described by POTTER and ELVEHJEM (1936). The powdered frozen tissue was transformed to the homogeniser tube which contained 6 per cent perchloric acid. It was necessary to wait for the tissue to thaw before homogenisation could be started. This took about 5 minutes. Powdered muscle was difficult to homogenise because it contained pieces of fibrous tissue which jammed the pestle. These pieces were cut up with scissors in order to make homogenisation possible. The tube was held in a plastic vessel containing ice and water; thus the extract was kept cool and the operator's hand was protected.

It was shown in a combined experiment (to be described

Table 10

Effect of homogenisation on yield of substrates from rat tissues

Tissue	Homogenisation (40 runs)	Yield of substrate µmM per g. fresh tissue			
		DAP	FDP	Pyruvate	Lactate
Liver*	NIL (8)	50	29	213	---
	carried out between 5 min. and 2 hrs. after sampling• (15)	54	26	226	---
Muscle*	NIL (6)	40	106	123	1163
	Immediate (10)	48	121	119	1280
	2-3 hours after sampling (8)	47	116	119	1689

None of the differences associated with delay in homogenisation is significant.

* Liver from a different rat used for each sample.

• Includes a comparison of effects of homogenisation immediately and after 2 hours (2 samples in each group): the differences were small.

* Powdered muscle from 1-3 rats was pooled and stored in powdered solid CO_2 for 30-45 minutes. Aliquots were then transferred to homogenisation tubes containing 6% perchloric acid, mixed with the acid and finally homogenised at the times indicated.

next) that the yield of substrates from muscle was the same after 40 or 160 runs of the tube against the rotating pestle. Therefore, in the experiments shown in table 10 only 40 runs were used. Both liver and muscle were tested in these experiments. There is no evidence that homogenisation carried out either immediately or after two hours affects the yield of the substrate examined. The high mean lactate levels in the muscle samples homogenised after standing in acid for two to three hours is anomolous; it is due to two very high results, the significance of which is unknown. It is of no importance in the present context. The lactate content of liver was not examined in these experiments. However, on other occasions when homogenisation was omitted, namely, in the guillotining and oxygen inhalation experiments, the lactate level was found to be similar to that obtained after homogenisation. These results confirm the observations of HOHORST and others (1959).

(b) Effect of a third extraction. Muscle was used for this experiment, approximately 3 gram being frozen and stored in a test tube standing in powdered solid carbon dioxide. Four aliquots were transferred to separate homogeniser tubes, each containing 5.0 ml. 6 per cent perchloric acid. The first two extractions were carried out in the usual way, and then two of the samples were extracted a third time with a second lot of 2.0 ml. 3 per cent perchloric acid. This experiment was combined with another in which

Table 11

Effect of number of extractions on yield of substrates from rat muscle

No. Extractions		Yield of substrate (µmM per g. fresh tissue) DAP	FDP	Pyruvate
2	a*	32	61	90
	b*	37	61	94
3	a*	38	68	101
	b*	36	64	92

* Homogenised, 40 vertical runs
* Homogenised, 160 vertical runs

see page

Table 12

Effect of pH on yield of substrates from rat liver

Experiment number	Final pH	Time occupied by addition of first 80% of 10 N KOH (0.4 ml.)	Yield of substrate µmM per g. fresh tissue		
			DAP	FDP	Pyruvate
1	3.0	3 min.	35	23	98
2	3.5	3 min.	37	22	100
3	3.5	1 min. 2 sec.	37	22	97
4	4.0	2 min. 45 sec.	37	21	98
5	6.0	3 min. 45 sec.	37	21	96
6	8.0	2 min. 45 sec.	35	24	94

the effects of homogenisation by 40 and 160 runs were compared. The results are shown in table 11; the difference in the yields, if any, due to the third extraction, is slight. It is concluded that 2 extractions are sufficient.

(c) Effect of pH. Three rat livers were sampled with the aid of the pre-cooled tongs, and pulverisation and extraction were carried out as usual. The final extracts were pooled and then 6 aliquots of 6.5 ml. were removed; each aliquot represented the extract from 1.05 g. liver. The pH was adjusted by the addition of 10 N potassium hydroxide, in the usual way, but to the values shown in table 12. Eighty per cent of the volume of potassium hydroxide required was added after intervals of time differing as indicated. The differences between the yields of each substrate obtained at a different final pH or after addition of alkali at different rates are minimal; neither the actual pH nor the rate of addition of alkali up to pH 8 are critical. However, pH 3.5 is convenient; further additions of strong alkali increase the pH rapidly and therefore increase the risk of reaching the high levels at which triosephosphates are unstable (HOHORST and others, 1959).

(d) Effect of delay between extraction and estimation. The livers of 4 rats were extracted as usual. Two extracts were taken to pH 3.5, 120 minutes after immersion in perchloric acid; the substrate concentrations were estimated

Table 13

Effect of delay between extraction and estimation on yields of substrates from rat liver

Amount of delay	RAT	Yield of substrate µmM per g. fresh tissue		
		DAP	FDP	Pyruvate
Nil*	1	32	18	51
	2	30	22	62
6 hours delay between taking extract to pH 3.5 and analysis	1	34	20	61
	2	32	20	64
5 hours delay between extraction and taking to pH 3.5. Analysis one hour later	3	44	24	220*
	4	35	18	66

* Time taken to obtain and extract samples, and to take extracts to pH 3.5, 120 minutes. Analyses of both samples completed 90 minutes later.

* These substrate levels are the only ones which are normal. The anaesthetic technique was inadequate at this stage and this rat was the only one of the four which had an adequate supply of air.

within a further 90 minutes and again after 6 hours. In the second 2 extracts the pH was taken to 3.5 after standing at 0° for 5 hours, and the estimations were carried out within one hour. The results are presented in table 13. There is no apparent correlation between the yield and the delay. The observation of a pyruvate level of 220 x 10^{-9} mole per gram of fresh tissue in the liver of rat 3, after 5 hours of exposure to pH $<$ 1 is not consistent with the suggestion that pyruvate is unstable at a low pH (HOHORST and others, 1959).

Lactate

Lactate can be estimated by enzymic and also by gas chromatographic methods. However, for many purposes the well-tried and reliable method of BARKER and SUMMERSON (1941) is suitable. This consists in the oxidation of lactate to acetaldehyde by concentrated sulphuric acid followed by colorimetric estimation of the acetaldehyde.

Reagents. 20 per cent (w/v) and 4 per cent (w/v) copper sulphate (AR quality). Calcium hydroxide, 'specially pure' (British Drug Houses Ltd., Poole, Dorset). Sulphuric acid S.G. 1.84 (AR quality, Hopkins and Williams). 1.5 per cent (w/v). p-hydroxydiphenyl in 0.5 per cent (w/v) sodium hydroxide (AR quality).

Lithium lactate (British Drug Houses, Poole, Dorset). The commercial product was re-crystallised twice. This was done by dissolving 5 g. in the minimum volume of distilled

water at approximately 60°. The solution was filtered through a Buchner funnel, and then cooled in an ice bath. The lithium lactate was then precipitated by adding approximately 8 volumes of ice-cold ethanol. The crystals were recovered by filtration on a Buchner funnel, washed with ethanol and then ether, and finally dried over calcium chloride in a vacuum desiccator. A stock standard solution of lithium lactate was prepared by first dissolving 106.6 mg. crystals in water. This solution was transferred to a 100 ml. standard flask. 20 ml. of approximately normal sulphuric acid solution was added and the volume was made up to the mark with water. The solution contained 1.00 mg. lactate per ml. It was stored at 4° and an aliquot was diluted to provide a working standard of 10 µg. per ml. as required.

Procedure. The Barker-Summerson method was followed except for the following modifications. (a) Removal of interfering substances by calcium hydroxide-copper sulphate treatment. The quantity of each substance used was reduced to one tenth of those suggested in the original method. Thus 0.1 to 0.8 ml. protein free extract containing 2 to 10 µg. lactate, and 0.1 ml. 20 per cent copper sulphate solution were used. The total volume was made up to 1.0 ml. with distilled water before adding approximately 100 mg. calcium hydroxide. (b) Conversion of lactate to acetaldehyde with sulphuric acid. One half of the quantities recommended in the original method were used from this stage onward, that

is, 0.5 ml. supernatant from (a) 0.025 ml. 4 per cent copper sulphate solution and 3.0 ml. concentrated sulphuric acid. The reaction was carried out at 60± 1° for 30 minutes in order to obtain a deeper colour when the acetaldehyde was allowed to react with p-hydroxy diphenyl (HULTIN and NOBLE, 1953).

Two standards, in duplicate, namely 1 and 3 µg. lactate, were used in order to dispense with a reagent blank; an apparent blank was used instead. The standard curve was linear up to an extinction of 0.7, corresponding to the highest concentration of lactate estimated. Each extract was also examined in duplicate at 2 concentrations, approximately those of the standards. In this way, differences in the apparent blanks of the standard and each of the test solutions were eliminated; each pair of test or standard samples provided its own apparent blank. The calibration curve prepared from the protein-free supernatant (see pages156 to156) was linear up to an extinction value of 0.7, again the highest value measured. Complete recovery of lactate added to the protein-free extract was obtained, both at low and high levels of added lactate and of lactate in the extract.

Calculation of the amount of lactate per gram fresh tissue. The concentration of lactate in µmM per gram of fresh tissue is obtained by substituting the appropriate values in the equation.

$$\frac{\text{Lactate in standard in } \mu g.}{\text{Mol. weight lactate} \times 10^{-9}} \times \frac{E_{test}}{E_{standard}} \times \frac{\text{Total volume extract} \times 2}{\text{Volume extract used}} \times \frac{1}{\text{Fresh weight of tissue extracted}}$$

The factor 2 is introduced in order to allow for the use of only half of the volume of extract after the removal of interfering substances.

Total Nitrogen

Homogenates of liver, kidney, testis, and tumours in water and samples of fresh muscle were used for the estimation of total nitrogen (see p107 for method).

Packed Cell Volume

The percentage of cells in blood was determined by centrifuging a Wintrobe tube, filled to the 100 mark with blood, for 30 minutes at 2250 x g at 25° (WINTROBE, 1951). The value obtained is corrected for any evaporation and for dilution by the anticoagulant (heparin plus saline).

RESULTS*

Assessment of Effect of Rate of Inactivation of Enzymes on Yields of Substrates

The means used to attain inactivation of enzymes were assessed by their effects on the yields of substrate from

* The significance of differences between results pertaining to the standard and other procedures is indicated in the tables as follows: $p < 0.05, S^{*}$; $p < 0.01, S^{**}$; $p < 0.001, S^{***}$.

Table 14

Effects of rate of inactivation of enzymes on yields of substrates

F - frozen to $<-183°$.
T - frozen by means of tongs.
P - frozen tissue pulverised.
H - homogenisation in 6 per cent perchloric acid

Tissue and procedure	Conditions: Thickness of samples	F	T	P	H	No. rats	Yield (µmM/g. fresh tissue): DAP	FDP	Pyruvate	Lactate
Liver										
Standard	1-2 mm.	+	+	+	+	27	52.6	27.4	219.4	1324^{x}
Freezing directly in liquid oxygen	lobe 5 mm.	+	-	+	+	6	39.8 S^{xxx}	20.4 S^{x}	161.8	1165
Thick leaf of tissue	2-4 mm.	+	+	+	+	8	30.0 S^{xxx}	13.3 S^{xxx}	120.3 S^{xxx}	916.6*
Homogenisation directly in perchloric acid	lobe 5 mm.	-	-	-	+	4	26.8 S^{xxx}	8.0 S^{xxx}	61.8 S^{xxx}	1108
No pulverisation	1-2 mm.	+	+	-	+	5	33.2 S^{xxx}	31.8	147.2 S^{x}	1122
Muscle										
Standard	1-2 mm.	+	+	+	+	9	36.4	86.4	78.3	1337
Freezing directly in liquid oxygen	2-3 mm.	+	-	+	+	8	41.6	94.0	94.3	1818 S^{x}

^{x} 17 rats * 3 rats

liver and muscle. The full standard procedure (page 145, pages 148 to 157) was used for reference and the effects of varying the parameters were observed.

Liver

The mean yields obtained with the standard procedure appear in the first line of table 14. They are expressed as μmM per gram of fresh tissue and are as follows: dihydroxyacetonephosphate 52.6, fructose diphosphate 27.4, pyruvate 219.4 and lactate 1324.

Effect of varying the rate of cooling. This was reduced by substituting liquid oxygen for the pre-cooled metal of the tongs. Part of a lobe of the liver was amputated, plunged into the oxygen and held submerged until the rate of bubbling was low. A significant decrease (25 per cent) occurred in the yields of dihydroxyacetonephosphate and fructose diphosphate. The apparent decrease in the yield of pyruvate was not significant and there was no change in that of lactate. The effects of changing the coolant were confused by the greater thickness of the sample obtained by amputation. In order to examine the effects of thickness alone, the closing pressure on the tongs was reduced. The leaves of tissue obtained were 2 to 4 mm. thick. The yields of dihydroxyacetonephosphate, fructose diphosphate and pyruvate were reduced to 45 to 60 per cent of those obtained with the standard procedure. There is insufficient data available to reach a conclusion about lactate. These experiments are

consistent with the view that the rate of cooling is important in fixing the concentration of labile substances in tissue.

Effect of varying the rate of penetration of perchloric acid into the tissue. Attempts were made to increase the rate of penetration of perchloric acid into liver by homogenisation of unfrozen tissue after immersion and by pulverisation of frozen tissue before immersion. Samples of unfrozen liver were disrupted in perchloric acid as rapidly as possible by means of a Potter-Elvehjem type of homogeniser. The results appear in the 4th row of the table; reductions in the yields of all substrates except lactate, relative to those obtained with the standard procedure, occurred to the extent of 50 to 70 per cent. HOHORST and others (1959) carried out a similar experiment with the exception that a homogeniser fitted with blades was used. They observed that pyruvate decreased by about 33 per cent, dihydroxyacetonephosphate increased to the same extent and lactate remained almost unchanged.

The pulverisation of the frozen tissue was a part of the standard procedure. Its omission (row 5, table 14) was associated with a significant reduction of the yields of dihydroxyacetonephosphate and pyruvate (about 33 per cent). The yields of fructose diphosphate and lactate were not affected. The reduced yields of dihydroxyacetonephosphate and pyruvate are attributed to the slower penetration of

perchloric acid into the cells when disruption is carried out by homogenisation alone. The ineffectiveness of homogenisation following pulverisation was demonstrated earlier (page177).

It is concluded that the highest yields of sugar phosphates and pyruvate are obtained only when the highest rates of cooling and of penetration of acid into tissues are achieved.

Muscle

The mean yields obtained with the standard procedure are as follows: dihydroxyacetonephosphate 36.4, fructose diphosphate 86.4, pyruvate 78.3 and lactate 1337 μmM per g. fresh tissue.

Effect of varying the rate of cooling. The yields obtained when the tongs were not used, liquid oxygen being the coolant, were similar. The differences are not remarkable with the exception of the yield of lactate which increased significantly by about 33 per cent. This is possibly a consequence of the contraction which occurs after physical stimulation such as cutting or exposure to low temperatures. The small or absent increase in the concentrations of the sugar phosphates and pyruvate, and the increase in the concentration of lactate illustrate the buffering and reservoir functions of the steady state.

Assessment of the Disturbance Produced in Animal Before and/or During Removal of Samples of Tissue

Comparison of Yields of Substrates Obtained by Use of Different Methods of Producing Unconsciousness

It is necessary to produce unconsciousness in an animal in order to obtain a sample of its tissue (page 72). The lack of adequate theoretical and experimental data concerning the production of unconsciousness necessitated examining and comparing the methods available. The criteria used to assess the methods were the production of apparently minimal physical disturbance of the animal and the agreement between results obtained by two or more different methods.

Ether anaesthesia. The major potential disadvantage of ether anaesthesia is an increased output of catechol amines from the adrenal medulla. This could result in increased glycogenolysis and increased formation of the intermediates being examined, in particular in liver and muscle. It is worth re-iterating here that the glucose resulting from glycogenolysis in the liver, induced by adrenaline, is believed to pass into the blood. The direct effects of the action of adrenaline in the liver may not be observed in the changes in the concentrations of metabolites being estimated. In muscle, on the contrary, the end result of the action of adrenaline is an increase in the concentration of lactate.

Table 15

Effects of anoxia on yields of substrates from liver

Conditions	No. rats	Yield (μmM per g. fresh tissue) DAP	FDP	Pyruvate	Lactate[§]
Standard[x]	27	52.6	27.4	219.4	1324
Oxygen[*]	4	45.8	27.8	214.5	1195
Nitrogen[•]	4	44.3	22.5	138.8 s[x]	4910 s[xxx]

[x] See also page . The partial pressure was such that the rat was apparently not disturbed. The induction time was usually 45 - 75 seconds. The total time of exposure to ether was usually 75 - 105 seconds. Rats taking longer than 120 seconds to reach the stage of surgical anaesthesia were excluded from this group. The depth of anaesthesia was judged by the depth and regularity of respiration. The rat often started to regain consciousness 15 - 30 seconds after sampling.

[*] Allowed to flow into desiccator at the rate of 1.5 litre per minute during anaesthesia.

[•] Allowed to flow into desiccator at the rate of 1 litre per minute for approximately 60 seconds before anaesthesia started. Flow stopped when rats became excited, ataxic or lethargic. Air admitted to desiccator and ether poured in through a tube in the bung. All rats became cyanosed.

[§] 17 rats only.

The results obtained from the oxygen experiments are included in those of the standard procedure in all tables. For the purposes of testing statistically the effects of breathing oxygen they were excluded.

Liver. The standard procedure of ether anaesthesia was carried out in 27 rats. The optimal conditions were found by trial and error, the criteria of most help being the lack of struggling and the speed of induction of anaesthesia. Especial care was taken to avoid anoxia and physical irritation by ether vapour.

(a) Effect of oxygen tension. The possibility of the yields of substrates being affected by low oxygen tension occurring in the desiccator as a result of displacement of the air by ether vapour was investigated. Two kinds of experiment were performed. In the first the desiccator was flushed with oxygen gas during anaesthesia. The results (table 15) show that the yields from liver, as determined by the standard procedure, were not altered. They are included, therefore, with those obtained with the standard procedure in all of the tables which follow. The second kind of experiment was carried out in order to define the amount of change in the yields of substrates which could be expected to accompany acute anoxia. Nitrogen gas was used to flush the air out of the desiccator after the rat was installed. Evidence of anoxia in the form of lethargy, ataxia and/or excitement was observed in approximately 60 seconds. The standard ether anaesthesia was then given. The yields of dihydroxyacetonephosphate and fructose diphosphate failed to alter significantly. The yield of pyruvate decreased by about 33 per cent and that of lactate increased by a factor of 3 to 4. Both of these changes are

Table 16

Effects of ether anaesthesia on yields of substrates

Tissue	Conditions	No. rats	Yield (μmM/g. fresh tissue) DAP	FDP	Pyruvate	Lactate
Liver	Standard	27	52.6	27.4	219.4	1324 (17)
	Low partial pressure of ether[x] during induction	5	41.8 S^{x}	20.9	59.6 S^{+++}	1055 (2)
	High partial pressure of ether[*] during induction	8	53.5	21.9	173.1	1578
	Prolonged anaesthesia[•]	6	36.2 S^{+++}	15.4 S^{+++}	177.5	1302
	Deep anaesthesia[§]	6	35.8 S^{+++}	22.0	98.3 S^{+++}	2368 (5) S^{x}
Muscle	Standard	9	36.4	86.4	78.3	1337
	Low partial pressure of ether[x] during induction	6	39.2	85.0	81.7	1047
	Prolonged anaesthesia[•]	6	34.3	68.3	100.3	1298

[x] The partial pressure was such that the rat did not lose consciousness for 4 - 11 minutes.

[*] The partial pressure was sufficiently high to be intensely irritating and to inhibit respiration. The rats often remained conscious for 120 - 135 seconds; they became excited and kept their heads above the level of the ether as long as possible. The results were very variable.

[•] After standard induction period an additional exposure of 95 - 240 seconds to ether vapour was allowed. Deep anaesthesia was avoided.

[§] Anaesthesia was taken to the stage where respiration almost stopped.

( ) Used to show numbers of rats used when they differ from the remainder of the series.

significant. It was concluded that substrate levels are affected by extreme anoxia only.

(b) Effect of speed of induction of anaesthesia. An attempt was made to induce unconsciousness with minimal amounts of ether. The results appear in table 16 in the second row as 'Low partial pressure'. The yields of dihydroxyacetonephosphate and pyruvate were reduced by approximately 25 and 70 per cent respectively. The reduction in the yield of fructose diphosphate is not significant at the 5 per cent level; the probability of the result occurring by chance is about 1 in 15. There was insufficient samples of lactate analysed to reach a conclusion.

The effects of high partial pressure were tried next, the object being to reduce the induction period. The results appear in the third row of the table. The ether vapour was so irritating that the rats became excited and they struggled, stopped breathing and became cyanosed. The yields of all substrates were variable; none of the mean yields differ significantly from those obtained with the standard procedure. Despite lack of significant changes in the yields of substrates, the behaviour of the rats is a reason sufficient to avoid high partial pressure of ether.

(c) Effect of dose of ether. The remaining two experiments were carried out with the object of producing a dose effect; it was argued that absorption of more ether would favour an increased release of adrenaline. This would activate glycogenolysis. Therefore, after a normal induction

one set of rats was exposed to low concentrations of ether for a long time, and another set was exposed to the normal concentration for a long time. In the second set of animals exposure to ether was prolonged until respiration became slow and irregular; this was usually about 60 to 90 seconds after the loss of consciousness. The results appear in rows 4 and 5. The effects of normal but prolonged anaesthesia were a reduction of 35 to 45 per cent in the yields of dihydroxyacetonephosphate and fructose diphosphate and no significant changes in pyruvate and lactate. The effects of deep anaesthesia were more striking. The yield of dihydroxyacetonephosphate decreased by 50 to 55 per cent, and that of pyruvate fell by 50 to 60 per cent. The lactate increased by approximately 75 per cent. The only change that can be construed as due to increased glycogenolysis is the increase in the yield of lactate. However, as an increase in lactate is inevitable when respiration is embarrassed there is no need to attribute the change to the action of adrenaline.

Muscle. The same standard anaesthetic procedure was used for obtaining samples of muscle. The effects of low partial pressure of ether during induction are shown in row 6 of the table 16. None of the changes in the yields are significant. The effects of prolonged anaesthesia of normal depth (row 9) likewise are not significant. There is no evidence of a dose effect. These results are of particular

significance when it is recalled that the action of adrenaline was detected by the production of increased amounts of glucose-6-phosphate (CORI and CORI, 1931) and of lactate (HEGNAUER and CORI, 1934) in muscle.

It was concluded from the experiments with ether anaesthesia that (a) it is possible to produce unconsciousness rapidly and without apparent disturbance in the rat, and (b) an increase in the dose of ether fails to produce evidence of increased release of adrenaline: indeed, with exception of lactate in liver, the only changes occurring were in the opposite direction to those expected from increased glycogenolysis.

Stimulation of adrenal medulla. However, it was still necessary to examine the possibility that some stimulation of the adrenal medulla is produced by the amounts of ether used in the standard procedure. This appeared unlikely because after guillotining (page 204), a procedure which is probably free from antecedant adrenal stimulation, the yields of pyruvate and lactate obtained were comparable to those observed after ether anaesthesia. Nevertheless, the possibility could not be excluded because the yields of dihydroxyacetonephosphate and fructose diphosphate obtained after ether anaesthesia were 50 to 60 per cent greater than those obtained after guillotining. A series of experiments, using the yields of substrates as indicators, was therefore conducted to test this possibility. On the one hand an

Table 17

Effects of changes in environment believed to alter the output of catechol amines and of adrenaline injections on yields of substrates from rat liver

Condition	No. rats	Yield µmM per g. fresh tissue			
		DAP	FDP	Pyruvate	Lactate
Standard	27	52.6	27.4	219.4	1324[§]
Training[⁂]	5	52.4	25.6	202.4	1400
NaCl injection (0.2 ml. 0.2 M) into peritoneal cavity 15 sec. before anaesthesia	6	42.5 s[*]	18.3 s[**]	228.5	1992 s[*]
Adrenaline[*]					
All experiments	14	39.4 s[**]	26.3	128.6 s[***]	2709 s[**]
20 µg./100 g. rat; anaesthesia and sampling 5-20 min. later	9	41.7 s[*]	27.8	135.7 s[**]	2811 s[***]
20-220 µg./100 g. rat; anaesthesia and sampling 10 min. later	8	42.8 s[*]	29.9	126.0 s[**]	3193 s[**]
Exposure to ether[⊕] vapour at high partial pressure	8	53.5	21.9	173.1	1578

⁂ This consisted of keeping the rats in the laboratory during the day for 2 weeks; they were removed from the cage, handled morning and afternoon for 5-10 minutes, weighed and placed in the anaesthetic chamber in the absence of ether. Thus they were exposed to noise and to people passing and to most of the conditions of the experiment; they became docile and were not frightened easily.

* Adrenaline HCl, B.P. 1 mg. per ml. diluted appropriately in sterile 0.2 M NaCl solution was injected into the peritoneal cavity. Doses of 10, 20, 50, 100 and 220 µg./100 g. rat were allowed to act for the times varying from 5 to 20 minutes. Doses up to 20 µg./100 g. rat allowed for 5 minutes produced no apparent change in behaviour. With larger doses and/or longer times of action many rats became lethargic and lay down; in several rats the respiratory rate increased. In the

Table 17 continued

* continued/
20 µg. group the adrenaline was allowed to act for 5 minutes in 2 rats, 10 minutes in 5, and 15 and 20 minutes in one rat each. In the 10 minute group the dose was 20 µg./100 g. rat in 5 rats, and 50, 100 and 220 µg./100 g. rat in one rat each.

• See table16 footnote for details.

§ 17 rats.

The results given under the standard procedure include those obtained from training experiments; however when the effect of training was tested statistically they were obviously excluded.

attempt was made to reduce adrenal medullary stimulation by training of the rats, and on the other, various measures designed to induce fear and thus to increase the amounts of catechol amines available to promote glycogenolysis were carried out (table 17).

(a) Effects of training. The details of the training experiments appear under the table. No effect was observed in the concentrations of the substrates in the liver. (These results are included among those obtained from normal liver in all tables). If fear is an important factor during the standard procedure it cannot occur before induction of anaesthesia.

