A STUDY OF VASCULAR ENZYMES

IN

EXPERIMENTAL HYPERTENSION

by

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A Thesis presented for the Degree of Doctor of Philosophy of the University of Edinburgh in the Faculty of Medicine, April, 1964.

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CHAPTER I

INTRODUCTION

Hypertension in man

Hypertension is the term used to describe the condition where elevated mean blood pressure is maintained continuously above the normal value for the age group. When there is no apparent cause of the raised blood pressure the condition is termed essential hypertension. In secondary hypertension the elevated blood pressure is a manifestation of a renal, neurological, vascular or endocrine disorder. The raised blood pressure may, for example, be a sign of glomerulonephritis, chronic pyelonephritis, polycystic kidney, urinary tract obstruction or radiation nephritis, or of coarctation of the aorta or polyarteritis nodosa, or of phaeochromocytoma, Cushing's syndrome or toxaemia of pregnancy (Pickering, 1955)..

Both essential and secondary hypertension may follow a benign or a malignant course. In the benign phase the condition of the patient remains stable for many years. Cardiac hypertrophy is found in such cases and the frequency of this finding is greatest in the groups with higher blood pressures (Bechgaard, 1946a). Of the deaths which occur in the benign phase 71 per cent are from causes regarded as being connected with hypertension. The majority are due to heart disease (45 per cent), 16 per cent to cerebral haemorrhage and 10 per

cent to renal insufficiency. The incidence of deaths from the above causes is twice that observed in the normal Danish population (Bechgaard, 1946b). The changes seen microscopically in the tissues of patients who die from causes associated with the existence of benign hypertension are arteriolar hyaline degeneration and elastic hyperplasia of large arterioles and medium-sized arteries in the kidney and other viscera (Moritz and Oldt, 1937). Many early cases of benign essential hypertension, however, show no arterial or arteriolar disease (Castleman and Smithwick, 1948).

In 2.8 per cent of large series of patients with essential and secondary hypertension the condition terminates in the malignant phase (Bechgaard, 1946c). This phase occurs most frequently in young adults who have suffered previously from benign hypertension and the incidence is highest in men (Milliez, Tcherdakoff, Samarcq and Rey, 1960). Clinically, the onset of the malignant phase is characterised by the appearance of papilloedema and albuminuria and the terminal picture is of simultaneous rapid functional failure of the kidney, heart and brain (Keith, Wagener and Kernohan, 1928). Death is most often caused by renal excretory failure, attributed to lesions of the afferent glomerular arterioles and glomeruli, although in some cases cerebral or cardiovascular accidents prove fatal (Heptinstall, 1953).

Focal necrosis of visceral arterioles ("fibrinoid change") is the microscopic hallmark of death due to malignant hypertension

in man (Heptinstall, 1953). The determination of the factors responsible for the appearance of this focal arteriolar necrosis leading to the functional failure of organs is the key to the understanding of the malignant (accelerated) phase of all forms of human hypertension.

Hypertension in animals

In the study of human disease the value of the discovery of a mechanism for producing the experimental counterpart in animals needs little emphasis. The counterpart of the benign phase of human hypertension has never been produced experimentally in animals although Smirk and Hall (1958) have bred a strain of rats in which raised blood pressure is transmitted genetically. Sustained elevated blood pressure was first produced in an animal by Goldblatt, Lynch, Hanzal and Summerville (1934) by constriction of both renal arteries of the dog. In the rat, it was demonstrated by Wilson and Byrom (1939) that experimental hypertension characterised by sustained high blood pressure, cardiac hypertrophy and focal necrosis of the visceral arterioles comparable to that seen in the malignant phase of hypertension in man, was produced in 65 per cent of the animals by constriction of one renal artery. The arteriolar lesions were not found in the clamped ischaemic kidney which was apparently protected from the damaging effects of the raised blood pressure, but they were present in the opposite kidney.

It has also been observed that elevated blood pressure developed in rats subsequent to other forms of renal damage.

Grollman and Halpert (1949) found that this occurred in 20 per cent of one to two year old rats subjected only to unilateral nephrectomy if glomerular lesions were present in the kidney removed. When renal damage was caused by choline deficiency in early life (Hartcroft and Best, 1949) elevated blood pressure developed in 16 per cent of the rats after approximately six months and focal necrosis of the renal arterioles was found in these animals. However Kolff and Fisher (1952) showed that elevated blood pressure and fibrinoid change in the arterioles developed in a small percentage of bilaterally nephrectomised rats treated with peritoneal lavage. This experiment demonstrated that the presence of damaged kidney tissue was not essential for the production of these symptoms and emphasised that extra-renal factors were important.

Wilson, Ledingham and Cohen (1958) observed raised blood pressure and focal necrosis of the renal arterioles in rats seven to nine months after their kidneys had been subjected to x-irradiation <u>in situ</u>. Prior to the finding of arteriolar necrosis the kidneys of these rats did not show any other microscopically visible anatomical lesion associated with the development of high blood pressure.

The importance of the adrenocortical hormones in the development of hypertension has also been studied experimentally in rats. The animals were subjected to unilateral nephrectomy and given one per cent salt to drink as such treatment was found to sensitise them to the effects of the hormones (Selye, 1943). In

rats sensitised in this way the administration of deoxycortone acetate (Selye and Pentz, 1943) produced elevated blood pressure and necrosis of arteriolar muscle cells in all treated animals within one month. Treatment of sensitised rats for a comparable time with aldosterone (Gross, Loustalot and Maier, 1957) or corticosterone (Skelton, 1958) produced elevated blood pressure but the vascular lesions were less pronounced. If one adrenal was removed and the other enucleated in a uninephrectomised, salt-loaded rat "adrenal-regeneration" hypertension developed (Skelton, 1955a). Treatment of sensitised rats with anterior pituitary extract (Masson, Hazard, Corcoran and Page, 1950) or methylandrostenediol (Skelton, 1955b) caused a rise in blood pressure and arteriolar lesions although the vascular lesions were less frequently reproduced than with deoxycortone.

Although uninephrectomy was not necessary for the successful production of hormonal hypertension in animals the increase in sodium intake was usually essential. To this rule cortisone was an exception (Knowlton, Loeb, Stoerk, White and Heffernan, 1952). The administration of deoxycortone acetate to salt-loaded rats with both kidneys untouched produced hypertension (Selye, Hall and Rowley, 1943) and the chronic treatment with aldosterone of intact and adrenalectomised rats resulted in elevated blood pressure and cardiac and arteriolar hypertrophy (Kumar, Anderson and Gornall, 1956). Hypertension was also induced when salt-loading was the only treatment. By feeding rats on a diet of high salt content raised blood pressures were induced and after several months necrosis of the smooth

muscle cells of the renal arterioles was found (Meneely, Tucker, Darby and Auerbach, 1953).

The physiological basis of hypertension

As blood pressure is determined by cardiac output, blood viscosity and peripheral vascular resistance the elevated values found in patients or animals with systemic hypertension may be maintained by an increase in cardiac output, a change in internal arteriolar cross-section or an increase in blood viscosity. There are few measurements of blood viscosity in hypertension but they suggest that it is unaltered in man (Pickering, 1936). Cardiac output in man is also apparently within normal limits when the hypertension is established (Goldring and Chasis, 1944; Brod, 1960). The peripheral resistance is increased in hypertensive subjects (Goldring and Chasis, 1944) and it has been demonstrated that this increased peripheral resistance is largely due to increased resistance of the vessels of the viscera and skin (Brod, 1960). Although, therefore, the available evidence supports the view that systemic hypertension is maintained by altered arteriolar contractility it has been suggested that there may be a prior change in cardiac behaviour (Borst and Borst-de Geus, 1963). Evidence to support this view has been obtained in the rat by Ledingham and Cohen (1963).

The arteriolar lesion in malignant hypertension

The cause of the acute focal necrosis which occurs in the arterioles of the viscera in the malignant phase of hypertension in man and in animals is still debated. Theories of the cause

of the arteriolar lesion have developed largely on the basis of evidence obtained from experiments with animals. Wilson and Pickering (1938) first suggested that the chief factor responsible for the acute arterial lesions was the greatly raised intra-arterial pressure. The critical evidence supporting this view is the work of Wilson and Byrom (1939). Their experiments on the rat demonstrated that the vascular lesions of hypertension in this species did not occur in the kidney protected from the high blood pressure by the clamp on the renal artery. These authors did not observe any relationship between the incidence of the arteriolar lesions in the other organs and the severity of the hypertension and they concluded that the determining factor in the production of necrosis was probably the sudden mechanical strain imposed on the vessel wall by the combination of severe vasoconstriction and the resultant rapid rise in blood pressure. It was envisaged that a more gradual rise in the arterial pressure produced compensatory hypertrophy of the vessel wall. When Byrom and Dodson (1948) reproduced the lesions of malignant hypertension in normal rats by brief over distension of the arterial tree with injections of warm Ringer's solution this appeared to be strong evidence in favour of the theory that a sudden rise in intra-arterial pressure was responsible. However others (Schaffenburg and Goldblatt, 1957) failed to duplicate these results which were, in fact, open to certain detailed criticism.

Byrom (1937) observed that repeated daily injections of vasopressin, each of which caused a sudden blood pressure

increase of approximately 50 mm. Hg lasting about one hour in normal young female rats, resulted in acute focal necrosis of the renal arterioles; focal spasm was seen to occur in these vessels. He suggested that the necrosis in this instance, was partly due to the focal spasm. That focal spasm of the vessels of the brain occurred in rats with severe experimental renal hypertension was shown clearly by Byrom (1954) and he postulated that this occurrence was an intermediate step between high intra-arterial pressure and the occurrence of arteriolar necrosis.

Remission and healing of the acute arteriolar lesions in the kidney was observed at autopsy when the malignant phase of human hypertension was treated with potent anti-pressor drugs such as hydralazine or hexamethonium, pentolium bitartrate, chlorisondamine, or mecamylamine, alone or in combination with reserpine (McCormack, Béland, Schneckloth and Corcoran, 1958). The effects of such drugs on the development of the acute arteriolar lesions was therefore studied further in experimental hypertension.

Masson, McCormack, Dustan and Corcoran (1958) showed that the visceral arteriolar lesion resulting after ligation of a renal artery branch in a uninephrectomised rat were delayed, modified or suppressed by treatment with the hypotensive drug hydralazine. It was claimed that constant control of the blood pressure was obtained in the rats by giving the drug in their drinking water. Arteriolar lesions did develop if treatment was stopped and if lesions were allowed to develop

initially they responded to delayed treatment by healing. This experiment was considered as further evidence that the acute vascular lesions were a direct consequence of raised blood pressure. McQueen and Hodge (1961) also observed that the arteriolar lesions of rat hypertension produced by clipping one renal artery were significantly less severe when the animals were treated by the oral administration of reserpine.

In the course of experiments on the toxicity of hydralazine (Gardner, 1958) when the drug was given daily as a single intramuscular injection to rats with deoxycortone hypertension it was observed that the acute vascular changes associated with the hypertension were altered. A close study was therefore made of the daily pattern of blood pressure levels in animals treated in this way and of the course of development of the arteriolar lesions of the steroid hypertension (Gardner, 1960). The results of this experiment showed that in spite of an abrupt daily fall in systolic blood pressure which, in some cases, was of more than 100 mm. Hg, and a more gradual rise to pre-injection hypertensive levels within twenty-four hours, the vascular lesions were almost completely prevented. These observations, which were subsequently confirmed in rats with adrenal-regeneration hypertension (Gardner and Brooks, 1962) suggested that the acute vascular disease in rats with deoxycortone hypertension could not be assumed to be the result of the critical rise in blood pressure alone. However, although the smallest doses of hydralazine tested caused a significant daily fall in blood pressure of the order of 60 mm. Hg the

effect lasted only one to four hours and this treatment did not prevent the occurrence of the vascular lesions. Thus hydralazine may have prevented the development of the vascular lesions by its discontinuous effect upon the mean blood pressure but its action may also have been directly or indirectly on some other more sensitive local factor such as the internal environment of the arteriolar smooth muscle cells. It was deduced that the continuous existence of raised blood pressure rather than the rapidity of the rise was the factor determining the susceptibility of the arterioles to necrosis and it was suggested that the necrosis, possibly precipitated by focal spasm, occurred in smooth muscle cells whose internal environment was already disturbed in a manner predisposing the cells to injury. It was envisaged that the sequence of events was analagous to that which occurs in experimental carbon tetrachloride liver cell necrosis where a biochemical lesion precedes the microscopic finding of cell damage (Cameron and Spector, 1961).

The nature of the disturbance in the smooth muscle cells, it was suggested, might be an alteration in their inorganic ion content, a change in the permeability of the cell membrane or an abnormality in the metabolism of the cells. On this basis the decision to study selected aspects of arteriolar muscle cell metabolism was made.

As a working hypothesis, it was suggested that the supposed metabolic change in arteriolar smooth muscle cells which antedated necrosis might be related to the mechanisms supplying energy for maintaining the increased peripheral resistance and

determining cellular ionic regulation, the sodium pump, and cell membrane permeability and potential. It had been suggested (Tobian, Janecek, Tomboulian and Ferreira, 1961) that in the hypertensive rat the visceral arterioles exhibited a raised sodium content and it seemed reasonable to think that this might be related to an underlying disturbance of cellular energetics, perhaps itself precipitated by the primary cause of the hypertensive state e.g. excess deoxycortone.

The enzymes selected for study were therefore those involved in the energy-producing steps of the major metabolic pathways.

The plan of the present experiments

This present study of the enzymes in arterioles of hypertensive rats was carried out as a preliminary to an investigation of the metabolism of the isolated smooth muscle cells. As the initial observation that discontinuous control of the blood pressure was adequate for the prevention of arteriolar necrosis was made in deoxycortone hypertension in the rat this experimental form of the disease was adopted for the present investigation. It was essential to investigate the enzymes in arteriolar tissue of the hypertensive animal before structural changes occurred in the muscle cells since these structural changes produced complicating, secondary effects. In deoxycortone hypertension in the rat the earliest evidence of fibrinoid necrosis of the arterioles was found sixteen to eighteen days after commencing treatment (Gardner, 1963). The work

described in this thesis was conducted therefore at an earlier period during what has been called the prenecrotic phase.

The only previous study of enzymes in rat arterioles was reported by Dubach and Recant (1960) and it was apparent that specialised ultramicro methods were necessary for the analyses of such small quantities of tissue. The techniques which these authors used for the preparation and analysis of arteriolar tissue were suitable for adaptation to the analyses of isolated smooth muscle cells dissected from arterioles. Prior to the study of arterioles however it was necessary to develop the methods of analysis with a more easily obtainable tissue. In the hypertensive animal the cardiac muscle undergoes hypertrophy and an increased amount of work is required of the heart in maintaining a constant output in the presence of an increased peripheral resistance. It was, therefore, considered to be of interest to compare any changes in the enzymes in arterioles in the early phase of rat hypertension with any observed in heart muscle. A study of selected enzymes in the rat heart in the prenecrotic phase of hypertension was therefore undertaken and forms the first part of this investigation.

Both qualitative and quantitative methods were available for the assessment of the enzymes chosen for investigation. Alkaline phosphatase activity was determined in this study because of its well known association with vascular tissue. Glucose-6-phosphate dehydrogenase was estimated as a representative of the pentose phosphate pathway, lactate dehydrogenase as a measure of glycolysis and malate dehydrogenase as a measure

of the importance of the citric acid cycle in cardiac and arteriolar tissue.

CHAPTER II

AN INTRODUCTION TO THE TECHNIQUES REQUIRED

1. The selection of the methods

The tissue to be analysed in this study was required in a state in which the enzymes were known to be stable and in a condition of cellular preservation such that the dissection of the smooth muscle cells from arterioles would ultimately be possible. Suitable methods for assaying enzyme activity in such small portions of tissue were needed. The techniques of analysis of freeze-dried sections of tissue developed by Anfinsen, Lowry and Hastings (1942) from the original work of Linderstrøm-Lang and Mogensen (1938) in Copenhagen provided for all these requirements.

Lowry, Roberts, Wu, Hixon and Crawford (1954) found that the enzyme activity measured in freeze-dried sections of brain was as great, or greater, than that in fresh homogenates of the same tissue. They also showed that freeze-dried sections of brain could be stored for as long as one year without loss of enzyme activity. Lowry, Roberts and Chang (1956) demonstrated that the freeze-dried sections could be obtained in a condition of cellular preservation such that the dissection and analysis of single large cell bodies of 0.01 μg. dry weight from rabbit brain was possible. The use of freeze-dried sections of tissue thus makes it possible to obtain and store tissue in a

condition in which enzyme activity and cellular structure are preserved. Assays of the activity of a number of different enzymes can thus be carried out on tissue from one animal.

Lowry et al. (1956) estimated the activity of several enzymes in 0.01 µg. portions of freeze-dried brain tissue. In order to weigh this minute sample it was necessary to construct a balance of the quartz fibre "fish-pole" type (Lowry, 1941) in the laboratory and the addition of the sample to the reagents was carried out with a mechanical loading device under a microscope. As the methods of enzyme assay to be used were all new to this laboratory it was considered essential to gain experience with them using larger samples of rat heart tissue. However, Lowry et al. (1956) pointed out that macro samples of tissue of 1 mg. dry weight cannot be analysed by the same procedures as those used for samples of 0.01 µg. dry tissue as the volumes of reagents required become of the order of one litre.

Dubach and Recant (1960) adapted Lowry's assay methods to the estimation of enzyme activity in 0.2 µg. dry weight of arteriole dissected from freeze-dried sections of kidney. They measured the activity of the enzymes in this weight of kidney arteriole by incubating the tissue with 10 µl. volumes of substrate solution. It seemed reasonable to assume that the enzyme activity in heart muscle and vascular tissue would be of a similar order to that in kidney arterioles and that 10 µg. of dry heart tissue would therefore require 500 µl. of substrate solution. One hundred assays of glucose-6-phosphate

dehydrogenase activity with 100 µl. of substrate solution or twenty assays with 500 µl. would require twenty-five milligrams of Sigma's nicotinamide-adenine dinucleotide phosphate (NADP) which cost fifty shillings. Thus, although it may seem more practical to develop the analytical methods with as large a portion of heart tissue as could be obtained, the volumes of solution involved, and the cost of the reagents, made it necessary to use a dry weight of tissue of the order of 10 µg. for the analyses.

The available commercial balances capable of weighing 10 µg. of dry tissue were considered and in order to be able to weigh dry sections of less than 10 µg. the method for construction of a quartz fibre "fish-pole" balance was studied. The use of freeze-dried sections of the tissue for the quantitative estimation of enzyme activity had the advantage that a selected number of the frozen sections could be used to determine the localisation of enzymes by histochemical techniques. Although alkaline phosphatase can be demonstrated in paraffin sections the use of cold microtome sections is necessary for the successful demonstration of the dehydrogenases by the methods such as those of Hess, Scarpelli and Pearse (1958).

2. Freeze-drying tissue

Freezing or quenching the tissue should be carried out immediately after the death of the animal as Bartelmez (1940) demonstrated that vacuolation of the cytoplasm of secretion-laden uterine epithelial cells occurred within 10 minutes and

de Duve and Beaufay (1959) found that the progressive release of lysosomal hydrolases and a loss of activity of enzymes such as cytochrome oxidase and glucose-6-phosphatase occurred within thirty minutes in ischaemic rat liver tissue.

The main factors which must be considered in order to obtain successful cellular preservation with this technique are those which govern the formation of ice crystals. If tissue is allowed to freeze in air at -20° C large ice crystals are formed distorting the cellular arrangement. The ice separates out as a pure substance until the eutectic point in the region of -45° C is reached where the remaining solution freezes out as a mixture (Rey, 1960). The proteins and salts in solution in the tissue are subsequently precipitated at the edges of the crystals formed. The best picture of the original positions of the components of the tissue will be obtained when the smallest possible ice crystals are produced. The size of the crystals formed at any temperature depends on the number of crystal nuclei present and the rate of growth of the crystals (Bell, 1952). Fisher, Holloman and Turnbull (1949) calculated that the rate of nucleation of pure water is very slow until temperatures of -33° C to -43° C are reached. Here the rate of nucleation increases by a factor of 10^{18}. As the rate of growth of ice crystals in tissue also becomes appreciable above -40° C (Rey, 1960) the specimen must be cooled rapidly to temperatures below -40° C to obtain a microcrystalline or vitreous state.

The rate of cooling of the tissue is determined by the

temperature difference between the tissue and the quenching liquid, the thermal conductivity of the quenching liquid and the size of the piece of tissue. Liquid nitrogen, oxygen, or air with temperatures in the range -195° to -183° C may seem to be most suitable but a pocket of vapourised liquid forms round the warm tissue decreasing the thermal conductivity greatly. Iso-pentane or pentane may be used with liquid nitrogen to give temperatures of -165° C but great care must be taken to ensure that the liquid does not solidify. Difluorodichloromethane, known commercially as Arcton-6 (I.C.I. Ltd.), m.p. -158° C can be safely used with liquid air or oxygen.

Lowry (1953) quenched blocks of brain tissue in liquid nitrogen for the preparation of freeze-dried sections for quantitative enzyme analysis. However, Scarpelli, Hess and Pearse (1958) achieved good preservation of cellular structure of rat stomach and kidney using a mixture of solid carbon dioxide and acetone at -65° C, and Nachlas, Walker and Seligman (1958) quenched these tissues in iso-pentane cooled to -70° C. As the sections of tissue obtained were used for the qualitative demonstration of enzymes their quantitative preservation under these conditions was not shown. A study of the quantitative preservation of the enzymes in cardiac tissue quenched by the method preferred by Nachlas et al. (1958) was undertaken as carbon dioxide is safer and more convenient to use than liquid nitrogen.

Freeze-drying tissue involved subliming the ice present in the tissue without giving an opportunity for the rearrangement of

the ice crystals present which occurs above -40° C (Rey, 1960). The solid ice has a definite vapour pressure at each temperature and if the vapour pressure above the tissue is maintained below the equilibrium vapour pressure for that temperature the molecules pass directly from the solid to the vapour phase (Bell, 1952). As this change is accompanied by an adsorption of heat it is difficult to state the exact temperature of the tissue being dried although it can be ensured that it does not rise above a stated temperature (Rey, 1960).

The essentials of a freeze-drying apparatus are therefore a container for the tissue at low temperature and a means of removing water vapour from the space above the tissue. The water vapour is removed from the tissue by evacuating the system and removed from the system by providing a water trap. This water trap can be a chemical such as phosphorus pentoxide, or a cold surface maintained at such a low temperature that the water condensing on it has a much lower vapour pressure than the water in the tissue. If a cold trap is used in the design of a freeze-drying apparatus the "mean free path" i.e. the distance which any molecule in the gas-containing space of the drying chamber and manifold can travel before it hits another molecule at the pressure established, must be taken into account. At 10^{-3} mm. Hg this distance is about 5 cm. (Bell, 1952). The time required to dry a block of tissue of 5 mm. diameter was seven hours in the most efficient design of this type of freeze-drying equipment (Mendelow and Hamilton, 1950).

Alternatively, freeze-drying apparatus can be of the

Moving-Air type as designed by Jensen (1957) in which dry nitrogen at -30° C and 1.5 cm. pressure is drawn over the specimen. In this apparatus 1 mm. sections of plant tissue were brought to dryness in four hours.

Tissue which is already cut into sections dries more rapidly than tissue in blocks as it has a larger surface area. Lowry (1953) dried 5-50 μ sections of brain tissue at -40° C under 0.1 mm. Hg pressure in one to six hours and Dubach and Recant (1960) also followed this procedure for kidney tissue. Bonting, Pollack, Muehrcke and Kark (1960) however, obtained cellular preservation adequate for dissection of the individual units of the nephron and estimation of their enzyme activity when kidney tissue was freeze-dried overnight at -20° C. They found that the drying of 16 μ kidney sections was complete in four to five hours at this temperature. The method of drying tissue at -20° C has the advantage that this temperature can be maintained in the cryostat. This technique was therefore investigated.

As different tissues have different physical properties, water contents and chemical constitution the methods of quenching and freeze-drying which give successful cellular preservation and water removal for one type of tissue cannot be assumed to be directly applicable to another. It was therefore necessary to show that the technique of drying tissue at -20° C under vacuum removed the water completely from rat heart tissue with satisfactory preservation of cellular structure and enzyme activity.

3. Commercial ultramicro balances available

E. Mettler, Pelikanstrasse 19, Zurich, Switzerland supply an ultramicro balance with a capacity of 2 mg. and a sensitivity of 0.1 µg. priced at £455 and available through A. Gallenkamp and Co. Ltd. The pan of this balance is mounted on top of the beam which has sapphire knife-edge bearings. The balance housing has a base area of 18 in. x 16 in. a height of 20 in. and is triple-walled to eliminate heat influences due to radiation and convection. The scale is read with the aid of a microscope with a built-in optical micrometer and is illuminated by a collecting mirror. As the pan is mounted on the top of the beam, loading is done through an opening in the roof of the balance housing. On examination of this balance it was found to be impossible to load the pan while sitting in the position required to read the scale. Apart from the inconvenience of having to stand up each time to load the balance pan the air currents produced by this movement were sufficient to cause a freeze-dried tissue section to float away. Another apparent disadvantage of this balance was that in order to read the scale with the microscope eyepiece it was necessary to sit almost touching the balance case. The temperature effects produced while carrying out a number of serial weighings would be considerable although the triple-walled housing may provide adequate protection.

L. Oertling Ltd., Orpington, Kent, also supply a balance with a sensitivity of 0.1 µg. and a range of 1 mg. at a cost of £395. The beam is constructed of quartz fibres and is linked

to an aluminium casting by two very fine quartz filaments and two quartz torsion filaments so that the torsion filaments do not carry the weight of the beam. The rear torsion filament is attached to a quartz bow which maintains a small but constant tension in the torsion filament and the front torsion filament can be rotated by means of a calibrated gearing system. The torsion head has coarse and fine adjusting knobs and is provided with three counting wheels and a reading drum on which the divisions are approximately 2.7 mm. apart. The value of one division is approximately 0.1 µg. but the true value depends on the dimensions of the torsion filament and must be determined by calibration.

Aluminium pans, 0.5 in. wide, in quartz fibre cradles are attached to the beam by 5 µ quartz fibres. The moving parts of the balance do not swing in large volumes of air. They are housed in narrow channels in an aluminium casting. Equilibrium of the balance beam is detected by the image of a pointer attached to the beam projected on to a ground glass screen. The base area of the instrument is 10 in. x 10 in., the height 14 in. This balance is a commercial development of the one described by Astbury, Belcher and West (1956) who reported that temperature, humidity and vibration had little effect on the performance of quartz torsion balances.

This quartz fibre balance was seen at Oertling's factory in Kent. The loading of the pans was simply done and the reading of the weight on the counting wheels could be carried out from the same sitting position. The disadvantage of this balance was

its extreme fragility. Great care must be taken to avoid touching the quartz fibre pan cradles with the forceps or with the weighed object.

Mr. J. Rodder of Microtech Services Co. Los Altos, California manufactures quartz fibre torsion balances of 0.001 µg. sensitivity with a price of £1300. The cost and the difficulty of servicing the instrument in this country made this balance of theoretical interest only.

Oertling's decimicrobalance was therefore selected as being the most suitable available at the time for weighing large numbers of 10 to 100 µg. samples of dried tissue.

4. Quartz fibre "fish-pole" balances

The original quartz fibre "fish-pole" balance described by Lowry (1941) was made by mounting a hollow quartz fibre, 20 cm. long, horizontally inside a metal cylinder 25 cm. long and 18 cm. in diameter. The samples were supported in quartz hooks of known weight. The displacement of the fibre tip was measured with a cathetometer which could be read to 0.01 mm. This balance had a sensitivity of 0.03 µg., a reproducibility of 0.1 µg. and a maximum permissible load of 200 to 300 µg. Later, Lowry (1953) modified the original balance. A pan made of glass blown thin enough to show interference colours was attached to the end of the fibre. This balance, made with a fibre 10 cm. in length, had a sensitivity of 0.01 µg. and could be used to measure weights of 2 to 15 µg. A balance with a sensitivity of 0.0001 µg. was also constructed by Lowry et al.

(1956) with a maximum permissible load of 0.05 µg. by mounting a 1 cm. fibre in a glass tube of 8 mm. diameter.

The sensitivity of a balance of this type i.e. the weight on the pan which produces unit displacement depends on the length and diameter of the fibre used (Lowry, 1941) but also on the size of the smallest unit of displacement which can be measured.

The great advantage of these balances is the simplicity of their construction. Their disadvantage is the narrow range of weights which can be measured on any one balance. The dimensions of the fibre required can be calculated from the weight range to be measured but because of the difficulties involved in producing quartz fibres and glass pans of exact and uniform diameter (Bonting and Mayron, 1961) the construction of this type of balance is partly done by trial and error.

5. Dissection of arterioles

Arterioles of less than 100 µ diameter were dissected from 30 µ freeze-dried sections of rat kidney for enzyme analysis by Dubach and Recant (1960) during a study of experimental nephrosis. These dissected portions of arteriole weighed from 0.075 µg. to 3.5 µg. If larger samples of arteriolar tissue could be obtained their analysis would be simplified.

The vascular lesions of the fibrinoid arteriolar type characteristic of severe hypertension in the rat (Wilson and Byrom, 1939) are observed after four weeks of induced steroid hypertension most frequently in the mesentery, pancreas, omentum and kidney (Gardner, 1960). As the behaviour of the mesenteric resistance vessels in culture was being studied in

this laboratory (Faed, Macgregor and Gardner, 1964), a method for dissection of these vessels under conditions designed for cellular and functional preservation was available. The preparation of freeze-dried mesenteric arterioles for quantitative enzyme analyses was, therefore, attempted.

6. Histochemical localisation of enzymes

In parallel with the quantitative estimation of enzyme activity in freeze-dried sections of cardiac and arteriolar tissue the localisation of the enzymes was to be studied in sections of the same tissue. The method of Gomori (1939) demonstrates alkaline phosphatase activity and the methods of Hess et al. (1958) demonstrate the presence of dehydrogenases which require the coenzyme nicotinamide-adenine dinucleotide (NAD) (previously known as diphosphopyridine nucleotide or DPN) or the coenzyme nicotinamide-adenine dinucleotide phosphate (NADP) (previously known as triphosphopyridine nucleotide or TPN). These methods are applicable to cold microtome sections of various rat tissues.

In the reaction catalysed by a coenzyme-linked dehydrogenase the natural substrate of the enzyme is oxidised and the coenzyme is reduced. In the living animal the reduced forms of the coenzymes are oxidised by the cytochrome system in the presence of their respective cytochrome reductases. In the presence of the enzyme diaphorase the reduced coenzymes can be oxidised by colourless tetrazolium salts which are reduced to coloured formazans. The insoluble formazan deposit thus localises the site of the original dehydrogenase activity

Hess et al., 1958).

The amount of reduction of the tetrazolium salt is increased by blocking the pathway for normal oxidation of the reduced coenzymes with cyanide, azide or amytal (Scarpelli, Hess and Pearse, 1958). As the NAD and NADP used in the incubation media are stable in solution for only a few hours, high initial reaction velocities are required for successful demonstration of the dehydrogenases. This is achieved (Hess et al., 1958) by using high substrate concentrations and by removing the product of the substrate oxidation with cyanide if it is an aldehyde or a ketone.

Nitro blue tetrazolium (Nitro-BT), a ditetrazolium salt, and MTT, the thiazolyl derivative of a monotetrazolium salt are used in the methods chosen for the demonstration of dehydrogenases. When N-thiazolyl-substituted tetrazolium salts are used alone the formazan deposit produced rapidly aggregates and crystallises (Pearse, 1960a) whereas in the presence of cobalt ions the formazan produces a chelate complex which is non-crystalline. Pearse (1960b) pointed out that this chelation method cannot be employed when cyanide is present in the incubating medium as an unstable cobalt cyanide complex is formed which rapidly reduces the tetrazolium salt to its formazan.

No attempt to prove or disprove the validity of these methods was considered but the possible interpretation of results obtained was studied. The mode of operation of the methods for dehydrogenase enzymes requires that the oxidation

of the reduced coenzyme formed in the reaction and the corresponding reduction of the tetrazolium salt, is catalysed by the enzyme diaphorase. The location of diaphorase can be demonstrated by incubating the tissue with reduced coenzyme and tetrazolium salt and it is shown to be present in the mitochondria (Scarpelli et al., 1958). As the location of the formazan deposited when the methods for dehydrogenases are employed depends on the presence of the diaphorase a dehydrogenase can be demonstrated only where diaphorase is also present. Nevertheless, if the deposition of formazan in a tissue after incubation with L-malate is different from the pattern obtained by incubation with DL-lactate this can be considered to show a genuine difference in the location of the dehydrogenase enzymes.

The quantity of formazan deposited in a given time also depends not only on the rate of the dehydrogenase-catalysed reaction but also on the rate of the diaphorase-catalysed reduction of the tetrazolium salt. This will have a definite maximum value. A certain rate of reduced coenzyme production will cause a maximum rate of diaphorase catalysed reduction of the tetrazolium salt and amounts of dehydrogenase enzymes giving reaction rates greater than this will not be distinguishable. Very careful control of the time of incubation of tissues with the substrate is therefore necessary if different concentrations of an enzyme are to be seen clearly.

Recently Van Wijhe, Blanchaer and Jacyk (1963) have shown that phenazine methosulphate can replace the enzyme

diaphorase in coupling the oxidation of the reduced coenzymes and the reduction of the tetrazolium salt in these histochemical methods. Their use of this technique brought their results for the distribution of lactate dehydrogenase into agreement with their quantitative estimations which showed that white muscle of the guinea pig had more than twice as much lactate dehydrogenase activity as red muscle and that the enzyme was present in the cytoplasm (Blanchaer, Van Wijhe and Mozersky, 1963). When Van Wijhe et al. (1963) used the histochemical methods of Hess et al. (1958) without modification the red fibres appear much richer in lactate dehydrogenase than the white fibres which have the lower diaphorase activity and the enzyme appears to be located in the mitochondria.

The results obtained in this work with the methods of Hess et al. (1958) were therefore to be interpreted as illustrating only some of the probable locations of the dehydrogenase enzymes.

CHAPTER III

METHODS

1. Preparation of freeze-dried sections

(a) Equipment

The cryostat available when this work was begun was of Coons' design, modified and supplied by the Prestcold Company at Cowley, Oxford. A Cambridge rocking microtome was operated within a chamber maintained at -20° C by inserting gloved hands through the armholes provided. This equipment proved excessively awkward to use as the armholes are placed so far apart that operations requiring both hands could not be performed within the chamber by a person of average size. A "Pearse" refrigerated microtome, as manufactured by the South London Electrical Equipment Co. Ltd., London, was therefore obtained. This cryostat contains a Cambridge rocking microtome operated entirely by external controls. As this equipment was used throughout the work, the description of the techniques tried for mounting tissue and cutting sections is limited to those used with this machine.

Section holders were made of aluminium sheet 1¼ mm. thick, 93 mm. x 26 mm. in area in which sixteen holes 7 mm. in diameter were drilled in the area covered by a microscope slide. The drilled sheet was sandwiched between two slides which were held in position by rubber bands. These holders are illustrated

diagrammatically in Fig. 1. The rubber bands used retained their elasticity at -20° C under vacuum for six months but after a period of one year a number of them had broken. The brass springs used to keep the slides in place in Lowry's design (Lowry, 1953) would therefore have been preferable but their construction was not possible with the facilities available at the time.

The drying tube (Fig. 1) was a ten inch, Quickfit test tube, with a B 34 socket joint. This tube held eight section holders and was sealed off under vacuum by using a B 34, cone-to-rubber-tubing adaptor with a stopcock and right-angled connection.

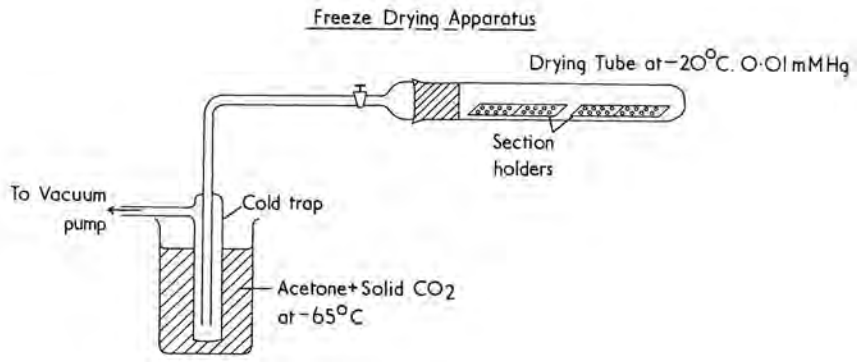


Fig. 1

Silicone high vacuum grease recommended for pressures down to 10^{-6} mm. Hg, and temperatures of -40° C to 200° C, by Edwards High Vacuum Ltd., Crawley, Sussex, was used to seal the ground glass joint and stopcock of the drying tube. After storing tubes at -20° C for six months they were still under vacuum but after a period of one year they were no longer under vacuum. They had been stored vertically and the silicone grease had run down the inside of the drying tube. Subsequently the tubes were stored horizontally.

A cold trap (Fig. 1) was designed in metal. The dimensions were such that one arm, of outside diameter 8 mm., could be inserted through the carbon dioxide inlet into the cryostat and attached by a short length of rubber tubing to the drying tube; the other arm, of outside diameter 16 mm., could be attached to the vacuum pump.

A "Speedivac" model 2SC 50B two stage, gas ballast pump capable of giving pressures of 0.0002 mm. Hg without air ballast flow, or 0.003 mm. Hg with air ballast was used. The accessory flanged tee piece with an air admittance valve, a half-inch, horizontally mounted vacuum connection and a vertical fitting to suit an M6A Pirani gauge head was fitted to the pump.

A thermal Pirani gauge was used to measure the pressure in the system. With the model B4 control unit pressures between 0.5 and 0.005 mm. Hg could be measured.

The vacuum pump and gauge were supplied by Edwards High Vacuum Ltd.

(b) Freezing and mounting the tissue block

The most rapid cooling of a block of tissue was expected when all sides of the block were exposed to the quenching agent. When a block of heart muscle 2 mm. x 3 mm. x 4 mm. in size was plunged directly into iso-pentane in a beaker cooled to -65° C with a mixture of solid carbon dioxide and acetone fracturing of the tissue block occurred. This cracking was attributed to excessively rapid cooling taking place, causing the outer frozen zone to compress the inner, yet unfrozen, tissue. Attempts were made to mount this frozen block of tissue directly on the holder by using an almost frozen paste of heart tissue as suggested by Lowry (1953). It was very difficult to avoid thawing a large proportion of the frozen block during this process. This technique of mounting the tissue block was also found unsatisfactory if the specimen holders supplied with the microtome were used as there were insufficient grooves on the holder within this small area to provide satisfactory adhesion. When a block of such small dimensions was eventually successfully mounted directly on the holder, a large part of it could not be cut as the arc described by the microtome arm was such that the knife would have been damaged by the metal holder.

A technique for quenching tissue mounted on a cork disc was found in the handbook supplied with the cryostat which overcame all these difficulties and it was used throughout this work. The block of fresh tissue, 2 mm. x 3 mm. x 4 mm., was placed on a piece of cork 2 mm. thick and 12 mm. in diameter. These cork discs were obtained by sawing up an ordinary cork

and selecting the pieces with a sufficiently rough surface to give adequate adhesion of the tissue block. The tissue on the cork was plunged into iso-pentane in a beaker cooled to -65° C with a mixture of solid carbon dioxide and acetone and held underneath the surface of the liquid for at least 10 sec. to allow the specimen to reach the temperature of the liquid. The cork was then placed in the cryostat and left for a few minutes to allow the iso-pentane to evaporate from the surface.

A piece of liver was made into a paste and spread evenly on the underside of the cork which was kept at -20° C inside the cryostat. The cork was then held firmly on top of a holder precooled to -20° C. Within five minutes the liver paste was frozen hard and the holder was fixed on the microtome arm and the tissue sectioned.

Heart tissue sections were cut 15 μ thick, thawed on to a slide, and stained with haematoxylin and eosin. Examination of the sections with the light microscope confirmed that good preservation of cellular structure was achieved with this method for quenching the tissue.

This technique had the additional advantage that when frozen sections were required for histochemical methods a number of blocks of different tissues from the same animal could be mounted on the one piece of cork and cut together. The composite sections obtained in this way demonstrated dehydrogenases in different tissues under identical conditions of incubation. After the sections required for freeze-drying were cut, the cork was easily detached from the holder and

stored in a small polythene bag at -20° C sealed with adhesive tape. Drying out of the tissue was minimal under these conditions and further frozen sections could be cut up to 7 days later if required.

(c) Cutting and freeze-drying sections

When sufficient skill in adjusting the angle of the knife and the position of the teflon guide plate was acquired, flat, undistorted sections of heart tissue could be cut from 2 to 20 μ in thickness. The sections required for freeze-drying were cut 15 μ thick and collected at the foot of the shute in a section holder. They were randomly distributed into the holes of the section holder with the aid of a long-handled brush of squirrel's hair. When the required number of sections was collected the covering slide was fixed in position and the holder immediately placed in the drying tube which was kept at -20° C inside the cryostat. This tube was stoppered with a rubber stopper until all the holders with sections to be dried at one time had been collected. Drying under vacuum was carried out immediately after the collection of the sections as they shrank and curled if they were allowed to lose moisture at -20° C under atmospheric pressure.

The equipment was designed so that freeze-drying could be carried out with the drying tube in the cryostat as the thin sections thawed instantaneously on exposure to warm air. When all the sections to be dried together had been collected, the greased cone adaptor, cooled to -20° C in the cryostat, was connected firmly with the socket of the drying tube. As a safety precaution a fine mesh wire gauze jacket was fitted

round the glass tube. The cold trap was cooled to -65° C with a mixture of acetone and solid carbon dioxide, then attached to the drying tube and to the vacuum pump. The system was evacuated for two hours at 0.01 mm. Hg. The drying tube was then isolated from the system by closing the stopcock and stored under vacuum until required.

When the sections were needed the tube was re-evacuated before and during warming to room temperature in case leakage had occurred. Dry air was then admitted to the tube through a calcium chloride trap. After cleaning the silicone grease off the ground glass joint the section holders were extracted from the tube and transferred to a box containing anhydrous calcium sulphate, to protect them from excess humidity and to standardise the conditions of exposure to room temperature.

(d) Estimation of efficiency of drying technique

In this laboratory the dry weight of a tissue is accepted as the constant weight reached after heating to 105° C. The weight of the sections of heart tissue after freeze-drying for 2 hr. at -20° C under 0.01 mm. Hg pressure was compared with the weight after further drying at 105° C for 24 hr. In any method involving heating of the tissue there is a danger of losing volatile constituents and the water bound to proteins so that the discrepancy in the apparent water losses found in this experiment was maximal.

In a preliminary experiment, heart tissue from twelve rats was dried to constant weight at 105° C, and the value of 4.1 was found for the ratio of wet heart tissue weight to dry heart

tissue weight. This value was used in the calculation of the results given in Table I. They show that the additional weight loss after heating freeze-dried heart tissue at 105° C was < 0.9 per cent of the total water content, or < 2 per cent of the dry weight of the section.

These results agree with those of Mendelow and Hamilton (1950) who found that rat heart tissue blocks lost 99.1 per cent of the water content during freeze-drying when the constant weight reached after heating in a vacuum oven at 50° C was accepted as the dry weight of heart tissue. Hoerr (1936) also found that even after freeze-drying for 5 days tissue lost a further ½ to 1 per cent of its wet weight when dried for 24 hr. at 37° C.

Lowry (1953) investigated the possibility that freeze-dried brain sections gave a falsely high "dry" weight when weighed in room air, because of the porous and hygroscopic nature of the tissue. He weighed freeze-dried brain sections under vacuum, in dry air and in air of increasing relative humidity at 25° C. The weight of a section in air of 75 to 100 per cent relative humidity was 108 per cent of the weight measured under vacuum and the weight in dry air was 102 to 104 per cent of that value due to air being absorbed by the tissue. These observations show that the comparison of heat-dried and freeze-dried tissue is further complicated by possible changes produced in the hygroscopic properties of the tissue.

The one ideal method for determining the drying point was evolved by Jansen (1954) who incorporated a steel wire torsion

TABLE I

Estimate of the percentage of the total water removed from rat heart tissue by freeze-drying 15 μ sections at -20° C and 0.01 mm. Hg for 2 hr.

Weight of section				
After freeze-drying	After drying to constant weight at 105° C	Water removed from freeze-dried section by heating to 105° C	Total weight of water present initially	Percentage total water remaining after freeze-drying
μg.	μg.	μg.	μg.	%
41.70	41.21	0.49	127.75	0.38
38.15	38.16	-0.01	118.30	0
38.18	37.25	0.93	115.48	0.81
42.67	42.64	0.03	132.18	0.02
51.38	50.17	1.21	155.53	0.78
64.99	64.17	0.82	198.93	0.41
82.93	81.02	1.91	251.16	0.76
70.20	69.09	1.11	214.18	0.52

balance in his freeze-drying apparatus and determined when constant weight was reached by the specimen but this method was too elaborate to be considered.

The observations of Lowry (1953) demonstrated that increasing the drying time or otherwise trying to improve the 99 per cent efficiency of water removal obtained with the freeze-drying technique was useless when the relative humidity of the balance room was not controlled. Humidity regulation in this room was not a practical proposition at the time of these experiments. The dry weight measurements in this work were required as a basis for comparison of the enzyme activity in vascular tissues of different groups of animals. The validity of the comparisons can therefore be improved by drying the tissues to be compared together, and weighing the sections at the one time, so that any variation in the drying technique, or in the relative humidity prevailing at the time of weighing, can be assumed to affect the weight of the sections from the different animals to the same extent.

2. Measurement of dry tissue weight

(a) Assessment of Oertling's decimicrobalance

In order to minimise local vibration effects the balance was mounted on a heavy slate bench, 2 in. thick, supported from an outside wall of the building. Although the accuracy of the balance was not permanently affected by vibrations (Astbury et al., 1956) the image of the index fibre oscillated until they died away making it impossible to use the balance. This effect was observed on a few days in the year when the high gales

blowing caused the outside walls of the building to vibrate.

The temperature and humidity of the balance room were not regulated. The heavy aluminium casting protected the quartz beam from sudden temperature changes and the presence of a fairly high humidity served to protect the balance from the accumulation of electrostatic charges. Any variability in the resting point of the balance, introduced by changes in either of these factors, was compensated for by subtracting the mean value of the resting point readings before and after each weighing from the reading given for the sample. The usual precautions necessary to balance any local temperature effects were taken. Both pan compartments were opened even if only one pan was to be loaded, and when operating the control knobs with one hand, the other arm was placed in a comparable position on the other side of the balance.

The errors most likely to be introduced when using this balance were the gross errors caused by specks of dust and by the accumulation of static electricity on the pans. Absolute cleanliness was essential. As the design of the pan compartments was such that when they were opened any dust in the vicinity was drawn into them, the bench and balance case were wiped with a slightly damp Kleenex before opening the pan compartments each day. The pans themselves were cleaned by drawing a single human hair across the surface. When electrostatic charges accumulated on the balance, the pan affected stuck firmly to the pan arrest and weighing had to be delayed until the balance discharged. The accumulation of static electricity was minimised by earthing both the balance and the

forceps. The remaining source of electrostatic charges was the operator. It was essential to avoid wearing synthetic materials such as orlon, nylon and terylene while weighing as these non-absorbent fabrics accumulate static electricity.

Freeze-dried sections were easily weighed directly on the balance pan. Any sudden gross (0.1 µg.) change in the resting point was taken to indicate that dust or a fraction of section had been left on the pan and it was swept clean again before proceeding. Microgram quantities of standard substances were also required for the analytical methods. The load limit of each pan was 250 mg. Two pieces of aluminium foil, 5 mm. x 9 mm., weighing 6.1 mg. each, were matched for weight as closely as possible and one was shaped as a boat to contain the samples and the other was used as a tare.

A series of eleven readings of the resting point were made. The pan compartments were opened after each reading. An average deviation from the mean value of 0.61 divisions, the equivalent of 0.061 µg., was found (Table II). At least four resting point readings were taken before commencing any series of weighings. These preliminary readings varied appreciably and were presumably made in the period required for the air inside the balance case to equilibrate with the room air.

The instrument was manufactured so that one division on the scale of the drum was approximately equivalent to 0.1 µg. but calibration was necessary. Although the large size of the divisions on the drum (2.7 mm.) made it easy to read the scale to 0.01 µg. the manufacturers quote the sensitivity as 0.1 µg.

per scale division and it was assumed that this was their estimate of the lowest reading compatable with the possible precision of the balance. Readings were nevertheless made with an estimate of fractions of the scale division. A 1 mg. weight was weighed sixteen times. The mean value of the resting point determined before and after each weighing was subtracted. One scale division was found to be equivalent to 0.1004 µg. (Table II). As all weighings in these experiments were to be made on this balance the value was approximated to 0.1000 so that the scale divisions were read directly in micrograms. The possible 0.4 per cent error introduced into the calculations by this approximation was negligible compared with the accuracy of tissue analysis on this scale. The average deviation from the mean value in the sixteen weighings of 1 mg. was equivalent to 0.05 µg. The number of divisions equivalent to the 1 mg. weight was also read on twenty-three separate days over a five month period. The average deviation from the mean value was the equivalent of 0.20 µg. (Table II). The possible variation in the calibration factor was, therefore, 0.02 per cent.

As the dry tissue to be weighed was in the range 0 to 100 µg. a piece of aluminium of approximately 50 µg. was also weighed sixteen times. The mean weight was 53.39 µg. with an average deviation from the mean of 0.06 µg. This average deviation was used by Astbury et al. (1956) as an estimate of the precision of weighing. The maximum error in weighing 50 µg. was thus 0.12 µg. or 0.24 per cent. The maximum error in weighing samples of 10 to 100 µg. with Oertling's decimicrobalance was 1 per cent. The performance and accuracy of the balance was,

TABLE II

Assessment and calibration of Oertling's decimicrobalance

	Readings made	Mean divisions	Average deviation from the mean divisions	Sensitivity per scale division µg.	Precision of weighing µg.
No load resting point	11	633.6	0.61		0.06
1 mg. load	16 within 2 hr.	9955.7	0.46	0.1004	0.05
1 mg. load	23 on separate days over a 5 month period	9958.3	1.99	0.1004	0.20
Approx. 50 µg. load	16	533.9	0.55		0.06

therefore, satisfactory with the maximum possible control over vibration, dust and static electricity, but none over temperature or humidity.

(b) Construction and calibration of a quartz fibre balance

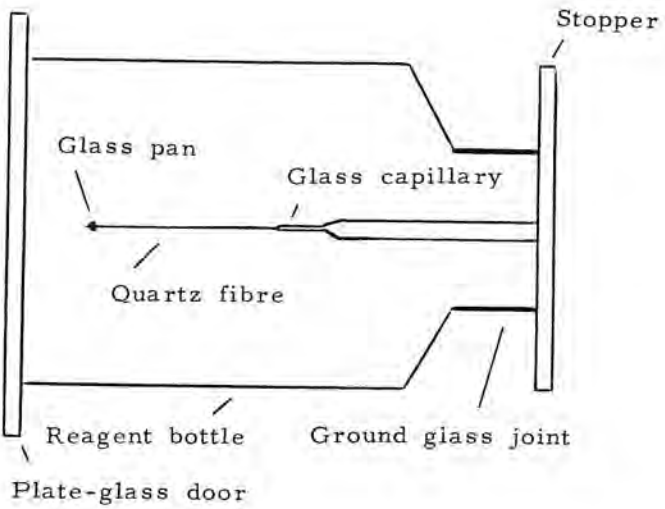
A quartz fibre balance which could be used to measure 0.1 to 10 µg. of dry tissue weight was constructed in the Biochemistry Department of the Chemical Defence Experimental Establishment at Porton Down, Wilts., under the guidance of Mr. D. Nicholls.

A number of fine quartz fibres were flame blown. When the narrowed end of a quartz rod was held in the flame of a gas and oxygen cannon burner, the melting quartz was blown across the room by the flame as a fine fibre. The fibres were collected and their diameters measured with the calibrated ocular scale of a microscope. One of 12 µ diameter was selected and a strip of gummed paper was attached to either end to simplify handling it. The fibre was straightened by suspending it vertically from one of the pieces of paper and warming it with a low bunsen flame for about 5 sec.

A glass tube 3 mm. in diameter was drawn out to a fine capillary. One end of the quartz fibre was pushed into this narrow opening and sealed in place with a one-in-five dilution of durofix in amyl acetate. Glass thin enough to show interference colours was made by heating a glass tube until it was red and rapidly blowing a glass bubble. The thin glass required for the balance pan was cut into a shape 1 mm. square with a razor blade. A tiny speck of araldite was placed near the end of the fibre and the pan was picked up with the

adhesive. The glass tube, with the capillary holding the fibre, was then centred inside the ground glass stopper of a wide-mouthed, 125 ml., reagent bottle, and sealed there with picien wax (Edwards High Vacuum Co., Ltd.).