(b) Production or simulation of fear. Two kinds of experiment were performed with the intention of producing fear. (i) Injection of sodium chloride solution intraperitoneally immediately before exposure to ether vapour. The yields of dihydroxyacetonephosphate and fructose diphosphate decreased, respectively by 20 to 25 per cent and 33 per cent, and the yield of lactate increased by 40 to 45 per cent. These changes are all statistically significant. No change occurred in pyruvate. The other kind of experiment which was aimed at the production of fear was exposure to a high partial pressure of ether vapour; none of the changes in the yields was significant.

(ii) Injection of adrenaline. A final attempt was made to define the amount and direction of the changes in the

yields of substrates in rat liver which might occur following adrenal medullarystimulation; this consists of injecting adrenaline into the peritoneal cavity. The details of the experiments appear in the footnotes to table 10. The results are divided into 2 groups in order to simplify the presentation. One group was obtained from rats injected with 20 µg. adrenaline hydrochloride per 100 g. rat, followed by anaesthesia and sampling of the liver 5 to 20 minutes later. The other group includes rats which were injected with 20 to 220 µg. per 100 g. rat and whose livers were sampled 10 minutes later. The differences between the results in the two groups are small. The differences between the yields obtained with the standard procedure and the combined results following the injection of adrenaline are significant for all substrates except fructose diphosphate. There was a decrease in the yields of dihydroxyacetonephosphate and pyruvate, respectively, of 27 and 42 per cent, and an increase in that of lactate of 99 per cent. The increase in the yield of lactate is the only observation that could be interpreted as evidence of increasedglycogenolysis. However, it is difficult to account for the decreases in the yields of dihydroxyacetonephosphate, fructose diphosphate and especially of pyruvate, on this basis. An increase in the yield of pyruvate, or a constant yield comparable to that following muscular exercise would be expected to follow increasing muscular glycogenolysis due to the action of

Table 18

Effects of guillotining on yields of substrates from liver

Conditions	No. rats	Yield (μmM/g. fresh tissue) DAP	FDP	Pyruvate	Lactate
Standard ether anaesthesia	27	52.6	27.4	219.4	1324 (17)
Guillotine without ether anaesthesia ※					
Mean time before freezing 11.2 sec.	8	34.9 S※※※	16.2 S※※※	206.9	1480
Mean time before freezing 24-30 sec.	4	49.5	28.0	117.3 S※※	2113 S※※※
Decapitation with scissors and without anaesthesia※					
Mean time before freezing 60-90 sec.	4	44.8	33.0	69.3 S※※※	3050 (2)
Guillotine during ether anaesthesia*					
Mean time before freezing 10.2 sec	8	47.5	20.6 S※	192.0	1299

※ Repeated muscular spasms of whole carcase for 40-60 seconds after neck severed.

* No muscular spasms after neck severed.

( ) Numbers in brackets given when number in group differs from remainder of series.

N.B. The results obtained for guillotining plus and minus ether anaesthesia differ only for DAP, $p < .05$, S※

adrenaline. The difference between the yields of the sugar phosphates obtained during ether anaesthesia and after guillotining would appear to have some explanation other than adrenal medullary stimulation.

Guillotining. The yields from liver following guillotining are presented in table 18. They are as follows: dihydroxyacetonephosphate 34.9, fructose diphosphate 16.2, pyruvate 206.9 and lactate 1480 µmM per gram fresh tissue.

Effect of delay before freezing tissue. Although the tissue was chilled to a temperature of less than -100° following guillotining within a mean time interval of 11.2 seconds, there was a possibility of changes occurring in the concentrations of reactants during this time (page 88). If it is assumed that any changes which might occur after guillotining are monophasic, then it should be possible to extrapolate back to the time of guillotining. The results of an attempt to provide data of this kind are shown in rows 3 and 4 of the table. The yields in row 3 were obtained by delaying the application of the pre-cooled tongs for 24 to 30 seconds after guillotining; those in row 4 were obtained after decapitation, carried out by means of scissors, and samples were not taken until after the reflex spasms had stopped some 40 to 60 seconds later. The effects of the delay in chilling are progressive increases in the yields of fructose diphosphate and lactate and a continuing fall in the yield of pyruvate. The content of dihydroxyacetonephosphate in

the liver increased during the first delay, but no further change occurred subsequently. If the initial assumption is correct, the interpretation of these results is that the resting levels of dihydroxyacetonephosphate, fructose diphosphate and lactate are lower and that of pyruvate is higher than those observed 10 to 11 seconds after guillotining. This interpretation is not wholly supported by the results obtained with the standard procedure; the yields of pyruvate and lactate are consistent with this interpretation whereas those of dihydroxyacetonephosphate and fructose diphosphate are not. The yields of the latter two substrates are approximately 50 per cent higher after the standard procedure. It was shown earlier that this difference cannot be attributed to adrenal medullary stimulation caused by ether.

Effect of antecedant ether anaesthesia. An attempt was made to resolve the difficulty as follows. It was postulated that if the yields of the sugar phosphates obtained with ether anaesthesia represent true resting <u>in vivo</u> concentrations, then the superimposition of guillotining on to exposure to ether would produce a reduction in the yields; contrariwise, if the results obtained after guillotining represent the true resting levels, that is, if the act of guillotining does not affect the <u>in vivo</u> concentrations of reactants, then guillotining after giving ether should produce the same yields as ether alone. The results of this attempt appear in row 5. The yields of dihydroxyacetonephosphate and fructose diphos-

phate were both increased compared with those obtained after guillotining alone, but only the increase in dihydroxy-acetonephosphate was significant (p is almost 0.025). The result is not conclusive; but it is clear that the act of guillotining reduces the yield of fructose diphosphate. The yields of pyruvate and lactate were not significantly different from those obtained with ether anaesthesia or with guillotining alone.

In view of the doubt remaining as to the effects of guillotining a further assessment of the two methods used to produce unconsciousness was attempted. This was made by examining the inevitable side-effects of guillotining which include interference with respiration, exsanguination and repeated violent muscular spasms. These effects cannot be investigated in an animal which has been decapitated. Therefore the experiments were carried out in rats under the standard ether anaesthesia; the liver was used for analysis.

Side effects of guillotining. (a) Interference with respiration. The experimental details are given in the footnotes to table 19. The time taken between incising the diaphragm and freezing the liver is almost identical with that lapsing between guillotining and freezing. The mean yields of the substrates obtained from liver frozen immediately

Table 19

Appraisal of side effects of guillotining on yields of substrate from liver

Conditions		No. rats	Yield (μmM/g. fresh tissue) DAP	FDP	Pyruvate	Lactate
Standard		27	52.6	27.4	219.4	1324§
Exsanguination*	A	10	36.8 S***	20.8 S*	95.9 S***	1800 S**
	B	4	54.5	48.5 S***	54 S***	5875 S***
Respiratory stoppage* or inhibition	A	10	37.1 S***	19.3 S**	182.7	1413
	B	2	43.0	25.0	107	4400
Exercise•		8	45.0	18.0 S**	220.0	2091 S**

* Exsanguination was carried out, under standard ether anaesthesia, by dividing the aorta and inferior vena cava between the renal and common iliac arteries, and allowing the blood to drain for 5 - 10 seconds. Freezing by means of the pre-cooled tongs was usually carried out immediately in groups A and after a planned delay in group B. 'Immediate' refers to time intervals of 5 seconds. Group A includes 3 samples frozen after a delay of one minute and group B includes one sample frozen after a delay of 3 minutes and 3 samples frozen after a delay of 10 minutes.

* Stoppage was produced by incision of both diaphragms and inhibition by incision of one diaphragm under ether anaesthesia; these procedures occupied 3 - 6 seconds. Group A was obtained from 6 rats in which both diaphragms were incised and 4 rats in which the left diaphragm was incised. In both rats in group B only the left diaphragm was incised. In group A freezing was carried out immediately and in group B after a delay of one minute.

• The exercise consisted in swimming for 2 minutes in water at approximately 37°. The rat was transferred immediately to the anaesthetic chamber and the standard procedure was carried out.

§ 17 rats.

after incising the diaphragm and guillotining, are as follows, the values for incising the diaphragm appearing first for each substrate: dihydroxyacetonephosphate 37.1 and 34.9, fructose diphosphate 19.3 and 16.2, pyruvate 182.7 and 206.9 and lactate 1413 and 1480 μmM per gram fresh tissue. The differences are not significant. Delay in freezing the sample after incising the diaphragm (60 sec.) resulted in changes in the yields of all of the substrates of the same order of magnitude and in the same direction as those associated with delay in freezing after guillotining or decapitation. The yields of dihydroxyacetonephosphate, fructose diphosphate and lactate increased, and that of pyruvate decreased.

(b) Exsanguination. The details of the procedure are given at the foot of the table. The time required for exsanguination plus freezing is 10 to 15 seconds. This time also is comparable with that required for guillotining and freezing, namely, 9 to 14 seconds. The mean yields of dihydroxyacetonephosphate and fructose diphosphate after exsanguination were similar to those obtained after guillotining. The yield of pyruvate was decreased by 50 to 55 per cent and that of lactate was increased by 20 to 25 per cent. Both of these changes were significant. When the time interval between exsanguination and freezing the tissue is increased, further changes in the yields of the substrates occur; these are similar in amount and direction to those

occurring when there is delay after either decapitation or incision of the diaphragm. The yield of dihydroxyacetonephosphate increases to the levels associated with standard ether anaesthesia, and that of fructose diphosphate may reach considerably higher levels. The yields of pyruvate decrease and those of lactate increase progressively with delay before freezing.

(c) Muscular contraction. It is unlikely that the effects of muscular exercise on the concentrations of substrates in the liver of the intact rat would resemble closely the effects of muscular spasm in a decapitate rat. The absence of anoxia, the presence of a normal circulation and the more prolonged but less intense contractions are some of the relevant differences. Any effects in the liver due to release of substances from muscle or the adrenal medulla will probably be absent in the guillotined rat. However, as it seemed possible that there might be sympathetic nervous stimulation of the liver, both during exercise and after guillotining the effects of muscular contraction were examined.

Swimming was the form of exercise chosen. The results appear in table 19. The yield of dihydroxyacetonephosphate was 45 µmM/g. liver; this does not differ statistically from that obtained after ether anaesthesia alone ($0.05 < p < 0.1$). The yield of fructose diphosphate was 18.0 µmM/g. liver, a value close to that obtained after guillotining, and differing significantly from that obtained with ether

anaesthesia alone. The yields of pyruvate and lactate are respectively 246.8 and 2886 μmM/g. liver; these values are higher than those resulting from guillotining. However, the differences are misleading because the concentrations of these two intermediates in the plasma and therefore in the extracellular fluid increases during exercise (dihydroxyacetonephosphate and fructose diphosphate are absent from the plasma and therefore from the extracellular fluid, page239). The volume of the extracellular compartment, including the blood vessels, is approximately 25 per cent of the whole liver (page242). After allowing for the increases of pyruvate and lactate concentrations in this space following exercise, the values become 220 and 2091 μmM/g. liver respectively. The value for lactate only now differs from that obtained after guillotining.

An appraisal of the side-effects of guillotining, as revealed by the experiments just described, may now be made. In all 3 kinds of experiment there was a reduction in the yields of dihydroxyacetonephosphate and fructose diphosphate to values comparable to those obtained after guillotining. The yields of pyruvate and lactate, which are almost identical after ether anaesthesia and guillotining vary more with the kinds of experiment. The comparison of the results associated with the side-effects of guillotining, as reproduced under ether anaesthesia, with those of guillotining alone, is misleading. A more apposite comparison is with guil-

lotining after ether anaesthesia. However, even when this is done the yields of fructose diphosphate remain almost identical. Nor is there a statistical difference in the recoveries of dihydroxyacetonephosphate: p is only slightly less than 0.1 for the effects of both exsanguination and respiratory stoppage when compared with ether plus guillotining. Nevertheless, if either of these accompaniments of guillotining were the sole cause of the reduction in the yields of both dihydroxyacetonephosphate and fructose diphosphate after simple guillotining, then the effect should be more marked when guillotining is carried out during ether anaesthesia.

The effects of moderate exercise are superficially similar to those of guillotining during ether anaesthesia. The difference in the content of lactate is probably due to the stopping of the circulation; there is no doubt that the lactate concentration of muscle is raised after violent contractions (see tables 13 and 14). However, whereas the effect of exercise on the conscious rat is to reduce the yield of fructose diphosphate, the prevention of the muscular contraction following guillotining brought about by giving ether, failed to effect an increase. This contrasts with the increase in the yield of dihydroxyacetonephosphate which results from giving ether before guillotining is carried out. Thus there is no evidence indicating that the muscular contraction alone was responsible for the effects observed

Table 20

Effects of freezing the whole rat on the yields of substrates

The details of the procedure are described on p.148 .

E-, E+ - freezing in the absence or presence, respectively, of ether anaesthesia. The symbols relating to significance of tests refer to comparisons made with the results derived from the standard procedure.

Tissue	No. rats	Yield (μmM per g. fresh tissue)							
		DAP		FDP		Pyruvate		Lactate	
		E+	E-	E+	E-	E+	E-	E+	E-
Liver*	5	23.4 S***	19.8 S***	12.7 S***	19.7	83.2 S***	50.9 S***	1126	948 S*
Muscle	5	17.4 S**	76.4 S***	36.0 S**	219.0 S***	94.0	174.4 S***	1064	2952 S*
Kidney	5	26.2	25.7	24.0 S**	40.2 S***	91.2	83.2	1306	1548
Brain	5	24.0	36.4	75.4	113.6	125.4	122.2	1250	1418

* 9 livers analysed for pyruvate, with and without ether anaesthesia.

after simple guillotining.

Freezing the rat whole. The results of these experiments are presented in table 20. Freezing of the whole rat (40 to 60 g.) was carried out both in the presence and in the absence of ether anaesthesia. Four tissues were analysed, namely, liver, muscle, kidney and brain.

(a) Liver. The yields of all substrates, both with and without ether anaesthesia are diminished compared with those obtained after standard ether anaesthesia alone. The decreases were significant with the exceptions of fructose diphosphate in the absence, and lactate in the presence of ether anaesthesia.

(b) Muscle. The yields of all substrates obtained after freezing the whole rat were 2 to 5 times greater when the animal was conscious than when it was subject to ether anaesthesia. The mean levels of pyruvate and lactate observed after ether anaesthesia were similar with or without freezing; they were, respectively, 78.3 and 94, and 1337 and 1064 μmM/g. fresh muscle. The mean levels of dihydroxyacetonephosphate and fructose diphosphate as obtained after ether anaesthesia were reduced by 50 to 60 per cent, when freezing was used in addition, that is, to 17.4 and 36.0 μmM/g. muscle, respectively.

(c) Kidney. Freezing the animal whole with or without ether anaesthesia did not significantly alter the yields of dihydroxyacetonephosphate, pyruvate or lactate as compared with those obtained during ether anaesthesia alone. The yield

of fructose diphosphate after freezing the conscious rat was 66 per cent greater than that obtained after ether anaesthesia with or without freezing.

(d) Brain. There are no figures with which to compare those obtained from the frozen rat. Ether anaesthesia failed to affect the yields of pyruvate and lactate, whereas those of dihydroxyacetonephosphate and fructose diphosphate were reduced by approximately 66 per cent. In general the results agree with those given by McILWAIN (1955). These are fructose diphosphate 30 to 80, pyruvate 100 to 200 and lactate 2,100 μmM/g.

The results relating to freezing the rat as a means of obtaining samples of tissue may be summarised as follows. In the liver the yields of all substrates were reduced relative to those obtainable with ether anaesthesia alone; the administration of ether before freezing failed to prevent this effect. This diminution in yields of substrate is a common finding when the rat is subject to stress, for example prolonged ether anaesthesia (table 16), stunning (table 21), or when samples of tissue are not frozen sufficiently fast (table 14). In muscle the absence of anaesthesia is associated with very high yields of all four substrates; this occurs in other conditions where there is extreme muscular activity, for example, after stunning (table 21). In all tissues examined the yields of fructose diphosphate were higher when freezing was performed without anaesthesia. High or increasing levels

Table 21

Effects of Nembutal anaesthesia and of stunning on yields of substrates from liver and muscle

Tissue	Conditions	No. rats	Yield µmM per g. fresh tissue			
			DAP	FDP	Pyruvate	Lactate
Liver	Standard procedure	(27)	52.6	27.4	219.4	1324*
	Nembutal, 0.5 to 3.0 mg./100 g. rat intraperitoneally 5-10 min. before freezing	(4)	23.0 S***	14.0 S***	28.5 S***	---
	Stunning*, time before freezing, 60-75 sec.	(4)	29.3 S***	24.3	49.8 S***	---
Muscle	Standard procedure	(9)	36.4	86.4	78.3	1337
	Stunning*, time before freezing, 60-95 sec.	(4)	42.5	173.3 S**	232.5 S**	14200 S**

* Reflex muscular spasms occurred for 60 to 75 seconds.

* 17 rats.

of fructose diphosphate were observed in other conditions only in the presence of severe stress, for example, decapitation (table 18), stunning (table 21) and exsanguination (table 19).

Conclusions. (a) In the absence of ether anaesthesia the results do not represent the resting state; (b) even if the results obtained by freezing during ether anaesthesia represent the resting state, the need for anaesthesia defeats the object of freezing the whole rat.

Other methods of producing unconsciousness. (a) Barbiturate anaesthesia. Pentobarbital was given intraperitoneally without disturbing the rat, in order to obtain samples of liver for chilling in the pre-cooled tongs. The results are shown in row 2, table 21. The yields of dihydroxyacetonephosphate and fructose diphosphate are less than 50 per cent and that of pyruvate is less than 15 per cent of those obtainable with ether anaesthesia.

(b) Stunning. This was carried out by striking the head of the rat with a mallet. Reflex struggling prevented opening the belly for 60 to 75 seconds. Liver and muscle were analysed after the usual preliminary chilling by means of the tongs. In the liver the yields of dihydroxyacetonephosphate and pyruvate were reduced significantly, the percentage recoveries being, approximately 45 and 25 respectively. In muscle the yields of all substrates except dihydroxyacetonephosphate were increased significantly, the

factor varying from 2 to 10 fold.

The yields set out in this table were observed in animals undergoing obvious stress, or they are comparable to those obtained from such animals. These methods clearly disturb the resting state before samples can be taken.

Conclusions

Of all of the methods examined for the production of unconsciousness in rats, the only two which merit further consideration are ether anaesthesia and guillotining. The results obtained by these methods differ only with respect to the yields of dihydroxyacetonephosphate and fructose diphosphate, those associated with ether anaesthesia being 50 to 60 per cent higher. It has been indicated already that the results obtained with ether anaesthesia are less suspect. The evidence for this view is summarised as follows.

First, the only objection to the use of ether is that it causes an increase in glycogenolysis through adrenal medullary stimulation. The only tissue where adrenaline is known to produce an increase in the concentrations of the phosphorylated intermediates of glycolysis beyond glucose-1-phosphate is muscle.

Secondly, production of fear by injection of a solution of sodium chloride or simulation of fear by an injection of adrenalin both failed to increase the concentrations of sugar phosphates in the liver. In fact, a decrease occurred frequently.

Thirdly, increased doses of ether were not associated with increased yields of the sugar phosphates from liver; again the reverse effects occurred.

Fourthly, guillotining during ether anaesthesia was associated with a yield of fructose diphosphate which was significantly less than that obtained during ether anaesthesia alone. This suggests that guillotining reduces the yield of fructose diphosphate, rather than that ether increases it.

Fifthly, many stimuli applied to the rat, conscious or unconscious, decreased the yields of the sugar phosphates. None increased the yields, provided that sampling was carried out immediately. Even apparently mild stimuli such as swimming or the injection of a small volume of physiological saline into the peritoneal cavity, produced large and significant decreases in the yield of fructose diphosphate.

Lastly, the timing of the stimuli in relation to the anaesthetic appears to have no effect on the yields of the sugar phosphates. Thus exercise and the injection of sodium chloride solution took place before, and exsanguination, respiratory stoppage and guillotining during anaesthesia. If ether anaesthesia causes an increase in the yield of fructose diphosphate, there seems to be no reason why mild stimuli applied before anaesthesia should prevent this rise.

Investigation of Range and Sensitivity to Stress of Yields of Substrate.

It seems probable that the yields obtained during ether anaesthesia and after guillotining approximate to the concentrations at rest *in vivo*. Nevertheless, there remains the pos-

Table 22

Range of yields of substrates in liver

Conditions	No. rats	Yield µmM per g. fresh tissue			
		DAP	FDP	Pyruvate	Lactate
Standard	27	52.6	27.4	219.4	1324[x]
Associated with lowest yields					
Frozen conscious	5	19.8			948.
Frozen whole under ether anaesthesia	5		12.7		
Nembutal anaesthesia	4			28.5	
Associated with highest yields					
Ether, high partial pressure	23	53.5[§]			
Adrenaline*, liver sampled 10" later	8		29.9[§]		
NaCl injection	6			228.5[◎]	
N_2 anoxia	4				4910[§]

[x] 17 rats

* 2·0 - 220 µg./100g rat

§ The highest values for each of these substrates was found when samples were frozen 10 minutes after exsanguination (table 19). Such values are not relevant to the present purpose.

sibility that both ether anaesthesia and guillotining disturb the concentrations of reactants in the resting steady state. This uncertainty can be reduced by making two more enquiries. The first is to discover the range of the yields of each substrate after subjecting the animal to various acute stresses. If this were not large and if the values obtained under the standard conditions were within the range some of the uncertainty would be removed. However, if these values were at either extreme of the range, it could be argued that the in vivo levels were possibly beyond the range established. The second enquiry is to test the sensitivity of the yields of substrates to different conditions of stress including those relevant to the two chief means of producing unconsciousness, that is to optimal ether anaesthesia and to guillotining. If the yields were not readily altered by the application of stress it would be reasonable to infer that little disturbance had occurred during ether anaesthesia.

Liver was used to provide most of the data for these enquiries. However, there being no reason to assume that other tissues react in the same way to stress as does liver, the effects of a number of stresses on muscle, kidney and testis were examined.

Liver. Range of yields of substrates. The various stresses applied to rats have been described in passing (tables 15 to 21). The conditions associated with the highest and lowest mean yields of each substrate are summarised in table 22. The range observed (μmM/g. liver) for each of the four substrates were, dihydroxyacetonephosphate 19.8 to 53.5, fructose diphosphate 12.7 to 29.9, pyruvate 28.5 to 228.5 and lactate 948 to 4910. The yields of

the substrates obtained under standard conditions of ether anaesthesia fall within the range of values associated with the various stressing conditions. The range of values observed for the yields of dihydroxyacetonephosphate and fructose diphosphate is not large; there is correspondingly less uncertainty about the resting values of these intermediates. The ranges observed for pyruvate and lactate are large. This makes for a large amount of uncertainty, but it also adds weight to the agreement between the results obtained during ether anaesthesia and after guillotining.

The position of the values of the yield of a given substreate obtained under standard conditions within the range of values obtained under different conditions is of especial interest. The yields of dihydroxyacetonephosphate, fructose diphosphate and pyruvate obtained under standard conditions are at or near the upper extremes of their respective ranges, whereas the yield of lactate is at or near the lower extreme (see also table 23). These relationships appear to be explicable by considering the unique position of the liver in carbohydrate metabolism. The liver uses carbohydrate as fuel for its own endergonic reactions as do other tissues. In addition, it is the only centre in the animal for the manufacture and storage of glycogen for subsequent distribution in the form of glucose. The lactate pool is presumably replenished from the blood stream from lactate arising from

other tissues, especially muscle. Even under resting conditions the concentration of lactate is considerable. It seems unlikely that it will diminish in the normal liver unless the enzymes concerned with its supply are poisoned. However, it may increase whenever there is release of lactate into the blood from any tissue undergoing increased glycolysis or if there is increased hepatic glycolysis. Therefore it is concluded that, in the absence of enzyme poisons, the lowest concentrations of lactate observed in extracts of liver represent the resting state in vivo.

There are equally compelling reasons for believing that in the resting animal, the yields of pyruvate should be at the upper end of the range observed. Acute disturbances, such as those which were considered earlier, could affect the concentration of pyruvate in the liver in the following ways:

(a) There could be a sudden demand for more local energy production via the tricarboxylic acid cycle; this would tend to lower the concentration of pyruvate. Any replacement from the glycolytic pathway, from the lactate pool or by deviation of acetyl-CoA from fatty acid synthesis would occur after the concentration had diminished. The reverse effect, namely, inhibition of the reactions of the tricarboxylic acid cycle, such as occur during anoxia, would produce a sudden demand for conversion of pyruvate to lactate.

This is shown in table 15.

(b) There could be a sudden breakdown of glycogen in response to adrenal medullary stimulation initiated by fear or ether, or in response to demands by other tissues for replenishment of their glucose or glycogen stores. The available evidence indicates that glucose formed by the degradation of glycogen, as when primed by adrenaline, passes into the blood-stream. It seems improbable that glucose designated for these purposes would spill over into the Embden-Myerhof pathway; indeed it is possible that it is deviated away from this pathway.

(c) There could be increased amounts of pyruvate (or lactate) entering the liver from the blood-stream from other tissues, especially muscle. This reaction obviously cannot occur after guillotining; this is partly because the interval of time is too short and partly because of the exsanguination. Nor is there any evidence to suggest that it takes place after standard ether anaesthesia; the results so obtained are comparable to those observed after guillotining. Furthermore, even after muscular exercise the pyruvate content of the liver fails to change significantly.

(d) There could be transient inhibition of energy production and an increase in the concentration of pyruvate. However, as the animal usually responds in a positive way to the effects of stress, this is regarded as unlikely to occur.

Thus the changes most likely to be associated with the acute disturbances used would reduce the concentration of pyruvate in the liver. A high yield of pyruvate is therefore expected in the resting state.

Similar arguments can be advanced in support of the probability that the resting levels of dihydroxyacetonephosphate and fructose diphosphate are at least at the upper end of the range observed. The only difference is that the synthetic pathway which by-passes pyruvate, involves the sugar phosphates. Thus it could be argued that ether stimulates synthesis and that this increased the concentrations of the sugar phosphates. The most likely source of synthesis would by pyruvate or lactate returning via the blood-stream, chiefly from muscle. However, none of the stresses applied, including exercise and the injection of adrenaline, increased the levels of the sugar phosphates. Thus there is no obvious way in which ether could promote such an increase.