The base of the reagent bottle was removed and the cut end ground smooth. This served as the "case" for the balance. The stopper with the fibre attached was firmly screwed into the neck of the bottle and bound to it with cellotape. The balance "case" was held horizontally in a clamp and a sheet of plate glass served to close the open end. Wooden arm rests placed in front of the balance supported the plate glass door. A diagrammatic representation of this balance is given in Fig. 2.



A QUARTZ FIBRE "FISH-POLE" BALANCE

Fig. 2

The fibre and pan of the balance were illuminated with a focussing lamp. The binocular head of a Watson sterioscopic microscope (x 7 eyepieces,x 5 objective) attached to a long arm, rackwork, stand, was manoevred until the balance pan was seen. The samples were loaded on the pan with a hair mounted in glass tubing. A microscope slide was placed under the pan to avoid losing the sample if it failed to stick to the hair. The displacement of the pan was observed with a vernier measuring microscope (x 10 eyepiece, x 3 objective) supplied by Vickers Instruments Ltd., Croydon. The initial reading on the vernier scale of the microscope stand was taken when the microscope was adjusted so that the tip of the fibre coincided with the fifth division of the 10 mm. scale in the eyepiece. When the balance was loaded the microscope was moved in a vertical direction so that the tip of the loaded fibre again coincided with division five in the eyepiece. The displacement of the fibre was measured to 0.02 mm. by reading the distance the microscope was moved on the vernier scale.

For the calibration of the balance a highly purified, stable crystalline substance with a measurable absorption or fluorescence at a concentration of 0.1 µg. per ml. was required. The sodium salt of dihydroxypyrene disulphonic acid fluoresces at this concentration, is stable, can be obtained pure, and has black rod-shaped crystals. The displacement caused by a crystal was measured and it was transferred to a test tube and dissolved in a known volume of water. The fluorescence intensity of the solution was read on the Aminco-Bowman

spectrophotofluorometer at 460 mμ, with 360 mμ as the activating wavelength. At the same time the fluorescence intensity of a series of standard solutions of this salt, prepared by weighing macro quantities, was read. The weights of the single crystals were determined from the calibration curve prepared from these standards.

The displacement measured was directly proportional to the weight placed on the pan up to 6 μg. The curve shown in Fig. 3 illustrates the weight range for which the balance was actually used (0.7 to 0.9 μg.).

The sensitivity of the balance was 0.048 μg. when the vernier scale of the microscope stand was read to 0.02 mm. The vernier scale can be estimated to 0.01 mm. but a more sensitive method for measuring the displacement would extend the usefulness of this balance.

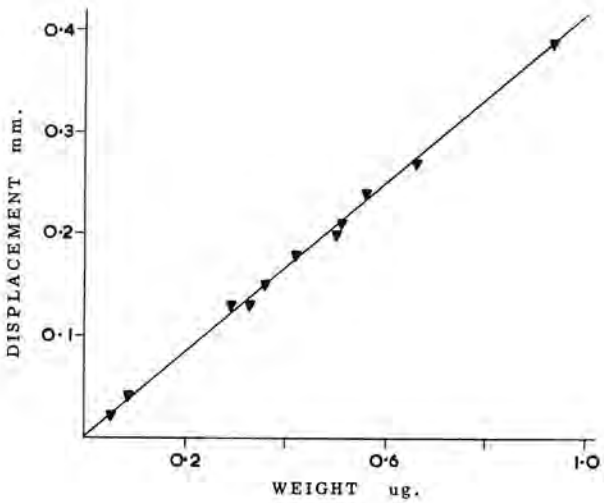


Fig. 3 Calibration curve for the quartz fibre balance.

3. Dissection of arterioles

Resistance vessels of approximately 200 μ diameter were obtained from the mesentery of a 200 g. male albino Wistar rat. As a few of the vessels were to be used for tissue culture (Faed et al., 1964) they were dissected under sterile conditions.

The animal was killed by exsanguination under ether anaesthesia. The whole intestine with its attached mesentery was quickly and aseptically removed and washed with Hanks' balanced salt solution (Hanks and Wallace, 1949) at 4° C. The dissection of the vessels and the removal of all traces of surrounding fat was accomplished under cold Hanks' solution with the aid of a dissecting microscope. The dissected vessels were then transferred briefly to 1 per cent NaCl solution at 4° C and the blood removed from them by perfusion of the main artery. Vessels less than 200 μ, free from blood and fat, were cut free from the artery and the excess fluid drained from them. Approximately eight vessels were coiled on a piece of cork, 4 mm. in diameter, to form a pile of vascular tissue 3 mm. in diameter and approximately 1.5 mm. high.

The cork was immediately plunged into iso-pentane at -65° C and the frozen block transferred to the cryostat. Fifteen micron sections were cut and freeze-dried for enzyme analyses by the method which proved satisfactory for heart tissue. The appearance of one whole section can be seen in Fig.32, p.154. Sections of 4 to 8 μ were cut for histochemical localisation of the enzymes and histological inspection.

Examination of sections, stained with Sudan IV (Pearse, 1960a) confirmed that negligible amounts of fat remained

after careful dissection. The appearance of these sections can be compared with that of sections of rat mesenteric arterioles which were not dissected free from fat (Fig. 28 and 29, p.154).

Approximately fifty sections were cut from the amount of vascular tissue which was dissected and freed from fat in forty-five minutes. As the dry weight of one section was in the region of 10 µg. the total amount of mesenteric arteriolar tissue available for enzyme analyses was approximately 500 µg.

4. Histochemical localisation of enzymes

After the sections for freeze-drying had been cut the blocks of tissue remaining were stored overnight in air-tight polythene bags at -20^{0} C in the cryostat. The following morning the sections, 8 µ thick, required for histochemical methods were cut and mounted by touching them lightly with a slide at room temperature. The adhesive forces operating when the frozen tissue thawed on to the glass were sufficient to keep the section in place. The term "cold microtome sections", recommended by Pearse (1960d), was applied to those prepared in this way.

Preliminary experiments showed that if these sections were stored in open racks in the refrigerator they were damaged by condensation occurring on the slide surface when the door was opened. The mounted sections were, therefore, stored in a closed box at 4^{o} C.

The histochemical methods were applied to the sections within 5 hr. The tissue was incubated for 5 min. with sodium

β-glycerophosphate as the substrate for the enzyme when the method of Gomori was used to localise alkaline phosphatase activity. These sections were counterstained with 1 per cent neutral red and mounted in Canada balsam. A black precipitate of cobalt sulphide indicated the site of activity of this enzyme.

The composition of the incubating media used to demonstrate the presence of glucose-6-phosphate dehydrogenase (G-6-PDH), lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) are given in Table III. The sections were covered with 0.1 ml. of the solution and incubated at 37° C in a closed box $1\frac{1}{2}$ in. deep, 10 in. x 4 in. in area to minimise evaporation. Those used to illustrate glucose-6-phosphate dehydrogenase activity were incubated for 30 min., rinsed for 30 sec. in 0.1 M HCl, fixed in 10 per cent neutral formalin for 10 min. and mounted in glycerine jelly containing 0.5 M cobaltous acetate. The coverslips were sealed in place with nail varnish. A black deposit of cobalt-formazan indicated the site of activity of the enzyme. The nuclei were counterstained when required with 0.5 per cent chloroform-washed aqueous methyl green.

Sections used to demonstrate lactate and malate dehydrogenase activity were incubated for 15 and 30 min. respectively, rinsed briefly, fixed in 10 per cent neutral formalin and mounted in glycerine jelly. They were counterstained, if necessary, with carmalum (Mayer's) diluted 1:10 with deionised water. A blue diformazan deposit indicated the site of enzyme activity.

TABLE III

Composition of the incubating media for the qualitative demonstration of glucose-6-phosphate dehydrogenase (G-6-PDH), lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) in cold microtome tissue sections

Stock solutions	Volumes mixed (ml.) for medium for G-6-PDH	LDH	MDH
1 M substrate *	0.1	0.1	0.1
0.1 M sodium cyanide		0.1	0.1
0.1 M sodium azide	0.1		
0.01 M sodium fluoride	0.05		
0.05 M magnesium chloride		0.1	0.1
0.06 M phosphate buffer pH 6.8-7.0		0.25	0.25
0.2 M tris buffer pH 7	0.25		
1 mg./ml. tetrazolium salt MTT	0.25		
Nitro-BT		0.25	0.25
0.5 M cobaltous chloride	0.05		
Deionised water	0.2	0.2	0.2
Coenzyme added to give 0.01 M concentration	8 mg. NADP	6.6 mg. NAD	6.6 mg. NAD
Polyvinyl Pyrrolidone (Mol.Wt. 12,000) added mg.	75	75	75

* Substrates for G-6-PDH disodium D-glucose-6-phosphate
LDH sodium DL-lactate
MDH L-malic acid neutralised with NaOH

The substrates, coenzymes, and Nitro-BT i.e. 2,2'-di-p-nitrophenyl-5, 5'diphenyl-3, 3'- (3,3'-dimethoxy-4, 4'-biphenylene) ditetrazolium chloride were obtained from the Sigma Chemical Co., St. Louis, M.O.

MTT i.e. 3- (4,5-dimethylthiazolyl-2) -2, 5-diphenyl tetrazolium bromide was obtained from the Nutritional Biochemical Corp., Cleveland, Ohio.

Polyvinyl Pyrrolidone was obtained from L. Light and Co. Ltd., Colnbrook, Bucks.

CHAPTER IV

DEVELOPMENT OF THE ENZYME ASSAY METHODS

1. Introduction

Enzymes are proteins whose biological function is the catalysis of chemical reactions in living systems. A catalyst is defined as a substance which changes the rate of a reaction without affecting the nature of the final products. The presence of an enzyme is detected by the occurrence of the specific reaction which it catalyses and the activity of the enzyme is measured by the amount of chemical change it catalyses under defined conditions. The measured rate of an enzyme-catalysed reaction is affected by the concentration of the enzyme and of the substrate, by the pH and temperature and by the presence of activators or inhibitors of the enzyme (Dixon and Webb, 1958).

In an enzyme assay method the substrate concentration chosen is that which is high enough to saturate the enzyme present so that the observed velocity becomes independent of the substrate concentration and approaches the maximum value which depends only on the concentration of enzyme present. The rate of the reaction is measured at the optimum pH of the enzyme and the temperature of 37° C is preferred. The presence of activators and inhibitors of the enzyme is avoided when these are known. The velocity measured cannot be assumed to be

constant with time. A decrease observed after a period of time may be due to inactivation of the enzyme by pH or temperature, inhibition of the enzyme by the products of the reaction, a fall in substrate concentration or the initiation of the reverse reaction. The reaction velocity measured in an enzyme assay should therefore be the initial value unless it is known that the velocity is unaffected by increasing the time of incubation.

The assay methods developed by Lowry and his co-workers for the estimation of alkaline phosphatase, glucose-6-phosphate dehydrogenase, lactate dehydrogenase and malate dehydrogenase were adopted. The pH and the substrate concentrations required for maximum reaction velocity in these enzyme assay methods had been determined by Lowry and his colleagues for the enzymes of rabbit brain. In each method for the estimation of enzyme activity the volumes of reagents used were adjusted to those suitable for the analysis of 10 to 100 µg. of heart tissue. As the method for alkaline phosphatase estimation was the simplest the micro techniques required were developed by assaying the activity of this enzyme in human sera.

The validity of each method proposed for the assay of the enzyme in rat heart tissue was then studied. To ascertain the validity of an enzyme assay the first essential was to verify that the analytical method for the estimation of the product formed in the reaction was valid. The relationship between the concentration of the product in solution and the

units of measurement was therefore established.

In the methods chosen, the enzyme activity was expressed as moles of substrate transformed by a kilogram of dry tissue in one hour. For this expression to have a practical meaning it was necessary to confirm that the velocity of the reaction was constant from 0 to 60 minutes. On the micro scale used, this was most easily done by incubating known quantities of tissue for different periods of time. The assumption made when this method was used was that the quantity of enzyme present was a constant proportion of the weight of tissue incubated. This assumption could not be made in the case of freeze-dried sections where the proportions of different cell types in each section might vary. To overcome this difficulty the preliminary tests of the validity of the methods for enzyme activity estimations were made with homogenates of heart tissue. When the time-velocity relationship was determined homogenised heart tissue was then used to establish the range of tissue weight over which the velocity measured was directly proportional to the quantity of enzyme present.

The assay method was then applied to freeze-dried sections in the weight range established. To determine whether freeze-drying the tissue had affected the activity of the enzymes the values measured in the sections of rat heart were compared with those measured in fresh homogenates of the same tissue. As freeze-dried sections could not be analysed on the day the fresh homogenate was made, because of the time involved in their preparation a preliminary investigation of the stability of the

enzymes in homogenates was necessary. The coefficient of variation of the analysis of sections was then compared with that of the analysis of a homogenate. A value of five per cent was considered acceptable for the analysis of homogenates.

The stability of the enzymes in stored, freeze-dried tissue was then studied. In the present experiment, as four enzymes were to be estimated in the tissue from one animal it would be necessary to bring the sections to room temperature and expose them briefly to room air each day, for four days. The stability of the enzymes in the freeze-dried sections was therefore studied under the appropriate conditions.

Each assay method used for heart tissue was subsequently adapted for the measurement of the enzyme in freeze-dried sections of arteriolar tissue. The valid range of incubation time and of tissue weight were again established and the coefficient of variation for the analysis of sections of arteriole was determined. The stability of the enzymes in stored, freeze-dried sections of arteriolar tissue was also studied.

2. Materials

Substrates and coenzymes used in the assay methods were obtained from the Sigma Chemical Co., St. Louis, M.O., U.S.A.

Analar grade reagents were used.

Deionised water from an "Elgastat" deioniser was used for the preparation of solutions.

Measurementsof pH were made with a Pye Universal pH meter.

Preparation of homogenates of heart tissue

A male rat of 200 to 250 g. was killed by bleeding from the aorta under ether anaesthesia. The heart was excised and the ventricles separated from the atria by an incision extending through the atrioventricular sulcus. The ventricles were opened and the cavities freed from blood. A block of tissue was taken from the anterior apical left ventricular myocardium, cut into thin slices and weighed on 1 sq.cm. of glass. The slices were picked up on the end of the pestle of a 10 mm. bore, all-glass homogeniser, and homogenised by hand in 1 ml. of a chosen medium which was kept ice-cold. Both tube and pestle had ground glass surfaces and the pestle fitted loosely in the tube. With this technique a homogenate was obtained in which no whole cells were found on microscopic examination.

3. Estimation of alkaline phosphatase activity

The method used was that described by Lowry et al. (1954) for the estimation of alkaline phosphatase activity in 0.1 to 10 µg. of freeze-dried brain, analysed with 10 µl. of substrate solution. This technique was a further application of the method developed for rapid alkaline phosphatase determinations in serum (Bessey, Lowry and Brock, 1946). The colourless substrate, p-nitrophenyl phosphate is hydrolysed at pH 10 by the enzyme to p-nitrophenol which exists as a yellow salt in alkali solution with an absorption maximum at 400 mµ. If the solution is then acidified the p-nitrophenolate is converted

to the colourless p-nitrophenol with an adsorption maximum at 318 mμ making it possible to correct for the adsorption of the yellow colour of the serum at 400 mμ. As the p-nitrophenol and any haemoglobin present absorb less light at 415 mμ than at 400 mμ the actual measurements of absorbancy are made at 410 mμ.

Reagents

Stock substrate solution 16 mM p-nitrophenyl phosphate

0.2104 g. of Sigma 104 phosphatase substrate was dissolved in 50 ml. of 0.001 N hydrochloric acid and the pH adjusted to 6.6. The solution was tested for the presence of free p-nitrophenol by diluting 1 ml. with 10 ml. 0.02 N sodium hydroxide and reading the transmittancy at 415 mμ in a 1 cm. cell. Throughout these experiments the light transmittancy was well above the limit of 83 per cent where extraction of the contaminating p-nitrophenol becomes necessary (Bessey et al., 1946). The reagent was divided into 1.5 ml. portions in plastic cups and stored at -20° C.

Stock buffer solution

1 M 2-amino-2-methyl-1-propanol, 4 mM magnesium chloride

8.9140 g. 2-amino-2-methyl-1-propanol was dissolved in water, a solution of 0.0813 g. $MgCl_2.6\ H_2O$ added, the pH adjusted to 10, and the volume made up to 100 ml. This reagent was stored in polythene at 4° C.

Buffered substrate solution

Equal volumes of stock substrate and stock buffer solution

were mixed immediately before use. It was verified that the mixture was of pH 10.

The above solutions were prepared fresh every six weeks.

Standard solutions of p-nitrophenol

0.1391 g. p-nitrophenol was dissolved in 100 ml. water to give a 10 mM solution. Solutions containing 1, 2, 4 and 6 m-mole per litre were prepared by dilution and stored in the dark. They were found to be stable for one year.

Development of micro technique

Macro method for human serum

0.1 ml. fresh serum was incubated for 30 min. at 37° C with 1 ml. of buffered substrate. The reaction was terminated by adding 10 ml. 0.02 N NaOH and the absorbancy of the solutions read on a Unicam SP.600 spectrophotometer. 0.05 ml. concentrated HCl was added and the absorbancy of the reagents read. The quantity of p-nitrophenol liberated was determined from a calibration curve prepared from p-nitrophenol standards treated in an identical manner. The results were expressed in "millimole units" defined as the phosphatase activity which will liberate 1 m-mole of nitrophenol per litre of serum per hour (Bessey et al., 1946).

Micro method for human serum

The volumes used in the macro method were reduced by a factor of ten and the absorbancy of the final solution read on a Beckman/Spinco spectro-colorimeter.

(a) Measurement of p-nitrophenolate

The Beckman/Spinco spectro-colorimeter measures the

absorbancy at wavelengths from 400-650 mμ of 100 μl. of solution in a stationery cuvette with a light path length of 6.4 mm. The wavelength, of 15 mμ bandwidth, is selected with a wedge interference filter.

In the present study it was confirmed that the absorbancy maximum of the p-nitrophenol standard in alkali solution on the Unicam SP.600 spectrophotometer with a 1 cm. cell was 400 mμ (Fig. 4). The absorbancy-wavelength curve of the same solution in the Beckman/Spinco instrument (Fig. 4) appears to level off to a maximum value at 400 mμ. A comparison was also made of the absorbancy of 1.0 ml. of buffered substrate solution diluted with 10 ml. of 0.02 N NaOH. It was noted that in this case the absorbancy reading at 410 mμ was greater on the Beckman/Spinco instrument.

A series of standards was prepared on the macro scale and the calibration curves obtained on the two instruments were compared (Fig. 5). The absorbancy readings in the Beckman/Spinco were approximately half the values obtained on the Unicam SP.600 so that the sensitivity of the method was decreased by using the instrument with the shorter light path. A linear relationship existed between the p-nitrophenol concentration and the absorbancy readings on both instruments. As the greatest difference in sensitivity of the two machines was in the range where the absorbancy due to the colour of the serum was estimated it was confirmed that the assay of alkaline phosphatase activity in serum on a macro scale was within the limits of 2 per cent reproducibility whether the absorbancy

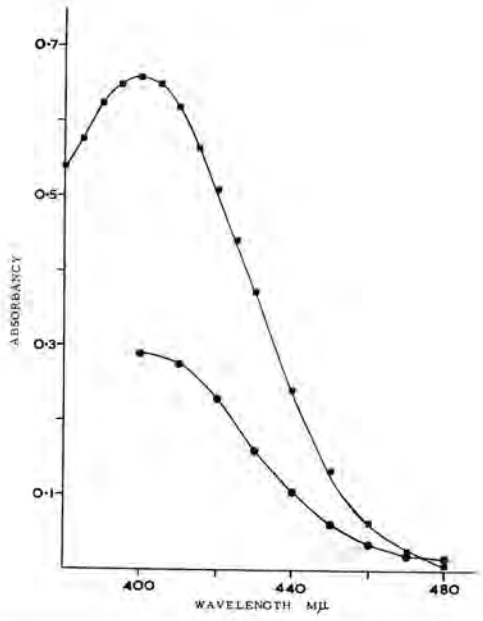


Fig. 4 Absorbancy-wavelength curve for p-nitrophenol in alkaline solution.
The solution contained 400 μm-mole p-nitrophenol in 11.1 ml. 0.02 N NaOH.

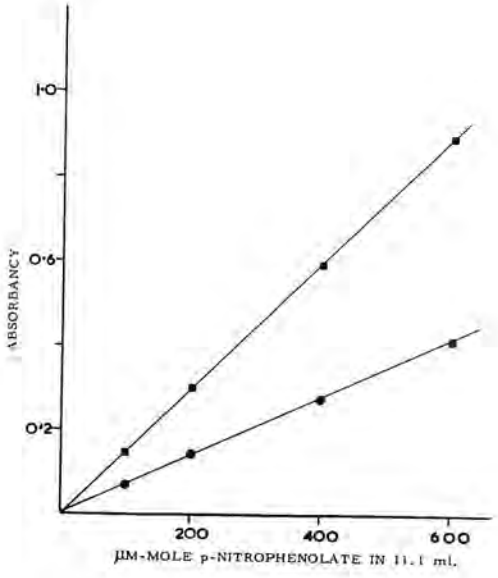


Fig. 5 Relationship between the absorbancy at 410 mμ and the concentration of alkaline p-nitrophenol solutions.
0.1 ml. of p-nitrophenol standard solution was added to 1 ml. of the buffered substrate solution for alkaline phosphatase assay and diluted with 10 ml. 0.02 N NaOH.

Key for Figs. 4 and 5

■ Absorbancy measured in a 1 cm. cell on a Unicam SP.600 spectrophotometer.

● Absorbancy measured in a 6.4 mm. cell on a Beckman/Spinco spectro-colorimeter.

readings were made in the Unicam SP.600 or on the Beckman/ Spinco (Table IV).

TABLE IV

Alkaline phosphatase activity estimated in human serum using macro and micro methods

Instrument used for absorbancy measurements		Alkaline phosphatase activity estimated m-mole/l/hr. Unicam SP.600	Beckman/ Spinco	Beckman/ Spinco
Serum no.	1	3.46	3.44	3.08 *
	1	3.46	3.44	3.08
	2	4.26	4.14	3.90
	2	4.16	4.08	3.90
	3	9.10	9.10	8.80
	3	8.94	8.98	8.64
	4	11.40	11.50	11.54
	4	11.40	11.52	-
Volume of serum incubated µl.		100	100	10
Duplicates reproducible within		2.5%	1.5%	1.9%

* The maximum difference between estimations made with 10 µl. and 100 µl. serum was 10 per cent of the mean value.

When absorbancy readings were made on the Beckman/Spinco it was observed that it was necessary to rinse the cell with at least two 100 µl. portions of the solution to obtain the true

values. This fault has been observed by other users of this instrument.

If at least 600 µl. of solution were available the Beckman/Spinco spectro-colorimeter was considered satisfactory for alkaline phosphatase activity measurements. The volumes used in the macro method could therefore be reduced by a factor of ten to give a micro method for 10 µl. of human serum.

Pipettes

Ten microlitre, "E-mil" brand, automatic constriction pipettes with a tolerance of 5 per cent were available but the Spinco precalibrated, polyethylene, ultramicro pipettes were considered more suitable for the measurement of a number of 10 µl. portions from different solutions. One pipette can be used for all 10 µl. measurements if it is rinsed three times with the solutions to be sampled. As these pipettes are made of non-wettable material the danger of denaturation of protein at the surfaces was decreased. Absorbancy readings of a standard solution of p-nitrophenol measured with a Spinco pipette differed from those prepared with a 0.1 ml. pipette with a 2 per cent tolerance, by 2 per cent. The need, however, was for a pipette with a highly reproducible performance rather than one with a high degree of absolute accuracy. The absorbancy reading of a standard solution measured daily with a Spinco pipette diverged by < 2 per cent, but by 4.6 per cent when measured with a 0.1 ml. pipette (Table V).

This one pipette was therefore used throughout this work for all measurements of ten microlitres.

TABLE V

Absorbancy readings of a solution of p-nitrophenol when the volume of standard used was measured daily with pipette (a) 0.1 ml. (glass) (b) 10 µl. (polyethylene)

Day	Absorbancy of solution prepared with pipette a	b
1	0.283	0.283
2	0.282	0.288
3	0.278	0.283
4	0.274	0.288
5	0.276	0.286
6	0.280	0.286
7	0.287	0.288
8	0.285	0.286
Mean	0.281	0.286
Range	0.274-0.287	0.283-0.288
Variability	4.6%	1.7%

Test tubes

Containers of non-wettable material with a capacity greater than 0.4 ml. were not available. Glass tubes 2 in. x 5/16 in., of 1.4 ml. capacity, were used. In order that the small volumes used reached, and remained, in the bottom of such tubes a perfectly grease-free surface was necessary. Accordingly, throughout this work, all glass tubes were soaked overnight in "pyroneg" detergent to remove any protein present and then in chromic acid cleaning solution prepared with concentrated sulphuric acid. The complete removal of all chromic

ions was ensured by rinsing the tubes ten times in tap water and three times in deionised water.

Accurate and reproducible results were achieved in the micro estimations only if this cleaning procedure was carried out.

Assessment of skill

On the macro scale, duplicate estimations of alkaline phosphatase activity in human serum had a variability of 2 per cent. Estimations were carried out on a micro scale until sufficient skill was acquired to produce duplicate analyses differing by less than 2.5 per cent. The assays of activity on the micro scale carried out simultaneously with those on the macro scale differed from them by approximately 10 per cent (Table IV). This difference was attributed to the different order of mixing of serum with buffered substrate necessitated by the difference in the two techniques and it emphasized the need for standardisation of every detail of enzyme assay methods.

Alkaline phosphatase activity values in normal adult serum

The serum analysed in these preliminary tests were received for routine alkaline phosphatase determinations in the Biochemical Laboratory of the Royal Infirmary, Edinburgh, where the results were given in King-Armstrong units with a normal range of 3 to 13 units. The normal range found in m-mole units for fifty adult sera was 1.8 to 7 units. Sigma give the normal range, estimated in glycine buffer, as 0.8 to 2.3 m-mole units. However, Lowry et al. (1954) observed that when 2-amino-2-methyl-1-propanol was used as the buffer, as in the

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experiments reported here, the measured activity was approximately doubled.

Proposed method

10 µl. of rat heart tissue homogenate was analysed by the micro method for human serum.