Sensitivity yields of substrates to stress. The simplest definition of sensitivity is the amount of displacement from a stable position brought about by a given stimulus. This definition can be applied to complex systems also, for example, to the open chemical system; but here the response is more complicated. The main kinetic features of the open system were discussed briefly earlier (pages 40 to 44). One of these features is adaptability. A sudden transient change in flux from the source does not produce a large change in

Table 23

Summary of effects of acute stress on yields of substrate from liver

Conditions	Yield µmM per g. fresh tissue			
	DAP	FDP	Pyruvate	Lactate
Standard ether anaesthesia	52.6	27.4	219.4	1324
Mild stress				
NaCl injection	42.5 S*	18.3 S**	228.5	1992 S*
Exercise	45.0	18.0 S**	220.0	2091 S**
Prolonged ether anaesthesia	36.2 S***	15.4 S***	177.5	1302
High concentration ether vapour	53.5	21.9	173,1	1578
Guillotine	34.9 S***	16.2 S***	206.9	1480
Ether anaesthesia + guillotine	47.5	20.6 S*	192.0	1299
Mean	43.3	18.4	204.1	1756
Moderate stress				
Anoxia due to N_2 inhalation	44.3	22.5	138.8 S*	4910 S***
Incision of diaphragm	37.1 S***	19.3 S**	182.7	1413
Adrenaline injection	39.4 S**	26.3	128.6 S***	2709 S**
Mean	40.3	22.7	150.0	3011
Severe stress				
Exsanguination	36.8 S***	20.8 S*	95.9 S***	1800 S**

the concentrations of the reactants in a metabolic sequence. The change is distributed over all of the reactants. Another feature is the stability of the steady state. After a transient disturbance of the concentrations of the reactants, the rates of the individual reactions alter in such a way as to restore the original concentrations of the steady state. Thus it is conceivable that the sensitivity of the system could be masked by the stabilising mechanism unless the displacement were measured within a sufficiently short interval of time.

The sensitivity of the system under consideration is obtained from a knowledge of the magnitude and direction of (a) the displacing force, that is, of the stress, and (b) the response, that is, of the yield of substrate. A summary of this information pertaining to all of the stresses and changes in the yields of substrates is given in table 23. The least severe stresses are the most valuable in trying to assess the sensitivity of the response. Furthermore, they are closer to the stress imposed by the standard procedure and to the stresses normally met in the animal. The range of the yields of substrates associated with these milder stresses is less than that resulting from the severe stresses. The stress of guillotining or of exsanguination is the ultimate; however, in the instance of guillotining, the samples of liver were frozen before extreme changes in concentrations could occur. The stresses of exercise, injection of sodium chloride,

prolonged exposure to anaesthetic doses of ether or exposure to high concentrations of ether are mild also. Those associated with incision of the diaphragm, anoxia from breathing nitrogen and injection of adrenalin are of moderate severity.

The direction of response, that is, the rise or fall, in the yields of substrate was considered when the range of the response was examined (page221). It was pointed out that the yields of lactate tend to rise whereas the yields of the 3 other substrates tend to fall when stress is applied. Thus the yields of dihydroxyacetonephosphate, fructose diphosphate and pyruvate were reduced significantly in 6, 7 and 3 respectively, of the 10 stresses applied. The yield of lactate was increased significantly after 5 of the 10 stresses.

When the results are considered in 3 groups, corresponding to the amount of the stresses, it is evident that the yields of dihydroxyacetonephosphate and fructose diphosphate were reduced to the same degree after mild, moderate or severe stress, and that of lactate was increased maximally after moderate stress. The effects of stress on the yields of the sugar phosphates are sufficiently similar to justify considering their sensitivity together. The yields of these 2 substrates differ from those of pyruvate and lactate in their response to stress. Even mild conditions may produce almost the same maximum response. However, the maximum response was not excessive; when compared with the yields

obtained after standard ether anaesthesia the maximum decreases in the yields of dihydroxyacetonephosphate and fructose diphosphate were, respectively, 35 per cent (after guillotining) and 44 per cent (after prolonged ether anaesthesia). The mean decreases over all stresses were 22.7 and 27.4 per cent respectively. It is concluded that the in vivo concentrations of these two sugar phosphates are very sensitive to stress, but that the stabilising mechanisms are also efficient.

The reduction in the yield of pyruvate after stress is the only resemblance of its response to that of the substrates just discussed. The other features, namely, the sensitivity and stability to stress are more like lactate. Thus increasing stress produces increasing change in the yield; there is no evidence of stabilisation occurring after a certain amount of change. The level of sensitivity is more difficult to interpret. This is partly because several of the stresses, namely, the injection of sodium chloride and exposure to nitrogen gas cause the animal to struggle; the resultant muscular contraction produces an increased return of pyruvate and lactate to the liver via the blood-stream. The effects of the increases of the concentrations of these metabolites in the extracellular fluid can be allowed for, but the amount which enters the cells remains unknown. Thus the direct effects of some of the stresses of pyruvate concentration in the liver cells are uncertain. (There is no such difficulty with

dihydroxyacetonephosphate and fructose diphosphate, the plasma concentrations of which do not increase during muscular contraction; these intermediates do not normally occur in plasma, see p.239). Another reason for the difficulty in assessing the sensitivity is related to the large size of both the pyruvate and lactate pools. A sudden increase in the rate of removal from, or decrease in the rate of addition to either pool of substrate of the order of 5 per cent, would not be noticed. However, if the equivalent of 5 per cent of the pyruvate pool were suddenly removed from the pools of dihydroxyacetonephosphate and fructose diphosphate the concentrations would fall by approximately 20 and 40 per cent, respectively. For these reasons the effects of mild stress on pyruvate and lactate cannot be assessed. The observations on the effects of moderate stress indicate a sensitivity similar to that of the sugar phosphates.

Summary and conclusions on effects of stress on concentration of substrates in the liver. The contents of sugar phosphates and pyruvate in the liver of the resting rat were found to be at the upper end of the range observed after the application of stress, whereas that of lactate was at the lower end. These observations were shown to be consistent with an interpretation of the effects of the stresses based on present knowledge of the intermediate metabolism of carbohydrates. The concentrations of the sugar phosphates were found to be sensitive to stress; the possibility that the

Table 24

Effects of stress on yields of substrates from muscle

Conditions		No. rats	Yield (μmM per g. fresh tissue) DAP	FDP	Pyruvate	Lactate
Standard		9	36.4	86.4	78.3	1337
Prolonged ether anaesthesia[x]		6	34.3	68.3	100.3	1298
Respiratory stoppage or inhibition[*]	A	5	45.0	104.8	97.2	1158
	B	2	52.5	134.5	136.5	2850
Exsanguination[•]	A	4	46.3	153.8 S[xxx]	68.5	1198
	B	4	47.8	151.5 S[xxx]	88.0	5000 S[xxx]
Exercise[§] Swimming		6	31.2	37.8 S[xxx]	106.4	2008 S[xxx]
Frozen conscious[//]		5	76.4 S[xxx]	219.0 S[xxx]	174.4 S[xxx]	2952 S[x]
Stunning[#]		4	42.5	173.3 S[xxx]	232.5 S[xxx]	14200 S[xxx]

[x] Standard induction period followed by 95-240 seconds exposure to ether vapour; deep anaesthesia was avoided.

[*] As for liver, table 19. Group A includes 3 rats with both diaphragms incised and 2 rats in which only the left diaphragm was incised. Group B consists of 2 rats in which only the left diaphragm was incised. In group A freezing was carried out in 10-15 seconds and in group B after a delay of 1 minute.

[•] The same procedure as used for liver, table 19. Group A includes 3 samples frozen within 10-15 seconds and 1 sample frozen after a delay of 1 minute. Group B includes 1 sample frozen after a delay of 3 minutes and 3 samples frozen after a delay of 10 minutes.

[//] Procedure described on p.148.

[#] Reflex muscle spasms occured for 60-75 seconds.

[§] See footnote in table 19. Figures corrected for changes in concentrations of pyruvate and lactate in extracellular fluid.

normal contents are higher than those obtained during ether anaesthesia cannot be excluded. In contrast, the concentrations of pyruvate and lactate were found to be fairly stable in the presence of various stresses; it is considered unlikely that the normal contents differ greatly from those observed.

Finally the effects discussed in this section support the conclusion that the results of analyses obtained under ether anaesthesia are more likely to represent the resting in vivo content of fructose diphosphate and dihydroxyacetonephosphate than those obtained after guillotining.

Muscle. Range of yields of substrates. All of the data pertaining to the effects of stress on the yields of substrates from muscle are presented in table 24. The range of the yields (μmM/g. fresh muscle) of the four substrates is as follows: dihydroxyacetonephosphate 31.2 to 76.4, fructose diphosphate 37.8 to 219, pyruvate 68.5 to 232.5 and lactate 1158 to 14200. The values obtained under standard conditions were within the range associated with stress for all substrates. Many of the stresses used in order to obtain extreme values for the yields of each substrate are unlikely to occur during life, except perhaps during a fight. Hence it is probable that the range of values associated with normal activity is much smaller than that shown. This is of special importance in trying to assess the evidence relevant to the identification of in vivo concentrations at rest.

The relation of the position of the mean yields of each substrate, obtained under standard conditions, to the extreme values of the range obtained under conditions of stress is of interest once again. The yield of lactate is at the lower end of the range as it is in liver. However, the positions of the other 3 substrates are relatively different from those in liver. Thus the yields of dihydroxyacetonephosphate and pyruvate appear at or near the lower end, whereas that of fructose diphosphate is nearer the middle of the range. The occurrence of the yields at or near the lower extreme of the range of values is accountable in a similar way to the same kind of phenomenon in liver.

The general features of carbohydrate metabolism in muscle must be considered before such an attempt is made. It differs from that in liver in consisting chiefly of a degradative anaerobic glycolytic pathway. The tricarboxylic acid cycle is relatively unimportant in skeletal muscle, with the notable exception of the pectoral muscle of flying birds (PERRY, 1956). The synthetic pathway comprises chiefly the assimilation of glucose to glycogen. Lactate is the end product of glycolysis and most of it is removed via the bloodstream. The degradation of glycogen depends on the activity of adrenaline.

The concentration of lactate in resting muscle is presumably related to the minimum amount of degradation of ATP required to maintain tone. The only change which is

likely, therefore, in the absence of poisoning of the enzymes, is an increase in the concentration of lactate associated with muscular contraction and the consequent increase in glycolysis. The same tendency would be expected with pyruvate, but to a less extent, because it is not the end-product and the equilibrium favours the formation of lactate. The theoretical expectation and the experimental observation are in accord for these metabolites.

The position within the range of values observed of the yields of fructose diphosphate obtained from resting muscle is more difficult to account for. Increased muscular contraction might be expected to result in increased concentrations of all intermediates of glycolysis, and especially the concentration of fructose diphosphate. The level of ATP decreases after muscular work and this stimulates phosphohexokinase, the effect of which is to increase the formation of fructose diphosphate (LARDY and PARKS, 1956). However, the content of fructose diphosphate after mild exercise is less than half of that obtained under standard conditions. This may be because it is impossible to freeze a sample of muscle in less than two minutes after stopping the exercise. During this time degradation stops and anaerobic recovery starts; this includes the synthesis of glycogen from lactate. The transition between steady and/or transition states may be associated with oscillations in the concentrations of the reactants. It is suggested that the low concentrations of the

fructose diphsophate observed after exercise corresponds to one of the minima of the oscillation curve (BURTON, 1939; DENBIGH and others, 1948). The response of the yield of dihydroxyacetonephosphate to stress is similar to that of fructose diphesphate, but the effects are less marked. Mild exercise was followed by a relatively small decrease in the yield.

Although the effects of the exercise experiments confuse the picture somewhat, it appears that generally stimuli are associated with increased yields of the sugar phosphates, and observation in agreement with theoretical predictions. Thus the resting concentrations in vivo would be expected to be low rather than high.

Sensitivity of the yields to stress. The effects of gross stress such as stunning or freezing the conscious rat are not relevant to the assessment of sensitivity.

Summary. Neither prolonged ether anaesthesia nor respiratory impairment affected the yields of any of the four substrates significantly. The only significant changes were the increase in the yield of fructose diphosphate following exsanguination, and the decrease in yield of fructose diphosphate and the increase in that of lactate after exercise. Thus the concentrations of these metabolites in muscle do not vary greatly in the absence of considerable stress. The relative lack of sensitivity of the concentrations of the four substrates to stress is evidence in support of the view

Table 25

Effects of stress on yields of substrates from kidney

Conditions	No. rats	Yield μmM per g. fresh tissue			
		DAP	FDP	Pyruvate	Lactate
Standard	10	25.5	16.8	109.0	1166.
Exercise*	6	17.0 s*	16.4	99.0	2220 s*
Exsanguination	6	19.1	23.2 s**	50.8 s***	1877 s**
Frozen conscious	5	25.8	40.2 s***	83.2	1548.

* Pyruvate and lactate figures corrected for changes in concentrations in extracellular fluid.

that the values obtained under ether anaesthesia approximate to those present in the resting muscle.

Kidney. Range of yields of substrates. The results of the imposition of stress on the yields from the kidney appear in table 25. The range of the mean yields (μmM/g. fresh kidney) was dihydroxyacetonephosphate 17 to 25.8, fructose diphosphate 16.4 to 40.2, pyruvate 50.8 to 109 and lactate 1166 to 2220. The values associated with standard conditions were within the range with the exception of lactate which was much lower than any value observed after stress. The relatively small range of values, even under conditions of extreme stress, reduces the uncertainty regarding the effects of the stress of the standard procedure.

There appears to be no information available regarding the details of carbohydrate metabolism in the kidney. Therefore any predictions as to whether the yields of substrates from the resting animal should be at the low or high end of the range obtained, can only be based on analogy with other tissues, and especially those carrying out many functions. Thus a low yield of lactate is expected. The decrease in the yield of pyruvate following exposure to stress is comparable to that observed in the liver. The changes in the yields of fructose diphosphate and dihydroxyacetonephosphate were not predictable either from knowledge of carbohydrate metabolism or from experience obtained from other tissues.

Sensitivity of the yields to stress. After exercise

there was no change in the yield of fructose diphosphate and pyruvate, a decrease of 33.3 per cent in that of dihydroxyacetonephosphate and an increase of approximately 100 per cent in that of lactate. The effect of exsanguination was to produce decreases of 25 and 53 per cent respectively, in the yields of dihydroxyacetonephosphate and pyruvate, and increases of 38 and 61 per cent, respectively, in the yields of fructose diphosphate and lactate. The change in the yield of dihydroxyacetonephosphate just failed to reach the 5 per cent level of significance, but the other changes were all significant. Freezing the conscious animal failed to affect the yield of dihydroxyacetonephosphate and there was a decrease of 23 per cent in the yield of pyruvate and increases of 140 and 33 per cent, respectively, in the yields of fructose diphosphate and lactate. The change in the yield of fructose diphosphate only was significant. Clearly the kidney is sensitive to stress applied to the rat. However, when the intensity of the stresses of exsanguination and freezing the conscious rat is considered the amount of change with reference to the yields obtained under standard conditions is not large. The mechanisms which stabilise the intracellular concentrations are obviously efficient.

Summary. The relatively small range of yields, even under considerable stress, is interpreted as evidence that the mild stress of the standard procedure is unlikely to produce much disturbance of the resting values. The effects

Table 26

Effects of stress on yields of substrates from testis

Conditions	No. rats	Yield µmM per g. fresh tissue			
		DAP	FDP	Pyruvate	Lactate
Standard	6	12.6	12.3	66.2	1145.
Exercise*	6	10.0	10.7	59.3	1120
Exsanguination	6	11.7	11.4	64.3	1385

* Pyruvate and lactate figures corrected for changes occurring in concentrations in extracellular fluid.

Table 27

Effects of exercise on yields of substrates from blood

The exercise was the same as for other tissues.

The values for blood cells were determined as follows. The volume of plasma in 1 g. blood was obtained from the packed cell volume (PCV) which was measured and the specific gravity (S.G.) of rat blood (SHERRINGTON and COPEMAN, 1893). The amount of pyruvate in this volume of plasma was calculated and the amount in the cells was reckoned by difference. The mass of the cells present in 1 g. blood was derived in a similar way, that of the plasma being determined first from the PCV and the S.G. of plasma. The amount of pyruvate in 1 g. cells was then obtained by a simple calculation. An attempt to obtain the values for lactate in the cells by this method proved to be unsatisfactory because of the relatively small amount of lactate in the cells and the lack of precision associated with the analytical methods.

Tissue	Conditions	No. rats	Yield µmM per g. fresh tissue			
			DAP	FDP	Pyruvate	Lactate
Whole blood	Standard[x]	10	11.1	2.8	130.6	1880
	Exercise	7	8.3	1.9 S***	213.4 S***	4100 S**
Plasma	Standard[*]		0	0	126.9	2860
	Exercise	6	0	0	241.0 S***	6276 S***
Blood cells	Standard[•]	10	18.0	6.0	135.0	---
	Exercise[§]	7	17.7	4.1 S***	186.0	---

[x] Pyruvate 14 samples, lactate 8.

[*] Pyruvate 9 samples, lactate 6. All plasma values referred to 1·0 ml.

[•] Pyruvate 9 samples. The PCV was not determined in 4 of the samples analysed for DAP and in 3 samples analysed for pyruvate. The mean value of 12 determinations was used; the maximum error introduced by this device is ± 10 per cent.

[§] The mean PCV of 6 samples was used in order to calculate the contents of the substrates in the cells of the 7th sample.

of the stresses were to reduce pyruvate and to increase fructose diphosphate and lactate; therefore the tendency will be to underestimate the yields of pyruvate and to overestimate those of the two other substrates. No assessment of the direction of the disturbance with dihydroxyacetonephosphate is possible.

Testis. The results are given in table 26. After allowance is made for the changes in the plasma concentrations of pyruvate and lactate due to exercise there is no significant change in the yields of any of the substrates after either of the stresses is applied. It is concluded that the results obtained with the standard procedure represent the resting concentrations in the testis.

Blood. The only stress in which the concentrations of the substrates in blood were examined was that of exercise (table 27). The concentrations of dihydroxyacetonephosphate and fructose diphosphate in whole blood or in the cells do not change greatly following exercise. However, the concentrations of pyruvate in whole blood increased from 130.6 to 213.4 and that of lactate from 1880 to 4100 μmM/g. The pyruvate content of the cells increased from 135 to 186 μmM/g. These changes are all significant.

Dihydroxyacetonephosphate and fructose diphosphate are absent from the plasma during rest and after exercise. Pyruvate and lactate are normally present in plasma and they are increased as a result of exercise, pyruvate rising to 190 per

cent and lactate to 220 per cent of the resting value. Therefore struggling must be avoided when blood is collected for the estimation of the resting levels of these metabolites in plasma.

Correction of Values Obtained From Crude Extracts

The use of the uncorrected fresh weight of tissues for the reference of yields of its constituents is common practice, but it can be criticised on several grounds. That it fails to reveal the concentrations at the sites of reaction is taken for granted. The additional criticisms are that it includes extracellular structures, and that it does not allow for differing degrees of hydration of cells. At present there are no means of applying precise corrections to the values obtained from the crude extracts. This is because there is insufficient detailed information about the grounds for criticism. However, it is possible to correct, in some measure, for both of these sources of imprecision.

Extracellular Compartment

This is discussed on page 30 . It is doubtful whether correction can be made for the volumes and contents of the lumina of glandular tissues, although it is claimed that in frog kidney the inulin space includes the volume of the lumina of the tubules (CONWAY and others, 1946). The determination of the total content of the constituents of the lumina of organs in the resting animal appears to be beyond present resources. The failure to correct for this source of error probably does not lead to a large discrepancy

because the total volume of the lumina is relatively small.

However, it is possible to correct for the amounts of substances present in the interstitial and connective tissue spaces. These corrections are only approximate because there is no way of determining accurately the extracellular volume (MANERY, 1954; ELKINTON and DANOWSKI, 1955; ROBINSON, 1960). Furthermore, the precise meaning of the values obtained by the various methods is not clear; this is because it is not certain that the membranes separating the various compartments are completely impermeable to the substance used for the estimation. Nevertheless, there is broad agreement as to which methods are most useful and as to the kind and amount of error involved. Thus methods based on the estimation of the halogen content overestimate the extracellular space because the agent passes into the cells. The use of large molecules is associated with underestimates unless sufficient time is allowed for diffusion into the connective tissues (COTLOVE, 1954).

Where possible it is obviously better to use the results obtained by methods which are in substantial agreement. A selection of estimates of the extracellular space of rat tissues, chosen where possible on this basis, is presented in table 28. The values for liver agree fairly well, the average being approximately 24 per cent

Table 28

Estimates of volume of extracellular fluid of tissues of the rat

Tissue	Author	Method	No. rats	Estimated % of weight of tissue
Liver	Truax (1939)	Histology	5	24.2 ± 1.9 S.D.
		Cl^-	5	25.0 ± 2.5 S.D.
	Cole (1949)	SCN^-	14	22.49 ± 2.31 S.E.M.
Kidney	Robinson (1950)	Inulin	6	25.3 ± 1.5 S.D.
Cortex		Sucrose	12	26.4 ± 0.8 S.D.
Muscle				
Gastrocnemius	Cole (1949)	SCN^-	14	11.26 ± 0.53 S.E.M.
Gastrocnemius	Creese and others (1955)	Inulin	18	9.6 ± 1.5 S.D.
Anterior tibial	Flear and others (1960)	Inulin	17	11.2 ± 0.35 S.E.M.
		Cl^-	18	12.2 ± 0.35 S.E.M.
Rectus abdominus	do.	Cl^-	6	20.9 ± 0.86 S.E.M.
Deltoid	do.	Cl^-	8	15.9 ± 1.37 S.E.M.
Diaphragm	do.	Cl^-	6	22.8 ± 1.57 S.E.M.
Testis	Cole (1949)	SCN^-	14	20.93 ± 2.46 S.E.M.

of the whole mass of tissue. The values obtained for rat kidney by ROBINSON (1950) appear to be the only ones available. They are similar to those obtained for frog kidney (23 per cent) and a little lower than those derived from rabbit kidney (28 per cent) (CONWAY and others, 1946; CONWAY and FITZGERALD, 1942). In muscle, the 2 most significant observations are that the estimates based on the inulin and chloride spaces of the same sample agree and that the extracellular fluid content of different muscles differ considerably. The muscle used in this investigation, namely, the rectus abdominus, has a chloride space of approximately 21 per cent (FLEAR and others, 1960). Only one estimate appears to have been made for testis (COLE, 1949). However, as the values for liver and muscle obtained by the same author with the same method (thiocyanate) agree with those obtained by others, there is no reason not to accept the value obtained for testis. No estimates were found for tumours, but because the overall range for tissues in general is small it seems that no appreciable error is likely to be introduced by using the values relating to liver.

Corrections for the pyruvate and lactate content of extracellular fluid based on these estimates are shown in table 29. No correction is required for dihydroxy-acetonephosphate and fructose diphosphate because these substances are not present in the plasma. The first

Table 29

Pyruvate and lactate yields corrected for contribution from extracellular fluid

Tissue	Extra cellular volume %	Pyruvate			Lactate		
		µmM in 1 g. tissue	µmM* in ECF of 1 g. tissue	µmM in cells of 1 g. tissue	µmM in 1 g. tissue	µmM* in ECF of 1 g. tissue	µmM in cells of 1 g. tissue
Liver	24	219.4	29.8	189.6	1324	670.	654.
Kidney	26	109.0	32.2	76.8	1166.	725.	441.
Testis	21	66.2	26.0	40.2	1145.	585.	560.
Muscle (rectus abdominus)	21	78.3	26.0	52.3	1337	585	752
RD3 sarcoma	24•	255.3	66.0	189.0	6720	1240.	5480.
DAB hepatoma	24•	181.7	29.8§	151.6	3937.	670. §	3267.

* Calculated from levels of 124.0 and 275 µmM/g. plasma for normal (9) and RD3 sarcomata-bearing (1) rats, respectively.

* Calculated from levels of 2970 and 5150 µmM/g. plasma for normal (6) and RD3 sarcomata-bearing (1) rats, repectively.

• The values are not obtainable from the literature; the value given for liver is used.

§ Correction is based on values for normal plasma because no blood was obtainable for estimations.

column for each substrate contains the data obtained from the analyses already described, the second the amounts of each substrate estimated to be in the extracellular compartment and in the third, the residual amounts reckoned to be in the cells. The correction for the normal tissues is calculated from the mean plasma levels of normal rats. That for the RD 3 sarcoma is derived from the analysis of one sample of plasma only. This is unsatisfactory, but it is the only figure available. In the case of the hepatomata the position is more unsatisfactory: no plasma was available. The mean value for the plasma of normal rats is used with the recognition that the correction is probably too small.

The size of the extracellular compartments of all of the tissues are comparable; therefore the effects of the correction are relatively greater in tissues with low contents of substrate, for example, pyruvate in kidney, testis and muscle. The importance of making the correction in such tissues is correspondingly greater. With lactate, the plasma content is so much greater than those of the normal tissues, namely, 2790 compared with approximately 1,300 μmM/g. that the amounts reckoned to be present in the cells

are reduced by 45 to 60 per cent after the correction is made. The relative effect of the correction for lactate in the tumours is much less: it amounts to a reduction of about 20 per cent.

No correction for the content of substrates in blood cells in tissues will be attempted for several reasons. The amounts of dihydroxyacetonephosphate and fructose diphosphate are insignificant. Corrections for the pyruvate and lactate in plasma are included in the correction for the total extracellular fluid. The percentage of a tissue occupied by the blood cells is of the order to 2 to 5 per cent; therefore any correction applied for pyruvate or lactate will be minimal. Finally in view of the uncertainty of the exact correction for the volume of the extracellular fluid and of the amount of intracellular fluid, there is no obvious gain in applying trivial corrections.

It is emphasised that the values obtained for pyruvate and lactate in the plasma affect the apparent intracellular concentrations of these substances profoundly. Their chief source is muscle as shown by the effects of exercise. Any struggling during anaesthesia would produce high plasma levels of these metabolites, but unless the rate of diffusion into the extracellular space were very fast the concentrations at that site would remain low. Thus a correction based on

the plasma level could result in apparently low intracellular concentrations.

The magnitude of an effect such as this on the correction factor is seen in the corrected values for the liver given by HOHORST and others (1959). The extracellular volume was found to be 25 per cent and the plasma levels of pyruvate and lactate, respectively, were 234 and 2,960 µmM/ml. When the correction is applied to the liver pyruvate, the amount which is apparently in the cells of a gram of tissue is 94 µmM compared with 154 µmM, uncorrected. This is approximately one half of that reckoned to be in the cells by the present author. When the correction is applied to lactate the intracellular content becomes 810 µmM compared with 1,540 µmM per gram of tissue, uncorrected. This is about 30 per cent more than that reckoned to be intracellular in this investigation. It is not claimed that the plasma levels used to correct the values in table 29 are exactly those of the resting animal, but they are lower than those by Hohorst and his colleagues and are therefore more likely to be valid. The difference in plasma levels applies chiefly to pyruvate and it is concluded that the estimate of the intracellular concentration made by Hohorst and his associates is too low.

Total Nitrogen as a Measure of Metabolically Active Cytoplasm

The estimates of the size of the extracellular compartment can be used to obtain an estimate of the concentration of metabolites per gram of cells (HOHORST and others, 1959).

However, this does not overcome the difficulty of the varying content of water in the cells of different tissues. An index which is related to the functional mass of the tissue is of greater value, for example, deoxyribonucleic acid or total nitrogen. The deoxyribonucleic acid content is a measure of the number of cells, whereas the total nitrogen relates to the amount of cytoplasm. When comparisons are being made between different tissues containing mostly cells and little connective tissue the total nitrogen is probably a better measure of the functional mass of tissue than the content of DNA. The total nitrogen represents chiefly the protein of the cells, but it includes also extracellular cellular structural protein and small amounts of intraluminal nitrogen, for example, that of the urea of the nephron. If the contents of protein and water in the extracellular compartment bear the same relation to their intracellular counterparts, then the percentage of the total nitrogen in the extracellular compartment is 20 to 25; this is unlikely because the extracellular fluid is protein-poor. It is conceivable that the extracellular protein is no more than 10 per cent of the total tissue protein. Thus the use of total nitrogen appears to be justified, even if it is not the perfect index.

It seemed possible that the nitrogen content of tissues obtained by means of the pre-cooled tongs might differ from that obtained in the usual way. Therefore, the total nitro-

Table 30

Nitrogen content of rat tissues (mg. N per g. fresh tissue)

Tissue	Unfrozen	Frozen
Liver	32.08 ± 0.97 (6)	27.88 (6)
Kidney	27.06 ± 0.30 (8)	22.5 (6)
Muscle	32.92 ± 0.42 (6)	34.28 (5)
Testis	14.80 ± 0.15 (8)	14.05 (6)
Sarcoma RD3	22.36 ± 0.44 (7)	---
Hepatoma DAB	23.8 (3)	---
Blood cells*	46	---

* Content is calculated from the following information:- P.C.V. 50 per cent, Hb. 15.6 g. per cent (FARRIES and GRIFFITH, 1942); the erythrocytes contain 98-99 per cent of the nitrogen of all blood cells in man (DALE, 1960); S.G. rat erythrocytes = 1.082 [illegible] (calculated from S.G. 1.056 (SHERRINGTON and COPEMAN, 1893) of whole blood and assumed value of 1.026 for plasma).

gen was estimated in both unfrozen tissues and in samples which were frozen and compressed by the tongs. These experiments were carried out at room temperature, that is at approximately 23°. The frozen samples of liver, kidney and testis were transferred to stoppered, tared homogeniser tubes, re-weighed and finally homogenised in 9 volumes of water; they remained at room temperature between the sampling and weighing, a period varying in length from 30 to 120 minutes. The samples of frozen muscle were transferred directly to tared Kjeldahl tubes which were re-weighed immediately.

The results are presented in table 30. The yields of nitrogen obtained from both frozen and unfrozen testis and muscle are sufficiently close for the differences to be due to experimental variation. The total nitrogen of muscle is equivalent to a protein content of 20.5 g. per 100 g. muscle; this is within the normal range for mammalian muscle (HAMOIR, 1961). The yields of nitrogen obtained from frozen liver and kidney are 13 to 16 per cent lower than the values derived from the unfrozen tissues. The values for unfrozen liver, namely 32.08 mg. per g., is similar to that cited by ELSON (1961), namely, 32 mg./g.; likewise the content observed in unfrozen kidney (27.06 mg./g.) compares with that quoted by CRAWFORD (1961), that is, 28.8 mg./g. The estimate for hepatoma (unfrozen) agrees with that given by ELSON (1961), namely 23.2 per cent.

Table 31

Concentrations of substrates in rat tissues

Tissue	No. rats	Substrates: DAP µmM in cells* in 1 g. tissue	DAP µmM per mg. N	FAP µmM in cells* in 1 g. tissue	FAP µmM per mg. N	Pyruvate µmM in cells* in 1 g. tissue	Pyruvate µmM per mg. N	Lactate µmM in cells* in 1 g. tissue	Lactate µmM per mg. N
Liver	27	52.60 ±1.73	1.72	27.41 ±1.35	0.85	189.6 ±11.2	5.94	654● ±39	21.5●
Kidney	10	25.45 ±3.21	0.94	16.80 ±1.36	0.62	76.8 ±5.8	2.84	441 ±60	16.3
Testis	6	12.58 ±0.67	1.03	12.25 ±1.56	0.83	40.2 ±1.9	2.72	560 ±49	37.8
Muscle	9	36.44 ±2.99	1.10	86.44 ±8.99	2.62	52.3 ±6.5	1.59	752 ±78	22.9
Blood cells	10	18.0§ ±1.67	0.39	6.00 ±0.186	0.13	135.0 ±8.47	2.94	---	---
Sarcoma RD3	7	13.60 ±2.10	0.61	23.60 ±3.53	1.06	189.0 ±17.5	8.45	5480 ±285	244
DAB Hepatoma	3	31.67 ±5.61	1.33	30.00 ±6.61	1.26	151.6 ±22.2	6.40	3267 ±600	137

* DAP and FDP are absent from plasma (table 27) and therefore from extracellular fluid; the yield from the tissues is assumed to arise from the cells.

* These values are approximations only. See page 241 for discussion.

● 17 rats

§ 9 rats.

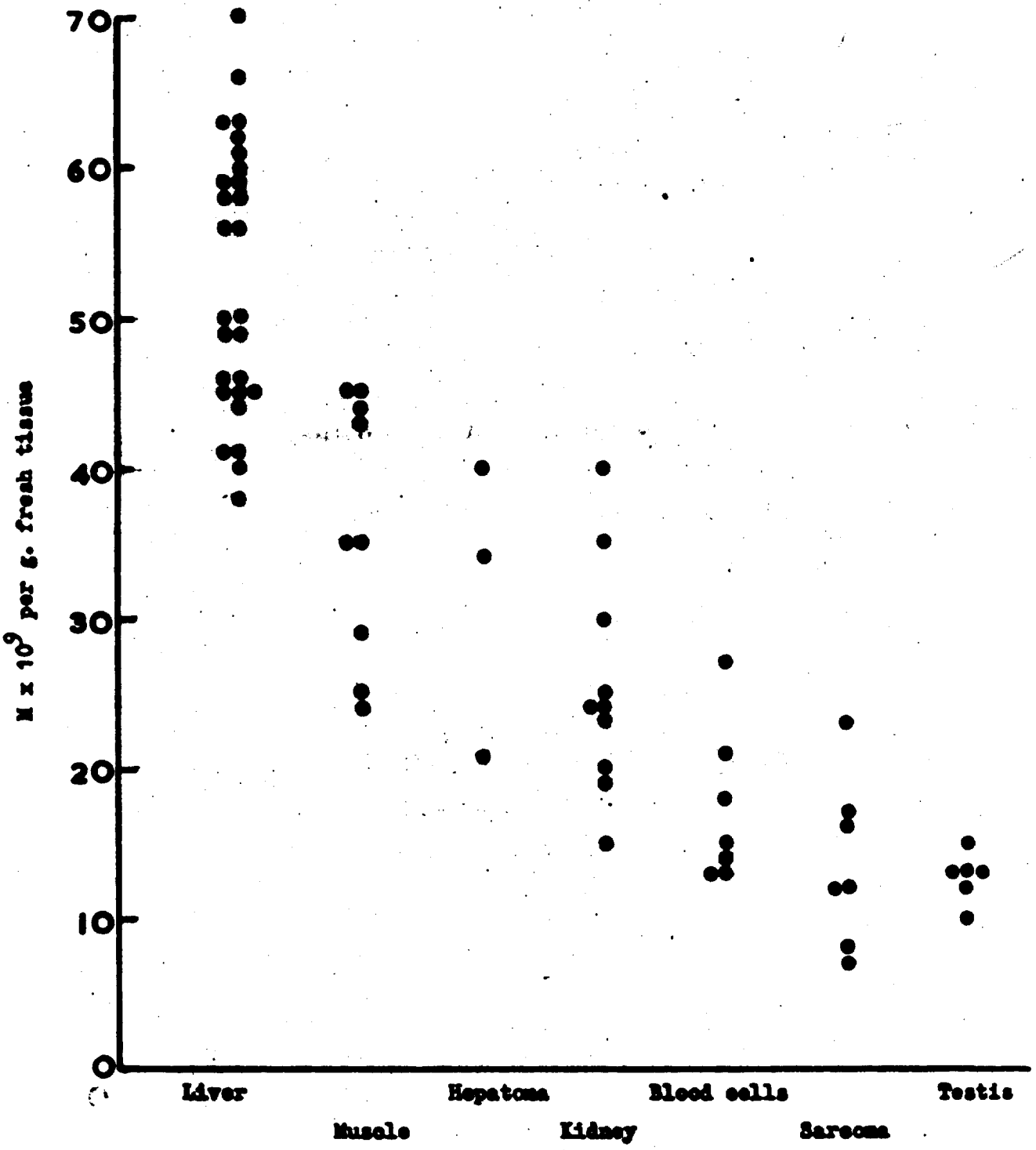

Fig. 19. Scatter diagram of content of dihydroxyacetonephosphate in tissues of the rat.

The lower values associated with the frozen samples of liver and kidney are attributed to condensation of water vapour from the atmosphere. There was no opportunity for this to occur with muscle and very little with testis. The only other explanation would be that a protein rich fluid was expressed by the tongs. The protein content of blood is approximately that of liver or kidney; the expression of such a fluid would not alter the total nitrogen content. No other component is readily expressed under the conditions of sampling. For these reasons the values derived from the unfrozen tissues will be used for reference of the concentrations of the substrates as well as the activities of the enzymes.

Final Statement of Results

The final statement of the yields of the 4 substrates from the tissues of resting rats is made in table 31. The mean yields are referred to both the total nitrogen content of the tissues and to the cells in one gram of fresh tissue. The latter is included so that it may be compared with the more correct nitrogen index. The standard error of the mean is given for the values referred to the cells in 1 g. tissue.

Dihydroxyacetonephosphate (fig. 19)

The highest yield comes from liver with 1.72 and the lowest from blood cells with 0.39 µmM/mg.N. The content in the tumours differs considerably, that for the sarcoma being second lowest and that in the hepatoma second highest.

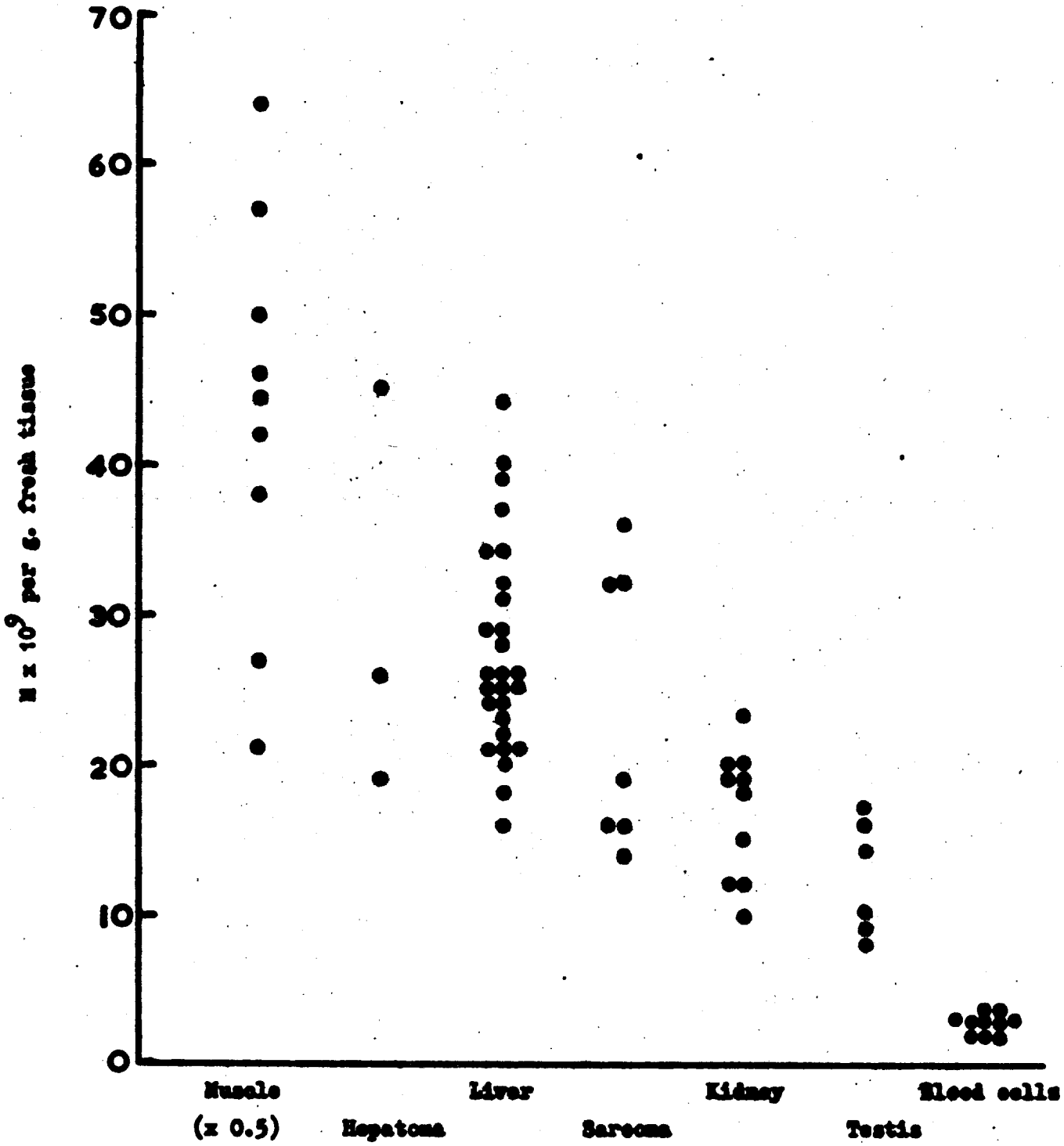

Fig. 20. Scatter diagram of content of fructose diphosphate in tissues of the rat.

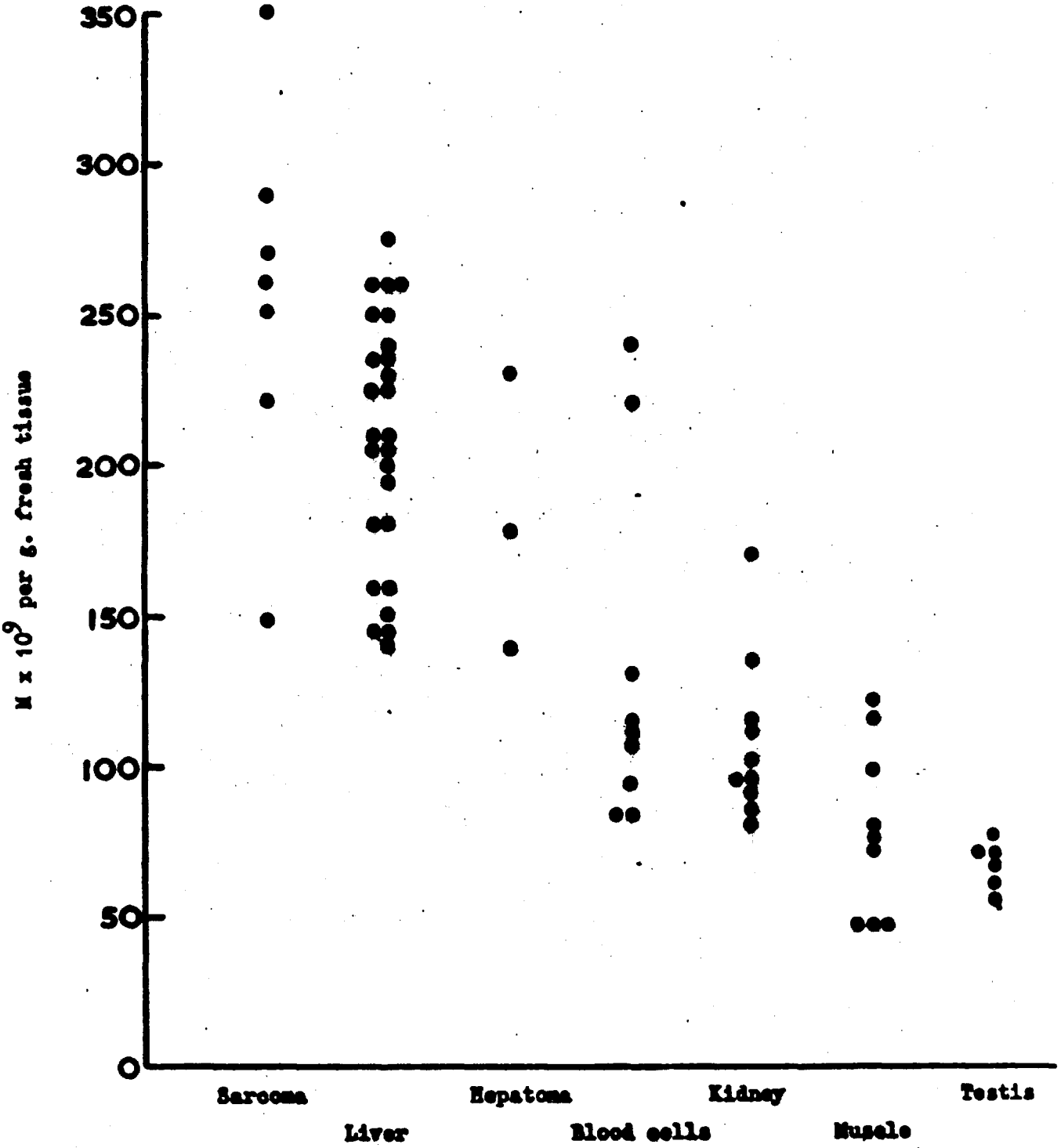

Fig. 21. Scatter diagram of content of pyruvate in tissues of the rat.

The mean concentrations in kidney, testis and muscle are similar, being, respectively, 0.94, 1.03 and 1.1 μmM/mg.N. When the cells of one gram of tissue are used for reference the order changes. The highest yield still comes from liver, but the lowest is now associated with testis and sarcoma. The remaining values are scattered as follows: blood cells 18.0, kidney 25.5, hepatoma 31.7 and muscle 36.4 μmM/g.

Fructose diphosphate (fig. 20).

The extremes are more divergent, the upper being muscle with a mean content of 2.62 and the lower, blood cells with a content of 0.13 μmM/mg.N. The remaining tissues fit into the range between 0.62 for kidney and 1.26 for hepatoma. The mean values for testis and liver are almost identical at 0.83 and 0.85 μmM/mg.N. The relative positions are a little different when the cells of 1 g. tissue are used as the index. The tissues at the extremes do not change, and the position of kidney remains low in the list. The most striking change is in the yield from testis which now occupies second lowest place.

Pyruvate (fig. 21)

The range of values extends from that of muscle to that of sarcoma with yields of 1.59 and 8.45 μmM/mg.N, respectively. The mean contents in liver and hepatoma are similar, namely 5.94 and 6.75 μmM/mg.N; and those of kidney, testis and blood cells are also similar lying between 2.72 and 2.94 μmM/mg.N.

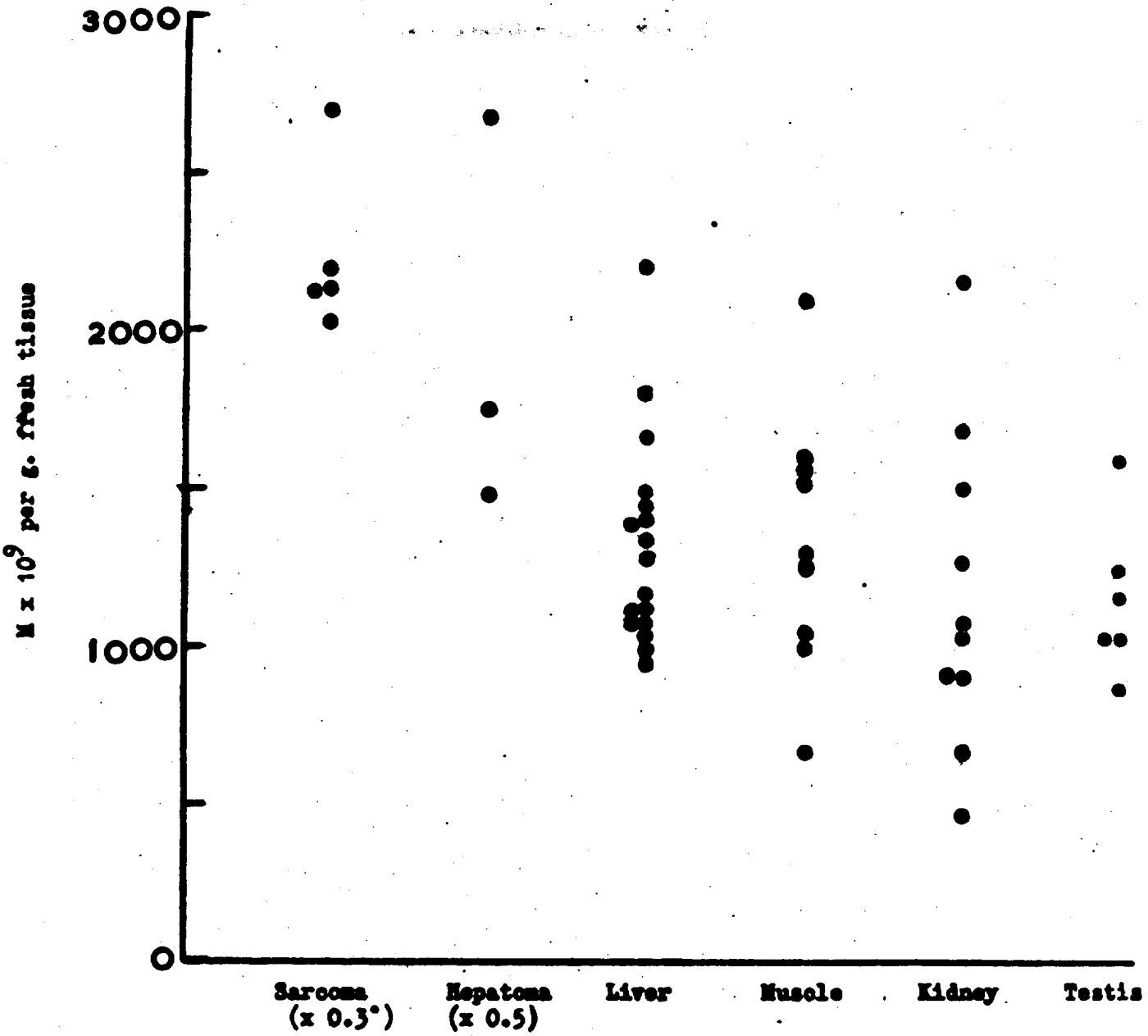

Fig.22. Scatter diagram of content of lactate in tissues of the rat.

The use of the cell content as the index results in the yield from liver and sarcoma sharing the maximum and that from testis becoming the minimum. The value for muscle remains low while that for blood cells shows a relative increase.

Lactate (fig. 22)

The concentration of lactate in the 4 normal tissues is well below that of the tumours; this is to be expected because tumours are characterised by their high level of glycolysis (WARBURG, 1926). The mean lactate content of liver, kidney and muscle are similar, being respectively 21.5, 16.3 and 22.9 μmM/mg.N. Testis contains relatively more lactate, namely, 37.8 μmM/mg.N. This may be related to the observation that the rate of anaerobic glycolysis of spermatozoa is approximately 66 per cent greater than that of liver (KRATZING, 1961). When cell mass is substituted for the total nitrogen the most marked change is the relative decrease in the value for testis. The concentrations in the tumours remain high.

VI

THE MAXIMUM ACTIVITY OF LACTATE DEHYDROGENASE AND ALDOLASE IN EXTRACTS OF TISSUES OF THE RAT

The objects of this section are to determine the maximum activities of aldolase and lactate dehydrogenase in the rat tissues in which the concentrations of the four substrates were examined.

MATERIALS AND METHODS

The analytical methods used for the determination of the activities of the two enzymes were described on pages The means of obtaining extracts of tissues without denaturing the enzymes are known in principle; but because some enzymes are more readily denatured than others, it was necessary to check the extraction procedure. In addition, it was necessary to inquire whether the tissue or extracts can be stored without loss of activity.

Procedure for Extraction of Enzymes

The procedure applied to human colon was used (page100); only the differences in detail are mentioned here. The rat was anaesthetised with ether as described for the determination of substrates (page145). The abdomen was opened by means of scissors and the appropriate tissue was removed and placed in a test tube standing in ice. Any fat or fibrous tissue was removed and the tissue was blotted before weighing out the aliquot to be homogenised. Forty runs of the pestle against

the tube were sufficient to obtain maximal extraction of the enzymes. A cuff of fibrous tissue often formed around the stem of the pestle just above the plastic head; it was necessary to cut this into smaller pieces with scissors in order to continue the homogenisation.

After the homogenisation was completed an aliquot of the suspension was removed for the estimation of the total nitrogen content. The remainder was spun in a refrigerated centrifuge at approximately 500 x g at 2° for 5 minutes. The supernatant was removed and stored in an ice bath until required for the estimation of aldolase. Subsequently it was stored at -20° overnight until required for the assay of lactate dehydrogenase. The enzymes were released from blood cells by lysis in four volumes of water. The extracts were diluted when necessary with the buffer appropriate to the assay. The only extract requiring dilution for the estimation of aldolase was that of muscle; this was diluted five fold. In the case of lactate dehydrogenase all of the extracts except for those of blood cells and testis were diluted by a factor of four.

Method of Obtaining the Sample

The possibility that ether might affect the enzymes to be extracted was examined by assaying the activities of aldolase and lactate dehydrogenase in the livers of 2 rats after decapitation, and of another 2 rats after ether

Table 32

Effect of method of obtaining tissue on activity of enzymes in liver

Enzyme	Rat	Ether* anaesthesia	Rat	Decapitation*
Aldolase µM FDP transformed per g. fresh tissue per min	1	5.0	3	5.1
	2	6.2	4	6.6
LDH µM pyruvate transformed per g. fresh tissue per min.	1	140.0	3	144.0
	2	141.0	4	168.0

The tissue was placed in a stoppered test tube and frozen in solid CO_2.

* Standard.

* By means of scissors.

anaesthesia. The results are presented in table 32. The differences in the yields of aldolase are minimal. The yield of lactate dehydrogenase from one of the liver samples after decapitation is higher than the yields from the 3 other rats. However, random variations of this magnitude between yields from different livers are common. It was concluded that the evidence did not contra-indicate the use of ether anaesthesia.