(b) Development of the method for rat heart tissue

Rat left ventricular muscle was homogenised in 0.1 M 2-amino-2-methyl-1-propanol pH 10.0. With a homogenate of heart tissue containing 132 µg. wet tissue in 10 µl. it was verified that a linear relationship existed between the amount of substrate transformed and the time of incubation over the range 0 to 60 min. (Fig. 6). Using a 30 min. incubation period a linear relationship was shown to exist between the amount of tissue incubated and the amount of substrate transformed from 5 to 100 µg. dry weight of tissue (Fig. 7). The stability of alkaline phosphatase activity in homogenates was demonstrated by assaying two homogenates when freshly prepared and after storing at 4° C for two days. The percentage loss in alkaline phosphatase activity determined was within the 2 per cent limit of reproducibility for the method (Table VI).

TABLE VI

Stability of alkaline phosphatase activity in homogenates of rat heart tissue stored at 4° C

Wet weight of tissue in 1 ml. homogenate *	Alkaline phosphatase activity mole/kg. dry weight/hr. estimated on		Loss in activity
mg.	day 1	day 3	%
35.0	1.10	1.10	nil
34.2	1.04	1.02	2

* Tissue homogenised in 0.1 M 2-amino-2-methyl-1-propanol.

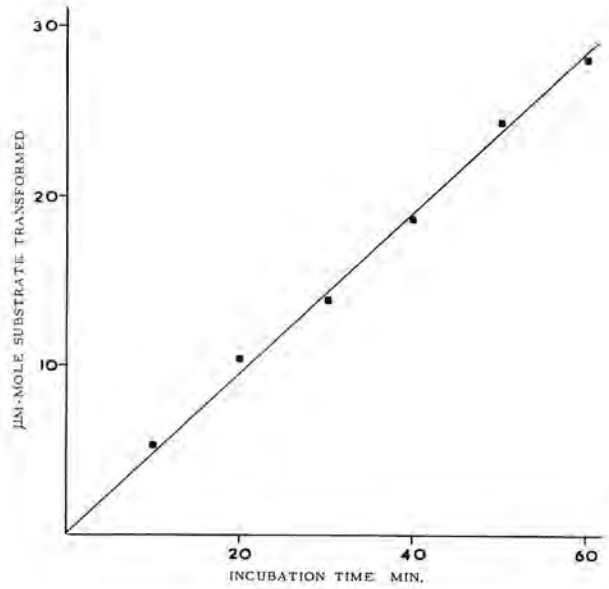

Fig. 6 Relationship between the quantity of p-nitrophenyl phosphate transformed and the time of incubation of rat heart tissue in alkaline phosphatase assay.

The 10 µl. of homogenate incubated contained 132 µg. wet weight of tissue.

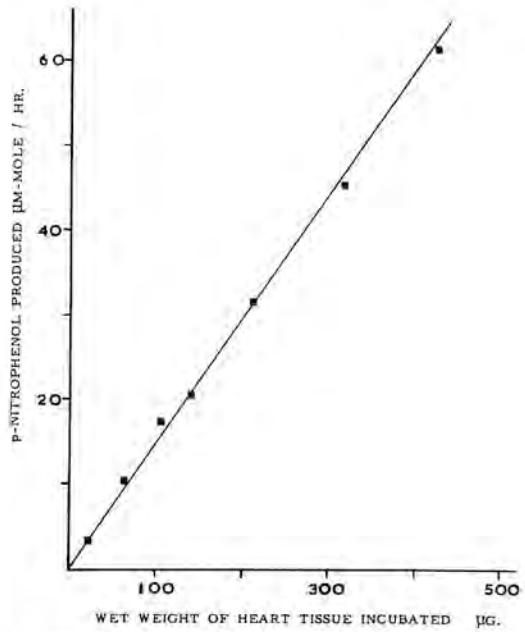

Fig. 7 Relationship between the rate of transformation of p-nitrophenyl phosphate and the wet weight of rat heart tissue incubated in alkaline phosphatase assay.

(c) Application of the method to freeze-dried sections

Method of analysis

Sections of 30 to 70 µg. dry weight were weighed and placed in dry tubes. When all the sections for analysis were collected 0.05 ml. 1 M buffer pH 10 was carefully added. The tubes were stoppered and stored at 4° C until required. To 10 µl. of each of the standard solutions of p-nitrophenol and to 10 µl. of water 0.05 ml. of 1.0 M buffer was also added. All the tubes were then placed in a water bath at 37° C and 50 µl. µl. warm substrate solution added to each tube with gentle mixing at 30 sec. intervals. After 30 min. 1 ml. 0.02 N NaOH was added, with immediate mixing, to each tube in turn. The absorbancy of the solutions at 410 mµ was read immediately. 5 µl. concentrated HCl was added to each solution and the absorbancy of the reagent blanks subtracted.

A homogenate of the tissue of the left ventricle apex was made immediately after killing a rat. Freeze-dried sections of the same left ventricle were prepared as described previously. The alkaline phosphatase activity estimated in freeze-dried sections was greater than that in homogenates of the same tissue (Table VII). Lowry et al. (1954) also made this observation when analysing brain tissue and attributed the increase to better disruption of the tissue.

The coefficient of variation of the alkaline phosphatase activity estimations made in six freeze-dried sections was 5.6 per cent. Twelve 10 µl. portions of a homogenate analysed in an identical manner gave a coefficient of variation of the

analysis of 3.1 per cent. The increase in variability of the activity estimated in freeze-dried sections was attributed to variation in the cellular composition of the sections. Alkaline phosphatase activity shown by the histochemical method was greatest in the adventitia of the coronary arteries (Fig.40, p.154) and the proportion of vascular tissue present varied in different sections.

TABLE VII

A comparison of alkaline phosphatase activity estimated in homogenates and in freeze-dried sections prepared from the same rat heart tissue

Rat weight	Alkaline phosphatase activity mole/kg. dry weight/hr. estimated in	
g.	Homogenates≠	Freeze-dried sections##
248	0.590	0.752 (4)*
205	0.607	0.721 (6)
248	0.599	0.750 (6)

* Figures in brackets denote the number of sections analysed.
≠ Homogenates were stored for 24 hr. at 4° C.
Freeze-dried sections were stored for 24 hr. at -20° C, under vacuum.

The mean alkaline phosphatase activity estimated in freeze-dried sections on the third day after their preparation was 1.9 per cent less than that estimated on the second day (Table VIII) but the activity estimated on the fourth day had decreased by 9.5 per cent. Lowry et al. (1954) have shown that alkaline phosphatase activity remained constant in freeze-dried sections of brain after at least twenty-four hours'

exposure to room temperature. Whether the 9.4 per cent loss in enzyme activity found in this experiment after exposure of the sections to room air for the four hours required for the two previous analyses was due to the adsorption of moisture, oxygen or contaminating fumes from the air was not established. The conditions of exposure of the dried sections were standardised as far as possible by keeping the section holders in a box containing anhydrous calcium sulphate when not actually in use. The sections, however were still exposed to room air during weighing. The validity of comparisons of alkaline phosphatase activity estimations in heart tissue from different animals would therefore be improved if the sections of tissue were cut and freeze-dried together so that they could be weighed and analysed under identical conditions.

TABLE VIII

Stability of alkaline phosphatase activity in freeze-dried sections of rat heart tissue

Time of storage at -20° C. days	Alkaline phosphatase activity mole/kg. dry weight/hr.	Loss in estimated activity %
2	0.752 (4) *	
3	0.738 (4)	1.9
4	0.681 (6)	9.4

* Figures in brackets denote the number of sections analysed.

4. Estimation of glucose-6-phosphate dehydrogenase activity

The method for the estimation of glucose-6-phosphate dehydrogenase activity was based on that used by Lowry, Roberts, Schulz, Clow and Clark (1961) to analyse 0.1 µg. dry weight of rabbit retina, with a 2 µl. portion of substrate in 2-amino-2-methyl-1,3-propanediol pH 9 to 9.4. In the reaction catalysed by the enzyme, glucose-6-phosphate is oxidised to 6-phosphogluconic acid and the coenzyme, nicotinamide-adenine dinucleotide phosphate (NADP) is reduced:

glucose-6-phosphate + $NADP^+$ + $H_2O \rightleftharpoons$ 6-phosphogluconic acid + NADPH + H^+

The reduced coenzyme formed was measured by its fluorescence in alkali solution.

The activity of this enzyme was determined at different pH by Glock and McLean (1953) but they showed that the pH optimum varied with the buffer used. They found maximum activity of the rat liver enzyme in glycylglycine buffer at pH 7.6, and in veronal buffer at pH 8.5. Kissane (1961) confirmed that maximum activity of the enzyme in rat kidney was found at pH 9.4 in 2-amino-2-methyl-1,3-propanediol.

The rate of reduction of NADP is often measured by the change in the absorption spectra which occurs at 340 mµ. However, Lowry, Roberts and Kapphahn (1957) have used the fluorescent properties of the dinucleotides to determine separately the oxidised and reduced forms in the same solution at concentrations of 0.01 µm-mole/ml., a thousandth of that required for colorimetry. Reduced NADP can be estimated by its native fluorescence in weak alkali solution from 0.1 µm-mole/ml. to

10 µm-mole/ml. For more dilute solutions, of 0.003 to 3 µm-mole/ml., it is oxidised to NADP and the induced fluorescence in strong alkali measured. These procedures of Lowry et al. (1957) were adapted from those of Kaplan, Colowick and Barnes (1951). As the most sensitive methods available were to be developed the reduced coenzyme was measured by its fluorescence in alkaline solution in this study.

Reagents

Stock Substrate solution 11 mM of the disodium salt of D-glucose-6-phosphate $C_6H_{11}O_9P\ Na_2.4\ H_2O$ Mol. Wt. 376.

0.0414 g. of glucose-6-phosphate was dissolved in 10 ml. of water. The solution was divided into 1 ml. portions in plastic cups, and stored at -20° C.

Stock buffer solution 0.2 M 2-amino-2-methyl-1,3-propanediol, 1 mM EDTA, pH 9.3

2.1028 g. 2-amino-2-methyl-1,3-propanediol, dissolved in 75 ml. water, was adjusted to pH 9.3; 0.0372 g. Na_2EDTA was added, and the volume made up to 100 ml.

Stock NADP solution 9.9 mM sodium salt of nicotinamide-adenine dinucleotide phosphate $C_{21}H_{27}N_7O_{17}P_3Na.6\ H_2O$ Mol. Wt. 873, 99 per cent pure.

26.2 mg. NADP was dissolved in 3 ml. water. The solution was divided into 0.3 ml. lots in polyethylene tubes and stored at -20° C.

The above solutions were prepared fresh every six weeks.

Buffered substrate solution

A solution of 0.1 M buffer, 5 mM substrate and 0.9 mM NADP was made by mixing 1 ml. of stock buffer solution with 1 ml. of stock substrate solution and 0.2 ml. stock NADP solution immediately before use.

Standard NADPH solution 800 µm-mole/ml. of the reduced form of nicotinamide-adenine dinucleotide phosphate $C_{21}H_{26}N_7O_{17}P_3Na_4.4\ H_2O$ Mol. Wt. 905, 90 per cent pure.

A standard solution of NADPH containing 8 µm-mole in 10 µl. was prepared when required by dissolving 240 µg. NADPH in 0.3 ml. 0.1 M 2-amino-2-hydroxymethylpropane-1,3-diol, pH 8.5. The weighed portion of NADPH was stored dessicated, in the dark, until immediately before use.

Carbonate buffer pH 10.9 0.05 M sodium carbonate, 0.005 M sodium hydrogen carbonate

This buffer was prepared by dissolving 1.3249 g. anhydrous Na_2CO_3 and 0.1050 g. $NaHCO_3$ in 250 ml. water.

Stock quinine solution 60 mg./100 ml. quinina B.P.C.

60 mg. quinine was dissolved in 100 ml. 0.1 N H_2SO_4 and stored in the dark.

Quinine solution 6 µg./ml.

1 ml. of stock quinine solution was diluted to 100 ml. with 0.1 N H_2SO_4 each week.

Proposed method

10 µl. of an aqueous homogenate of rat left ventricle apex was incubated with 100 µl. of buffered substrate solution for 30 min. 1.2 ml. of 0.01 N NaOH was added to terminate the

reactions, destroy excess NADP and develop the fluorescence of NADPH.

(a) Measurement of NADPH

Fluorescence measurements were made on the Aminco-Bowman spectrophotofluorometer, supplied by the American Instrument Co., Inc., Silver Spring, Md., U.S.A. In this instrument the activating light from a xenon lamp is dispersed by a grating into monochromatic radiation before reaching the sample. A similar monochromator disperses the fluorescent light emitted by the sample. The number 3 slit arrangement, as described in the manual for the instrument, was used throughout the present work. Although a current stabiliser was included in the instrument the intensity of the xenon lamp varied with fluctuations in the mains voltage. The intensity of light from the lamp also decreases with age. A simple method of ensuring that readings were reproducible from week to week was proposed by Sprince and Rowley (1957) who used a standard quinine solution to check the instrument. The fluorescence maximum for 0.005 to 10 µg./ml. quinine in 0.1 N H_2SO_4 is 450 mµ, with an activation maximum of 365 mµ and the corresponding values for the fluorescence of the dinucleotides in alkali are 460 mµ and 360 mµ respectively. The two solutions will thus be affected similarly by changes in light intensity.

For each experiment where fluorescence was measured the fluorometer was adjusted to give a constant reading with a freshly prepared standard solution of quinine. While reading

a series of unknown solutions the fluorescence given by the quinine solution was continually checked. A preliminary experiment showed that the fluorescence intensity of a solution of 0.05 µg./ml. quinine, exposed to light of 360 mµ for the time required to take twenty readings did not differ from that of unexposed portions of solution.

As NADPH had not arrived from the manufacturers at the beginning of this experiment the initial observations on the fluorescence of the reduced dinucleotides were made using NADH. Lowry et al. (1957) have shown that if interfering cations such as Mg^{++} were removed by EDTA, NADH and NADPH in dilute alkali gave the same fluorescence per mole and had indistinguishable fluorescence spectra.

Solutions containing known amounts of NADH were prepared in 0.1 M buffer, pH 9.3, 0.5 mM EDTA, and their fluorescence in 0.01 N NaOH studied. The fluorescence spectrum of NADH had a maximum at 465 mµ with 360 mµ as the activating wavelength. The induced fluorescence of NADP in 6 N NaOH had a maximum at 460 mµ (Fig. 8). A small peak caused by light scatter in the instrument was observed at 410 mµ when 360 mµ was the activating wavelength. The fluorescence intensity was proportional to the quantity of NADH in 1.31 ml. from 29.8 µm-mole to 0.258 µm-mole (Fig. 9). Intensity readings on the lower multiplier ranges of the photomultiplier microphotometer were unstable. On the 0.01 multiplier setting, when 10 units on the 0 to 100 transmission scale were considered as the minimum useful reading, the lowest concentration of NADH which could be read by its

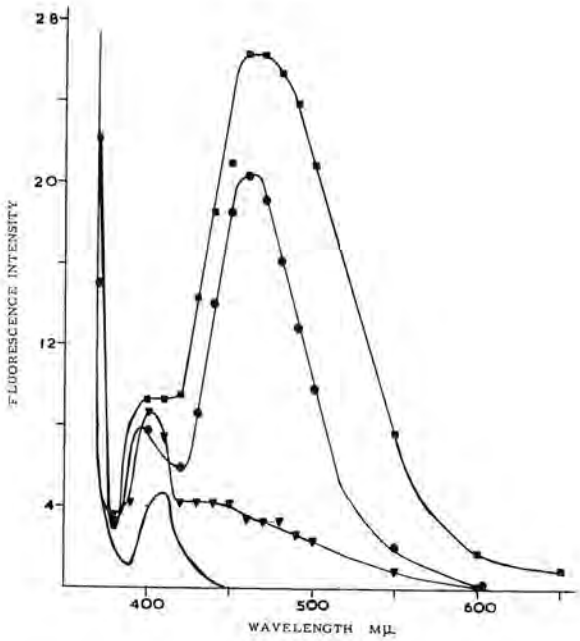

Fig. 8 Fluorescence spectra of NADH and NADP.
Activating wavelength 360 mμ.
▼ 1.21 ml. 0.01 N NaOH added to 100 µl. buffer, 0.1 M 2-amino-2-methyl-1 , 3-propanediol, 0.5 mM EDTA, pH 9.3.
■ 2.58 µm-mole NADH in the above solution.
——water.
● 0.3 µm-mole NADP dissolved in 100 µl. water was heated with 200 µl. 9 N NaOH for 10 min. at 60° C then diluted with 1 ml. water.

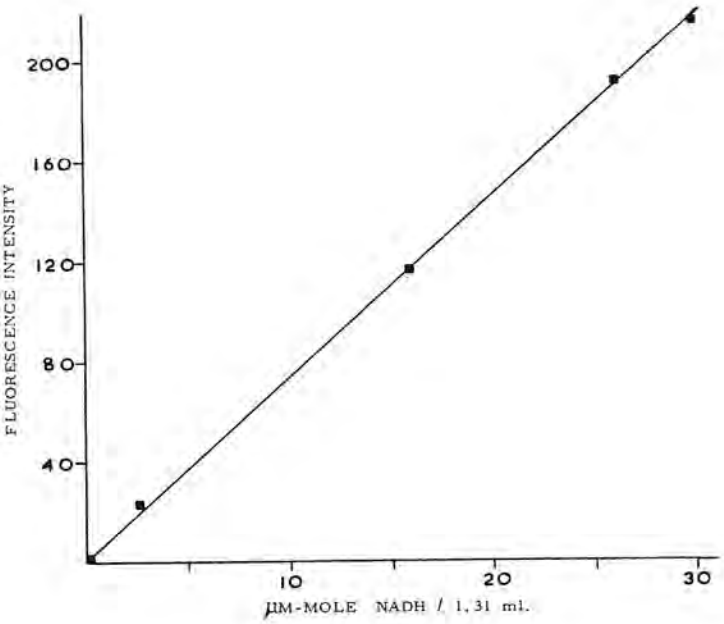

Fig. 9 Relationship between fluorescence intensity and NADH concentration.
NADH in 100 µl. buffer, 0.1 M 2-amino-2-methyl-1, 3-propanediol, 0.5 mM EDTA, pH 9.3, and 1.21 ml. 0.01 N NaOH added.

native fluorescence in weak alkali was 1.23 µm-mole/1.31 ml. By the same criteria the lowest concentration of NADP which could be read by the induced fluorescence in strong alkali was 0.15 µm-mole/1.31 ml. As readings made on different multiplier settings of the fluorometer were not always directly comparable it was noted that the upper limit of NADH concentration which could be read on the 0.01 multiplier was 12.0 µm-mole/1.31 ml.

Later, when NADPH became available it was verified that when standard solutions were incubated with 100 µl. buffered substrate and diluted with 1.2 ml. carbonate buffer pH 10.9 the fluorescence intensity was directly proportional to the concentration of NADPH in the range 2 to 16 µm-mole/1.31 ml.

(b) Development of the method for rat heart tissue

The stock solution of NADP used initially was 3 mM so that 27.3 µm-mole NADP were present in 100 µl. of buffered substrate solution. With a homogenate of rat heart tissue containing 100 µg. dry weight in 10 µl. the reaction velocity decreased after 45 min. incubation when 9.1 µm-mole NADP were reduced. The quantity of NADP in the 0.1 ml. of buffered substrate solution was increased to 54.5 µm-mole and 81.9 µm-mole. With 81.9 µm-mole NADP present the reaction rate produced by 57 µg. dry weight was constant for 0 to 60 min. (Fig. 10). The quantities of NADPH produced were plotted as fluorescence intensity readings but the line drawn through the points did not go through zero when the fluorescence of the homogenate incubated without NADP was subtracted. This fluorescence blank value did not increase with incubation time. The fluorescence

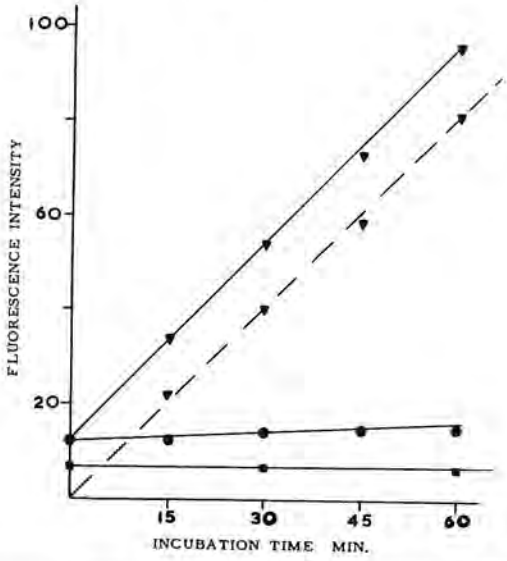

Fig. 10 Relationship between the fluorescence intensity produced and the time of incubation of heart tissue in glucose-6-phosphate dehydrogenase assay. 1.2 ml. 0.01 N NaOH was added to read the fluorescence produced when 57 µg. dry weight of rat heart was incubated with 100 µl. of:-
1. ▼ complete buffered substrate solution i.e. 0.1 M 2-amino-2-methyl-1,3-propanediol buffer pH 9.3, 0.5 mM EDTA, 5 mM glucose-6-phosphate, 0.9 mM NADP.
2. ■ buffered substrate solution without NADP.
3. ● buffered substrate without glucose-6-phosphate.
– ▼ – Subtracting fluorescence in '3' from that in '1' gave the fluorescence due to the NADPH produced by the activity of glucose-6-phosphate dehydrogenase.

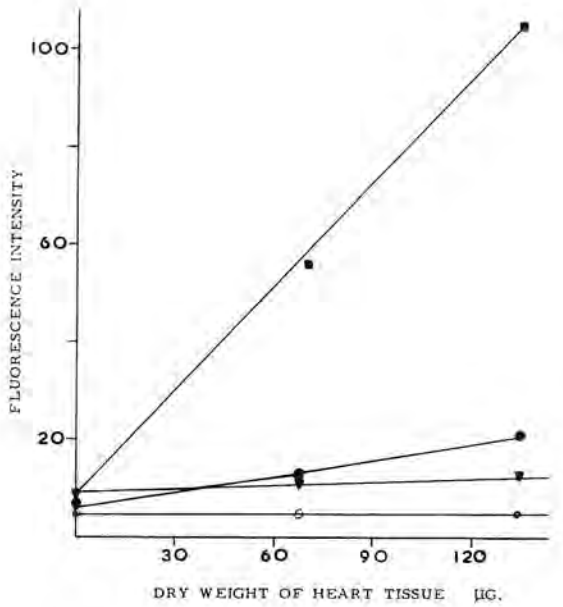

Fig. 11 Relationship between the fluorescence intensity produced and the weight of heart tissue incubated in glucose-6-phosphate dehydrogenase assay. Tissue incubated with 100 µl. of:-
■ complete buffered substrate solution pH 9.3 i.e. 0.1 M 2-amino-2-methyl-1, 3-propanediol, 0.5 mM EDTA, 5 mM glucose-6-phosphate, 0.9 mM NADP, for 30 min.
▼ complete buffered substrate solution for "zero time".
○ buffered substrate solution without NADP for 30 min.
● buffered substrate solution without glucose-6-phosphate for 30 min.
1.2 ml. carbonate buffer pH 10.9 was added to read the fluorescence produced.

produced by incubating 10 µl. of homogenate in a buffered substrate solution without glucose-6-phosphate was the greater and it increased with time (Fig. 10). When the appropriate value of this fluorescence blank was subtracted the line drawn through the points went to the origin. A dilution of a homogenate was prepared and the behaviour of the possible fluorescence blank values with increasing weight of tissue present were compared (Fig. 11). Incubating the homogenate in buffered substrate solution without NADP the blank value did not increase appreciably. Incubating the homogenate with the complete buffered substrate for "zero time" the blank value increased with increasing weight of tissue present. The greatest increase in the blank value however was that obtained by incubating the homogenates with buffered substrate solution prepared without glucose-6-phosphate. When this blank was subtracted from the fluorescence readings obtained by incubating the homogenates for 30 min. the values gave a straight line going through the origin. The reaction velocity was directly proportional to the weight of tissue incubated from 0 to 135 µg. dry weight.

In an attempt to find a means of eliminating this fluorescence blank the fluorescence intensity produced by incubating the homogenate with complete substrate at pH 6.5 to 10.8 was compared with that produced by incubating the tissue without glucose-6-phosphate at the different pH (Fig. 12). The pH optimum of the glucose-6-phosphate dehydrogenase was found near pH 8.8 and the reaction between heart tissue and NADP had an optimum

around pH 7.7. One possible explanation of the cause of this fluorescence blank is that sufficient endogenous substrate for some NADP-requiring enzyme with a pH optimum at 7.7 is present in the tissue to cause the production of small amounts of NADPH. The fluorescence produced in this way, in excess of that caused by the presence of tissue in the solution, was 5 to 10 per cent of the fluorescence produced by the NADPH resulting from the action of glucose-6-phosphate dehydrogenase.

The valid fluorescence blank reading for all glucose-6-phosphate dehydrogenase activity estimations by this method was therefore taken to be that value produced by incubating the tissue in buffered substrate without glucose-6-phosphate under otherwise identical conditions. As difficulties were foreseen in determining this blank value for freeze-dried sections the range of tissue weight which could be used in the assay was not determined with homogenates but with sections.

(c) Application of the method to freeze-dried sections

In a preliminary experiment in which sections were stored at 4° C in 0.2 M buffer in the period between weighing them and analysing them it was found that they did not remain in solution but rose to the surface and stuck to the sides of the tubes making accurate analyses impossible. As Lowry et al. (1956) have shown that moisture condensation on dry sections destroyed enzymes present, tissue kept in the dry state for a few hours was not stored at 4° C.