Medium for Extraction

A number of experiments was carried out in order to find a medium which would facilitate maximum extraction and produce minimum denaturation. Thus water was tested because it produces osmotic disruption not only of the cell membrane but also of the particles within the cell (de DUVE and others, 1955). Tris buffer (pH 7.4) was used in order to preserve the pH and ionic strength constant and at values known to be favourable to enzyme activity. Sodium chloride solution, 0.1 M, was used in order to preserve ionic strength without affecting the pH. Albumin and gelatin solutions were tried because of the stabilising effect of protein solutions on enzymes. Mannitol was examined because it is bland and non-ionic and it would not produce osmotic disruption. Finally, the effect of an anti-frothing agent, n-octanol, was observed. The results of these experiments which were carried out on extracts of liver appear in table 33.

None of the variations in the media affected the yields of lactate dehydrogenase. The yields of aldolase proved to

Table 33

Effect of medium used for extraction on activities of enzymes in liver

Enzyme	EXPT	Medium: Water	TRIS 0.1 M pH 7.4	NaCl 0.1 M	Albumin 1%	Gelatin 0.1%	Mannitol 0.25 M	Mannitol 0.25 M + n-octanol 1%
Aldolase µM FDP transformed per g.liver per min.	1		12.8		4.2	5.1	10.9	
	2		13.3	12.2			11.2	7.3
	3	6.6*						
LDH µM pyruvate transformed per g.liver per min.	1		266		294	276	280	
	2		250	240			220	230
	3	222*						

* These results are the mean of the yields from 4 livers.

Table 34

Effects of mincing muscle on activities of extracts

Amount of mincing	Aldolase μM FDP/min./g.	Lactate dehydrogenase μM pyruvate/min./g.
Pieces ~ 2 x 2 x 2 mm.	55.73 (11)	249.3 (11)
Pieces ~ 1 x 1 x 0.5 mm.	12.6 (5)	214.0 (5)

be sensitive to several of the media. Tris buffer favoured the highest yield, at approximately 13 μM fructose diphosphate split per g. liver per minute. Sodium chloride and mannitol were associated with yields of approximately 12 and 11 units, respectively. Water, albumin, gelatin and n-octanol all resulted in reduced yields.

Although the use of tris buffer was associated with a greater yield of aldolase, this advantage was offset by an important disadvantage. It was necessary to determine the total nitrogen content of the extracts; the presence of tris, a nitrogen containing molecule, would make the procedure too complicated to justify spending the time on it. Therefore mannitol, a non-ionic medium, was chosen because it could be used for other purposes than the present ones if desired.

Effect of Mincing Muscle on the Activities of Enzymes Extracted

It was necessary to cut up muscle in order to disrupt it in the homogeniser. This was made minimal because it was found that a fine mince was associated with a reduction of 75 to 80 per cent in the activity of aldolase in the extract (table 34).

Effect of Freezing and Storage on Activities of Enzymes

Freezing and thawing the colon appeared to reduce the activity of aldolase (page115); for this reason and also because it was hoped to store one or both enzymes overnight in order to stagger the assays, the effects of freezing

Table 35

Effect of freezing and storage on activities of enzymes in liver

Enzyme	State of Tissue: Fresh		Stored*		
				Brei	Whole
	Unfrozen	Frozen*	2°	-18°	-18°
Aldolase μM FDP transformed per g. fresh liver per minute	—	5.0	—	—	4.0
	—	5.1	—	—	4.2
	9.5	7.3	—	—	7.2
	9.0	6.4	—	—	5.5
	9.0	4.8	—	4.5•	—
	10.4	5.7	—	4.5•	—
	8.7	—	3.2	5.1	—
	9.8	—	3.9	6.1	—
LDH μM pyruvate transformed per g. fresh tissue per minute	—	140	—	—	158
	—	144	—	—	174
	178	174	—	—	174
	153	157	—	—	146
	129	120	—	98•	—
	142	138	—	118•	—
	157	—	162	163	—
	148	—	148	149	—

* Storage overnight, except as indicated

* Frozen whole in stoppered container in solid CO_2. Thawed within 30 minutes.

• Brei prepared from unfrozen liver, then stored at -18° for 3 days.

and thawing and of storage were examined.

The results of these experiments appear in table 35. The effects of rapid freezing of whole liver followed by thawing within 30 minutes and storage at -18° overnight were observed. Also the effects of storing a brei (1:9 in 0.25 M mannitol) at 2° and at -18° were investigated.

The activity of lactate dehydrogenase is approximately the same whether prepared from unfrozen or frozen liver, and whether stored whole or as a brei. The temperature of storage made no difference. The one possible exception is the brei stored for 3 days at -18°; the activity appears to have decreased by 15 to 25 per cent.

The activity of aldolase, on the contrary, was reduced on every occasion when either freezing or storage was carried out. Simple freezing and thawing was sometimes followed by a decrease of 40 to 50 per cent in activity. Storage of the brei at 2° resulted in a loss of 60 to 75 per cent of the activity.

These results demonstrated that the yields are not increased by freezing and thawing; more important, it is evident that aldolase is inactivated by this procedure and that its activity must be assayed as soon as possible. The estimation of the activity of lactate dehydrogenase can be conveniently carried out up to at least 18 hours after removing the tissue.

These results are supported by observations showing

Table 36

Maximum activities of aldolase and lactate dehydrogenase in rat tissues

Tissue	No. rats	Aldolase μM FDP transformed per min at 37°		Lactate dehydrogenase μM pyruvate transformed per min. at 25°	
		per g. fresh tissue	per mg. N	per g. fresh tissue	per mg. N
Liver	12	10.85± 0.38	0.338	242.5± 6.6	7.55
Kidney	11	5.41± 0.30	0.200	119.0± 3.9	4.40
Muscle	11	55.73± 3.59	1.69	---	---
	14	---	---	249.3± 10.8	7.58
Testis	11	2.73± 0.05	0.184	36.36± 0.87	2.46
RD3 sarcoma	6	5.65± 0.34	0.254	133.5± 7.1	6.04
DAB hepatoma	3	5.30± 0.57	0.223	138.3± 15.1	5.81
Blood cells	8	1.33± 0.04	0.029	41.60± 1.17	0.91

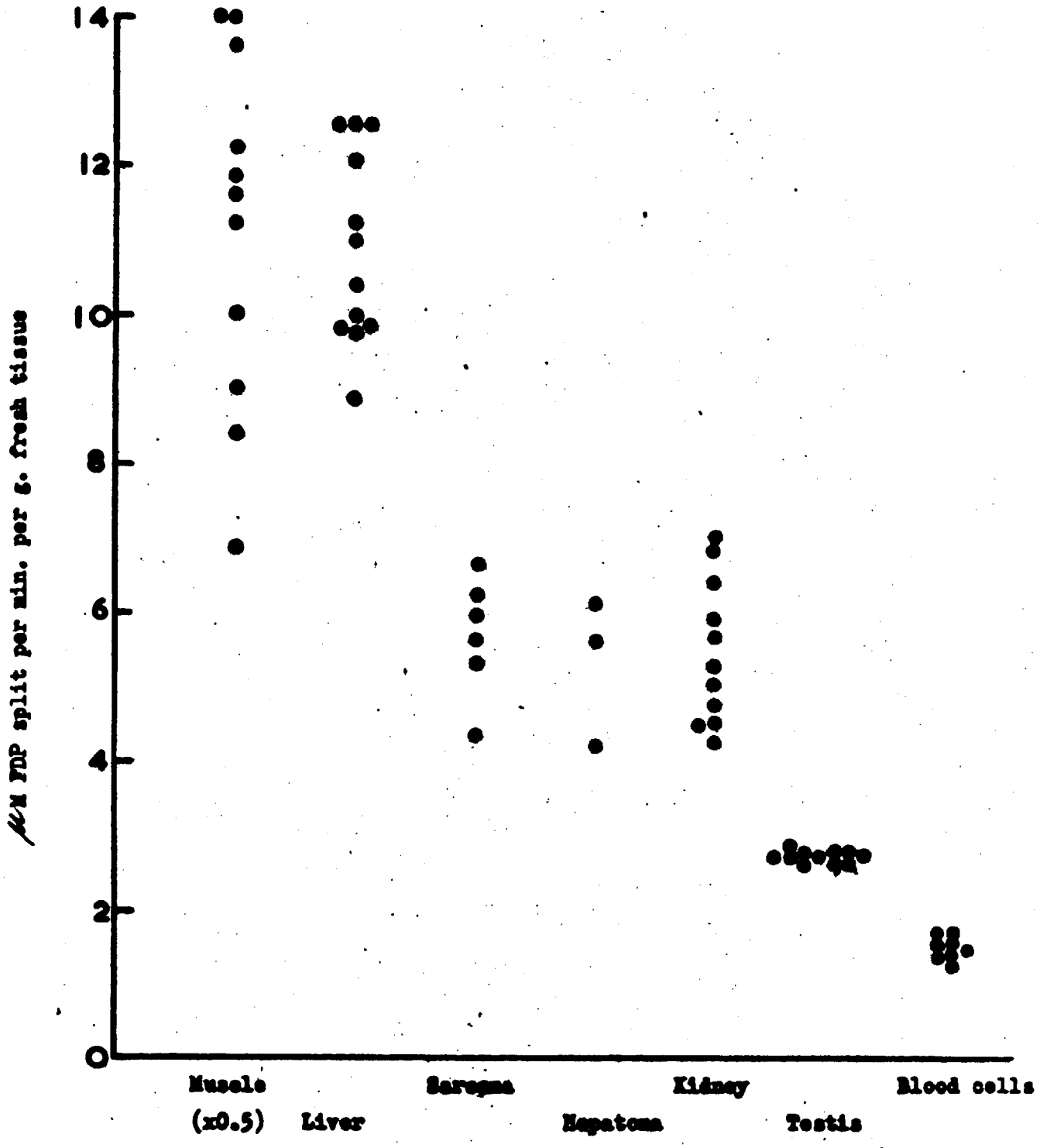

Fig.23. Scatter diagram of activity of aldolase in tissues of the rat.

that the activities of aldolase and of lactate dehydrogenase to some extent, decrease when tissue is stored for 24 hours at 4° (SCHMIDT and SCHMIDT, 1960). The percentage reduction varied with the tissue. The reduction in the activity of aldolase was 35 per cent in the rectus abdominis of man, and only 10 per cent in the brain of the guinea-pig. The decrease in the activity of lactate dehydrogenase was 18 per cent in the kidney and 10 per cent in the brain of the guinea-pig.

RESULTS

The activities of aldolase and lactate dehydrogenase obtained under optimum conditions, as revealed by the experiments described, are presented in table 36 and figs. 23 and 24. The units are micromoles of substrate transformed per minute and they are referred to both g. fresh tissue and mg. total N. in the tissue. It is of interest to have a record of the activities referred to fresh weight; but useful comparisons between different tissues can be made only when nitrogen or some other constituent related to the level of metabolism in the tissue is used as the index. The standard error of the mean accompanies the activities referred to wet weight.

Aldolase

The range of activities of aldolase, referred to fresh weight extends from 1.33 units in blood cells to 55.73 units in muscle. Most tissues have activities in the range 1 to 11

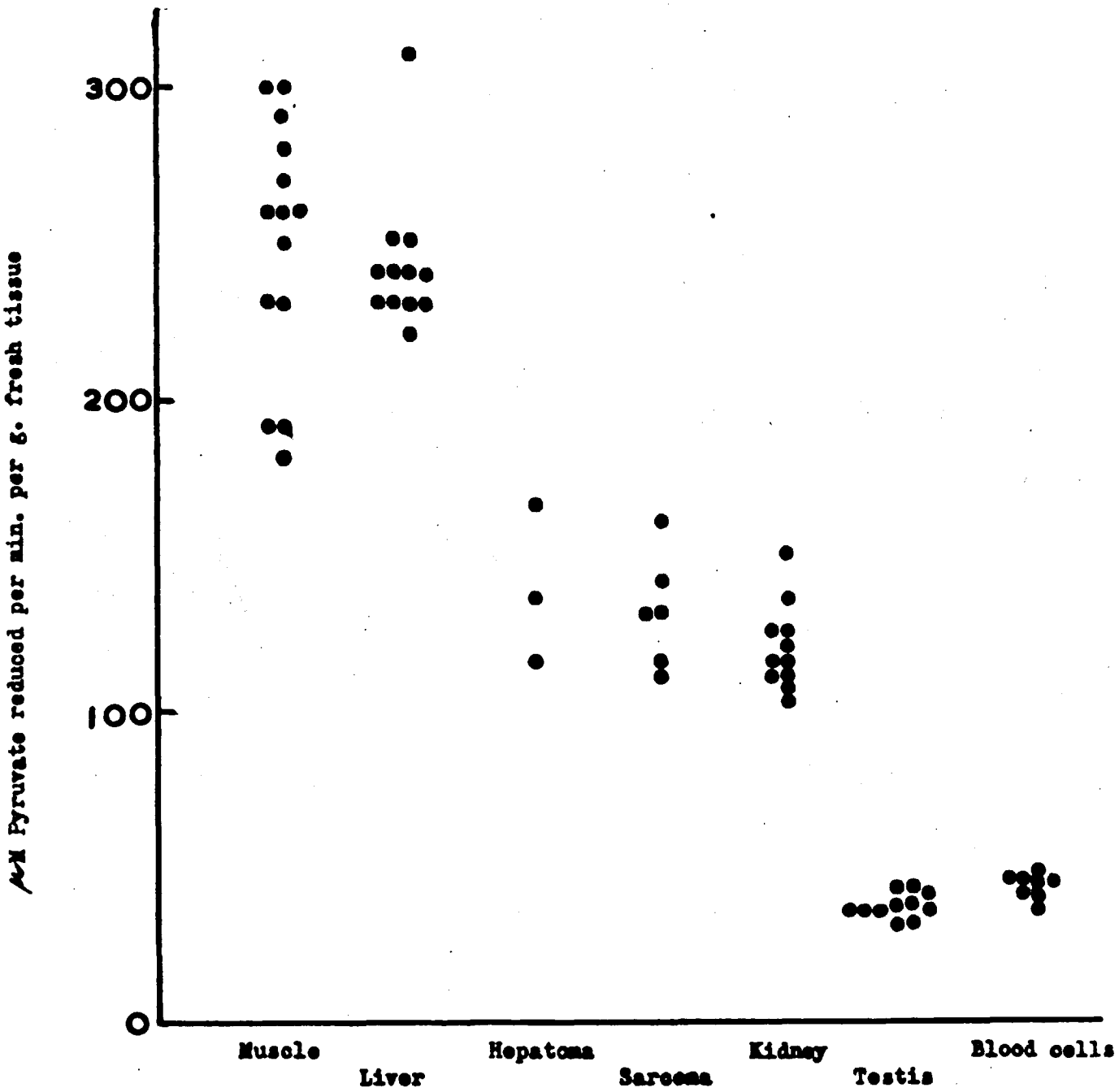

Fig. 24. Scatter diagram of activity of lactate dehydrogenase in tissues of the rat.

units, however, the value for muscle being unusually high by comparison. When the activities are referred to the total nitrogen content, the ratio of the extreme values is increased because of the relatively high content of nitrogen in erythrocytes. The mean levels for blood cells and muscle become, respectively, 0.029 and 1.69 units. There is another, and more important effect, namely the clumping of the values of the other tissues roughly in the middle of the range, namely between approximately 0.18 and 0.34 units. This is due to the relatively low content of nitrogen in testis, sarcoma and hepatoma.

Lactate dehydrogenase

The mean activities of lactate dehydrogenase per gram of fresh tissue range from 36.36 units in testis to 249.3 units in muscle. The value for blood cells is similar to that for testis, while the level of liver aldolase compares with that in muscle; the values for the remaining tissues lie in the middle of the range. Substitution of total nitrogen for the fresh weight again increases the ratios of the extreme values; thus the mean value for blood cells is now 0.91 units and that for muscle is 7.58 units. The activities of the other tissues again become more clumped together; as with aldolase the values obtained for the tumours are similar to those observed in tissues such as liver and kidney.

VII

DISCUSSION AND CONCLUSIONS

'One of the general working hypotheses in biochemical research, which has often proved correct, is the assumption that chemical substances and chemical reactions occurring in living matter are not as a rule accidental, but serve some purpose' (KREBS, 1946-48)

A. HUMAN COLON AND RECTUM

Comparison with Values in the Literature

Tissue in Man

The aldolase activities of the mucosa and the carcinomata of human colon were assayed as 4.1 and 7.0 µM, respectively, of fructose diphosphate split per minute per gram of fresh tissue by SIBLEY and FLEISHER (1955). Their value for mucosa is similar to that obtained in the present investigation, namely, 5.8 µM/min./g. Their value for the tumours is approximately twice that observed in this investigation; the difference could be due to differing degrees of cellularity of the tumours. Whatever the reasons for the differences between the results of the two investigations, the present results do not support the contention advanced by Sibley and Fleisher, namely, that the change from normal to neoplastic growth is accompanied by an increase in the activity of aldolase.

There appear to be no estimates of the activity of

lactate dehydrogenase or deoxyribonuclease in human colon in the literature.

SCHMIDT and SCHMIDT (1960) published a large number of results of determinations of activities of enzymes in human tissues obtained after death and at operation. The aldolase activities were less than 6 µM/min./g. fresh tissue with the exception of that of skeletal muscle which was 48 µM/min./g. The activities of aldolase in liver, renal cortex, gastric mucosa and erythrocytes respectively, were 5.7, 1.8, 1.1 and 1.0 µM/min./g. Thus the values obtained for the mucosa, polyps and carcinomata of colon are similar to that in liver but different from that in gastric mucosa. The values obtained for the activity of lactate dehydrogenase in liver, skeletal muscle, renal cortex, gastric mucosa and erythrocytes, respectively, are 145, 148, 113, 65 and 36 µM pyruvate reduced per min. per g. fresh tissue. In this case the values for colon and its tumours are near the lower end of the range occurring in human tissues. The proximity of the values for lactate dehydrogenase in colonic and gastric mucosa are in contrast to the differences in the activity of aldolase in these tissues.

There is relatively little information regarding the distribution of deoxyribonucleases in human tissue. Furthermore, the methods and units differ so much that comparisons are not profitable. The most useful results from the present viewpoint are those of COOPER and others (1950) who observed

that the activity in carcinomata of the stomach is twice that in gastric mucosa and approximately equal to that in carcinomata of the 'bowel'.

Tissues in Other Species

The results obtained for the activities of aldolase and lactate dehydrogenase in the tissues of the rat are given in table 36. The activities of aldolase in human colon are similar to those derived from rat kidney, hepatoma and sarcoma, but they are about twice the activity extant in the rat stomach (SIBLEY and LEHNINGER, 1949). The values obtained for the activity of lactate dehydrogenase in the colon are about one third of those observed in the tissues enumerated. It is worth noting the activities of lactate dehydrogenase in the mucosa of other parts of the alimentary canal. The level is low in the stomach of the mouse, namely, 60 µM/min./g. fresh tissue, but it is high in the intestine, 220 µM/min./g. fresh tissue (MEISTER, 1950).

The distribution of deoxyribonuclease in mammalian tissues has been investigated chiefly by GREENSTEIN (1941-42) and by ALLFREY and MIRSKY (1952). The units employed by these two groups differ from each other and from those being used in the present investigation; therefore it is impossible to make direct comparisons. It is noteworthy that the nuclease acting at pH 5 is apparently the most widely distributed (ALLFREY and MIRSKY, 1952). However, this conclusion may need to be revised because many tissues contain inhibitors

which may prevent the detection of the enzyme which acts at pH 7 and requires Mg^{2+} (LASKOWSKI, 1961). It is not even certain that all of the nucleases in tissues reported as deoxyribonuclease II are the same enzyme. In most cases only the pH optima and the requirements for Mg^{2+} are known. The enzymes obtained from spleen and thymus appear to be similar: the purified enzymes split deoxyribonuclease into the same products and have identical requirements for ions. The enzyme present in the extracts from the colon is of the deoxyribonuclease II type. Thus the optimal pH is 5, the optimal ionic strength is 0.15, and Mg^{2+} in a concentration greater than 10^{-2} is inhibitory.

Assays of the tissues of the calf show that the activity of intestinal deoxyribonuclease (pH 5) is relatively high, lying approximately midway between thymus and spleen (ALLFREY and MIRSKY, 1952). This high activity is doubtless related to the rapid replacement of the epithelial cells of the intestine. Thus in man all of the cells of the crypts of the acini in the colon are replaced every 3 - 4 days (LIPKIN and others, 1963).

Interpretation of Results

Epithelial Cells in Mucosa and Carcinomata

As a result of the failure to achieve a separation of the epithelial cells of the mucosa and carcinomata of the colon with preservation of their contents, it is not possible to make a comparison of the activities of the three enzymes

in these cells in the two kinds of tissue. There is a sufficiently high percentage of supporting tissue to dilute any real differences which might exist. In the case of deoxyribonuclease there is an additional reason why comparisons within tissues are not helpful; this is that the activity is higher in tissues which are proliferating (ALEFREY and MIRSKY, 1952). Thus the connective tissue of carcinomata which grows as the tumour cells grow probably has an increased activity of deoxyribonuclease. It is concluded that in order to decide whether the epithelial cells of a normal tissue and the tumour cells derived from them differ with respect to the activity of a given enzyme, it is necessary to take intracellular samples in the way suggested by LOWRY and his colleagues (1954). However, even this technique would fail to answer the question if the cells from which the samples were taken could not be identified. This is difficult enough in stained sections and smears of tumour cells; whether it is possible in untreated fresh freeze-dried tissues requires to be investigated.

The preceding observations are relevant to the detection of changes in the activities of enzymes in the polyps of multiple polyposis. Thus although samples taken from the polyps and from relatively normal mucosa in the same colon differed with respect to the activities of lactate dehydrogenase and deoxyribonuclease, no conclusions can be reached as to the cells of origin of the enzymes.

The greater activities of the two enzymes in the polyps could derive from active proliferation of either connective tissue or epithelial cells. The sampling of the content of single cells appears to be the only way of solving the problem.

There is no correlation between the activities of aldolase and lactate dehydrogenase over all of the samples of tissue taken in isolation. This need not be taken as evidence of a random relation between the activities of the enzymes. Thus when the activities of these enzymes are compared over the wide range of activities observed in a number of tissues of the rat a significant correlation is demonstrable (fig. 26). There are, as already indicated, many factors affecting the activities of enzymes and unless these are known, correlations within a small range may be obscured.

Likewise, there is no correlation between the activities of each enzyme in the mucosa and the tumour of the same specimen; this could be because of the different amounts of connective tissue associated with each tumour.

Ratio of Activity of Lactate Dehydrogenase to Aldolase

The ratio of the activity of lactate dehydrogenase to that of aldolase is 14.4 in the carcinomata, 8.1 in the mucosa and 10.5 in the polyps. These are not necessarily the ratios present in the epithelial cells: they refer to the whole tissue only. Any attempt to interpret this ratio is speculative

because information regarding the detail of the glycolytic pathway in the whole mucosa of colon and in its connective tissue is lacking. An obvious suggestion is that the potential for converting pyruvate to lactate is greater in tumour tissue. This is supported by the observation that tumours contain large amounts of lactate (LePAGE, 1948; this thesis, table 29). The deviation of pyruvate to lactate could be associated with the poor blood supply of tumours and a relative increase in the rate of anserobic glycolysis compared with that in normal tissue. Alternatively, the relatively high rate of oxidation of fat in tumours (AISENBERG, 1961) might result in the deviation of pyruvate to lactate.

The differences in the activities of aldolase in the mucosa of stomach and colon and the similarities in the activities of lactate dehydrogenase presumably reflect differences in function. In the absence of detailed information regarding the glycolytic pathway in these tissues, speculation as to the reasons for the differences is not likely to be profitable. All that can be said is that it is conceivable that the higher activity of aldolase in colonic mucosa may indicate that relatively more energy is derived from carbohydrate sources in this tissue.

Activity of Deoxyribonuclease in Tumours

The greater activity of deoxyribonuclease in the carcinomata and polyps (referred to DNA P) presumably reflects a greater turnover of deoxyribonucleate (ALLFREY and MIRSKY,

1952). It may be related to the increase in activity observed, after feeding azo dyes, in the mitochondria of rat liver (SCHNEIDER and others, 1953) and in the nuclei of pre-cancerous liver, hepatomata and adjacent non-neoplastic liver (LAMIRANDE and others, 1954). It is presumably associated with a larger amount of enzyme protein. This could be because there are more cells normally rich in deoxyribonuclease present, or because there is more enzyme present in cells which normally contain a smaller amount of the enzyme. If the latter alternative is the reason, then a control mechanism is involved, possibly of the induction-repression type observed in cultures of HeLa cells by deMARS (1958). This author remarked that the activity of glutamyl transferase increases about twenty-fold in the presence of glutamic acid and decreased in the presence of glutamine. The mechanism of the neoplastic process may be found to be due to breakdown in control of a mechanism of this kind.

Summary

1. The activity of aldolase in human colonic mucosa, polyps and carcinomata is at the upper end of the range for other human tissues, with the exception of skeletal muscle.
2. The activity of lactate dehydrogenase in these tissues is at the lower end of the range cited for human tissues.
3. The significance of any differences observed in the crude activities of enzymes in normal and tumour tissues will remain

obscure until the activities can be measured within single cells whose identity is known.

4. The ratio of the activity of lactate dehydrogenase to that of aldolase in the carcinomata was almost 1.8 times greater than that in the mucosa and almost 1.4 times greater than in the polyps.

5. The higher activity of deoxyribonuclease in the polyps of multiple polyposis could arise from any cells which are proliferating rapidly (see also point 3).

B. TISSUES OF THE RAT

1. CONCENTRATION OF SUBSTRATES

Comparison with Values in the Literature

The yields of the four substrates obtained in this investigation together with those obtained by others are presented for comparison in table 37. All results are referred to 1 g. fresh tissue.

Liver

The range of yields of dihydroxyacetonephosphate observed by various investigators falls within that observed in the present enquiry. This is partly because the analytical methods were similar; but the chief reason appears to be that a large range of values does not occur. Decapitation and ether anaesthesia for 3 to 5 minutes are associated with yields lower than those attainable with optimal ether anaesthesia; these are probably the reasons for the lower values obtained by HOLZER and others (1956) and by HOHORST and others (1959), respectively.