Method of analysis

After weighing them, five sections of 50 to 80 µg. dry weight were placed in dry 2 in. x $\frac{5}{16}$ in. tubes and stored in the

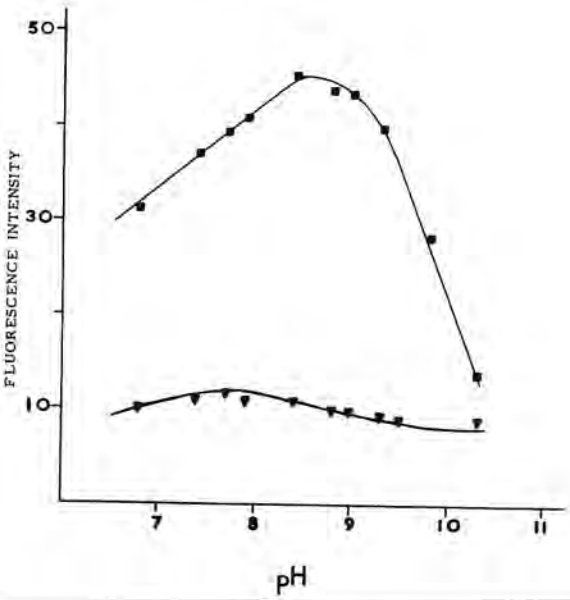

Fig. 12 Relationship between the fluorescence intensity produced and the pH of the buffered substrate solution used in glucose-6-phosphate dehydrogenase assay.
53 µg. dry weight of tissue incubated with 100 µl. of:-
■ complete buffered substrate solution i.e. 0.1 M 2-amino-2-methyl-1,3-propanediol, 0.5 mM EDTA, 5 mM glucose-6-phosphate and 0.9 mM NADP at different pH for 30 min.
▼ buffered substrate solution without glucose-6-phosphate at different pH for 30 min.
1.2 ml. carbonate buffer pH 10.9 was added to read the fluorescence produced.

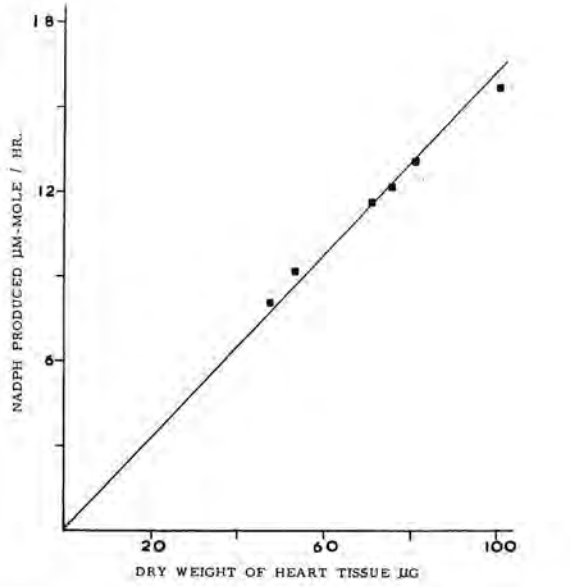

Fig. 13 Relationship between the quantity of NADPH produced and the weight of the freeze-dried sections incubated in glucose-6-phosphate dehydrogenase assay .
Tissue incubated with 100 µl. buffered substrate solution, 0.1 M 2-amino-2-methyl-1,3-propanediol pH 9.3, 0.5 mM EDTA, 5 mM glucose-6-phosphate, 0.9 mM NADP.

presence of anhydrous calcium sulphate desiccant at room temperature until required. 0.1 ml. of buffered substrate solution at 37° C was added to each of three sections at room temperature and the tubes were then incubated at 37° C for 30 min. 10 µl. of a standard solution of NADPH and 10 µl. of water were treated in an identical manner. After 30 min. 1.2 ml. carbonate buffer pH 10.9 was added with immediate mixing and the solutions were stored in the dark. The remaining two sections were incubated for 30 min. with 0.1 ml. of a solution prepared by mixing 0.5 ml. water with 0.5 ml. stock buffer and 0.1 ml. stock NADP. The reaction was terminated by adding 1.2 ml. carbonate buffer pH 10.9. The fluorescence intensity of the NADPH in the final solutions was read on the Aminco-Bowman spectrophotofluorometer. A quinine solution containing 0.05 µg./ml. was used to calibrate the fluorometer and readings were made with the meter multiplier set on 0.01.

The fluorescence produced by incubating freeze-dried sections with buffered substrate solution without glucose-6-phosphate also increased with increasing weight of tissue. This value was subtracted from the total fluorescence readings and the quantity of NADPH produced was calculated. A linear relationship was found between the weight of tissue incubated from 40 to 80 µg. and the quantity of NADPH produced (Fig. 13).

As five sections from each tissue was the maximum number it was possible to weigh within a reasonable time, they were selected within a narrow weight range so that the fluorescence blank values for the sections incubated with complete buffered

substrate solution were estimated as accurately as possible. The coefficient of variation for the assay method was 6.4 per cent when eight 10 µl. portions of homogenate were analysed and 2.7 per cent when the estimations were made in five freeze-dried sections.

It was verified that the glucose-6-phosphate dehydrogenase activity estimated in freeze-dried sections was equal to that estimated in an aqueous homogenate of the same tissue which had been stored for 24 hr. at -20° C (Table IX). As this enzyme was to be estimated on the first day after preparation of the freeze-dried sections, its stability in the sections of heart tissue was not further examined.

TABLE IX

A comparison of the glucose-6-phosphate dehydrogenase activity estimated in homogenates and freeze-dried sections prepared from the same rat heart tissue

Rat weight	Glucose-6-phosphate dehydrogenase activity mole/kg. dry weight/hr. estimated in	
g.	Homogenates+	Freeze-dried sections##
130	0.186	0.186 (5)*
151	0.214	0.216 (5)

* Figures in brackets denote the number of sections analysed.

\+ Homogenates stored for 24 hr. at -20° C.

Freeze-dried sections stored for 24 hr. at -20° C under vacuum.

5. Estimation of lactate dehydrogenase activity

The method for the estimation of lactate dehydrogenase activity in single rabbit nerve cell bodies (Lowry et al., 1957) was described with the details of the substrate concentrations required for kidney arterioles by Dubach and Recant (1960). They incubated 0.2 µg. of tissue with 20 µl. of substrate solution. In the reaction catalysed by the enzyme, pyruvate is reduced to L-lactate and the coenzyme NADH is oxidised to NAD.

$$\text{Na pyruvate} + \text{NAD} + H^+ \rightleftharpoons \text{Na L-lactate} + NAD^+$$

The NAD produced was measured by its fluorescence in strong alkali (Lowry et al., 1957).

Reagents

Stock buffer solution 0.1 M 2-amino-2-hydroxymethylpropane-1,3-diol (tris) pH 7.4.

3.028 g. tris were dissolved in water, adjusted to pH 7.4 with 1 N HCl, and the volume made up to 250 ml.

Stock substrate solution 1.65 mM sodium pyruvate, 0.1 M tris pH 7.4 Sigma reagent: $CH_3.CO.COONa$ 96 per cent pure.

0.0189 g. sodium pyruvate was dissolved in 99.8 ml. 0.1 M tris and 0.2 ml. 1 N NaOH added to adjust the pH to 7.4. This reagent was stored in glass at 4° C.

The above solutions were prepared fresh every six weeks.

Stock NADH solution 11 mM disodium β-dihydro-nicotinamide-adenine dinucleotide

Sigma reagent $C_{21}H_{27}O_{14}N_7P_2Na_2.2.5\ H_2O$ Mol. Wt. 754, 96 per cent pure

8.64 mg. was dissolved in 1 ml. 0.1 M tris pH 8.5. The solution was divided into 0.25 ml. lots in polyethylene tubes and stored at -20° C. This reagent was prepared every two weeks.

Buffered substrate solution

Immediately before use 0.4 ml. stock NADH solution was added to 4 ml. stock substrate to give a solution of 1.0 mM NADH, 1.5 mM sodium pyruvate and 0.1 M tris pH 7.4.

Standard NAD solution 7000 µm-mole/ml. β-nicotinamide-adenine dinucleotide

Sigma reagent $C_{21}H_{27}O_{14}N_7P_2.2.5\ H_2O$ Mol.Wt. 708.3, 99 per cent pure

A standard solution of NAD containing 70 µm-mole in 10 µl. was prepared when required by dissolving 500 µg. NAD in 0.1 ml. water. The weighed quantity of NAD was stored dessicated and dark until immediately before use.

6.6 N NaOH was stored in polythene.

0.42 N HCl was prepared.

Proposed method

10 µl. of an aqueous homogenate of rat left ventricle muscle was incubated for 30 min. with 100 µl. buffered substrate in 1 in. x $\frac{5}{16}$ in. pyrex tubes. 100 µl. 0.42 N HCl was added to terminate the reaction and destroy excess NADH. After centrifuging for 15 min., 20 µl. of the supernatant was added to 200 µl. 6.6 N NaOH in 2 in. x $\frac{5}{16}$ in. tubes and mixed at once. The fluorescence of NAD was developed at 60° C for 10 min. 1 ml. of water was then added and the fluorescence intensity of the NAD

produced read in the Aminco-Bowman spectrophotofluorometer.

(a) Measurement of NAD

It had been confirmed (Fig. 8, p73) that the fluorescence spectrum of the oxidised dinucleotides in strong alkali had a maximum at 460 mμ. Standard solutions of NAD were treated by the proposed method. The fluorescence intensity readings were proportional to the quantity of NAD present in the 1.22 ml. of the final solution from 0.4 μm-mole to 16.4 μm-mole (Fig. 14): the equivalent of 4.5 to 180 μm-mole of NAD in the incubated solution.

(b) Development of the method for rat heart tissue

When aqueous homogenates, containing 100, 10 and 1 μg. wet weight of tissue in 10 μl. were incubated with buffered substrate, it was found that, under the conditions of the proposed method, 1 μg. wet weight of tissue oxidised 11.7 μm-mole of the 100 μm-mole NADH present. A series of dilutions of a homogenate were prepared and the upper limit of the range for which the fluorescence intensity produced was proportional to the weight of tissue incubated was established as 5.8 μg. wet weight when 58 μm-mole of NADH were oxidised (Fig. 15). As this represented 1.4 μg. dry weight, which could not be measured accurately on Oertling's decimicrobalance, the proposed method was adjusted in an attempt to increase the weight of tissue which could be analysed.

The incubation period was shortened although this increased the percentage error in the timing. A series of dilutions of a homogenate were incubated for 10 min. and the quantity of NAD

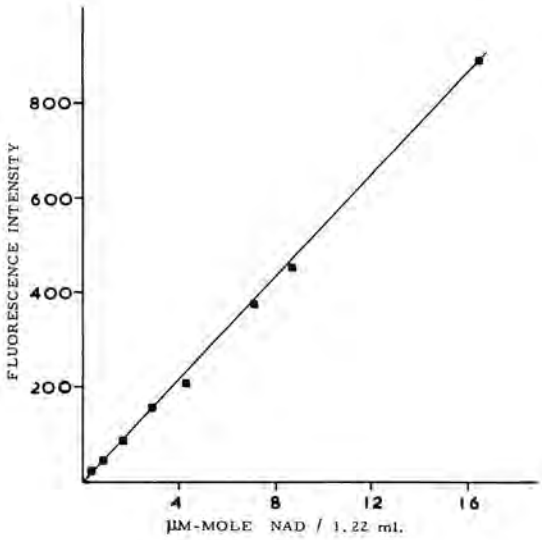

Fig. 14 Relationship between fluorescence intensity and concentration of NAD.

10 µl. of NAD standard solution was mixed with 100 µl. buffered substrate solution i.e. 0.1 M tris pH 7.4, 1.5 mM Na pyruvate, 1.0 mM NADH. 100 µl. 0.42 N HCl was added to destroy NADH and 20 µl. of this mixture was incubated at 37° C with 200 µl. 6.6 N NaOH for 30 min. to develop the fluorescence of NAD and then diluted with 1 ml. of water to read the fluorescence intensity.

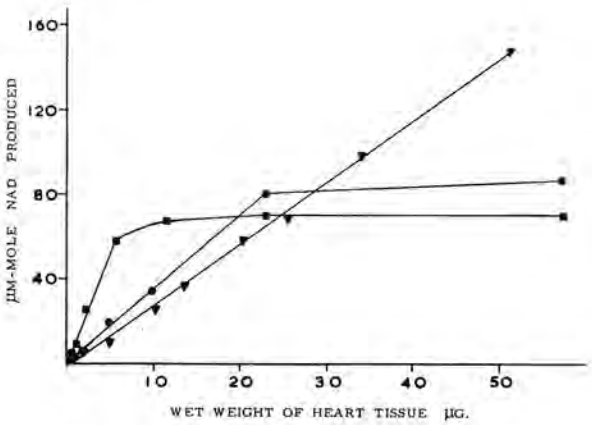

Fig. 15 Relationship between the quantity of NAD produced and the wet weight of heart tissue incubated in lactate dehydrogenase assay.

Tissue incubated with:-

■ 100 µl. buffered substrate solution i.e. 0.1 M tris pH 7.4, 1.5 mM Na pyruvate, 1 mM NADH, for 30 min.

● 100 µl. buffered substrate for 10 min.

▼ 200 µl. buffered substrate for 5 min.

produced was proportional to the wet weight of tissue incubated from 0 to 23 µg. A maximum of 80 µm-mole NADH were oxidised (Fig. 15) by this weight range.

In these preliminary experiments reported the fluorescence of the NAD was developed at 60° C for 10 min. The fluorescence can also be developed at 38° C for 30 min. or at room temperature for 1 hr. (Lowry et al., 1957). In the absence of a bath thermostatically controlled at 60° C no time was saved by the shorter heating period. The fluorescence of NAD was therefore developed at 37° C for 30 min. in all the remaining experiments on the estimation of lactate dehydrogenase activity.

As the maximum weight of sample which could be analysed was still too small to be weighed accurately the volume of substrate solution with which it was incubated was doubled and the incubation period shortened to 5 min. A constant value for the activity measured was found when 0 to 50 µg. wet weight (0 to 12 µg. dry weight) was incubated in 200 µl. buffered substrate solution for 5 min. The maximum value of the NADH oxidised by this weight range was 138 µm-mole of the total 200 µm-mole present (Fig. 15).

As the reaction velocity measured in this assay method was not constant up to 60 min. the results of the lactate dehydrogenase estimations were expressed as moles of substrate transformed by a kilogram of tissue in one minute. With a homogenate containing 10 µg. dry weight in 10 µl. it was verified that the amount of NAD produced was proportional to the time of incubation within the five minute period (Fig. 16).

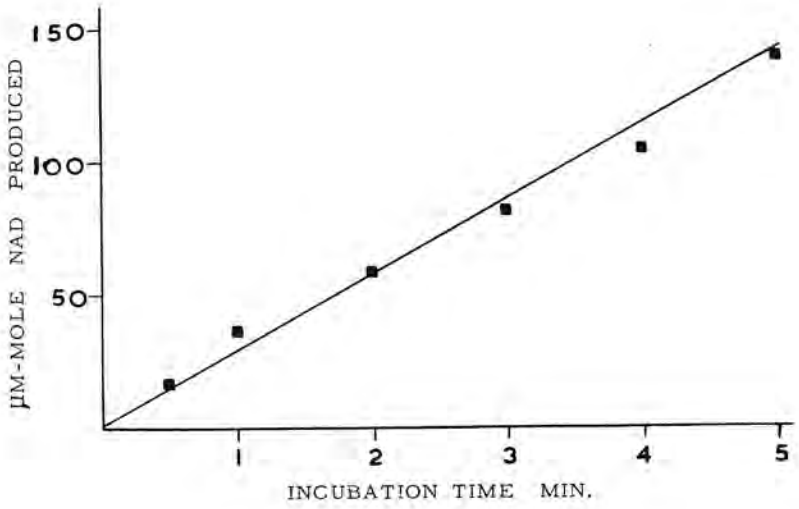

Fig. 16 Relationship between the quantity of NAD produced and the time of incubation of heart tissue in lactate dehydrogenase assay.

10 µg. dry weight of tissue was incubated with 200 µl. buffered substrate solution i.e. 0.1 M tris pH 7.4, 1.5 mM Na pyruvate, 1 mM NADH.

The value of the fluorescence produced by incubating this small quantity of tissue for "zero time", or in the absence of pyruvate, did not differ from the reagent blank.

Aqueous homogenates stored at -20° C often required rehomogenisation before reproducible analyses could be made. An experiment was planned to determine if lactate dehydrogenase was stable in any media at 4° C. Three homogenates of approximately 50 mg. wet weight/ml. were prepared from the same rat heart in water, 1 per cent NaCl and 0.1 M tris pH 7.4 respectively. They were each divided into two portions one of which was analysed immediately then stored at 4° C, and the other portion was stored at -20° C. Those stored at 4° C and at -20° C were analysed on the two following days. The homogenates stored at -20° C were thoroughly mixed after thawing. The activity of lactate dehydrogenase found in the different portions is shown in Table X.

TABLE X

Activity of lactate dehydrogenase in homogenates of rat heart tissue prepared in different media and stored at 4° C or -20° C

Homogenate prepared in	Estimated activity of lactate dehydrogenase mole/kg. dry weight/min. in homogenates				
	stored at 4° C for			stored at -20° C for	
	0 hr.	24 hr.	48 hr.	24 hr.	48 hr.
Water	3.02	2.04	1.29	2.40	3.06
1% NaCl	3.48	3.70	3.76	1.29	2.04
0.1 M tris pH 7.4	3.54	3.36	3.22	3.84	3.54

The variability in the duplicate estimations made with homogenates prepared in water and stored at -20° C indicated that they required rehomogenisation. The enzyme was unstable in water at 4° C, slightly unstable in tris pH 7.4 at 4° C and very unstable in 1 per cent NaCl at -20° C. The value found for the activity of the enzyme in fresh homogenates prepared in 0.1 M tris pH 7.4 and 1 per cent NaCl was 16.6 per cent greater than that for homogenates prepared in water. Homogenates used after this time therefore were prepared in 0.1 M tris pH 7.4 and stored at -20° C.

The maximum weight of tissue which could be analysed by the modified method was therefore calculated to be decreased from 50 µg. to 41 µg. wet weight or 10 µg. dry weight.

(c) Application of the method to freeze-dried sections

The modification of the proposed method made it possible to estimate the lactate dehydrogenase activity in less than 10 µg. dry weight of heart tissue. The performance of the decimicro-balance in this weight range could only be assessed by weighing tissue as all other materials were invisible to the eye in such quantities.

Portions of freeze-dried sections were weighed and an attempt made to estimate the enzyme activity in less than 10 µg. dry weight. The greatest difficulty encountered was that these minute pieces of tissue would not stay in solution. The sample floated on the surface of the liquid and the force of air required to expel the last few drops from a 0.2 ml. pipette calibrated to contain 0.2 ml. often blew the tissue up the side

of the tube where it stuck firmly. The use of an Ostwald-Folin pipette with a wide bore tip eliminated the latter difficulty but the values found for the activity of lactate dehydrogenase in dry sections were 50 per cent of those found in homogenates of the same tissue and were very variable.

The possibility that the dried tissue had to be in contact with an aqueous solution for a finite time before the active form of the enzyme became soluble was investigated.

Method of analysis

Sections < 6 µg. were weighed and transferred on a hair point to 10 µl. portions of 0.1 M tris pH 7.4 at the bottom of 1 in. x $\frac{5}{16}$ in. test tubes. Each tube was stoppered to avoid evaporation and contamination from the atmosphere, and held stationary to prevent the solution and section from spreading over the inner surface of the tube. The sections were stored in the buffer at 4° C for 90 min. before the analyses were performed. The tubes were then allowed to reach room temperature. At thirty second intervals 0.2 ml. buffered substrate solution at 37° C was added to each tube and it was placed in the water bath at 37° C. Exactly five minutes later 0.2 ml. 0.42 N HCl was added with rapid mixing. After centrifuging for 15 min., 20 µl. of the supernatant was added to 0.2 ml. 6.6 N NaOH with immediate mixing. The fluorescence of the NAD was developed at 37° C for 30 min. The solution was immediately diluted with 1 ml. of water and the fluorescence intensity read on the Aminco-Bowman spectrophotofluorometer. 10 µl. of standard solutions of NAD and 10 µl. of 0.1 M tris pH 7.4 were

treated in an identical manner. A quinine solution containing 0.146 µg./ml. was used to calibrate the fluorometer and the readings were made on the 0.03 meter multiplier of the instrument.

The activity estimated in sections treated in this way was similar to that found in stable homogenates of the same tissue (Table XI). A coefficient of variation of 4.7 per cent was established for the analyses of freeze-dried sections of 3 to 7 µg. dry weight. No loss in lactate dehydrogenase activity was found when freeze-dried sections were stored under vacuum at -20° C for 48 hr. (Table XI).

TABLE XI

A comparison of the lactate dehydrogenase activity estimated in homogenates and freeze-dried sections prepared from the same rat heart tissue

Time of storage at -20° C	Rat weight	Lactate dehydrogenase activity mole/kg. dry weight/min. estimated in	
hr.	g.	Homogenates+	Freeze-dried## sections
24	144	4.32	4.64 (10)*
24	185	3.96	3.86 (7)
48	185	4.36	4.24 (10)

* Figures in brackets denote the number of sections analysed.

\+ Homogenates prepared in 0.1 M tris pH 7.4.

\## Sections stored under vacuum at -20° C.

The actual values of the activity determined in homogenates and sections after storing them for 48 hr. were 10 per cent greater than those determined after 24 hr. As the difference was observed in both preparations of the tissue this suggested that some factor in the method of analysis was responsible for the apparent increase in activity. A very slight alteration in the standard NAD solution or in the incubation solution, time or temperature might have produced this effect. Although every precaution was taken to protect the dinucleotides required as the coenzyme and the standard from destructive agents as they are affected by light and heat their protection cannot be absolute. The final fluorescence developed is also affected by light and heat and on days when the fluorometer was unstable it was impossible to ensure that the readings were taken with the minimum exposure of the solution to activating light. The time and temperature of incubation were already controlled as rigidly as was practically possible. Further investigation of the factors responsible for the difference found in this experiment was considered unprofitable. The observation emphasised the necessity for analysing tissues whose enzyme activities were to be compared together so that they were analysed in the presence of identical conditions of light heat and laboratory atmosphere.

6. Estimation of malate dehydrogenase activity

The method for the estimation of malate dehydrogenase was developed by Strominger and Lowry (1955) for the measurement of the enzyme activity in brain tissue. In the reaction

catalysed by the enzyme malate is oxidised to oxaloacetate in the presence of the coenzyme nicotinamide-adenine dinucleotide (NAD) which is reduced.

$$Na_2L\text{-malate} + NAD^+ \rightleftharpoons Na_2\text{oxaloacetate} + NADH + H^+$$

The rate of reduction of NAD was determined by the change in absorbancy of the solution at 340 mμ. The NADH produced can also be measured by its fluorescence in weak alkali (Lowry et al., 1957). The latter technique was used in the present work.

Reagents

Stock buffer solution 0.2 M 2-amino-2-methyl-1-propanol pH 10.5 containing 0.1 per cent albumin

1.7828 g. 2-amino-2-methyl-1-propanol was dissolved in water, the solution adjusted to pH 10.5 with concentrated HCl and the volume made up to 100 ml. 100 mg. crystallised bovine albumin (British Drug Houses Ltd.) was added to 100 ml. buffer. This reagent was stored in polythene at 4° C.

Stock substrate solution 240 mM potassium L-malate

Sigma reagent L-malic acid was recrystallised three times from warm ethyl acetate by addition of petroleum ether to remove an inhibitor present in some manufacturers' samples (Strominger and Lowry, 1955). 804.54 mg. L-malic acid was dissolved in 15 ml. of water. 5 ml. 2.4 N KOH was added, the solution was adjusted to pH 7 and the volume made up to 25 ml.

The above solutions were prepared fresh every six weeks.

NAD required 4.5 mM β-nicotinamide-adenine dinucleotide Sigma reagent $C_{21}H_{27}O_{14}N_7P_2.2.5\ H_2O$, Mol. Wt. 708.3, 99 per cent pure: 3.2 mg. required per ml. of solution.

Buffered substrate solution

Immediately before use 2 ml. of stock buffer solution were mixed with 2 ml. stock substrate solution and 12.8 mg. of NAD were added to give a solution 0.1 M buffer, 120 mM malate, 4.5 mM NAD.

Standard NADH solution 3500 µm-mole/ml. β-dihydro-nicotinamide-adenine dinucleotide

Sigma reagent $C_{21}H_{27}O_{14}N_7P_2Na_2.2.5\ H_2O$ Mol. Wt. 754, 96 per cent pure

A standard solution of NADH containing 35 µm-mole in 10 µl. was prepared when required by dissolving 260 µg. of NADH in 0.1 ml. ml. of 0.1 M tris buffer pH 8.5. The NADH was stored dessicated and dark until just before use.

Phosphate buffer pH 7.0

60 ml. 0.067 M Na_2HPO_4 were mixed with 40 ml. 0.067 M KH_2PO_4.

Carbonate buffer pH 10.9.

A solution 0.05 M Na_2CO_3 and 0.005 M $NaHCO_3$ was prepared.

Proposed method

10 µl. of a homogenate of rat left ventricle muscle, prepared in 0.1 M tris buffer pH 7.4 was incubated with 200 µl. of buffered substrate solution in 2 in. x $\frac{5}{16}$ in. tubes. The NADH produced was estimated by its fluorescence intensity in dilute alkali solution.

(a) Measurement of NADH

It had been confirmed that, with light of 360 mµ as the activating wavelength, the fluorescence spectrum of the

reduced dinucleotides in weak alkali (0.01 N NaOH) has an intensity maximum at 465 mμ (Fig. 8). Measuring the intensity of the light emitted at 470 mμ when the NADH was activated by 360 mμ a linear relationship existed between the fluorescence intensity and the NADH present in 1.31 ml. from 0.258 μm-mole to 29.8 μm-mole (Fig. 9, p.73).

In the proposed method the fluorescence of the NADH, produced by the action of malate dehydrogenase was to be measured in a solution having a pH between 8 and 12 (Lowry et al., 1957). 1.2 ml. 0.01 N NaOH added to 0.2 ml. buffered substrate solution gave a solution of pH 10.9. It was doubtful if this method of dilution would be adequate to terminate an enzyme reaction with a pH optimum of 10.5. As the presence of carbonate ions has been shown to accelerate the rate of destruction of NAD (Lowry, Passonneau and Rock, 1961) a carbonate buffer of pH 10.9 was preferred. There was the possibility that under the conditions in the experiment the destruction of NAD might be accelerated to such a rate that its presence would be eliminated instantaneously thus bringing the enzymic reaction to a halt. Stronger alkali solutions of 0.02 N NaOH, and 0.04 N NaOH, which gave mixtures of pH 11.7 and 12.2 respectively, were also considered although it seemed possible that they would produce a large amount of fluorescence from the unused NAD in the buffered substrate solution.

Standard solutions of NADH were treated by the proposed method and diluted with 1.2 ml. 0.02 N NaOH and 1.2 ml. carbonate buffer, pH 10.9 respectively. The results of this

test are shown in Fig. 17. When 0.02 N NaOH was used for dilution, the fluorescence intensity from NAD in the reagent was as much as that of 75 µm-mole NADH. If this blank was subtracted the fluorescence intensity produced was proportional to the quantity of NADH present from 0 to 25 µm-mole in 1.41 ml. However, the unused NAD in the buffered substrate solution after incubation with heart tissue would not be a constant quantity so that 0.02 N NaOH could not be used for the development of the fluorescence of NADH in the enzyme assay method. When carbonate buffer was used for dilution the fluorescence intensity of the buffered substrate solution was very similar to that of the same solution prepared without NAD. The fluorescence intensity of NADH under these conditions was proportional to the quantity present from 0 to 25 µm-mole in 1.41 ml. solution.

A letter asking for further details of the method of terminating the reaction catalysed by malate dehydrogenase was sent to the Washington University School of Medicine, St. Louis, M.O., where the assay was developed. Dr. Irene Karl explained that they terminated the enzyme reaction by adding a portion of the buffered substrate solution to 0.05 M phosphate buffer pH 7 and measured the fluorescence of the NADH at neutral pH. 0.1 ml. of the 0.21 ml. of incubated solution was added to 2.5 ml. of 0.067 M phosphate buffer pH 7. A linear relationship existed between the fluorescence intensity and the quantity of NADH in solution from 0 to 25 µm-mole in 2.6 ml. of neutral solution (Fig. 18). This range of concentration in the solution read, was the equivalent of 0 to 50 µm-mole NADH produced in the 200 µl.

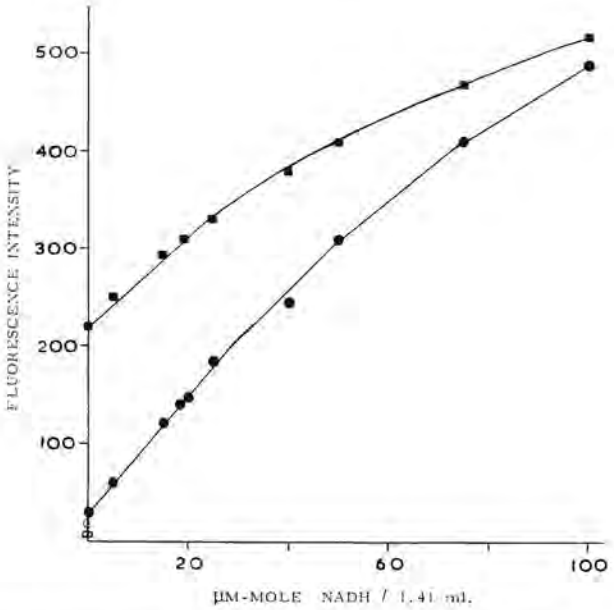

Fig. 17 Relationship between the fluorescence intensity and the concentration of NADH in weak alkali solution.

● NADH in 0.21 ml. buffered substrate solution i.e. 0.1 M 2-amino-2-methyl-1-propanol pH 10.5, 120 mM malate, 4.5 mM NAD, diluted with 1.2 ml. 0.02 N NaOH.

● NADH in 0.21 ml. buffered substrate solution diluted with 1.2 ml. carbonate buffer pH 10.9.

Fluorescence of 0.2 ml. buffered substrate solution prepared without NAD

□ diluted with 1.2 ml. 0.02 N NaOH,

⊙ diluted with 1.2 ml. carbonate buffer pH 10.9.

The fluorescence measurements were converted to the equivalent readings on the 0.01 meter multiplier of the Aminco-Bowman spectrophotofluorometer.

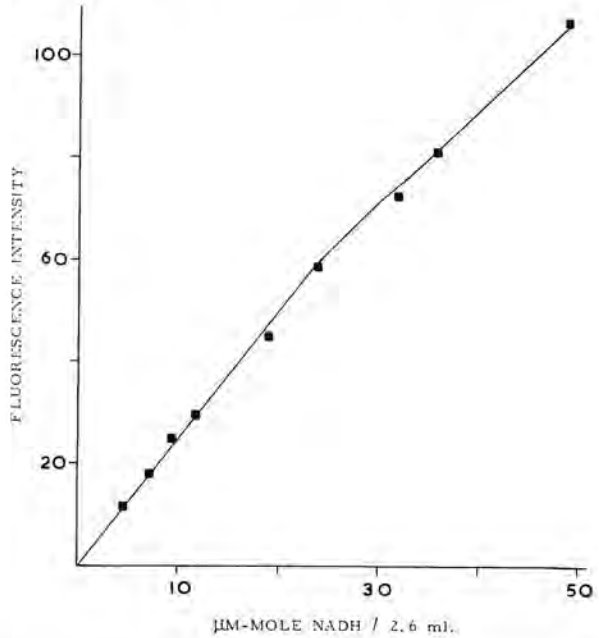

Fig. 18 Relationship between the fluorescence intensity and the concentration of NADH in neutral solution.

0.1 ml. of 0.21 ml. buffered substrate solution i.e. 0.1 M 2-amino-2-methyl-1-propanol, pH 10.5, 120 mM malate, 4.5 mM NAD containing NADH was added to 2.5 ml. 0.067 M phosphate buffer pH 7.0.

of buffered substrate solution incubated.

(b) Development of the method for rat heart tissue

A series of dilutions of a homogenate of rat heart tissue were prepared and 10 µl. incubated with 0.2 ml. buffered substrate for different times. 1.2 ml. carbonate buffer was added with the purpose of terminating the reaction. The fluorescence from the NADH produced by incubating more than 2.74 µg. of the tissue for "zero time" was as great as that produced by incubating the tissue for 5 or 20 min. (Table XII) showing that the addition of carbonate buffer did not, in fact, terminate the enzyme-catalysed reaction. When 0.55 µg. dry heart was incubated the amount of fluorescence produced indicated that there was a measurable rate of enzyme-catalysed reaction with this weight of tissue.

TABLE XII

Fluorescence intensity measured after incubating 10 µl. of homogenates of rat heart tissue with the buffered substrate solution for malate dehydrogenase assay and developing the fluorescence of the NADH produced by adding 1.2 ml. carbonate buffer pH 10.9

Dry weight of heart tissue in 10 µl. homogenate	Fluorescence intensity readings converted to equivalent value on 0.01 meter multiplier			
	Tissue incubated with 200 µl. buffered substrate* solution for min.			
ug.	0	2	5	20
0	10.4			
0.55	56	67	98	156
2.74	174	216	219	249
5.48	249	282	258	-
9.14	264	282	264	-
13.7	282	255	-	243

* 0.1 M 2-amino-2-methyl-1-propanol pH 10.5, 120 mM malate, 4.5 mM NAD

A series of dilutions of a homogenate of heart tissue, containing 0.18 to 2.7 µg. dry weight in 10 µl., were prepared in 0.1 M tris pH 7.4. 10 µl. portions were incubated for 30 and 60 min. with 0.2 ml. of buffered substrate solution. At the end of the incubation period 20 µl. samples of the solutions were taken with a Spinco pipette and added to 1.2 ml. 0.067 M phosphate buffer pH 7. The reaction velocity measured was constant with time and directly proportional to the weight of tissue incubated when less than 1.26 µg. dry weight of heart tissue was present (Fig. 19). As the fluorescence measured when the homogenates were incubated for "zero time" did not differ from that of the reagents the enzyme reaction was terminated by the dilution in phosphate buffer. The 1.26 µg. dry weight of heart tissue reduced 116 µm-mole of NAD of the 900 present in the incubation mixture in 60 min. As 20 µl. of this incubation mixture was taken for the fluorescence measurement 11.5 µm-mole NADH were read in 1.22 ml. solution. This amount of NADH was near the limit of 11.7 µm-mole/1.22 ml. where the fluorescence intensity was no longer directly proportional to the concentration in neutral solution.

In this experiment the time required to rinse the 20 µl. polyethylene pipette adequately with each solution and to measure the volume accurately was not readily standardised so that error was introduced in timing the incubation period when a Spinco pipette was used. Sufficient numbers of 0.1 ml. glass automatic constriction pipettes were available to permit the use of a clean pipette for each sample. It was estimated that if the incubation

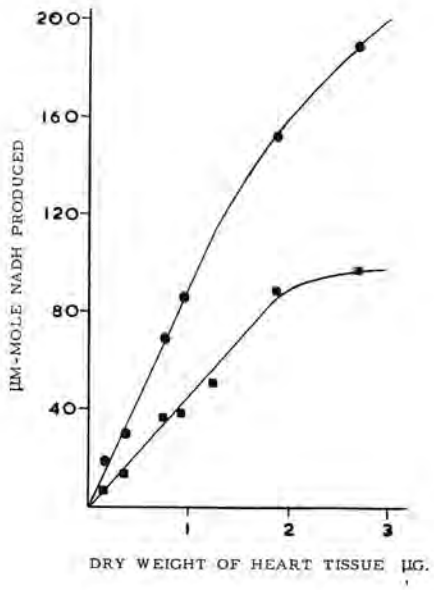

Fig. 19 Relationship between the quantity of NADH produced and the dry weight of rat heart tissue in the homogenate incubated in malate dehydrogenase assay.
■ µm-mole NADH produced in 30 min.
● µm-mole NADH produced in 60 min.
The tissue was incubated with 200 µl. 0.1 M 2-amino-2-methyl-1-propanol, 120 mM malate, 4.5 mM NAD.

time was limited to 30 min. and 0.1 ml. of the incubated solution was added to 2.5 ml. phosphate buffer pH 7 a quantity of NADH in the range 0 to 25 µm-mole/2.6 ml. would be obtained. It was confirmed that under these conditions the fluorescence produced by incubating 10 µl. portions of homogenates containing 0 to 1.0 µg. dry weight of heart tissue for 30 min. in buffered substrate solution prepared with no malate present or for "zero time" in the complete substrate did not differ from the fluorescence of the reagents. It was thus verified that the enzyme-catalysed reduction of NAD occurred only in the presence of malate and was satisfactorily terminated by this dilution.

A quartz fibre fish-pole balance had, by this time, been constructed (p. 44) which could be used to weigh these small quantities of dry tissue. The tissue weight range which could be assayed by this method was finally established with freeze-dried sections of heart as the range was partly limited by the weight which could be determined with this balance.

(c) Application of the method to freeze-dried sections

Method of analysis

0 to 0.9 µg. dry weight of freeze-dried sections of heart tissue were weighed on the quartz fibre "fish-pole" balance and placed in dry 1 in. x $\frac{5}{16}$ in. tubes. When all the sections to be analysed were collected, 10 µl. 0.1 M tris pH 7.4 was added and the tubes were stoppered, and stored vertically at 4° C until required. 0.2 ml. buffered substrate solution at 37° C was added to each tube in turn. After exactly 30 min. at 37° C each tube was shaken gently and 0.1 ml. of the solution extracted

and added to 2.5 ml. 0.067 M phosphate buffer pH 7.0 with mixing. The fluorescence intensity of the NADH produced was read on the fluorometer and compared with that of a standard solution of NADH treated in an identical manner.

A quinine solution containing 0.1 µg./ml. was used to calibrate the fluorometer on the 0.01 meter multiplier.

The quartz fibre "fish-pole" balance for the measurement of 0 to 6 µg., had a sensitivity of 0.048 µg. per unit of displacement measured (0.02 mm.). The maximum error in weighing 0.5 to 1 µg., the required dry weight of tissue, was therefore estimated as 10 per cent. However, by limiting the weight of tissue to 0.7 to 0.8 µg. and estimating the displacement measured to 0.01 mm. a minimum possible error of 3.3 per cent might be achieved.

Freeze-dried sections were prepared from the apex of the left ventricle of a rat heart and a homogenate was made from tissue from the same heart. The homogenate was divided into five portions; one was analysed immediately and the remainder were stored at -20° C in plastic cups. The necessary dilutions were made immediately prior to analysis. Portions of freeze-dried sections of 0 to 1.2 µg. dry weight were weighed and 10 µl. 0.1 M tris pH 7.4 was added to the tissue with great care. The tubes were stoppered and stored at 4° C until required. The values of the malate dehydrogenase activity estimated in homogenates and freeze-dried sections from the same rat heart after increasing periods of storage are shown in Table XIII. The malate dehydrogenase activity estimated in the freshly prepared

homogenate was 40 per cent lower than that in a homogenate which had been frozen and thawed. This increase in activity was attributed to increased disruption of the tissue caused by freezing and thawing. The enzyme activity estimated in freeze-dried sections after 1, 6 and 14 days was as great as the activity found in homogenates and no loss in activity occurred with increasing time of storage.

TABLE XIII

A comparison of the activity and stability of malate dehydrogenase estimated in homogenates and freeze-dried sections prepared from the same rat heart tissue

Time of storage at -20° C	Malate dehydrogenase activity mole/kg. dry weight/min. estimated in	
days	Homogenates*	Freeze-dried sections
0	2.572	
1	3.071	3.246 (1)+
6	3.458	3.494 (7)
14	3.790	3.582 (5)

+ Figures in brackets denote the number of sections analysed.

* Stored as 11.0 mg. wet weight of heart tissue in 1 ml. 0.1 M tris pH 7.4; diluted for analysis with 0.1 M tris pH 7.4.

When freeze-dried sections of heart tissue were analysed the upper limit of the range of tissue weight for which the reaction velocity was shown to be directly proportional to the dry weight incubated for 30 min. was 1.00 µg. (Fig. 20), when 90 µm-mole of NADH was produced. The 45 µm-mole NADH in the solution read was estimated from a calibration curve. The weight of tissue used in the assay of malate dehydrogenase activity in heart was therefore restricted to 0.7 to 0.8 µg. dry weight. The results of the malate dehydrogenase estimations were expressed as moles of substrate transformed by a kilogram of dry tissue in one minute.

A coefficient of variation of 6.7 per cent was found for the analyses of five freeze-dried sections compared with 6.2 per cent for the analyses of twelve 10 µl. portions of a homogenate of heart tissue. Thus the use of the quartz fibre balance to measure dry weight with a probable error of approximately 5 per cent did not greatly increase the variation in the analysis. Further modification of this method of enzyme assay to decrease the variability of the estimates was not possible in the time available.

As this assay method was not developed at the time when the freeze-dried sections from animals with experimental hypertension were prepared the malate dehydrogenase activity was to be estimated in rat heart tissue which had been stored under vacuum at -20° C for one year. Strominger and Lowry (1955) found that the malate dehydrogenase activity of freeze-dried sections of rabbit brain stored for one year at -20° C was 90 per cent of the

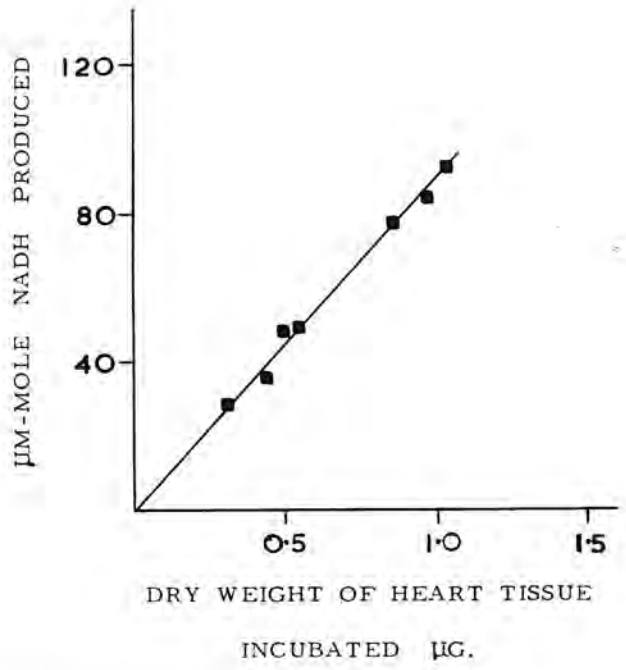

Fig. 20 Relationship between the quantity of NADH produced in 30 min. and the dry weight of freeze-dried sections of heart tissue incubated in malate dehydrogenase assay.

estimate in fresh tissue but the stability of the enzyme in heart tissue over such a long period has not been shown in the present work. Thus, although the coefficient of variation of the assay of malate dehydrogenase activity in heart tissue was slightly greater than five per cent the method was considered adequate for the purpose for which it was required.

7. Adaptation of the enzyme assay methods for the analysis of arteriolar tissue

Alkaline phosphatase activity in rat mesenteric arterioles

Freeze-dried sections

The method used for the estimation of alkaline phosphatase activity in arterioles was that developed for freeze-dried sections of rat heart tissue. Four sections of arteriolar tissue weighing approximately 10, 20, 30, 40 µg. were analysed for alkaline phosphatase activity. The absorbancy readings of the p-nitrophenol produced showed that the weight of arteriolar tissue which could be assayed by this method without further modification of the volumes of solutions was in the region of 10 µg.

No p-nitrophenol was produced by arteriolar tissue incubated for "zero time". When 0 to 8 µg. sections of arteriole were incubated for 30 and 60 min., comparable mean values of the enzyme activity were found (Table XIV). However, the coefficient of variation of the analysis of sections incubated for 30 min. was 12 per cent. The coefficient of variation for this method with homogenates of heart tissue was 3 per cent.

This large variation in the alkaline phosphatase activity found in freeze-dried sections of arterioles was attributed to the variation in the proportion of cell types present in a section.

Resistance vessels which had been cut transversely and longitudinally were present in a dried section. Alkaline phosphatase activity was demonstrated by histochemical techniques to be mainly in the adventitia (Fig.32) so that the amount estimated in any one section would depend on the proportion of adventitia present. If the arterioles were all cut transversely the proportion of adventitia present would be fairly constant. When the arterioles were cut longitudinally the portion analysed might be 0 to 100 per cent adventitia. The circular portions of the arterioles, found in the freeze-dried sections where the vessels were cut transversely, were therefore collected for analysis for alkaline phosphatase activity.

When such tissue collections were analysed the rate of the enzymic reaction measured was directly proportional to the weight of tissue present (Fig. 21) from 0 to 8 µg. A coefficient of variation of 2.4 per cent was found for the analysis of five freeze-dried arteriolar sections. As the dissection of three 5 µg. collections of the circular portions of the arterioles took 30 min., no attempt was made to increase the weight of tissue which could be analysed by the method, by increasing the volumes of solutions. Any theoretical improvement in the technique from an increase in the accuracy of the weight measurement would be cancelled out by increasing the possibility of the loss of enzyme activity from prolonged

TABLE XIV

Alkaline phosphatase activity in rat mesenteric arterioles estimated in 0 to 8 µg. dry tissue incubated for thirty and sixty minutes

Mean alkaline phosphatase activity ± S.D. mole/kg. dry weight/hr.	
7 sections incubated for 30 min.*	3 sections incubated for 60 min.
7.242 ±0.915	7.365 ±0.350

* Coefficient of variation was 12.6 per cent.

TABLE XV

Stability of alkaline phosphatase in freeze-dried sections of rat mesenteric arterioles stored at -20° C under vacuum

Stored at -20° C for days	Alkaline phosphatase activity mole/kg. dry weight/hr.
1	8.621 (5)*
4	7.543 (7)

* Figures in brackets denote the number of sections analysed.

TABLE XVI

Alkaline phosphatase activity in mesenteric arterioles of rats of different weights

Rat weight g.	Alkaline phosphatase activity (estimated after storage at -20° C for 5 days) mole/kg. dry weight/hr.
283	3.39
206	9.81
180	10.02
152	12.55
207	11.29

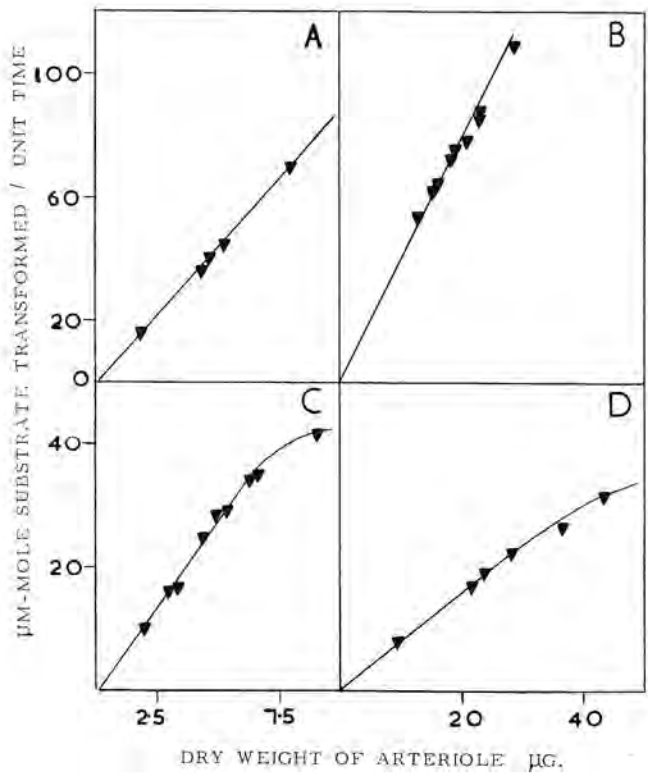

Fig. 21 Relationship between the reaction velocity measured and the dry weight of rat mesenteric arteriole assayed for enzyme activity.

A Alkaline phosphatase estimation.
The reaction velocity is expressed as µm-mole p-nitrophenol produced in 1 hr.

D Glucose-6-phosphate dehydrogenase estimation.
The reaction velocity is expressed as µm-mole NADPH produced in 1 hr.

B Lactate dehydrogenase estimation.
The reaction velocity is expressed as µm-mole NAD produced in 10 min.

C Malate dehydrogenase estimation.
The reaction velocity is expressed as µm-mole NADH produced in 30 min.

exposure of the sections to room air. The alkaline phosphatase activity estimated on the fourth day after the first analysis was decreased by 12.5 per cent (Table XV), when the sections were brought to room temperature and exposed to room air for 2 hr. in the intervening days. The circular portions of the vessels in the freeze-dried sections were therefore collected on the first day after preparation of the sections in a section holder and stored at -20° C under vacuum in a separate drying tube until required.

In these preliminary observations it was noted that the alkaline phosphatase activity estimated in rat mesenteric arterioles showed a tendency to decrease with increasing weight of the animal used (Table XVI). This observation drew attention to the need for taking the precaution of matching as closely as possible, the weight of the animals used to assess the effect of any treatment on the enzyme activity of rat mesenteric arterioles.

Glucose-6-phosphate dehydrogenase activity in rat mesenteric arterioles

Freeze-dried sections

The method used for the estimation of glucose-6-phosphate dehydrogenase activity in arterioles was that developed for freeze-dried sections of rat heart tissue. A preliminary experiment indicated that arteriolar sections of 0 to 30 µg. dry weight could be analysed by the method.

Six sections from 0 to 45 µg. in weight were incubated for

30 min. with the complete buffered substrate solution. Another six sections, of 0 to 40 µg. dry weight, were incubated for 30 min. with buffered substrate solution prepared without the glucose-6-phosphate present. No fluorescence in excess of the reagent blank was found when tissue was incubated in the absence of glucose-6-phosphate. The factor which caused the production of a measurable fluorescence when NADP was incubated with heart tissue without glucose-6-phosphate present was therefore not present in arteriolar tissue. The reaction velocity was directly proportional to the weight of arteriolar tissue incubated for a 30 min. period from 0 to 30 µg. dry weight (Fig. 21). The NADPH produced in 30 min. by 28 µg. of dry tissue was 11.1 µm-mole which was close to the limit (12 µm-mole) which can be read in 1.31 ml. of solution on the 0.01 multiplier of the fluorometer.

The apparent decrease observed in the reaction velocity when >30 µg. dry weight of arteriole was incubated may therefore be caused by the limitations of the method for measuring the product of the reaction.

The amount of arteriolar dry tissue was therefore limited to less than 30 µg. and the glucose-6-phosphate dehydrogenase activity estimated by incubating this weight of tissue for 30 or 60 min. was compared (Table XVII). The reaction velocity was constant within the sixty minute incubation period. A coefficient of variation of 4 per cent was found for the estimation of glucose-6-phosphate dehydrogenase activity in dry arteriolar tissue incubated for 30 min.

No decrease in the measured activity of the enzyme in freeze-dried sections was found after storage at -20° C under vacuum for 2 days (Table XVII).

TABLE XVII

Glucose-6-phosphate dehydrogenase activity estimated in rat mesenteric arterioles in less than 30 µg. dry weight of tissue incubated for thirty and sixty minutes, after storing the tissue at -20° C under vacuum

Mean glucose-6-phosphate dehydrogenase activity ± S.D. mole/kg. dry weight/hr.

Stored for 1 day	Stored for 2 days	
4 sections incubated for 30 min.	7 sections incubated for 30 min. *	4 sections incubated for 60 min.
0.802	0.800 ±0.032	0.806

* Coefficient of variation for analysis was 4 per cent.

Lactate dehydrogenase activity in rat mesenteric arterioles

Freeze-dried sections

The method used for the estimation of lactate dehydrogenase activity in arterioles was that developed for freeze-dried sections of rat heart tissue. A preliminary experiment showed that the method developed for lactate dehydrogenase activity estimations in rat heart tissue could be used without alteration for the analysis of 0 to 60 µg. of freeze-dried sections of rat mesenteric arterioles. The incubation period in the method for heart tissue was limited to 5 minutes. It was appreciated that if a longer incubation period could be used with arteriolar tissue the percentage error in timing would be reduced.

When sections of 0 to 60 µg. were incubated in buffered

substrate solution for "zero time", and when 0 to 8 µg. sections were incubated in buffered substrate solution prepared without pyruvate, for 30 min., no fluorescence in excess of the reagent blank was produced.

The amounts of NAD produced per kg. per min. by 0 to 40 µg. in 5 min., 0 to 25 µg. in 10 min., 0 to 16 µg. in 15 min., or 0 to 8 µg. in 30 min., were comparable (Table XVIII) A decrease was observed in the activity estimated in 0 to 4 µg. tissue incubated for 60 min. (Table XVIII).

TABLE XVIII

Lactate dehydrogenase activity estimated in rat mesenteric arterioles by incubating different weights of tissue for different times and after storing the tissue at -20° C under vacuum

Sections stored at -20° C in vacuo for days	Range of tissue weight incubated µg.	Time of incubation min.	Lactate dehydrogenase activity mole/kg.dry weight/min.
1	20 to 60 (3)*	5	0.362
	0 to 8 (3)	30	0.330
	0 to 5 (3)	60	0.297
2	0 to 40 (4)	5	0.343
	0 to 25 (4)	10	0.342
	0 to 16 (3)	15	0.340
	0 to 8 (3)	30	0.356

* Figures in brackets denote the numbers of sections analysed.