The case of fructose diphosphate is more complex. The range of values which appears in the literature extends from 22 to 263 µMm/g. fresh tissue. The higher values, obtained by LePAGE (1948, 1950) and by THRELFALL and STONER (1961), were the results of chemical analyses. The amount of separation of the sugar phosphates during analysis affects the result; if it is not precise the result is misleading. The

Table 37

Content of substrates in tissues of the rat as obtained by various investigators

Tissue	Author	Conditions of sampling	Metabolite µmM/g. fresh tissue DAP	FDP	P	L
Muscle	LePage, 1948, 1950	See under liver	---	70	81	1880
	Frohman & others, 1951	See under liver	---	---	250	920
	Threlfall & Stoner, 1961	Sodium pentobarbitone anaesthesia; frozen in liquid nitrogen	---	220	---	---
			---	250	113	4710
	Dale, 1963	Ether anaesthesia 90 sec.; frozen with pre-cooled tongs	36	86	78	1337
Whole Blood	Ronzoni, 1950	Sodium pentobarbitone anaesthesia	---	---	---	833
		No anaesthesia	---	---	---	1020
	Frohman & others, 1951	See under liver	---	---	148	1510
	LePage, 1950	Arterial blood	---	---	135	---
		Venous blood	---	---	360	---
	Goodwin & Williams, 1952	Sodium pentobarbitone anaesthesia, aortic blood	---	---	103	---
	Holzer & others, 1956	See under liver	15	10	240	---
	Hohorst & others, 1959	See under liver. Blood collected from aorta after sample taken from liver	25	12	190	2230
	Dale, 1963	Ether anaesthesia 90 sec.; mixed arterial and venous blood	11	3	131	1880
Plasma	Hohorst & others, 1959	See under liver	0	0	234	2960
	Dale, 1963	See under 'whole blood'	0	0	127	2860
Blood cells	Bartlett, 1959	Blood centrifuged 20 min. Cells washed twice in 0.15 M NaCl	---	60 to 120	---	---
	Hohorst & others, 1959	Calculated from results given above	55	27	135	1360
	Dale, 1963	See 'whole blood'	18	6	135	710
Liver	LePage, 1948, 1950	Sodium pentothal anaesthesia; frozen in liquid air	---	170	16	2300
	Frohman & others, 1951	Fasted 24 hours. Sodium pentothal anaesthesia, exsanguination; removal of brain and then liver, kidney and muscle; frozen in solid CO_2	---	---	330	1350
	Holzer & others, 1956	Decapitation; extracted into perchloric acid	43	33	60	---
	Lamprecht & Trautschold, 1958	12 hours fasting. Extracted into cold perchloric acid	51	47	26	---
	Thorn & others, 1958	Urethane anaesthesia. Extracted into perchloric acid	35	28	81	1750
	Hohorst & others, 1959	Ether anaesthesia 3-5 min.; frozen with pre-cooled tongs	38	22	154	1540
	Threlfall & Stoner, 1961	Sodium pentobarbitone.	---	263	47	3010
		Guillotine	---	160	33	1060
	Dale, 1963	Ether anaesthesia 90 sec.; frozen with pre-cooled tongs	54	27	220	1362
Kidney	LePage, 1948, 1950	See above	---	40	33	1550
	Frohman & others, 1951	See above	---	---	280	1200
	Dale, 1963	See above	25	17	109	1166
Sarcoma	LePage, 1948	See under liver	---	50	180	6370
	Dale, 1963	See under liver	14	24	255	6720
Hepatoma	Le Page, 1948	See under liver	---	200	---	6000
	Dale, 1963	See under liver	32	30	182	3940

yields obtained by THRELFALL and STONER contain an error of this kind (personal communication). The result obtained by LePage is subject to the additional complications produced by freezing an adult rat during sodium pentobarbital anaesthesia. The value given by LAMPRECHT and TRAUTSCHOLD (1958) namely, 47 µmM/g. fresh liver, differs greatly from that obtained by the present investigator under similar conditions, namely, 8 µmM/g. fresh liver (table 14). It resembles that obtained when samples were taken 3 minutes after exsanguination. The ratio of the concentration of dihydroxyacetonephosphate to that of fructose diphosphate obtained by these workers, is approximately one and is similar to that obtained by the writer when pulverisation of the frozen tissue was omitted (table 14). The results of HOLZER and others (1956) and of HOHORST and his colleagues (1959) are comparable to those obtained by the present investigator under the conditions of stress associated with these sampling techniques. Reasons were given for rejecting results associated with such conditions in the last paragraph.

The yields of pyruvate also cover a wide range, namely from 16 to 330 µmM/g. fresh tissue. This is attributable chiefly to the means used to produce unconsciousness and to the inefficient methods of cooling the tissue. It was shown that stressing the rat (table 23) or delay in cooling the tissues (table 14) results in low yields of pyruvate. The value obtained by FROHMAN and others (1951) is difficult to

account for. Most of the steps used by them were found to produce low yields by the present investigator. It seems possible that the method of separating the substrates (partition chromatography on silica gel) was not sufficiently specific.

The yields of lactate agree reasonably well, with the exception of the high values obtained by LePage after freezing the whole rat, and by Stoner and Threlfall after sodium pentobarbital anaesthesia.

On the whole the results obtained by the present investigator agree with those of Hohorst, Kreutz and Bucher whose method was followed closely. The differences are almost certainly due to the shorter period of ether anaesthesia used in the present investigation.

Muscle

There do not appear to be other estimates of the content of dihydroxyacetonephosphate in muscle. The yield of fructose diphosphate obtained by LePage agrees with that observed in the present investigation. It is difficult to understand why it should because the two lots of results relating to liver are disparate. It is conceivable that the difference in the rates of freezing of muscle and liver in the whole rat account for the discrepancy. A number of factors could explain the difference between the yields of Threlfall and Stoner and the present ones. These include the different anaesthetic, the time required to dissect out

the limb muscle and inadequate chromatographic separation of the sugar phosphates.

The yields of pyruvate agree, with the exception of that obtained by Frohman and others. However, as already indicated, the method of obtaining and freezing the tissue used by these investigators leads to anomolous results. The only occasion when a pyruvate level of this magnitude was observed by the present investigator was after muscular spasm.

The lactate results are all different. The high values observed by Threlfall and Stoner after pentobarbital anaesthesia can be rejected. The difference between the results obtained by LePage and the present author is attributed to the slow rate of cooling of the whole animal. It is not possible to say which of the remaining two results is more likely to represent the normal resting level.

Kidney

Very few analyses have been published. The yield of fructose diphosphate obtained by LePage is probably too high; freezing the whole rat is associated with high yields (table 20). The yield of pyruvate observed by the same author is probably too low, for the same reason; that of Frohman and others must be suspect because of the conditions of analysis. The figures cited for lactate agree fairly well; the highest is probably too high because it was obtained from a whole frozen rat.

Sarcoma

There is overall agreement between the analysis carried out on two different types of sarcoma by different methods of sampling and estimation. This may be because tumours are not likely to be affected by homeostatic mechanisms and because they normally depend on anaerobic glycolysis more than respiration for their supplies of energy. Both of these factors would make them less sensitive to the procedure used for sampling.

Hepatoma

There are insufficient data to make a comparison. The apparent yield of fructose diphosphate obtained by LePage was large, presumably for the same reasons as that in normal liver.

Whole Blood

The yields of both dihydroxyacetonephosphate and fructose diphosphate obtained by the present investigator are the lowest observed. In the case of Hohorst and his colleagues the explanation probably lies in the longer period of ether anaesthesia, and in particular, in the delay in removing the blood until after removing samples of liver. There were insufficient details provided by Holzer to reach any conclusions as to the values obtained.

There are several estimates of the pyruvate content of whole blood which indicate that the normal level is less than 200 μmM/g. The difference between the arterial and venous

concentrations may account for some of the discrepancies; LePAGE (1950) gives the arterial content as 135 and venous content as 360 µmM/g. blood. Another factor may be muscular contraction which causes an increase in blood pyruvate (see page 239; LU, 1939). The lower values are more likely to be those in the rat at rest.

The values for lactate vary. Again, the lowest figure is more likely to represent the in vivo level at rest. The site of removal of the sample may be of importance; the concentration in arterial blood would be expected to be low and that in venous blood, especially that draining muscles, to be high. The relatively low value obtained by Ronzoni may be due to her sampling from the tail vein; the tissues of the tail do not have a high rate of metabolism, but there is a large blood flow, presumably in order to keep the tail warm or the rat cool.

Plasma

The results of this investigation confirm those of Hohorst and others. The only important difference is the lower concentration of pyruvate; a possible reason for this is that already suggested for whole blood. Both values for lactate are very high - 2 to 3 times those apparently occurring in the cells. The possibility was considered that this was due to passage of lactate from the cells into the plasma after removal of the blood from the rat. The likelihood that this is an important source of error was reduced by the

observation that even after allowing whole blood to stand for as long as 5 minutes at 37° no increase was detectable in the plasma lactate level.

Blood Cells

The only agreement here is on pyruvate. Reasons for the differences between the results of Hohorst and his colleagues and the present investigator have been given. The value for fructose diphosphate observed by BARTLETT (1959) was obtained after centrifuging for 20 minutes, followed by washing twice in 0.15 M sodium chloride solution; these appear to be sufficient reasons for the difference between his result and the two others.

Interpretation of the Results

The Sugar Phosphates

The most striking feature of each of these substances is the similarity of the amounts present in the different tissues; this is clearer when the total N is used for reference (table 31). Only in the case of fructose diphosphate are there large differences in the values; these occur in muscle and in the blood cells. Another feature is that there is relatively little of these substrates in most tissues; with the exception of muscle the concentrations are less than 5×10^{-5} M. This is presumably due to the large excess of enzyme which can catalyse the transformations of the metabolite as soon as it is formed (RACKER, 1954; KREBS and KORNBERG, 1957). These comments apply to tumour tissue as well as to

the normal; this is not surprising because the sugar phosphates are important in the production of energy in all tissues.

The relative magnitude of the amounts of either substrate in the different tissues cannot be used as a pointer to the relative flux through the glycolytic pathway without further information. This is because the components and the direction of metabolism are not necessarily the same in each tissue. With dihydroxyacetonephosphate this is especially true because there are several sources and sinks. Fructose diphosphate is involved in few reactions and it is conceivable that the relative magnitude of the amounts present in the different tissues do reflect the size of the flux. Thus it might be expected that the blood cells, which contain little fructose diphosphate, would have a small flux and that muscle, which has a large content of fructose diphosphate would have a large flux. This point will be taken up again later.

Ratio of $\frac{FDP}{DAP}$. There is no correlation between the amounts of fructose diphosphate and dihydroxyacetonephosphate in the different tissues. But the ratio in each tissue is of interest in showing the amount of deviation from the thermodynamic equilibrium; it may also provide some idea of the dominance of degradation or synthesis. In the reaction catalysed by aldolase there is 89 per cent of fructose diphosphate and 11 per cent of the triosephosphates at equilibrium (MYERHOF

Table 38

Ratio of concentrations of reactants and products

Tissue	FDP / DAP	Lactate / Pyruvate
Liver	0.52	3.4
Kidney	0.66	5.7
Testis	0.97	14.0
Skeletal muscle	2.36	14.4
Blood cells	0.33	---
Sarcoma	1.73	29.0
Hepatoma	0.95	20.0

and JUNOWICZ-KOCHOLATY, 1943), that is, the ratio is about 16:1 for each triosephosphate. The nearest approach to this is in muscle at 2.36, and the next is in RD 3 sarcoma at 1.73 (table 38). This is perhaps to be expected in view of the importance of degradation of glycogen in muscle, and the high rate of glycolysis in tumours. Presumably the lower values in blood cells, liver and kidney indicate that degradation is less important or that synthesis is occurring. The only value available for comparison is that of HOHORST and his colleagues (1959) who found the ratio to be 0.58 in liver. This compares with 0.52 as observed by the present writer. The change in the ratio, $\frac{FDP}{DAP}$, from 1:2 to 1:1 in liver after the omission of pulverisation (table 4), sampling 10 minutes after exsanguination (table 19) and stunning (table 21) could be due to cessation of synthesis. It could be due also to a change to a closed system and the tendency to the establishment of equilibrium conditions. In none of these three cases was the amount of dihydroxyacetonephosphate increased; therefore it is unlikely that the changed ratio is due to slowing of the triosephosphate dehydrogenase reaction.

Pyruvate and Lactate

The ratio of the highest to lowest pyruvate content in the different tissues is comparable to the corresponding ratio of the sugar phosphates. With lactate there is a large ratio owing to the very large amounts present in the tumours. In the normal tissues the amounts appear to be comparable;

this may be an artefact because of the large concentration of lactate in the extracellular fluid and the correspondingly large correction required in order to obtain the intracellular moiety. The concentrations of pyruvate and lactate are, on the whole, considerably higher than those of the sugar phosphates. The chief exceptions are the pyruvate content of muscle when referred to wet weight or to total N, and that of testis when referred to wet weight.

The relative magnitude of the pyruvate contents of the different tissues cannot be taken as it stands, as a guide to the flux of pyruvate through the tissue. Thus muscle, which presumably has the highest flux even at rest, has a low pyruvate content. This may be because in muscle pyruvate arises chiefly from one reaction. In other tissues, and especially in liver, pyruvate enters many reactions, and in particular, the tricarboxylic acid cycle. If each of these reactions contribute to the pyruvate content the amount would conceivably be larger than that in a tissue where only one pathway contributed. Another reason for the difference in pyruvate content is that pyruvate formed in muscle is converted rapidly to lactate and eliminated from the cell; the same lactate is taken up by the liver and heart and possibly by other tissues, and converted to pyruvate. The small amount of pyruvate in kidney and testis is not readily accounted for; it could be attributed to rapid removal into the tricarboxylic cycle. This does not appear to be likely in view

of the numerous observations indicating that the ratio of the $-Q_{O2}$ in kidney to that in testis is approximately 4:1. The rates of glycolysis are no more helpful: they are approximately the same as in liver. The correlation between pyruvate concentration and the activity of lactate dehydrogenase may be relevant in this respect (fig. 24). The activity in both tissues is lower than it is in liver and if it represents the rate of transformation of lactate to pyruvate then lower concentrations of pyruvate would be predicted (see page 319 for a further discussion of this function of lactate dehydrogenase).

A possible reason for the greater content of pyruvate than sugar phosphates in many tissues was given in passing: pyruvate is produced in more reactions in pathways of high flux, for example, from glycogen and glucose, lactate and amino acids. The reason for the very high concentration of lactate is presumably that the equilibrium constant of the reaction favours the formation of lactate.

The ratio of lactate to pyruvate. This differs considerably in different tissues (table 38). It is lowest in liver and kidney, being 3.4 and 5.7, respectively; it is highest in tumours at 20 to 27, and it is approximately midway between these values in testis and muscle. The value for liver given by HOHORST, KREUTZ and BUCHER (1959) is 9.6. The difference is attributable chiefly to the higher concentration of pyruvate observed by the present writer. This

difference is important because the ratio was used by the authors referred to in order to calculate the ratio of $\frac{NAD^+}{NADH}$. This is given by the ~~first~~ equation on page 159 and it is equal to

$$\frac{(\text{pyruvate}) \cdot (H^+)}{(\text{lactate}) \cdot K_{eq}} \sim \frac{10^{-7}}{3.4 \times 53 \times 10^{-13}} \sim 5600,$$

using the values for (H^+) and K_{eq} given by Hohorst and his colleagues. Their values for the ratio is ~ 2000. The question of the ratio oxidised/reduced form of nicotinamide-adenine dinucleotide has been argued by both CHANCE (1954) and by HOLZER (1959b) (see also page 58). HOLZER (1959) claims that the ratios obtained by his method are a true measure of the thermodynamically active co-enzyme; he regards the values obtained by Chance as representing the total amount of co-enzyme including a large inactive fraction bound to protein (DUYSENS and KRONENBERG, 1957).

Substrates and Tumours

There is almost no information regarding the concentrations of the labile substances occurring during glycolysis in tumours. This is partly because only one extensive investigation appears to have been carried out (LePAGE, 1948, 1950), and partly because the methods available until recently were unsatisfactory. The information arising from the present investigation extends the knowledge of the concentrations of the sugar phosphates and pyruvate in tumours. The high content of lactate is familiar and may be due to dependence on a high rate of anaerobic glycolysis (WARBURG, 1926). This is not

a wholly satisfactory reason. Tumours have a rate of respiration comparable to that of most normal tissues (AISENBERG, 1961); therefore such an excessive production of lactate is not to be expected on the grounds of defective respiration. Furthermore, after exercise, the content of lactate in muscle (produced by glycolysis) reached only 40 to 60 per cent of that observed in the two tumours examined. Another reason is suggested for the higher lactate level in tumours, namely, the effects of a poor blood supply; this would not only favour anaerobic glycolysis but it would allow the accumulation of the lactate so produced. The amounts of the other three metabolites are similar to their concentrations in liver or kidney; this is not surprising in the case of the hepatoma, but it is of considerable interest in the sarcoma. It is not long since GREENSTEIN (1954) stated that the level of pyruvate is low in normal tissues and high in tumours. The similarity of the composition of the tumours and normal tissues is but another manifestation of the resemblance of the kinds and amounts of enzymes present in the two types of tissue. Likewise the similarity of the composition of the two types of tumour is a sign of the uniformity of the composition of all tumours. According to GREENSTEIN (1954) "Whatever the cause or origin of the tumour may be, the tumour ends by possessing a chemical pattern very largely the same as that of other tumours of quite different etiology or histogenesis".

Effects of Stress

The only consistent effect of stress observed in the

different tissues was that usually a large amount of stress was needed to produce a significant change in the concentrations of the four substrates. It was suggested that this is attributable to the properties of the open system, namely, to the buffering capacity, the stability and the accumulator function. The factors responsible for the diverse changes in magnitude and direction of the amounts of the substrates in all of the tissues examined are largely unknown and are too numerous to even begin to discuss; but it is worth making an attempt to do so for liver and testis in both of which the changes are internally consistent.

Liver. In the case of liver all of the stresses applied to the animal, whether it was conscious or unconscious, produced either no change or a decrease in the concentrations of the sugar phosphates and pyruvate, and either no change or an increase in the concentration of lactate. The increase in the concentration of lactate was associated with conditions of anoxia and/or muscular contraction. These stresses produce an increase in the concentration of blood lactate; therefore it is probable that the accompanying large content of lactate in the liver arises from the blood.

The decrease in the concentrations of the other three metabolites could be due to diminished formation, or to enhanced usage. It is unlikely that there is a reduced rate of formation during stress, a state when the reserves of the animal are called forth. The increased return of lactate to

the liver and the activation of phosphorylase via the release of adrenaline might be expected to produce an increase in the amounts of the other intermediates. It is possible, however, that the products of glycolysis are deviated to the blood-stream in order to replenish the glucose which is oxidised in tissues affected by the stress. This would, in effect, reduce the amounts available for local consumption in the tricarboxylic acid cycle. On the other hand, there is no obvious reason why the liver should not participate in the response to stress; it is conceivable that the reduction in the concentrations of the sugar phosphates and pyruvate are due to an increased flux initiated in the tricarboxylic acid cycle in the liver.

An increased demand for fuel in the tricarboxylic acid cycle would result in an increased consumption of the lactate derived from the blood stream and therefore no increases in the amounts of other intermediates examined need be expected from this source. It is possible that increased demands for energy both in the liver and in other tissues during stress contribute to the reduction in the concentrations of dihydroxy-acetonephosphate, fructose diphosphate and pyruvate.

Testis. The testis showed no response to the two stresses applied, namely exercise and exsanguination; but in liver, muscle and kidney both stresses were sufficiently severe to produce changes in the concentrations of the metabolites. The reasons for the insensitivity to stress

presumably include the low blood flow, the low rate of metabolism and the lack of involvement of the metabolism of the testis with other tissues or with general homeostatic mechanisms.

Fructose diphosphate. The effect of stress on the content of fructose diphosphate is of especial interest because of the potential importance of the phosphofructokinase reaction in the control of glycolysis. The relatively small range of values for the content of fructose diphosphate in the different tissues in all but extreme conditions emphasises the importance of establishing the resting content with the greatest possible certainty. Unless this is known a change in the content of fructose diphosphate would not be detected and evidence relating to the control of the phosphofructokinase reaction would be missed.

Evidence of activation of the enzyme is provided by an increase in the concentration of fructose diphosphate coupled with decreases in the concentrations of the hexose-6-phosphates. This occurs in the perfused heart in anoxic conditions (PARK and others, 1961; NEWSHOLME and RANDLE, 1961). It may be the reason for the increased concentration of fructose diphosphate in muscle, kidney and brain of the frozen conscious rat, in muscle, liver and kidney after exsanguination and in muscle after stunning. All of these conditions are associated with reduced concentration of ATP, a condition which favours the activation of phosphofructokinase (LARDY and PARKS, 1956).

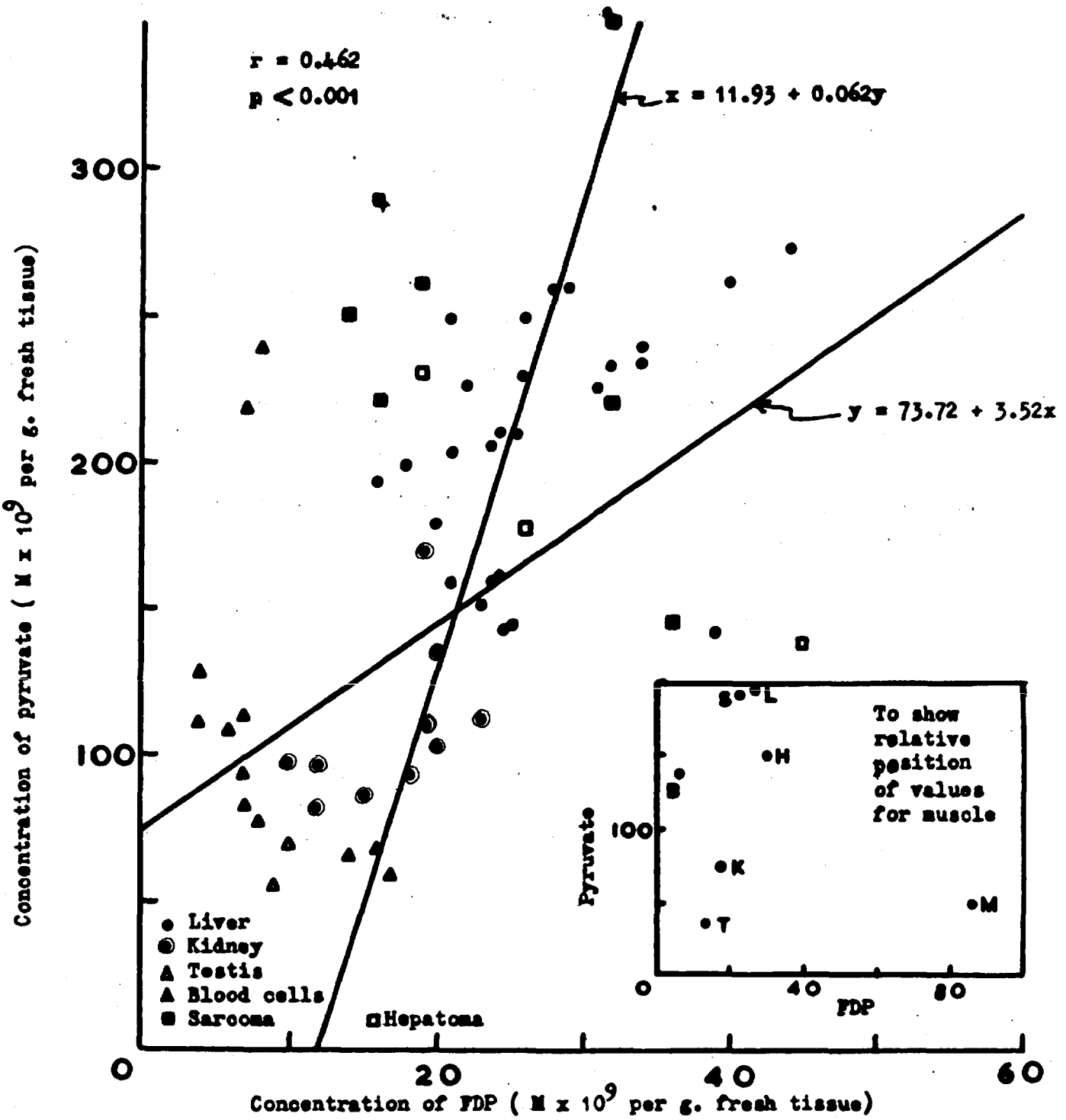

Fig. 25. Correlation of concentrations of pyruvate and fructose diphosphate (FDP) in tissues of the rat. In figs. 25-30 the names of the tissues are abbreviated as follows: liver, L; kidney, K; skeletal muscle, M; testis, T; sarcoma, S; hepatoma, H.

The reduction of the content of fructose diphosphate in the liver was associated with many forms of stress and it is unlikely that inhibition of the phosphofructokinase reaction is the reason for the decrease in all cases.

The Correlation Between the Amounts of Fructose Diphosphate and Pyruvate in the Tissues of the Resting Rat

It was pointed out in Chapter II that it is impossible to measure the concentrations of reactants in cells at the sites of reaction; and sufficient has been written in this chapter to indicate that the comparison of the gross concentrations of a particular metabolite from a number of tissues is not justified unless the metabolic sequences from which they arise are believed to be similar in all respects. One way of trying to establish the similarity of a given sequence in different tissues is to correlate the amounts of the reactants at different points in the sequence; if the correlation were significant this would be presumptive evidence of the similarity of the sequence in the tissues examined.

With this object, the paired values for the concentrations of fructose diphosphate and pyruvate, as observed in all tissues examined except muscle, are plotted in fig. 25. There is a highly significant positive correlation ($r = 0.462$ $p < 0.001$). The exclusion of muscle is justified because its metabolism differs from that in other tissues. Some of the differences were mentioned in passing and others will be discussed presently. The differences are associated with the

the unique function of muscle, namely, the rapid production of large amounts of energy by glycolysis. As a result, the amounts and/or flux of the components of the glycolytic pathway dominate the metabolism of skeletal muscle; furthermore, the reactions are concerned chiefly with the degradation whereas in the glycolytic pathway in non-muscular tissues, as far as is known, there is not such a large discrepancy between the degradative and synthetic functions. The correlation of the concentrations of two metabolites, separated by a number of steps, in a metabolic pathway is consistent with the view that the pathway in the tissues concerned is similar in most respects. The large difference in the results obtained for muscle (insert, fig. 25) confirms its exclusion on the grounds of a different metabolism. The concentrations in the different tissues are dependent on the flux and the activities of the enzymes catalysing their reactions; therefore further comment will be deferred until the activities have been discussed.

The amounts of dihydroxyacetonephosphate and lactate fail to show any correlation with those of fructose diphosphate or pyruvate. In the case of dihydroxyacetonephosphate the lack of apparent correlation is ascribed to its participation in several reactions other than that catalysed by aldolase. With lactate the absence of a correlation could be due to exchange with the large amounts present outside the cells; any differences in the intracellular concentration in different tissues would be obscured readily.