Incubating 0 to 25 µg. dry tissue weight for 10 min. was selected as the technique of analysis having the minimum percentage error in timing and weighing. The reaction velocity measured under these conditions was directly proportional to the weight of tissue incubated (Fig. 21). A coefficient of variation of 2.4 per cent was found for the analysis of 10 freeze-dried arteriolar sections with this technique.

The activity of lactate dehydrogenase estimated with a 30 min. incubation period, in tissue stored for 2 days at -20° C under vacuum was not appreciably different from the estimate made after one day's storage at -20° C (Table XVIII).

Malate dehydrogenase activity in rat mesenteric arterioles

Freeze-dried sections

The method used for the estimation of malate dehydrogenase activity in arterioles was that developed for freeze-dried sections of rat heart tissue. A preliminary experiment showed that the weight of arteriolar tissue which could be analysed without further modification of the method was less than 10 µg. dry weight.

The fluorescence produced by incubating 10 to 40 µg. arteriolar tissue for "zero time" in complete buffered substrate solution, or for 30 min. in buffered substrate solution prepared without malate, or without NAD, was no greater than the fluorescence of the reagents. The reaction velocity measured was directly proportional to the dry weight of tissue incubated for 30 min. from 0 to 6 µg. (Fig. 21). The velocity of the

enzyme-catalysed reaction was constant with time within the thirty minute period used for the incubation (Table XIX).

The activity of malate dehydrogenase estimated in freeze-dried sections of arteriolar tissue which had been stored under vacuum at -20° C for 2 days was 12.6 per cent less than the value of the enzyme activity estimated after 1 day's storage. The measurements of malate dehydrogenase activity in freeze-dried sections of rat mesenteric arterioles should therefore be made on the day after preparation of the tissue.

A coefficient of variation of 6 per cent was found for the analysis of eleven freeze-dried arteriolar sections.

TABLE XIX

Malate dehydrogenase activity in rat mesenteric arterioles estimated in 0 to 7 µg. dry tissue incubated for ten, twenty and thirty minutes after storing the tissue at -20° C under vacuum

Sections stored at -20° C for days	Mean malate dehydrogenase activity mole/kg. dry weight/min. estimated in		
	3 sections incubated for 10 min.	4 sections incubated for 20 min.	3 sections incubated for 30 min.
1	-	-	0.246
2	0.218	0.213	0.215

8. Summary of suitable methods

The conditions of incubation used in the methods for the assay of the activity of alkaline phosphatase, glucose-6-phosphate dehydrogenase, lactate dehydrogenase and malate dehydrogenase in freeze-dried sections of rat heart tissue, are shown in Table XX.

The proofs of the validity and reproducibility of the methods described for analysis of heart tissue are summarised in Table XXI. The validity and reproducibility of the methods of analysis when applied to rat mesenteric arteriolar tissue are demonstrated in Table XXII.

Addendum

The final units used in these experiments to express enzyme activity do not conform with the standard unit proposed in the "Report of the Commission on Enzymes of the International Union of Biochemistry" 1961 but the conversion is simple and will be illustrated in the results.

TABLE XX

Conditions of enzyme assay for rat heart tissue

Enzyme	Buffer	Substrate	Cofactors	Dry wt. of tissue required ug.	Incubation at 37° C in volume µl.	Incubation at 37° C for time min.
Alk. p'tase (Lowry et al., 1954)	0.5M AMP_1 pH 10.0	8mM disodium p-nitrophenyl phosphate	2mM $MgCl_2$	50-100	100	30
G-6-PDH (Lowry et al., 1961)	0.1M AMP_2 pH 9.3 with 0.5mM EDTA	5mM disodium salt of glucose-6-phosphate	0.9mM NADP	50-80	100	30
LDH (Lowry et al., 1957)	0.1M tris pH 7.4	1.5mM sodium pyruvate	1mM NADH	2-5	200	5
MDH (Strominger and Lowry, 1955)	0.1M AMP_1 pH 10.5 with 0.05% albumin	120mM potassium L-malate	4.5mM NAD	0.7-0.8	200	30

Abbreviations used:

AMP_1 ≡ 2-amino-2-methyl-1-propanol. (Light and Co.)

AMP_2 ≡ 2-amino-2-methyl-1,3-propanediol. (B.D.H.)

tris ≡ 2-amino-2-hydroxymethylpropane-1,3-diol. (B.D.H.)

NADP ≡ nicotinamide-adenine dinucleotide phosphate.

NADH ≡ reduced nicotinamide-adenine dinucleotide.

NAD ≡ nicotinamide-adenine dinucleotide.

The dinucleotides required as cofactors and standards, and the substrates were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.)

TABLE XXI

Determination of validity and reproducibility of enzyme assay techniques of Table XX with rat heart tissue

	Alk. p'tase	G-6-PDH	LDH	MDH
Substrate transformed directly proportional to incubation time. min.	0-60	0-60	0-5	0-30
Reaction velocity directly proportional to dry weight of tissue incubated. µg.	5-100	40-100	0-7	0-0.9
Coefficient of variation of assay technique.* %				
with 10 µl. homogenate	3.1 (12)	6.4 (8)		6.2 (12)
with freeze-dried sections	5.6 (6)	2.7 (5)	4.7 (10)	6.7 (5)
Enzyme stable in freeze-dried sections+ analysed daily for: days	3	1	2	4

* Number of replicate analyses in brackets

\+ Stored at -20° C under vacuum and brought to room temperature daily for 2 hr. for analysis.

TABLE XXII

Determination of validity and reproducibility of enzyme assay methods described in Table XX when applied to freeze-dried rat mesenteric arteriolar tissue

	Alk. p'tase	G-6-PDH	LDH	MDH
Substrate transformed directly proportional to incubation time. min.	0-60	0-60	0-10	0-30
Reaction velocity directly proportional to dry weight of tissue incubated. µg.	0-8	0-30	0-25	0-6
Coefficient of variation of assay technique.* %	2.4 (5)	4 (7)	2.4 (10)	6 (11)
Enzyme stable in freeze-dried sections+ analysed daily for: days	Unstable	2	2	Unstable

* Numbers of replicate analyses in brackets.

\+ Stored at -20° C under vacuum and brought to room temperature daily for 2 hr. for analysis.

CHAPTER V

THE DETERMINATION OF THE ACTIVITY OF ALKALINE PHOSPHATASE, GLUCOSE-6-PHOSPHATE DEHYDROGENASE, LACTATE DEHYDROGENASE AND MALATE DEHYDROGENASE IN THE VASCULAR TISSUE OF RATS IN THE PRENECROTIC PHASE OF EXPERIMENTAL HYPERTENSION

1. Experimental Plan

(a) Introduction

The possible effects of humidity on the weight of freeze-dried tissue and of air and the time of exposure to a temperature of 25° C, on the stability of the enzymes to be studied suggested that the validity of any comparisons of enzyme activity would be increased if the estimates in hearts to be compared were made on tissue dried simultaneously and analysed together. When tissue from one animal in each group was analysed in one batch any error in the measurements caused by the unstable nature of the enzyme, the substrates, or the dinucleotides required as standards, was distributed equally between the groups. The maximum number of animals from which tissue could be obtained, sectioned, and freeze-dried within one day was four. If three sections from the one tissue of each animal were analysed the twelve sections thus obtained were also the maximum number which could conveniently be analysed in one day.

Practical considerations thus limited to four the number of groups of animals which could be studied. When steroid hypertension was induced in the rat deoxycortone acetate (DOCA) was implanted subcutaneously in an animal subjected to left nephrectomy and salt-loading. Normal untreated animals were required as one control group and, of the other possible controls, the two chosen were salt-loaded animals and uninephrectomised animals drinking tap water. No information was therefore obtained in these experiments on the effect of DOCA implantation on normal animals or the effect of salt-loading on uninephrectomised animals.

The metabolism of vascular tissue in hypertensive animals was to be studied in the prenecrotic phase of hypertension. The earliest histological change in arterial muscle cells was seen fourteen days after the hypertensive regime was begun (Gardner, 1963). The tissues from the animals used in the present experiments were therefore studied seven days after commencing treatment.

(b) Treatment of animals

Male, albino Wistar rats of a laboratory-bred strain were kept in individual cages in a room maintained at 73-77° F. They were fed on standard rat cubes (British Oil and Cake Mills Ltd., diet 41) and their drinking fluid was contained in calibrated bottles so that their daily fluid intake could be measured.

Each week ten male rats in the weight range 140 to 280 g. were selected, closely matched for weight and their systolic

blood pressures were measured. Blood pressure was measured indirectly by tail cuff and microphonic manometer (Friedman and Freed, 1949) with the animal under light ether anaesthesia, warmed by infra-red radiation. The ten selected animals were divided into four groups:

Group N Two rats were kept as untreated controls.

Group S Two rats were given 1 per cent w/v NaCl solution to drink in place of water.

Group U Three rats were subjected to left nephrectomy. The animal was anaesthetised with ether, the hair on the left side was clipped and a lateral incision made. The kidney was mobilised and exteriorised and the renal pedicle was clamped with an artery forcep and ligated. The kidney was excised with a scalpel preserving the left adrenal. The muscle layers were approximated with two interrupted sutures and the skin closed with three 12 mm. Michel clips. These animals were given tap water to drink.

Group H Three rats were subjected to left nephrectomy and at the same time a 50 mg. pellet of deoxycortone acetate (DOCA) was implanted subcutaneously through a separate cervical incision. These animals were given 1 per cent w/v NaCl solution to drink.

The animals were kept under careful observation for seven days.

(c) Procedure in the study of heart tissue

The procedure in the experiment in which heart enzymes were studied differed slightly from that in the experiment in which

arteriolar enzymes were estimated. In the former experiment a study of the behaviour of the mesenteric arterioles of hypertensive and uninephrectomised animals in tissue culture was being carried out simultaneously making it necessary to kill the animals of these groups at specified times.

The daily fluid intake of the ten rats were measured for seven days. After six days of treatment the systolic blood pressures of the animals were recorded. On the seventh day the animals were weighed and one rat was taken at random from each group. The rats chosen from groups H, S, N and U were killed at 90 min. intervals in that order. Each rat was killed by exsanguination under ether anaesthesia and the blood obtained collected in a tube for analysis of urea nitrogen. The heart was removed immediately and the ventricles separated from the atria by an incision extending through the atrioventricular sulcus. The ventricles were opened, the cavities freed from blood and the tissue quickly weighed. A block of ventricular muscle 2 mm. x 3 mm. x 4 mm. was taken from the anterior apical left ventricular myocardium, and quenched at once in iso-pentane cooled to -65° C. The remainder of the wet heart tissue was weighed and dried to constant weight at 105° C. The percentage dry weight found was used to calculate the total dry heart weight.

The frozen sections of heart required for quantitative enzyme analysis were then cut. When the sections from the four animals had been collected they were freeze-dried at -20° C and 0.010 mm. Hg for 2 hr. and stored at -20° C under vacuum.

Glucose-6-phosphate dehydrogenase, lactate dehydrogenase and alkaline phosphatase were estimated in the heart tissue on the first, second and third days respectively after drying. Malate dehydrogenase was estimated in these tissues after storing them for 1 yr. at -20° C. Three sections of tissue from each animal were taken for each enzyme assay. The tissues were weighed and incubated in the order in which the animals were killed. The methods used for the quantitative analysis of freeze-dried sections of heart tissue were summarised in Table XX, p. 116.

The localisation of the enzymes was demonstrated by the histochemical methods described on p.48 in cold microtome sections of the same tissue.

This procedure was carried out each week for twelve consecutive weeks.

(d) Procedure in the study of arteriolar tissue

The daily fluid intake of the ten rats was measured for seven days. On the morning of the seventh day the systolic blood pressures were measured, the animals weighed, and one rat chosen from each of the four groups by selecting those most closely matched by weight. The rats chosen from groups N, U, S and H were killed at 90 min. intervals in that order and their mesenteric arterioles dissected and freeze-dried for quantitative enzyme analysis as described previously. The wet heart weight was recorded and this tissue was then dried to constant weight at 105° C. In the following weeks the animals were killed in the order U, S, H, N and S, H, N, U etc., to equalise

within the groups the effect of any possible diurnal variation in enzyme activity. The tissues prepared for quantitative estimations were weighed and analysed each week in the order in which the animals were killed.

Three portions of freeze-dried mesenteric arteriole from each animal were analysed for each enzyme. On the first day after drying the tissue the transverse sections required for alkaline phosphatase activity estimations were collected and stored separately at -20° C under vacuum. Malate dehydrogenase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase and alkaline phosphatase were estimated on the first, second, third and fourth day respectively after drying by the methods summarised in Tables XX and XXII, p.116 and p. 118.

The localisation of the enzymes was demonstrated by histochemical methods in cold microtome sections of the same tissue.

This procedure was carried out each week for twelve weeks.

2. Results in the study of heart tissue

(a) General changes in the animals' condition

The results are summarised in Table XXIII.

The values of the measurements for the individual animals are given in Tables XXIV to XXVI in Appendix I, p. 198.

TABLE XXIII

Body weight, systolic blood pressure, fluid intake, blood urea nitrogen and heart weight of rats before treatment and seven days after commencing treatment
(mean of 12 animals ± S.D.)

N = normal, S = salt-loaded, U = uninephrectomised, H = DOCA hypertensive

	Group N			Group S			Group U			Group H		
	Initial	Final	p*	Initial	Final	p*	Initial	Final	p*	Initial	Final	p*
Body weight g.	217 ±37	226 ±31	n.s.	220 ±38	227 ±36	n.s.	214 ±41	224 ±39	n.s.	220 ±25	215 ±31	n.s.
Systolic blood pressure mm.Hg //	113 ±9	115 ±9	n.s.	111 ±10	119 ±7	<0.05	111 ±7	118 ±6	<0.02	109 ±8	134 ±8	≪0.001
Daily fluid intake + ml.	27 ±12	33 ±9	n.s.	43 ±21	60 ±22	<0.1	20 ±9	29 ±7	<0.02	40 ±20	78 ±22	<0.001
Blood urea nitrogen mg./100 ml. //		13 ±2			14 ±3			16 ±3			14 ±2	
Wet heart weight g.		0.674 ±0.057			0.711 ±0.074			0.634 ±0.084			0.679 ±0.085	
Dry heart weight g.		0.162 ±0.010			0.169 ±0.017			0.152 ±0.021			0.169 ±0.025	
Relative heart weight %		0.302 ±0.043			0.319 ±0.048			0.286 ±0.030			0.318 ±0.027	

+ Measurement of initial intake was for the first 24 hr. after commencing treatment, the final intake quoted was that for the fourth day of treatment.

* Significance of the difference of the mean values before and after treatment n.s. = not significant.

// Final measurement made on the sixth day.

Mean value for seven animals.

Blood pressure (Tables XXIII and XXIV)

In groups S, U and H the mean blood pressure measured 6 days after treatment was significantly greater than the initial mean blood pressure. The mean increase was greatest and most significant in group H. The mean blood pressure in group H after six days did not reach 150 mm. Hg, the level at which hypertension is generally considered to be established in rats. From previous experience in this laboratory (Gardner, 1960) however, it was known that the mean systolic pressure of rats treated in this way will exceed the arbitrary upper limit of normal within three weeks. The animals of group H in the present experiment were accepted as hypertensive in this sense.

Body weight (Tables XXIII and XXV)

The mean weight of the rats of groups N, S and U increased during one week but that of the animals of group H decreased slightly. This slight weight loss early in the development of experimental hypertension has been observed previously (Gardner, 1963).

Fluid intake (Table XXIII)

As a few readings of the fluid intakes on the fifth, sixth and seventh days were unfortunately unrecorded the mean values for the groups of animals on the fourth day were considered. The mean fluid intake of the rats of group U and H on the fourth day after commencing treatment was significantly greater than that on the first day. The mean fluid intake of group S was already appreciably greater than normal on the first day

and by the fourth day the mean fluid intake was significantly ($p < 0.001$) greater than that of the normal group. The mean fluid intake of group U on the fourth day was not significantly greater than normal, that of group H was very significantly greater ($p << 0.001$).

Blood urea nitrogen (Table XXIII)

The blood urea nitrogen of the treated animals was not significantly different from that of the normal group.

Heart weights (Tables XXIII and XXVI)

After 7 days of treatment the mean heart weights of the animals in groups S and H were greater than normal. The mean heart weight of the rats of group U was less than normal. The extent of the changes did not reach conventional levels of significance. When wet heart weight was expressed as a percentage of body weight and when the dry heart weight was calculated similar changes were observed which also failed to reach conventional levels of significance.

(b) Enzyme activity in rat heart tissue

Quantitative estimations

The mean value of the activity of alkaline phosphatase (alk. p'tase) glucose-6-phosphate dehydrogenase (G-6-PDH), lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) found in the heart tissue of the different groups of animals are shown in Table XXVII.

As the experiment was planned so that in each week one animal in each of the four groups had been kept under the same conditions and their cardiac tissue analysed in an identical manner the value of the enzyme activity estimated in the normal animal in the group for week 1 could be considered as the best estimate for a normal value with which to compare the values for the salt-loaded, uninephrectomised and DOCA hypertensive animals of that week. On this basis an estimate was obtained of the change in enzyme activity for each treated animal. The mean values of the differences in enzyme activity in rat hearts calculated in this way are shown in Table XXVIII.

The values of the measurements of the enzyme activities for the individual animals are given in Tables XXIX to XXXII in Appendix I, p. 205.

TABLE XXVII

Enzyme activity in rat left ventricle apex
(mean of 12 animals ± S.D.)

N = normal, S = salt-loaded, U = uninephrectomised
H = DOCA hypertensive

Enzyme*	Group N	Group S	Group U	Group H
Alk. p'tase	1.335	1.103	0.992	1.044
mole/kg./hr.	±0.226	±0.202	±0.359	±0.337
p+		<0.02	<0.02	<0.05
G-6-PDH	0.214	0.257	0.253	0.251
mole/kg./hr.	±0.033	±0.049	±0.050	±0.033
p+		<0.02	<0.05	<0.02
LDH	4.063	4.087	4.025	4.067
mole/kg./min.	±0.331	±0.470	±0.464	±0.373
p+		n.s.	n.s.	n.s.
MDH #	2.73 (11)	2.44 (8)	2.42 (6)	2.42 (11)
mole/kg./min.	±0.08	±0.12	±0.13	±0.17
p+		n.s.	n.s.	n.s.

* The enzyme activity is expressed as moles of substrate transformed by one kilogram of dry tissue weight per unit time at 37° C.

\+ Significance of the difference of the mean value from that for group N.

\# Estimated after storing the freeze-dried sections for 1 yr. at -20° C.

Figures in brackets denote the number of animals from which tissue was still available.

TABLE XXVIII

Differences between enzyme activity in rat left ventricle apex of normal and treated animals (mean of 12 animals ± S.E.M.)

N= normal, S = salt-loaded, U = uninephrectomised
H = DOCA hypertensive

Enzyme*	Groups compared	Mean difference ± S.E.M.		Significance
Alk. p'tase mole/kg./hr.	N-H	0.291	±0.094	$0.01 < p < 0.02$
	N-U	0.343	±0.085	$0.001 < p < 0.005$
	N-S	0.232	±0.097	$0.02 < p < 0.05$
G-6-PDH mole/kg./hr.	H-N	0.037	±0.009	$0.001 < p < 0.005$
	U-N	0.039	±0.020	$0.05 < p < 0.1$
	S-N	0.043	±0.011	$0.001 < p < 0.005$
LDH mole/kg./min.	N-H	-0.005	±0.076	-
	N-U	+0.038	±0.097	-
	N-S	-0.025	±0.076	-

* The enzyme activity is expressed as moles of substrate transformed by one kilogram of dry tissue weight per unit time at 37° C.

Alkaline phosphatase (Tables XXVII and XXVIII; Table XXIX, p.201.)

In each abnormal group there was a significant decrease in alkaline phosphatase activity. The mean reduction was greatest in the uninephrectomised group, least in the salt-loaded group.

The enzyme estimates made in the preliminary experiments had suggested that there might be a relationship between alkaline phosphatase activity and animal weight but no such association was found in the results of the present experiment

when the activity was plotted against the final body weight.

As the enzyme activity was measured per unit of heart weight the estimated value of the activity was plotted against the wet heart weight to determine if any relationship existed between enzyme activity and the size of the organ. The scatter diagram obtained did not show any correlation between the two measurements. The total alkaline phosphatase activity per heart was determined by multiplying the activity per unit dry weight by the total calculated dry weight. The mean values for groups N, S, U and H were 216, 186, 152 and 170 µ-mole substrate transformed/hr./organ. As the activity of this enzyme was localised mainly in the adventitia of coronary arteries whose distribution throughout the heart is certainly not constant per unit weight these figures will not represent a true value of the total activity per heart. They do, however, serve to indicate that the observed decrease in activity cannot be explained entirely by a decrease in the proportion of vascular tissue present per unit weight if the assumption is made that the increase in heart weight in the hypertensive animal is due to an increase in the mass of muscle; the total amount of vascular tissue per heart remaining constant.

The values of alkaline phosphatase activity were also plotted against the final blood pressure and the relationship found in the different groups are shown in Fig. 22. In group N the trend was for alkaline phosphatase activity to decrease as the blood pressure increased although the regression coefficient

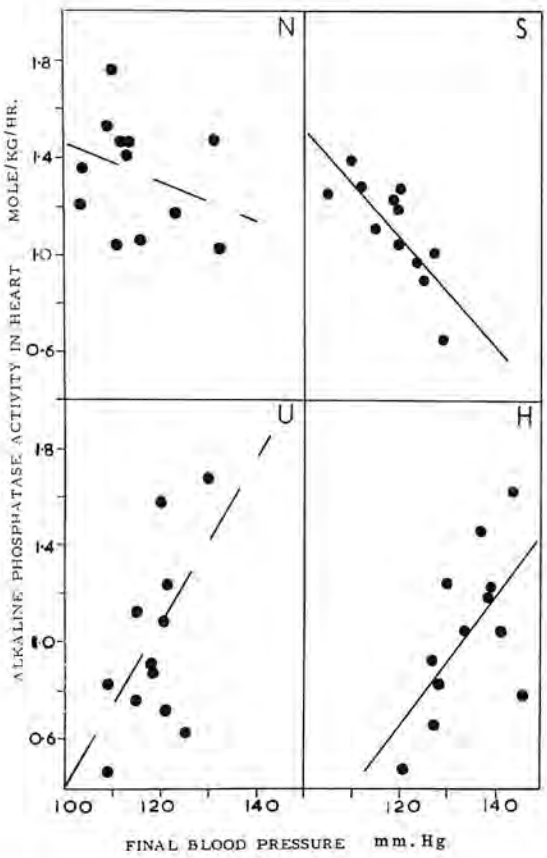

Fig. 22 The sample regressions of cardiac alkaline phosphatase activity on the blood pressure measured after 6 days treatment of the four groups of rats.
N = normal animals. The regression coefficient b = -0.008 ± 0.008 is not significantly different from zero. The unit of b is mole per kg.per hr./mm. Hg ± S.E.
S = salt-loaded animals; b = -0.022 ±0.005; $0.001 < p < 0.005$.
U = uninephrectomised animals; b = +0.033 ±0.016; $0.05 < p < 0.1$.
H = DOCA hypertensive animals ; b = +0.026 ±0.011; $0.02 < p < 0.05$.

(Snedecor, 1956) was not significant in this group. The negative value of the regression coefficient for group S however, was significantly different from zero. In contrast there was a trend for the alkaline phosphatase activity to increase as the blood pressure increased in group U and group H. The regression coefficient was not significantly different from zero in group U but was so in group H.

The estimated change in alkaline phosphatase activity for each treated animal was plotted against the increase in blood pressure of the animal after one week of treatment. No association between the two values was suggested.

Glucose-6-phosphate dehydrogenase (Tables XXVII and XXVIII; Table XXX, p. 202.)

In each abnormal group there was a significant increase in glucose-6-phosphate dehydrogenase activity. An association between glucose-6-phosphate dehydrogenase activity and wet heart weight was suggested only in group S where there appeared to be a trend for the enzyme activity to decrease with increasing heart weight. This association was not confirmed statistically. The regression coefficient for the sample regression of glucose-6-phosphate dehydrogenase activity on heart weight in group S was $b = -0.171 \pm 0.203$ mole per kg. per hr./g. wet weight of cardiac tissue.

No relationship was suggested between enzyme activity and final blood pressure in group N or group U. In group S there was a trend for glucose-6-phosphate dehydrogenase activity to be higher in animals with higher blood pressure and in group H the

enzyme activity tended to be lower in animals with higher blood pressure (Fig. 23, p.136). The regression coefficient was not, however, significantly different from zero in either group.

The estimated change in glucose-6-phosphate dehydrogenase activity for each treated animal was plotted against the increase in the blood pressure after one week of treatment. No association between the two values was found .

Lactate dehydrogenase (Tables XXVII and XXVIII; Table XXXl, p.203.)

There was no difference in the activity of lactate dehydrogenase per unit dry weight in the hearts of the rats in the different groups.

In group U the enzyme activity increased with increasing wet heart weight (Fig. 24, p.137) and the regression was significant. When all 48 animals were considered together a significant positive regression coefficient was found (Table XXXIII).

There was no suggestion of any relationship between lactate dehydrogenase activity and blood pressure.

Malate dehydrogenase activity (Table XXVII; Table XXXII, p. 204.)

No difference in the activity of malate dehydrogenase in the cardiac tissue of the animals in the different groups was found. There was no indication of any relationship between malate dehydrogenase activity and heart weight or blood pressure.

Localisation of enzymes

No differences between the four groups of animals were found when the activities and the localisations of the four enzymes observed in the cardiac tissue by histochemical methods were studied.

Alkaline phosphatase activity was found in the adventitia of coronary arteries with the short incubation time used in this experiment. Glucose-6-phosphate dehydrogenase, lactate dehydrogenase and malate dehydrogenase activity were distributed throughout heart muscle cells and the endothelium and media of coronary arteries. The activity of lactate dehydrogenase (Fig. 30) and the activity of malate dehydrogenase (Fig. 31) were apparently of a high order both in heart muscle cells and in coronary arterial media and endothelium. In comparison the activity of glucose-6-phosphate dehydrogenase was low and could not be illustrated photographically.

(Figures 28 to 41 are colour transparencies and they are contained in the holder attached opposite p.154).