Summary: Concentration of Labile Substrates

1. As intimated in the introduction

(a) the values obtained by different investigators for the concentrations of labile substances in rat tissues differ greatly,

(b) the methods of sampling the tissues disturb the animals from the resting state, and

(c) little or no data is available for many tissues.

2. The rapid freezing technique introduced by ERANKO (1954), and developed by WOLLENBERGER and others (1958) and HOHORST and others (1959), made the values obtained by analysis of extracts credible as estimates of the total amounts of labile substances present in tissues.

3. Even with this advance there was no information as to the magnitude and direction of the effects of sampling on the concentrations.

4. Evidence is provided in this thesis which indicates that the total amounts of certain labile metabolites of carbohydrate metabolism in the tissues of the resting rat can be estimated with reasonable certainty. The tissues include liver, kidney, skeletal muscle, testis, blood cells, sarcoma and hepatoma. The need to make a correction for the amounts of the metabolites in the extracellular fluid is emphasised.

5. The recent publication (AISENBERG, 1961) of figures purporting to be the normal concentrations of the intermediates of glycolysis in rat tissues, but which are no longer

acceptable (they were obtained in 1948 by LePAGE) underlines the necessity for examining the means used in order to obtain and to analyse samples of animal tissue.

6. The significance of the results is discussed:

(a) The relatively large amount of pyruvate in most tissues may be due to its formation from several reactions; the very large amount of lactate in all tissues is probably determined by the equilibrium constant of the lactate dehydrogenase reaction.

(b) The ratios of fructose diphosphate to dihydroxyacetonephosphate and lactate to pyruvate may reflect the relation of degradation to synthesis.

(c) The similarity of the concentrations of the substrates in tumours and normal tissues is further evidence consistent with the view that the neoplastic process is one of degree and not of kind.

(d) No obvious reasons can be found to account for the consistent response to stress in the liver, namely, the tendency for the concentrations of the sugar phosphates and pyruvate to decrease and that of lactate to increase.

(e) The concentrations of fructose diphosphate and pyruvate are correlated significantly in the tissues examined, muscle being excluded. This is regarded as evidence of internal consistency within the Embden-Myerhof pathway in these tissues; it justifies the suggestion that it is valid to make comparisons between the amounts

of substances in a comparable metabolic sequence in different tissues. The difference between the results obtained for muscle and other tissues confirms the need to exclude from such comparisons a tissue in which important differences exist, despite the presence of many similarities.

Table 39

Comparison of activities* of aldolase obtained by various authors in rat tissues

Author	Tissue					Conditions
	Liver	Kidney	Muscle	Testis	Whole Blood	
Myerhof & Lohman (1934)	---	Very low	4.2	---	0.012	Triosephosphates trapped by cyanide, and determined as alkali-labile phosphate, pH 7.5. Re-calculated for 38°
Warburg & Christian (1943)	Very low	Very low	2.7	---	0.011	Spectrophotometric estimation, pH 7.6. Re-calculated for 38°
Dounce & Beyer (1948)	0.09	0.20	2.8	---	---	Triosephosphates converted to acetaldehyde which was estimated by method of Barker & Summerson (1941). Re-calculated for 38°
Myerhof & Wilson (1949)	0.17	---	3.7	---	---	Alkali-labile phosphate determined after 1 to 5 min. incubation at pH 8.2. Re-calculated for 38°
Sibley & Lehninger (1949)	0.28	0.21	1.7	0.15	0.01	Triosephosphates trapped by hydrazine and determined as dinitrophenylhydrazines. Initial reaction carried out at pH 8.6, 37°
Trautschold (1956)	0.24	---	---	---	---	Quoted by Delbruck and others (1959).
Holzer & others (1956)	0.26	---	---	---	0.009	Spectrophotometric determination at 21° to 23°
Delbruck & others (1959)	0.17	---	---	---	---	Extraction in 0.25 M sucrose. Spectrophotometric estimation at 25°
Schmidt & Schmidt (1960)	0.29	---	---	---	---	Extracted into 0.15 M NaCl solution. Spectrophotometric estimation.
Dale (1963)	0.34	0.20	1.7	0.18	0.02	Extracted into 0.25 M mannitol solution. Estimated by method of Sibley & Lehninger (1949)

* µg. FDP split per mg. N per minute

2. ACTIVITIES OF ENZYMES

Comparison with the Values in the Literature

The comparison of the activities as obtained by other authors is unsatisfactory. This is largely because relatively few assays have been made. However, even the values available are not always easy to interpret because of the use of different methods of extraction of the enzymes, and of analysis, different temperatures for reaction, variation in the diet and/or state of nutrition of the animals, and finally storage of the enzymes. These difficulties are more important than two others which it is usually possible to overcome, namely the units employed and the index of reference. The variations in technique, as far as could be ascertained, are indicated in the tables 39 and 40, in which activities of aldolase and lactate dehydrogenase, respectively, as obtained by various authors, are collated.

Aldolase

With the exception of the earliest assays (MYERHOF and LOHMAN, 1934; WARBURG and CHRISTIAN, 1943) the results obtained for the few tissues examined agree fairly well; this is in spite of the relatively lower specificity of the enzymic method which was used by most of the investigators. The agreement between the two sets of results obtained by the method of Sibley and Lehninger is excellent. Some of the differences appearing in the table may be due to the ease with which aldolase is inactivated (see tables 33, 34 and

Table 40

Comparison of activities* of lactate dehydrogenase obtained from rat tissues by various authors

	Liver	Kidney	Muscle	Testis	Whole blood	Conditions
Wenner & others (1952)	1.9	1.2	5.1	---	---	Acetone powder extract. Method of Kubowitz & Ott (1943). 22° to 26°
Trautschold (1956)	9.1	---	---	---	---	Quoted by Delbruck and others (1959)
Holzer & others (1956)	2.5	---	---	---	0.13	Method of Kubowitz & Ott (1943). 21° to 23°
Delbruck & others (1959)	9.6	---	8.6	---	---	Method of Kubowitz & Ott (1943). 25°
Schmidt & Schmidt (1960)	10.4	---	---	---	---	Extracted into 0.15 M NaCl solution. Method of Kubowitz & Ott (1943)
Dale (1963)	7.6	4.4	7.6	2.5	0.64	Ether anaesthesia. Extracted into 0.25 M Mannitol. Method of Kubowtiz & Ott (1943). Corrected to 25°
Meister* (1950)	4.3	3.7	9.7	2.0	---	Cervical dislocation. Extracted into water. Method of Kubowitz & ott (1943). 26°

* µM pyruvate transformed per min. per mg. N.

* Mouse tissues.

Table 41

Comparison of activities* of aldolase and lactate dehydrogenase in tumours of the rat and the mouse as obtained by various authors

Author(s)	Animal	Tumour: Sarcoma		Hepatoma		Carcinoma	
		ALD	LDH	ALD	LDH	ALD	LDH
Warburg & Christian, 1943	Rat	0.25	---	---	---	---	---
Dounce & Beyer, 1948	Mouse	---	---	---	---	0.5	---
Myerhof & Wilson, 1949	Rat	0.4	---	---	---	0.4	---
	Mouse	---	---	---	---	0.4	---
Sibley & Lehninger, 1949	Rat	0.43	---	---	---	---	---
	Mouse	---	---	---	---	0.45	---
Meister, 1950	Mouse	---	4.3	---	6.7 3.8	---	3.3● 3.0*
Wenner & others, 1952	Rat	---	---	---	2.3	---	---
	Mouse	---	---	---	1.6	---	1.8*
Delbruck & others, 1959	Rat	---	6.7	---	---	---	4.8*
Dale, 1963	Rat	0.25	6.0	0.22	5.8	---	---

* µg. substrate split per mg. N per minute.

● Gastric carcinoma.

* Mammary carcinoma.

35). The only result which is outstandingly astray is that in liver obtained by Dounce and Beyer; the reason is partly because the method was not standardised against one of those known to be reliable, and partly because no trap for the carbonyl radicles was provided. Thus the triosephosphates formed could be degraded during assay.

Lactate Dehydrogenase

There are fewer assays available for this enzyme and there is only moderately good agreement. This is surprising because all investigators used the method of Kubowitz and Ott; in addition lactate dehydrogenase is a stable enzyme (see tables 33, 34 and 35). However, if the results derived from the acetone powder extracts of Wenner and his associates are excluded, most of the remaining results are in agreement. It is of interest that the activities of the enzyme in the tissues of the mouse (MEISTER, 1950) are similar to those in the rat.

Activities in Tumours

Although the activities of many enzymes in many tumours have been examined, there are few results pertaining to particular enzymes in particular tumours. The results of the assays of mouse tumours are included in table 41 in order to produce a more comprehensive picture. In general, there is agreement within enzymes and tumours and between species. The lowest values are again those of WENNER and others (1952) who used extracts in the form of acetone powders.

The small range of values is consistent with the tendency to a uniform enzymic composition of tumours (GREENSTEIN, 1954).

Interpretation of the Values Obtained

The potential difficulties associated with the interpretation of the maximum activities of enzymes determined *in vitro* were discussed in Chapter II. Apart from the amount and K_m of the enzyme, the chief factors are the concentrations of substrates and coenzymes. The effective concentration of a substrate depends ultimately on the rate of release of a precursor and competition with other pathways. There is insufficient information available to make a definite statement about the effects of the contributions of these factors on the maximum activity of aldolase and lactate dehydrogenase in tissues. However, if some conclusions can be reached it is worth examining the data obtained.

In determining the activities of enzymes the conditions are made optimal with respect to all known parameters. Therefore it is probable that the activities reflect the number of active centres of the enzyme in the different tissues. There is presumably determined ultimately by the chromosomes, but it is probable that environmental changes can affect the amount of enzyme protein (LAWRIE, 1953b). Such changes have widespread effects on enzyme activity; they were reviewed by KNOX and others in 1956.

Just as with the concentrations of substrate there is not

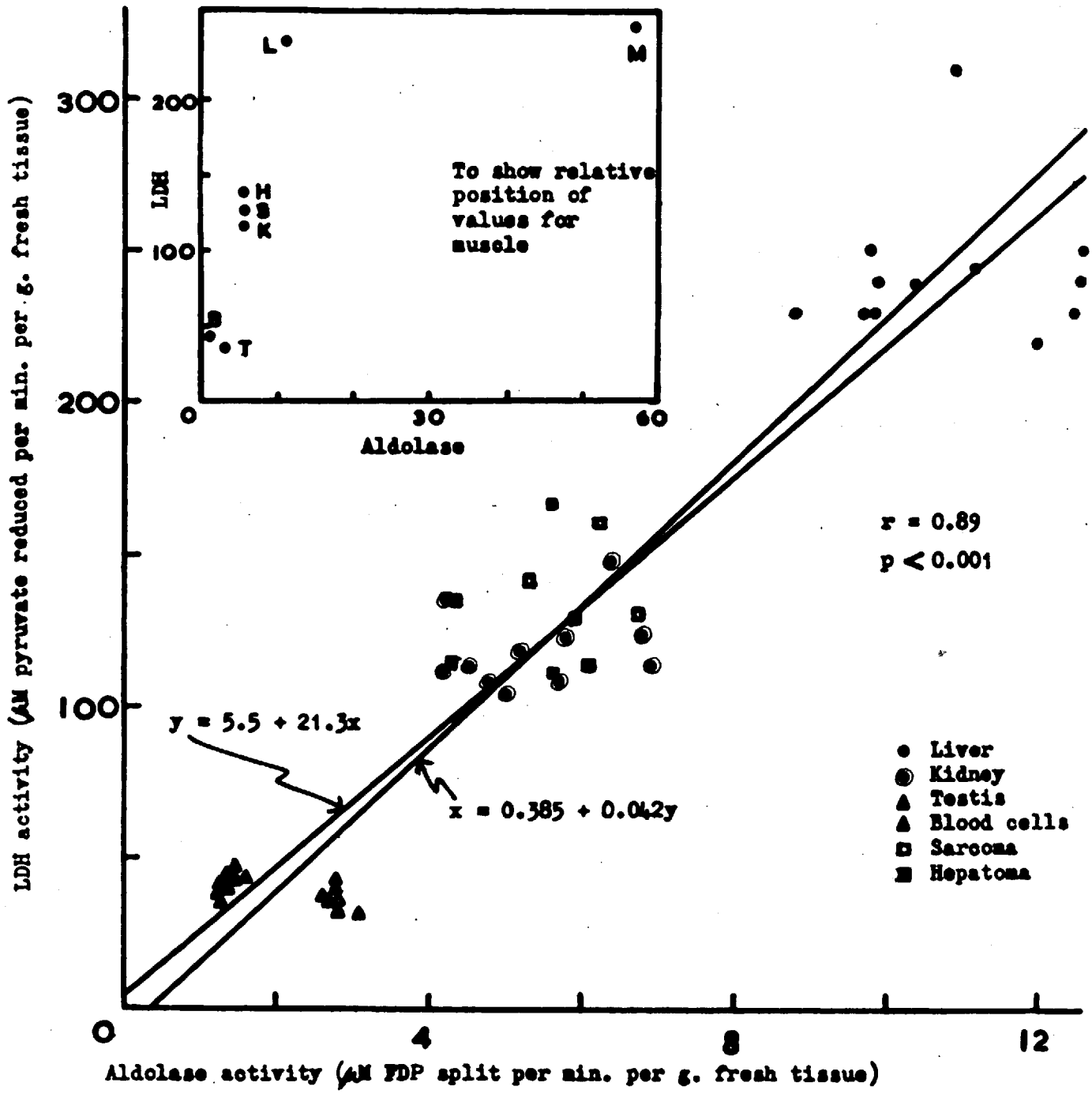

Fig. 26. Correlation of activities of aldolase and lactate dehydrogenase in tissues of the rat.

a very wide range of activities of a given enzyme in most of the tissues examined. Thus for both enzymes the values at the extremes of the range do not differ by more than one order of magnitude, when referred to the total nitrogen content, except for the activities of aldolase in muscle and aldolase and lactate dehydrogenase in the cells in blood. In these cases the values differ by eightfold or more from those at the other extreme of the range.

Correlation of Activities of Aldolase and Lactate Dehydrogenase

It was suggested (page 69) that the relative maximum activities of an enzyme in a metabolic sequence may represent the relative functional capacities of those tissues. This suggestion would receive support if it could be shown that the maximum activities of two or more enzymes in the sequence were correlated. With this object in view, the results of the paired assays of aldolase and lactate dehydrogenase are plotted against each other for all of the tissues examined except muscle (fig. 26). There is a highly significant positive correlation ($r = 0.89$, $p < 0.001$). Reasons were given for the exclusion of the results pertaining to muscle from the calculation of the correlation coefficient on page 302 They receive confirmation from the difference between the results obtained for muscle and other tissues (insert, fig. 26). This difference is discussed on page 319.

Ratio of activities in different tissues. The correlation between the activities of the two enzymes can be expressed

Table 42

Activity $\frac{\text{LDH}}{\text{Aldolase}}$ in tissues of the rat

Author	Tissue							
	Liver	Hepatoma	Skeletal muscle	Heart	Sarcoma	Kidney	Testis	Blood cells
Holzer & others, 1956	9.6							14.5
Trautschold, 1956	38.0							
Delbruck & others, 1959	56.0			11.3				
Lamprecht, 1959				12.2				
Schmidt & Schmidt, 1960	36.0							
Dale, 1963	22.0	26.0	4.5		24.0	22.0	14.0	32.0

Table 43

Activity $\frac{\text{LDH}}{\text{Aldolase}}$ in tissues of species other than the rat

Author	Species	Tissue					Colon		
		Liver	Muscle	Heart	Kidney	Blood cells	Mucosa	Carcinoma	Polyps
Beck, 1955	Man[x]					3.9			
Delbruck & others, 1959	Locust[*]		0.046						
Schmidt & Schmidt, 1960	Man	25.0[•]	3.1[•] 21[•]// 27[•]#	25.0[§]	62.0[§]	36			
Dale, 1963	Man						8.1[•]	14.4[•]	10.5[•]

[x] Leucocytes, washed. Vmax.
[*] Flight muscle.
[•] Operation material.
[§] Postmortem, 24 hours.
// Smooth muscle, stomach.
Uterus.

N.B. The values obtained by Schmidt & Schmidt (1960) for human cerebral cortex have a ratio of 10·0

in another way; this is the ratio of the activities of lactate dehydrogenase and aldolase. It gives an estimate of the excess functional capacity of the reaction catalysed by lactate dehydrogenase over that catalysed by aldolase. It is obviously of fundamental importance; therefore it was calculated for all of the tissues of the rat for which data could be gathered. The result is presented in table 42. The ratio in skeletal muscle is the lowest. The difference between muscle and other tissues was so striking that the results of assays made in other species were sought. These are given in table 43. With the exception of the ratio for human leucocytes obtained by BECK (1955), all of the ratios relating to mammalian tissues, including that for the cerebral cortex of man, are considerably higher than that extant in normal skeletal muscle of the rat or man. The ratio for leucocytes was observed in washed cells, and it is calculated from the theoretical V_{max}; its significance is not clear in the present context. The results of Schmidt and Schmidt are of especial interest because they show in man that the ratio in skeletal muscle is lower than that in heart muscle and in the smooth muscle of uterus or stomach; it is close to that of the skeletal muscle of the rat. The other interesting ratio is that of the flight muscle of the locust; this is quite different from that in mammalian skeletal muscle, ~~but it is similar to that in the human heart~~. It is associated with a different kind of metabolism,

namely, one in which respiration predominates. It is concluded that the ratio of the activity of lactate dehydrogenase to that of aldolase in skeletal muscle differs from the ratios observed in the tissues referred to in man and the rat.

Significance of the Correlation Between the Activities of Aldolase and Lactate Dehydrogenase in Different Tissues

Lack of relation to glycolytic and respiratory rates. The existance of a highly significant correlation between the activities of two enzymes in the Embden-Myerhof pathway of a number of tissues would perhaps be expected to be manifest in their rates of glycolysis and/or respiration. This is not the case; there is no evidence of any relation between these rates (KRATZING, 1961), and the activities measured in any of the tissues of the rat. One of the reasons for the absence of any apparent relation is that most rates determined *in vitro* relate to non-phosphorylated substrates. Thus the rate-limiting effect of hexokinase can come into play (LePAGE, 1950a; KREBS and KORNBERG, 1957). The difficulties of reconciling maximum velocities of individual enzymes and maximum glycolytic rates were remarked by BECK in 1955. He found that the maximum velocity of lactate dehydrogenase in human leucocytes was about 8 times greater than the maximum glycolytic rate and 2 - 4 times greater than the maximum rate of aldolase. He attributed these discrepancies to rate-limitation imposed by the hexokinase reaction *in vivo* (1958). This difference in the activities of lactate dehydrogenase and aldolase is

comparable to the observations made in this investigation.

Relation of ratio of activities to degradation or synthesis: function of lactate dehydrogenase. There is another and fundamental reason for the apparent lack of relation between the rates of individual enzymes and respiratory and/or glycolytic rates. The determination of the rates of individual enzymes is a gross measurement which does not take into account the direction, that is, the presence of degradation or synthesis, or the differing components of the energy-producing system, namely those associated with respiration and glycolysis. If a large fraction of the activities of one or both of the two enzymes examined were associated chiefly with synthesis, any potential correlation with respiratory or glycolytic rates might be masked. There is evidence in favour of this view. This is derived from the high ratio of the activity of lactate dehydrogenase to aldolase in most tissues in contrast with the low ratio in muscle, the release of lactate from muscle and its uptake by other tissues, and the entry of small molecules into the triosephosphate pool.

The ratio of the activities of lactate dehydrogenase to aldolase must be halved in order to obtain the ratio of the numbers of 3-carbon molecules transformed by the two enzymes (the activity of aldolase is defined in terms of moles of fructose diphosphate split). When this is done the ratio of the catalytic capacities of lactate dehydrogenase to

aldolase in muscle is 2.25. In other tissues examined it varies between 6.7 and 15.7, the values for liver, kidney and the two tumours lying between 11 and 13. The relative excess of lactate dehydrogenase in different tissues is presumably related to its function in each tissue.

(a) Muscle: glycolysis. The lactate dehydrogenase in skeletal muscle appears to be concerned with glycolysis only, muscle glycogen being formed from blood glucose (CORI, 1931). Further evidence in support of this view is the failure to find appreciable activity of hexose diphosphatase in rat heart and diaphragm (NEWSHOLME and RANDLE, 1962). Thus, if required, all of the enzyme in muscle would be available for competition with pyruvate dehydrogenase. The relatively low respiratory rate in skeletal muscle compared with that in other tissues including testis, would not offer much competition; this may be the reason for the low ratio of the activity of lactate dehydrogenase to aldolase in muscle. In this case the higher ratio in other tissues may simply reflect the greater respiratory rate and greater competition by pyruvate dehydrogenase.

(b) Liver and other tissues: glycogenesis and/or provision of fuel for tricarboxylic acid cycle. On the other hand the lactate released from muscle into the blood is available as a fuel and it is a satisfactory substrate for many tissues <u>in vitro</u> (KRATZING, 1961). The synthesis of glycogen in the liver from ingested labelled lactate, via the tricarboxylic acid cycle was demonstrated by TOPPER and HASTINGS (1949) and by LORBER and others (1950).

The probable synthetic pathway was indicated by KREBS and KORNBERG (1957). Finally, the amount of lactate formed by a liver homogenate from labelled pyruvate is not more than 10 per cent (HASLAM and KREBS, 1963). Thus there is no doubt that the lactate released from muscle enters the tricarboxylic acid cycle of the liver and that it can be transformed into glycogen; in addition there is the probability that pyruvate is not converted to lactate extensively. The lactate/pyruvate ratio in different tissues offers support for this view (table 38). The ratio in muscle and other tissues in which glycolysis is of major importance is relatively high. In liver, where lactate from blood-stream is converted to pyruvate, the ratio is low.

If a high percentage of the lactate were synthesised into glycogen, an aldolase activity of similar magnitude to that of lactate dehydrogenase might be expected. This is not extant in any of the tissues examined. A possible reason is that the rate of formation of pyruvate from lactate may be slower than the reverse reaction, NEILANDS (1952) believes that this is to be anticipated because the affinity of pyruvate for lactate dehydrogenase is about 100 times greater than that of lactate; this would have the effect of increasing the rates of formation and breakdown of the enzyme-substrate complex from pyruvate and of decreasing the corresponding rates from lactate. Thus, even if the conditions were in favour of the formation of pyruvate, more enzyme would be required to produce adequate rates. The

converse would apply during degradation, that is, relatively less lactate dehydrogenase would be required. The differences in ratios of the activities of lactate dehydrogenase to aldolase in muscle and other tissues could be accounted for on this basis.

Another explanation may be that the function of the excess of lactate dehydrogenase over aldolase is to convert lactate to pyruvate for use as fuel in the tricarboxylic acid cycle; this would reduce the need for the degradative action of aldolase. Indeed it is conceivable in the liver that the 3- and 6-carbon carbohydrates arriving via the blood-stream and those derived from degradation of fats and amino acids provide most of the fuel for the tricarboxylic acid cycle. The purpose of glycogenolysis may be solely the release of glucose to the tissues in general. In this case aldolase would be concerned chiefly with the assimilation of trioses formed in excess of the requirements of the tricarboxylic acid cycle; these arise during the conversion of fructose-1-phosphate to fructose-1,6-diphosphate in the liver and from glycerol residues in the liver and other tissues. The relatively low ratio of fructose diphosphate to dihydroxyacetonephosphate in the liver (table 38) may be due to such syntheses as these; conversely the relatively high ratio in muscle is to be expected in tissues where degradation is prominent.

An example of the utilisation of lactate and residues

derived from fatty and amino-acid degradation exists in heart muscle. The evidence for this was reviewed by EVANS (1939) who, together with several colleagues, carried out a lot of the early work. They showed that the heart consumes lactate in preference to glucose and that the oxidation of glucose plus lactate accounts for only 40 per cent of the total oxygen consumption. RANDLE (1963) reviewed the more recent evidence which indicates that, in the heart, fatty acids and ketone bodies are oxidised in preference to pyruvate and lactate. He makes the suggestion that the uptake of glucose by the heart may be chiefly for the purpose of replenishing glycogen stores which may be used mainly in order to provide small molecules for the synthesis of phospholipids, purines and pyrimidines. Thus it is conceivable that the metabolism of heart and liver are comparable, both with respect to the oxidation of small molecules and to the formation and degradation of glycogen for purposes other than providing fuel for the local tricarboxylic acid cycle. There is also evidence indicating that brain uses lactate as fuel. McILWAIN (1953b) observed that in brain slices lactate does not accumulate until the glucose content of the medium increases to 1 mM; he observed also when lactate is the sole substrate that an increase in the concentration is associated with an increase in the rate of respiration.

Relation of molecular form of enzyme to function in different tissues. The existence of several molecular forms

of lactate dehydrogenase (KAPLAN and CIOTTI, 1961) and of two varieties of aldolase (reviewed by RUTTER, 1961) are of interest in this connection. Kaplan and his associates (CAHN and others, 1962) have tried to link these different molecular forms to differences in function. Thus they find that the enzyme extracted from chicken heart is readily inhibited by pyruvate whereas that obtained from skeletal muscle is inhibited less. They suggest that a high concentration of pyruvate in skeletal muscle is necessary in order to ensure plentiful supplies of NAD for the action of glyceraldehyde-3-phosphate dehydrogenase. This suggestion receives no support from analyses of rat skeletal muscle (table 24): the pyruvate content is the lowest of the tissues examined with the exception of testis. Even after exercise the content increased by only about 30 per cent. Furthermore, according to Kaplan, the enzymes from liver and muscle are similar. Once again, the pyruvate content of liver is at variance with this suggestion; it is approximately 3 times higher than that of skeletal muscle.

The aldolase in muscle and liver have different specificities; that from muscle splits fructose diphosphate almost exclusively, whereas the one in liver splits fructose-1-phosphate also. The need for the latter reaction in the liver is obviously related to the relatively large amounts of fructose arriving from the intestine. But possibly more interesting is the present context is that the liver enzyme

has about 10 per cent of the activity of the muscle enzyme towards fructose diphosphate. This difference might reflect differences in degradative function in the two tissues.

Summary: Activities of Enzymes

1. There is relatively little information available on the activities of most enzymes in most tissues of every species of mammal. This lack plus the differences in conditions and technique of assay make it difficult to compare the maximum activities of the two enzymes examined with the activities described by others. On the whole the results agree with those observed where there are no obvious objections to the technique employed.

2. The activities of the two enzymes in the tumours are within the range observed for normal tissues.

3. There is a significant correlation between the activities of aldolase and lactate dehydrogenase in the tissues examined, muscle being excluded because of its different metabolism. The difference in muscle was emphasised by the low ratio of the activity of lactate dehydrogenase to that of aldolase. As with substrates, the correlation supports the suggestion that it is valid to compare the amounts of the constituents of a similar metabolic pathway in different tissues.

4. The maximum activities presumably reflect the functional capacities of the system, but they give no information about the basal or resting flux.

5. There is no relation apparent between the activities of

the two enzymes and the glycolytic or respiratory rates of the tissues. It is suggested that this is possibly because of the enzymes are concerned with synthesis more than with degradation in tissues other than muscle.

6. The different molecular forms of aldolase and lactate dehydrogenase present in different tissues may have different functions; this may account for the differences witnessed in the ratio of their activities in muscle compared with the ratios in other tissues.

3. CORRELATION BETWEEN THE ACTIVITY OF AN ENZYME AND THE CONCENTRATION OF ITS SUBSTRATE

The values for the concentrations of substrates and the activities of enzymes were obtained from different lots of rats. Therefore it was not possible to use paired results in order to calculate the correlation coefficient. Instead, the mean values for each substrate and enzyme were used and the equation giving the correlation coefficient was weighted, as indicated by the factor "w".

Thus:

$$r = \frac{S\ w(x-\bar{x})(y-\bar{y})}{\sqrt{\left[S\ w(x-\bar{x})^2\right]\left[S\ w(y-\bar{y})^2\right]}}$$

where r = the correlation coefficient

$$w = \frac{1}{\text{product of the standard error of the two means}}$$

The remaining term in the numerator is related to the covariance and the remaining two terms in the denominator are related to estimates of the variance of the two variables. This device ensures that appropriate weight is given to the number and variance of the determinations of each variable in each tissue.

The results for aldolase and fructose diphosphate and for lactate dehydrogenase and pyruvate are presented in figures 27 and 28, respectively. In both cases muscle is excluded from the calculations for reasons given already.

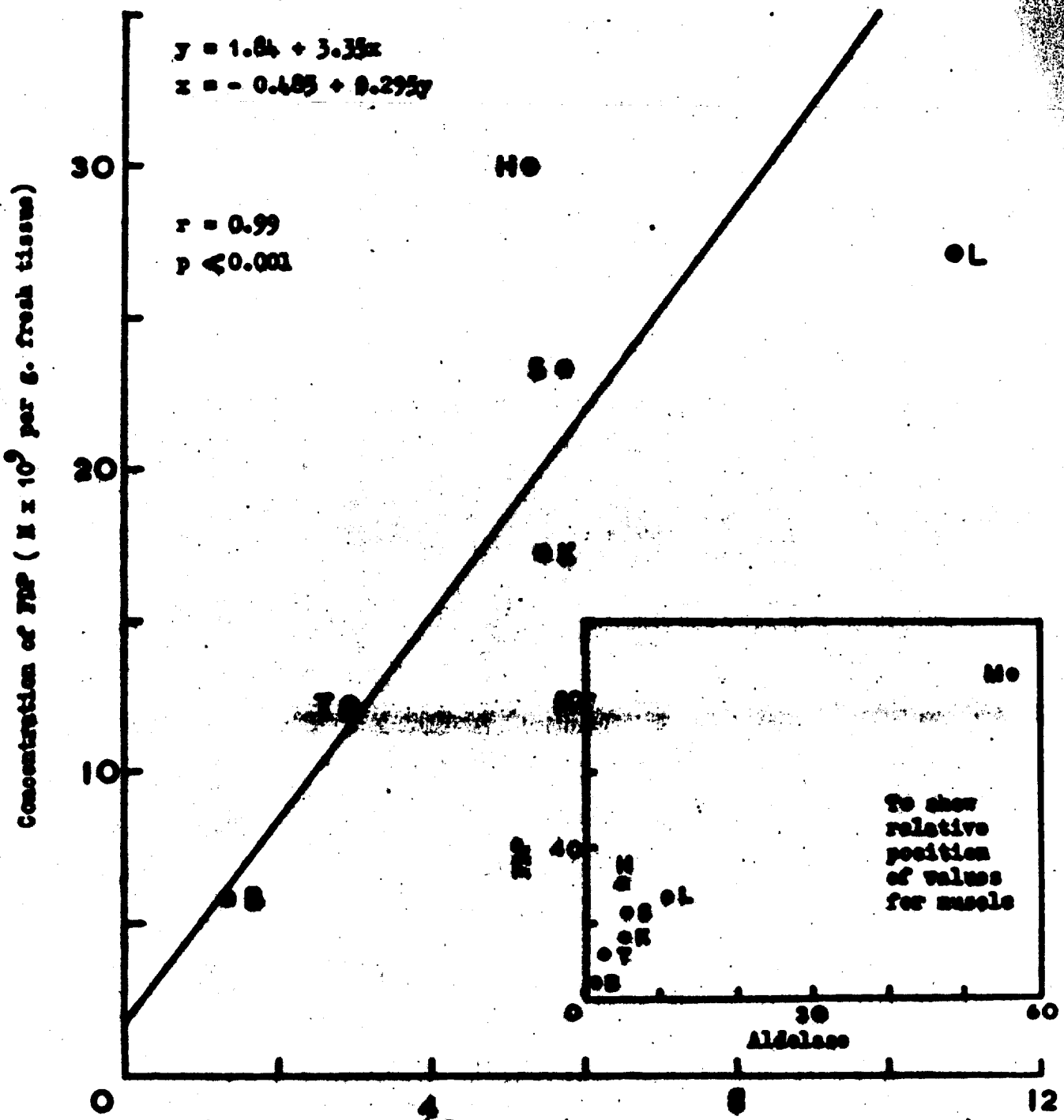

Fig. 27. Correlation of activity of aldolase and concentration of fructose diphosphate (FDP) in tissues of the rat. For key to symbols see fig. 25.

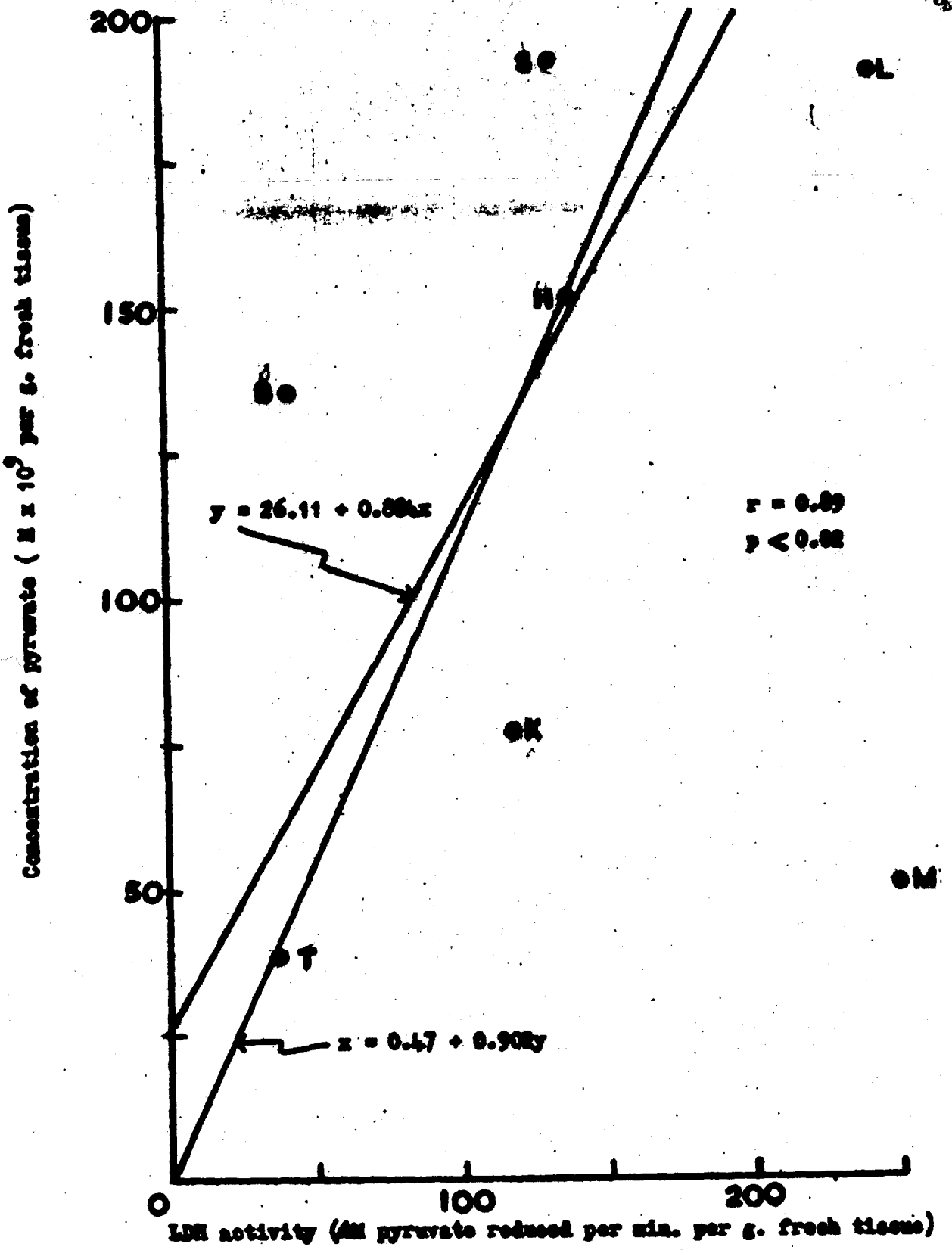

Fig. 28. Correlation of activity of lactate dehydrogenase (LDH) and concentration of pyruvate in tissues of the rat. For key to symbols see fig. 25. Results for muscle are not included in the correlation (see page)

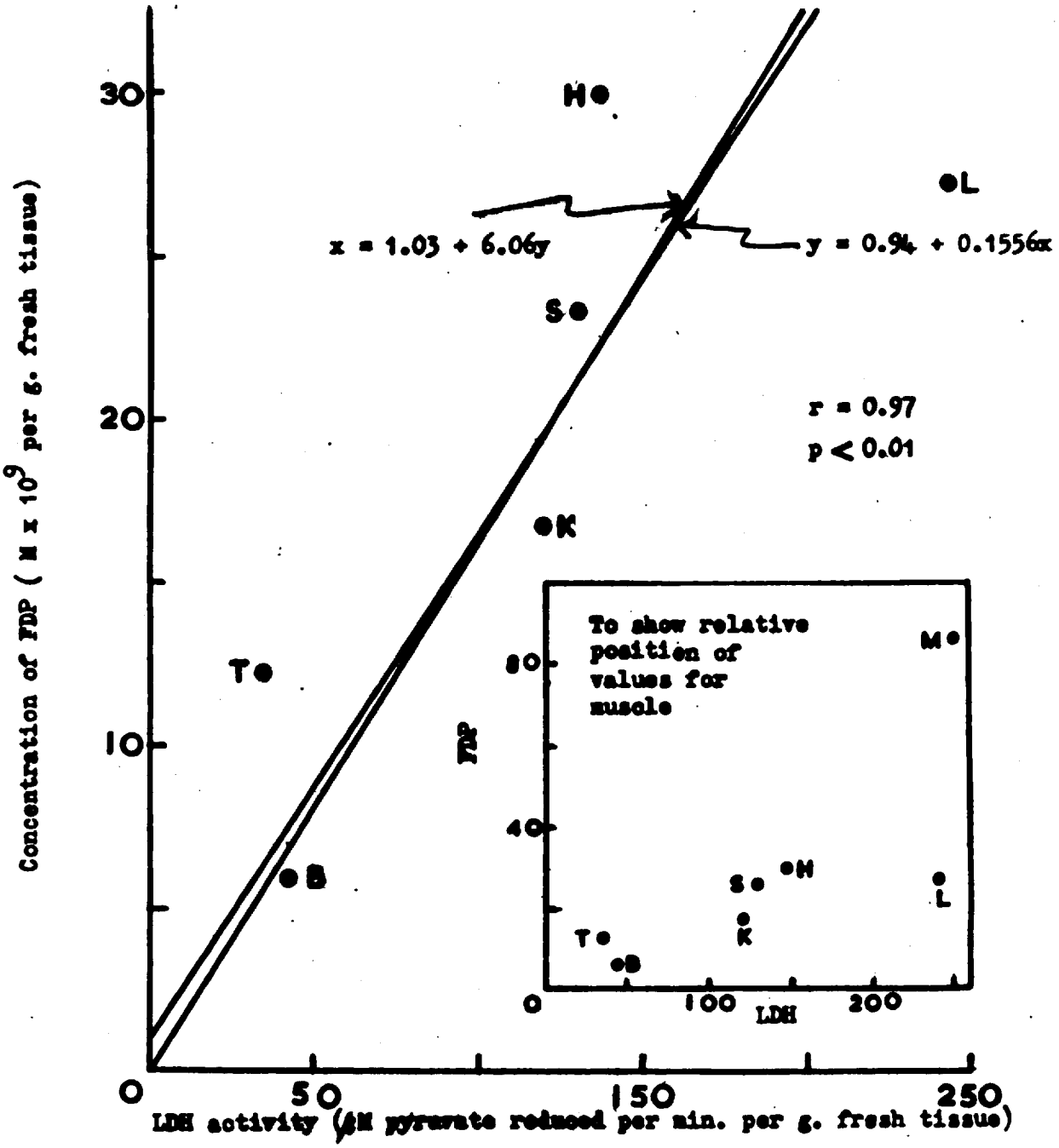

Fig. 29. Correlation of activity of lactate dehydrogenase (LDH) and concentration of fructose diphosphate (FDP) in tissues of the rat. For key to symbols see fig. 25.

The correlation coefficient between the activity of aldolase and the concentration of fructose diphosphate is 0.994, $p < 0.001$; for lactate dehydrogenase and pyruvate the correlation coefficient is 0.893, $p < 0.02$. Both of these correlations are significant. They are also positive, that is, the concentration of the substrate in the tissues of the resting rat is large when the activity of the enzyme is high.

Consistence within Embden-Myerhof Pathway in Different Tissues

This correlation is further evidence of the internal consistence in the relations between the components of the Embden-Myerhof pathway. The other evidence is the highly significant correlation between the activities of aldolase and lactate dehydrogenase. Additional confirmation of this consistence is the significant correlation between the mean concentration of fructose diphosphate and the mean activity of lactate dehydrogenase: $r = 0.97$, $p < 0.01$ (fig. 29)*. No correlation was demonstrable between the activity of aldolase and the concentration of pyruvate; this is attributed to the numerous reactions in which pyruvate appears. It is obvious that the concentrations of reactants and the activities of enzymes must be integrated within the cell; but it remained to be shown that this is true after dislocation

* The mean values were used and the equation for the correlation coefficient was modified as indicated already, by the inclusion of the factor "w".

of the cell. At the same time the results demonstrate that the apparent similarity of the steps of the glycolytic pathway can be misleading: the values obtained for muscle are different from those in the other tissues examined. This is not surprising in view of the unique function of the Embden-Myerhof pathway in muscle.

Flux of Material Through Embden-Myerhof Pathway in Different Tissues

The tissues with high activities of enzymes have high concentrations of substrates and vice versa. Presumably the large concentrations of substrate are associated with a large flux: if the flux through all of the tissues examined were the same, then the concentrations of the substrates would be related inversely to the activities of the enzymes or at most, they would remain the same also. In this connection it is worth noting that DENBIGH and his colleagues (1948) showed theoretically that an increase in the inflow to an open system results in a small increase in the concentrations of the reactants. The way in which a large flux could be associated with a large concentration of substrate and a high maximum activity is worth considering. In vitro the larger the activity of enzyme, the smaller is the concentration of substrate. Therefore the association of a high activity of enzyme and a large content of substrate raises the question of the physical relation between the substrate and enzyme within the cells. There are at least two ways of accounting for the observation.

One way involves the percentages of substrate which are free and bound to enzyme within the cell. The information on this matter is scant, but there is at least one substrate which is chiefly bound to enzyme; this is hydrogen peroxide (CHANCE, 1954). If a large percentage of each of the substrates assayed in this investigation were bound, then in the presence of a high flux there would be a large amount of substrate bound to enzyme. The other way concerns the distribution of enzyme in different kinds of cell. Thus differing amounts of enzyme can be accomodated either at constant volume or at constant concentration. If larger amounts of enzyme were accomodated at constand concentration then a larger flux would be associated with a larger content of substrate. This would be true for the free and bound substrate. Whether the different amounts of an enzyme in different cells are accomodated at constant volume or at constant concentration is unknown for most enzymes. But there can be little doubt in specialised cells such as muscle that the large amounts of the enzymes in the Embden-Myerhof pathway must occupy a larger percentage of the total volume than in most types of cell. It is conceivable that the correlation between activity and substrate content is accountable in both of the ways discussed.

Causal Relations Between the Activity of an Enzyme and the Concentration of the Substrate

There are at least three kinds of causal relation possible between activity and concentration. Thus the maximum activity

may determine the magnitude of the flux and the concentrations of substrates. The flux of substrates present at rest in the glycolytic pathway is presumably related to the energy requirements of the tissue. It is difficult to understand how this could be determined by the maximum rates of the enzymes. The second possibility is that the larger amounts of the enzymes are induced by high concentrations of substrates as occurs in some systems in micro-organisms. The evidence is consistent with such a mechanism, that is, an increased activity occurs only in associated with an increase in the concentration of substrate. However, the increases are observed in different tissues and this raises the third reason for the relationship. The characteristics of each tissue, including the amounts of enzymes and the resting and maximum rates of function are controlled ultimately through the characteristics of the deoxyribonucleic acid of its cells. It is probable that induction functions within such a frame of reference in micro-organisms (HALVORSON, 1960), and it is conceivable that similar mechanisms operate in mammals. There is some evidence in support of this possibility. LAWRIE (1953a) showed that the myoglobin and cytochrome oxidase contents of the psoas muscle of a number of mammals were related over a large range of values. This could occur through a hereditary mechanism, or because of a greater demand for the two oxygen carriers. It probably involves

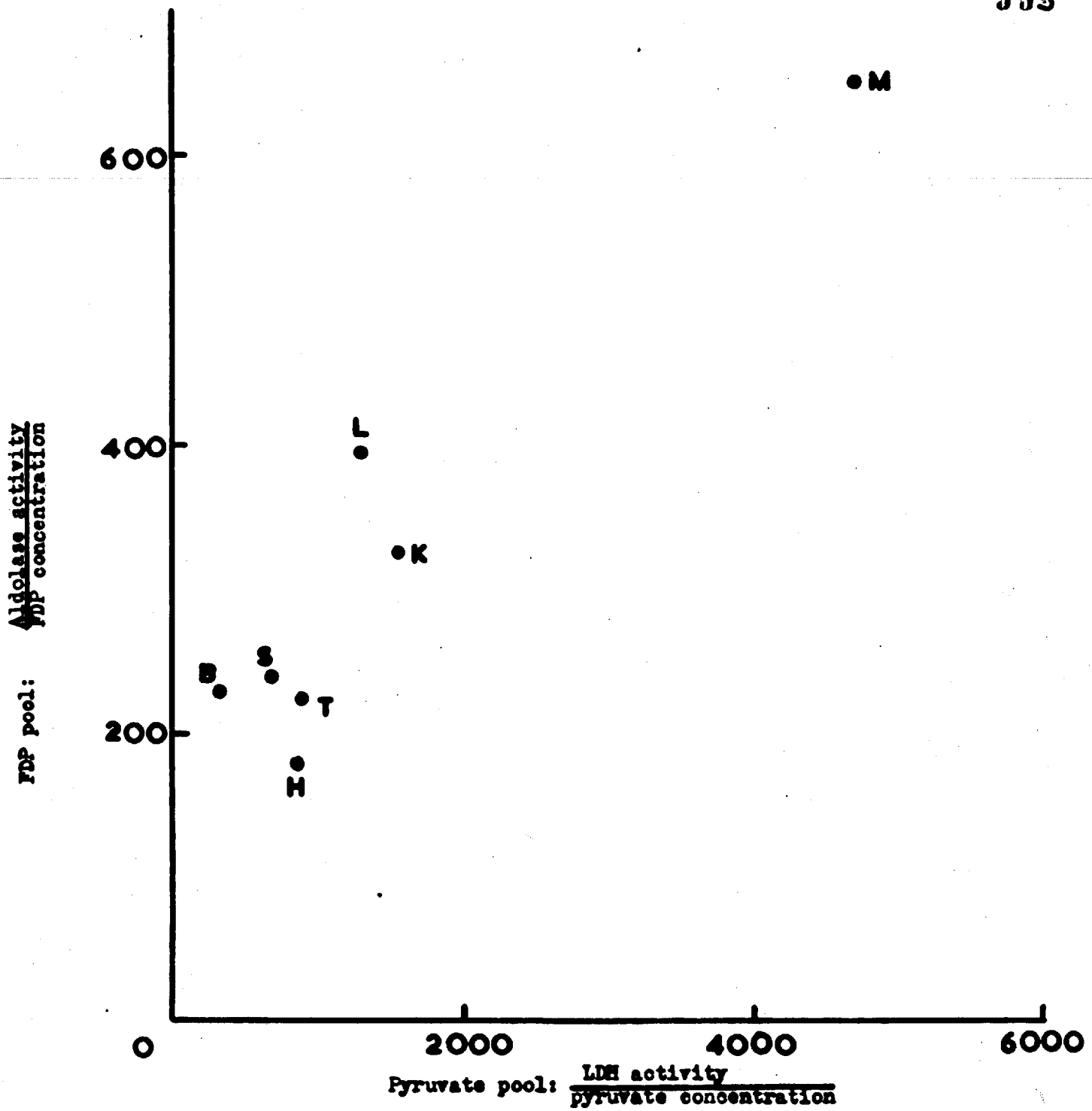

Fig. 30. Potential maximum rate of clearance of pools of pyruvate and fructose diphosphate (FDP) in tissues of the rat. (Number of times per minute). For key to symbols see fig. 25.

both mechanisms and certainly appears to involve the second one: the content of myoglobin in hind-limb muscles of rats and fowls increases by 40 to 50 per cent when the daily exercise is increased for periods of one to two months (LAWRIE, 1953b). Additional evidence of the same kind was provided by LAWRIE (1953a). He showed that the activities of cytochrome oxidase and succinic dehydrogenase, and of the succinic oxidase system in the heart, psoas and diaphragm of the horse increase commensurately from foetal life until the age of two years. Although evidence such as this is consistent with the occurrence of induction, it is only a beginning. Evidence of an increase in the amount of the enzyme protein produced in response to stimulation by the specific substrate is needed in order to demonstrate the existence of induction.

Potential Reserve Function

The data relating the activities of the two enzymes and the concentrations of their substrates can be used in order to obtain an estimate of the potential reserve function of the system in the tissues examined. The ratios of the activities of aldolase to the concentrations of fructose diphosphate, and the activities of lactate dehydrogenase to the concentration of pyruvate in the tissues examined are plotted in figure 30. The lowest ratios of both enzyme-substrate pairs occur in cells which might be expected to metabolise relatively slowly, namely blood cells, testis

and tumours. The potential maximum rates of clearance of the pools of fructose diphosphate and pyruvate in the testis are approximately 900 and 220 times per minute respectively. The highest ratios occur on muscle with potential rates of clearance of the pools of fructose diphosphate and pyruvate of approximately 4,700 and 650 times per minute respectively. The rates for liver and kidney fall in between. It is not suggested that these are the actual rates of clearance, but it is conceivable that the relative rates in the different tissues are represented by these values. If this is true, then muscle has not only a greater potential maximum rate of glycolysis, but it has two to three times the reserve of function present in other tissues.

Summary

1. The highly significant positive correlation between the activities of aldolase and lactate dehydrogenase and the concentrations of fructose diphosphate and pyruvate, respectively, confirms the previous evidence of consistence between the amounts of the components of the Embden-Myerhof pathway in different tissues.

2. It is suggested that this correlation indicates that in the glycolytic pathway of the tissues examined, with the exception of muscle, the flux of material through the resting tissues is related to amounts of the enzymes, that is, the resting flux is greater in the tissues with more enzymes.

4. FUTURE DEVELOPMENTS

The most important outcome of this work is that it appears to be possible to measure the gross amounts of labile substances in the tissues of the rat with some assurance that they represent the levels present in resting tissues. This is especially important now that enzymic and chromatographic methods make it possible to measure the concentrations of most substances in the Embden-Myerhof pathway and the tricarboxylic acid cycle and many of the intermediates of carbohydrate and fat metabolism.

The identification of rate-limiting steps behind which a metabolite may accumulate is simplified by any evidence which reduces doubt regarding the effects of sampling and analytical procedures. One of the simplest systems in which to investigate such effects is the exercising muscle because it has a large range of function. A great deal of work has been carried out on isolated heart and diaphragmatic muscle. The time is now ripe to start investigating the control mechanisms of glycolysis in skeletal muscle.

It would be of considerable interest to investigate the correlation of the concentration of substrate with other parameters which appear in the Michaelis-Menton equation. This could be done by measuring the concentration of the substrates in a given metabolic pathway in different tissues and determining the K_m of each of the enzymes. Although

there is no reason to doubt that the Michaelis theory (or variations on it) describe the kinetics of enzyme-catalysed reactions in tissues, there is very little evidence on this point. It consists of the demonstration of the formation of complexes between an enzyme and its substrate in suspensions of single cells such as described by CHANCE (1954).

The different ratio of the activities of aldolase and lactate dehydrogenase in muscle as compared with that in other tissues raises the question of the precise function of these enzymes, and indeed of the whole glycolytic pathway in all tissues. There is sufficient collateral evidence which indicates that it is by no means as simple as it appears. The relative amounts of energy contributed by the oxidation of triose phosphates, lactate and acetate in the form of acetyl CoA in all tissues, but especially in the liver of the intact animal, require to be ascertained. One way of providing this information would be to inject each of the intermediates of the pathways to be investigated, labelled with C^{14}, into the portal vein and to determine the distribution in the pathways after a suitable interval of time. Thus problems such as the transformation of glucose-6-phosphate to triose phosphate or to glycogen and the percentage of lactate entering the tricarboxylic acid cycle and/or being converted to glycogen could be investigated.

The comparison of the composition of different tissues is bedevilled by the number of different kinds of cell

present. The measurement of the amounts of the components of different kinds of cell in normal tissues is a prerequisite to understanding their function. This is equally important in the case of neoplastic cells because differences between the amounts of their constituents and those of normal tissues may provide evidence as to their development. Thus there are compelling reasons for the extension of Lowry's techniques for sampling the content of single cells to the examination of neoplastic cells and their cells of origin. The chief technical problem requiring investigation is the identification of the malignant cells in vivo before sampling.

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