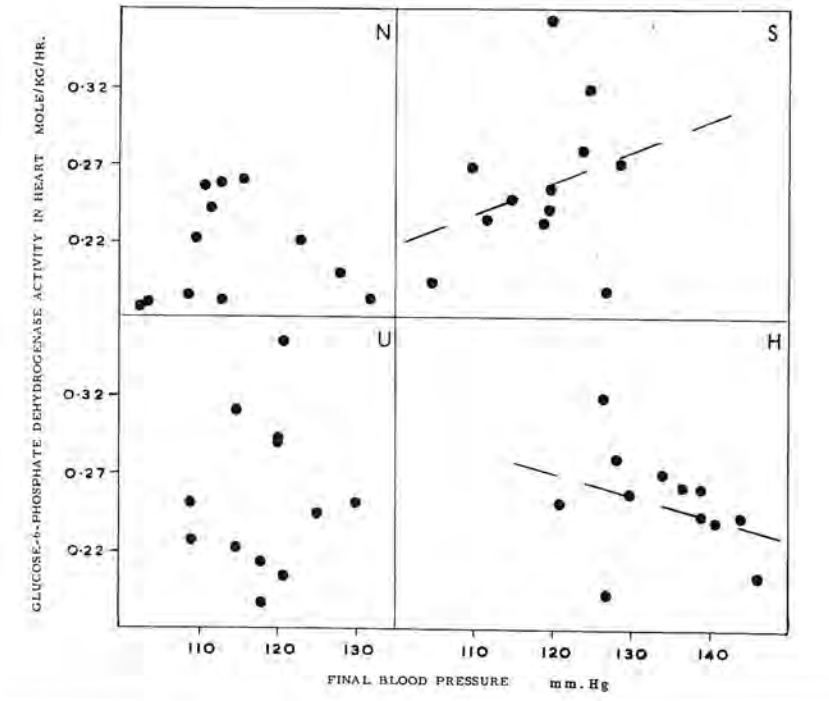

Fig. 23 Relationships between cardiac glucose-6-phosphate dehydrogenase activity and blood pressure measured after 6 days treatment in the four groups of rats.

N = normal animals.

S = salt-loaded animals. The regression coefficient b = +0.0019 ±0.0021 is not significantly different from zero. The unit of b is mole per kg. per hr./mm. Hg ± S.E.

U = uninephrectomised animals.

H = DOCA hypertensive animals. The regression coefficient b = -0.0014 ±0.0013 is not significantly different from zero.

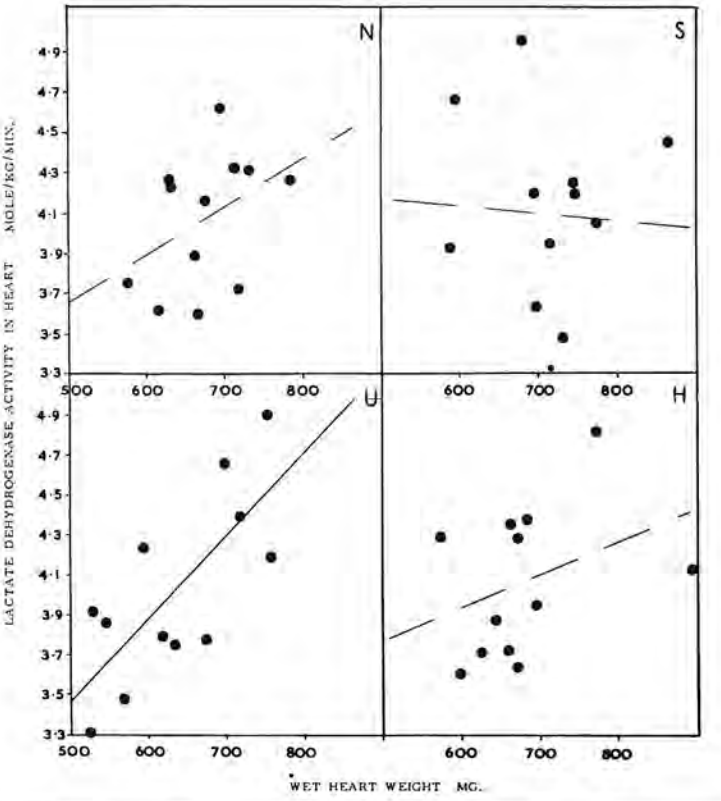

Fig. 24 The sample regressions of cardiac lactate dehydrogenase activity on the heart weight of the rats in the different groups after treatment.

The calculated regression lines (Table XXXIII) are drawn for the different groups of animals but the regression coefficient is significantly different from zero only in group U.

TABLE XXXIII

The regression coefficients of the sample regressions of lactate dehydrogenase activity on total heart weight in the separate groups of animals, and when considered together (12 animals per group)

N = normal, S = salt-loaded, U = uninephrectomised, H = DOCA hypertensive

	Group N	Group S	Group U	Group H	N+S+U+H
b*	+2.45	-0.37	+4.12	+1.55	+1.87
S_b+	±1.66	±2.01	±1.17	±1.30	±0.70
p	n.s.	n.s.	$0.005 < p < 0.01$	n.s.	$0.01 < p < 0.02$

* The units of b are mole per kg. dry weight per min./g. wet heart weight.

\+ The standard error of b.

3. Results in the study of arteriolar tissue

(a) General changes in the animals' condition

The results are summarised in Table XXXIV, p.140.

Blood pressure (Tables XXXIV and XXXV)

The final mean blood pressure was significantly greater than the mean value before treatment only in group H. There was a slight increase in the mean value in group S and in group U.

Body weight (Tables XXXIV and XXXVI)

The mean weight of the rats in all groups increased slightly in seven days although none of the increases were statistically significant. The observed increase was smallest in group H greatest in group N.

Fluid intake (Table XXXIV)

The mean fluid intake of all four groups increased significantly. The mean fluid intake on the seventh day in groups S and H was significantly greater than that in group N ($p \ll 0.001$).

Blood urea nitrogen (Table XXXIV)

The mean blood urea nitrogen values of the animals of groups H and S were not significantly different from the mean for group N. The mean value for group U was significantly ($p < 0.05$) greater than the mean value for group N. None of the individual values however was greater than 21 mg./100 ml., the upper limit of the range for normal rats being 16.3 mg./100 ml. (Spector, 1956).

Heart weight (Table XXXIV)

After 7 days of treatment the mean heart weight of the animals in group H expressed as wet weight, and as per cent of

body weight was greater than normal. The differences observed were not significant. The mean dry heart weight measured after drying the hearts to constant weight at 105° C was not different in the four groups of animals.

Tables XXXV and XXVI give the values of the measurements for the individual animals and they are contained in Appendix II, p. 205.

TABLE XXXIV

Body weight, systolic blood pressure, fluid intake, blood urea nitrogen, and heart weight of rats before treatment and seven days after commencing treatment
(mean of 12 animals ± S.D.)

N = normal, S = salt-loaded, U = uninephrectomised, H = DOCA hypertensive

	Group N			Group S			Group U			Group H		
	Initial	Final	p*	Initial	Final	p*	Initial	Final	p*	Initial	Final	p*
Body weight g.	212 ±15	223 ±14	n.s.	212 ±18	222 ±16	n.s.	218 ±13	224 ±13	n.s.	219 ±12	222 ±13	n.s.
Systolic blood pressure mm.Hg	113 ±6	115 ±6	n.s.	107 ±7	111 ±7	n.s.	113 ±6	117 ±6	n.s.	114 ±7	125 ±7	<0.001
Daily fluid intake + ml.	23 ±6	32 ±9	<0.02	33 ±10	55 ±13	<0.001	21 ±6	35 ±10	<0.001	25 ±9	83 ±24	<0.001
Blood urea # nitrogen mg./100 ml.		14(10) ±3			16(12) ±3			17(11) ±3			15(10) ±3	
Wet heart weight g.		0.671 ±0.073			0.673 ±0.086			0.673 ±0.065			0.687 ±0.055	
Dry heart weight g.		0.154 ±0.017			0.154 ±0.020			0.155 ±0.014			0.156 ±0.013	
Relative heart weight %		0.300 ±0.018			0.303 ±0.028			0.301 ±0.025			0.310 ±0.022	

+ Measurement of initial fluid intake was for the first 24 hr. after commencing treatment.
* Significance of the difference of the mean values before and after treatment n.s. = not significant.
# Figures in brackets denote the number of measurements made.

(b) Enzyme activity in rat mesenteric arterioles

Quantitative estimations

The mean value of the activity of alkaline phosphatase (alk. p'tase), glucose-6-phosphate dehydrogenase (G-6-PDH), lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) found in the mesenteric arterioles of the different groups of animals are shown in Table XXXVII, p.142.

The change in the activity of the four enzymes in each of the treated animals was also estimated by subtracting the value for the normal animal used that week as in the previous experiment. The mean values of the estimated increases are given in Table XXXVIII, p.143.

Alkaline phosphatase (Tables XXXVII and XXXVIII; Table XXXIX, p.207.)*

The mean alkaline phosphatase activity in mesenteric arterioles in the hypertensive group of rats was significantly greater than that in groups N, S and U. The mean value of the enzyme activity in group S was slightly greater than that in group N. The values of alkaline phosphatase activity in rat arterioles were plotted against the final body weight of the animals and the relationships found are shown in Fig. 25, p.144. In groups N and U the alkaline phosphatase activity in arterioles decreased with increasing body weight. The negative value of the regression coefficient in group U was significantly different from zero, that for group N just failed to reach a significant value. No relationship was indicated between the

* The enzyme activity values for the individual animals are given in Tables XXXIX to XLII in Appendix II.

TABLE XXXVII

Enzyme activity in rat mesenteric arterioles
(mean of 12 animals ± S.D.)

N = normal, S = salt-loaded, U = uninephrectomised
H = DOCA hypertensive

Enzyme*	Group N	Group S	Group U	Group H
Alk. p'tase	5.50	6.20	5.40	9.05
mole/kg./hr.	±1.84	±1.84	±2.13	±3.65
p+		n.s.	n.s.	< 0.01
G-6-PDH	0.777	0.829	0.797	0.922
mole/kg./hr.	±0.089	±0.118	±0.089	±0.098
p+		n.s.	n.s.	< 0.001
LDH	0.312	0.306	0.318	0.318
mole/kg./min.	±0.024	±0.039	±0.030	±0.046
p+		n.s.	n.s.	n.s.
MDH	0.198	0.185	0.187	0.204
mole/kg./min.	±0.030	±0.040	±0.019	±0.041
p+		n.s.	n.s.	n.s.

+ Significance of the difference of the mean value from that for group N.

* The enzyme activity is expressed as moles of substrate transformed by one kilogram of dry tissue weight per unit time at 37° C.

TABLE XXXVIII

Differences between enzyme activity in the mesenteric arterioles of normal and treated rats (mean of 12 animals ± S.E.M.)

N = normal, S = salt-loaded, U = uninephrectomised
H = DOCA hypertensive

Enzyme *	Groups compared	Mean difference ± S.E.M.		Significance
Alk. p'tase mole/kg./hr.	S-N	+0.700	±0.728	n.s.
	U-N	-0.100	±0.400	n.s.
	H-N	+3.547	±1.225	$p < 0.02$
G-6-PDH mole/kg./hr.	S-N	+0.0522	±0.040	n.s.
	U-N	+0.0322	±0.041	n.s.
	H-N	+0.146	±0.028	$p << 0.001$
LDH mole/kg./min.	S-N	-0.006	±0.011	n.s.
	U-N	+0.005	±0.012	n.s.
	H-N	+0.006	±0.012	n.s.
MDH mole/kg./min.	S-N	-0.013	±0.008	n.s.
	U-N	-0.011	±0.008	n.s.
	H-N	+0.006	±0.009	n.s.

* The enzyme activity is expressed as moles of substrate transformed by one kilogram of dry tissue weight per unit time at 37° C.

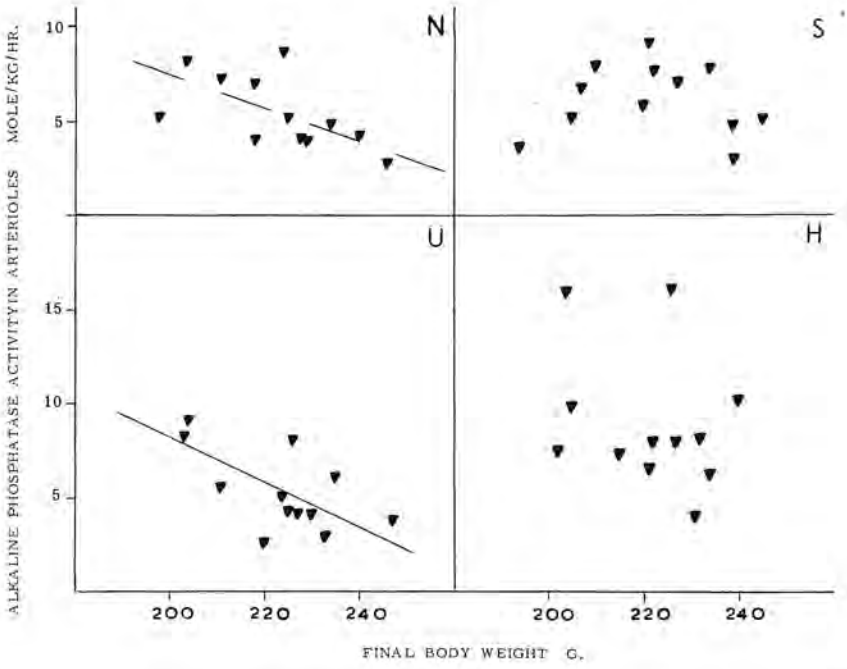

Fig. 25 The sample regressions of arteriolar alkaline phosphatase activity on the body weight of the animals after seven days treatment of the four groups of rats.
N = normal animals. The regression coefficient b = -0.078 ±0.035; $0.05 < p < 0.1$. The unit of b is mole per kg. per hr./g. body weight.
S = salt-loaded animals.
U = uninephrectomised animals. b = -0.103 ±0.041; $0.02 < p < 0.05$.
H = DOCA hypertensive animals.

two measurements in the animals of groups S and H.

When alkaline phosphatase activity was plotted against the blood pressure of the animals measured after one week of treatment no association between these measurements was observed. The estimated increase in alkaline phosphatase activity in the treated animals was also plotted against the blood pressure increase in one week (Fig. 26, p. 146). No relationship was suggested between the two measurements in groups U and H. The negative value of the regression coefficient of enzyme activity increase on blood pressure increase calculated for group S did not reach the conventional levels of significance.

Glucose-6-phosphate dehydrogenase (Tables XXXVII and XXXVIII; Table XL, p. 208.)

The mean value of arteriolar glucose-6-phosphate dehydrogenase activity estimated in group H was significantly greater than that in groups N, S and U. The values of the mean in groups S and U were slightly greater than that in group N.

There was no indication of any relationship between glucose-6-phosphate dehydrogenase activity in arterioles and the final body weight of the animal. When the value for arteriolar glucose-6-phosphate dehydrogenase was plotted against the final blood pressure an association between the two measurements was suggested only in group H. The regression coefficient $b = 0.0058 \pm 0.0044$ in units of mole per kg. per hr./mm. Hg, was not significant. When the estimated increase in glucose-6-phosphate dehydrogenase activity was plotted against

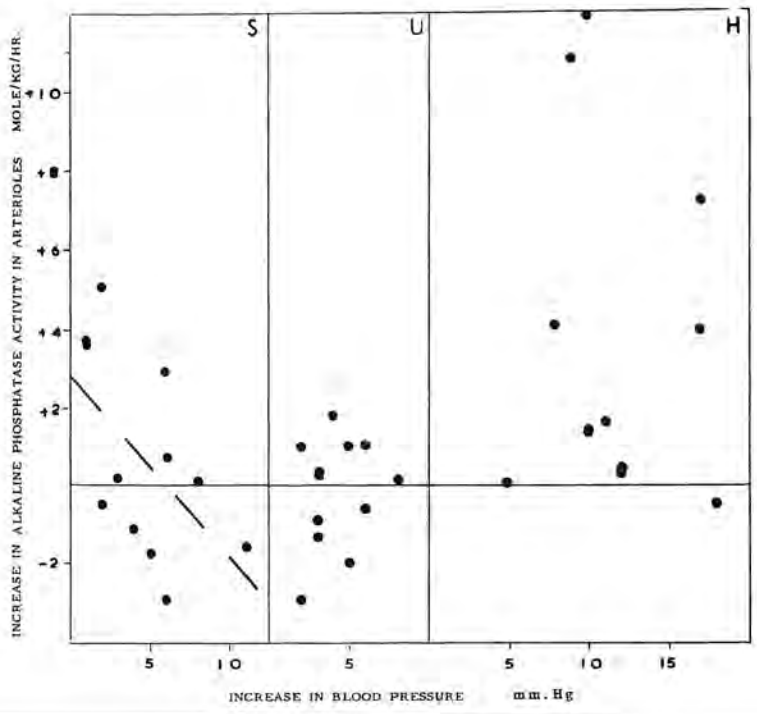

Fig. 26 The sample regressions of the estimated increase in arteriolar alkaline phosphatase activity on the blood pressure increase in rats after one week of treatment.
S = salt-loaded animals. $b = -0.462 \pm 0.229$; $0.05 < p < 0.1$.
The unit of b is mole per kg. per hr. increase in alkaline phosphatase activity /mm. Hg increase in blood pressure.
U = uninephrectomised animals.
H = DOCA hypertensive animals.

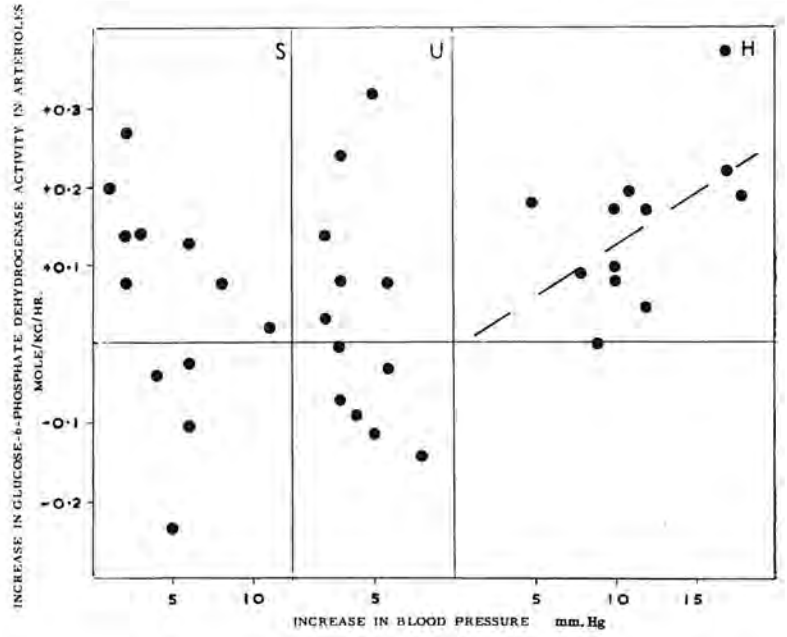

Fig. 27 Relationships between the estimated increase in glucose-6-phosphate dehydrogenase activity and the increase in blood pressure within one week in the treated groups of animals.
S = salt-loaded animals. $b = -0.020 \pm 0.013$; $p > 0.1$
The unit of b is mole per kg. per hr. increase in glucose-6-phosphate dehydrogenase activity/mm. Hg increase in blood pressure.
U = uninephrectomised animals.
H = DOCA hypertensive animals. $b = +0.0136 \pm 0.0064$;
$0.05 < p < 0.10$.

the increase in blood pressure in each of the treated animals the observed trend for the increase in enzyme activity to decrease with increasing blood pressure in group S was not significant. The positive value of the regression coefficient for group H just failed to reach conventional levels of significance (Fig. 27, p.147).

Lactate dehydrogenase (Tables XXXVII and XXXVIII; Table XLI, p.209.)

There was no significant difference in the mean activity of arteriolar lactate dehydrogenase measured in the different groups of animals. There was no suggestion of any association between lactate dehydrogenase activity and body weight or between the enzyme activity and the blood pressure measured after treatment.

Malate dehydrogenase (Table XXXVII and XXXVIII; Table XLII, p.210).

No difference of significance was observed in the mean value of arteriolar malate dehydrogenase activity measured in the four groups of animals. There was no indication of any relationship between malate dehydrogenase activity and animal weight or between enzyme activity and the blood pressure measured after treatment.

Localisation of enzymes

No differences between the four groups of animals were found when the activities and the localisations of the four enzymes observed in arteriolar tissue by histochemical methods were studied.

Alkaline phosphatase activity was localised in the adventitia of the mesenteric vessels. The distribution of the activity of this enzyme seen in this experiment by using Gomori's calcium-cobalt method with a five minute incubation period was identical to that seen in Figs. 32 to 34 where the activity is demonstrated by the coupling azo dye method described in section 4 of this chapter. Glucose-6-phosphate dehydrogenase, lactate dehydrogenase and malate dehydrogenase were distributed throughout the endothelium and media of the arterioles. The activity of lactate dehydrogenase (Fig. 35) and of malate dehydrogenase (Fig. 36) was of a high order. In comparison the activity of glucose-6-phosphate dehydrogenase was low. The activity of glucose-6-phosphate dehydrogenase is illustrated in a renal arteriole (Fig. 37) as the renal vessels were generally better preserved when the dehydrogenase enzymes were demonstrated. The low activity of this enzyme in the vessel shown can be compared with the high activity of lactate dehydrogenase seen in a similar vessel (Fig. 38).

4. The localisation of alkaline phosphatase in vascular tissue

When alkaline phosphatase was demonstrated with Gomori's method in the experiments reported, the cold microtome sections were incubated for five minutes with sodium β-glycerophosphate and the enzyme activity was shown clearly in the sites of maximum activity.

However in order to be able to interpret the changes observed in the quantitative work it was necessary to obtain a fuller picture of all the possible sites of activity of this enzyme in cardiac and arteriolar tissue.

Gomori (1941) found that the capillary endothelium throughout the body of the rat showed a high alkaline phosphatase activity. Zorzoli and Stowell (1947) reported that with Gomori's method and greatly prolonged incubation times (70 hr.) the nuclei, cross-striations, myofibrils, intercalated discs, capillaries and surrounding connective tissue of rat cardiac muscle showed a reaction for this enzyme but it is most probable that some false localisations would be seen after this treatment. Bourne (1953) however, also found alkaline phosphatase activity in the capillaries and intercalated discs. Localised areas of high activity were demonstrated in the rat heart and in the mesentery in the endothelium of vessels with luminal diameters of $< 25\ \mu$ at the point of their origin from the larger vessels by Romanul and Bannister (1962). These sites of alkaline phosphatase activity were all seen with Gomori's method but most of the work by Romanul and Bannister was done with a

modified simultaneous coupling azo dye method (Pearse, 1960e) using ∝-naphthyl phosphate and 4-amino-2,5-diethoxybenzaniline (fast blue BBN) in 0.1 M tris buffer at pH 9.5. The simultaneous azo dye method had the advantage that as it was carried out at room temperature by covering the tissue section with the incubating solution the reaction could be observed with the microscope.

Procedure

A normal male albino Wistar rat, in the weight range 200 to 250 g. was killed by exsanguination under ether anaesthesia and a block of tissue of the left ventricle apex and a block of soleus muscle were frozen immediately at -65° C as described previously. Most of the fat was dissected from the resistance vessels of the mesentery but they were left attached to the main artery, mounted on cork and frozen rapidly. 15 μ sections of these tissues were cut and they were incubated with 0.2 ml. of a filtered solution of 20 mg. ∝-naphthyl phosphate and 20 mg. blue BBN in 20 ml. of 0.1 M tris buffer pH 9.5, for increasing periods of time. Similar sections were incubated in 0.2 ml. of a filtered solution of 10 mg. ∝-naphthyl phosphate, 30 mg. fast blue B (o-dianisidine) and 50 mg. magnesium chloride in 0.7 per cent borax solution for increasing periods of time (Gomori, 1952). The sections were fixed in 10 per cent neutral formalin, counterstained with haematoxylin or Mayer's carmalum and mounted in glycerine jelly. Alkaline phosphatase in the heart and soleus muscle of the normal rat was also demonstrated by the calcium-cobalt method

of Gomori (1939) with increasing periods of incubation.

In addition to such normal material, the tissues from the eleventh and twelfth groups of animals used in the experimental study of arterioles were also treated by the coupling azo dye method with fast blue B.

Results

In normal rat cardiac tissue alkaline phosphatase activity was seen in the adventitia of the coronary arteries by all three methods used for the demonstration of this enzyme after 5 minutes' incubation with the substrate. With the three histochemical methods very slight activity of this enzyme was demonstrated in the capillaries until the sections had been incubated with the substrate for 20 min. The variability of the results among sections treated in the same way made it impossible to compare and contrast those treated by the different methods for different times.

In normal rat soleus muscle alkaline phosphatase activity was not seen in the capillaries by any of the three methods used until the sections had been incubated for 20 to 40 min.

In the normal rat mesentery alkaline phosphatase activity in the adventitia was clearly shown by all three methods after as little as 3 minutes' incubation with the substrate (Figs. 32 to 34).

No differences in the activity of alkaline phosphatase were detected in the mesenteric arterioles of the animals from the different groups studied by the coupling azo dye method.

Small branching arteries were most easily found in the rat soleus muscle and high alkaline phosphatase activity was

found in these vessels at their point of origin from the larger artery (Fig. 41). It was not, however, clear whether the activity was in the endothelium, the media or the adventitia. Branching arteries were more difficult to find in the heart and mesentery and the finding by Romanul and Bannister of high alkaline phosphatase in the small arteries where they branched from larger vessels was confirmed in the heart but not in the mesentery. It was not possible, however, to determine if the activity was in fact located in the endothelial cells in this area (Fig. 40) in the coronary vessel.

CAPTIONS FOR FIGURES 28 to 41

To view in detail:- extract the transparencies from the holder and project.

The sections photographed are all cold microtome sections. The tissue was prepared by quenching a small block immediately after the death of the animal in iso-pentane cooled to -65° C and cutting the required sections at -20° C on a "Pearse" refrigerated microtome. The sections were mounted by touching them with a slide at room temperature.

In the photographs taken to show the activity of the dehydrogenase enzymes the photographic film has been focussed on the particulate deposit which lies immediately above the tissue section.

Fig. 28 The appearance of a normal rat mesenteric arteriole before dissection. The vessel has not been freed from the surrounding fat which can be seen as bright orange-red droplets.

Sudan IV and haematoxylin. x120.

Fig. 29 Transverse section of a normal rat mesenteric arteriole to show the appearance seen following dissection and removal of the surrounding fat. Note the prominent refractile internal elastic lamina.

Sudan IV and haematoxylin. x120.

Fig. 30 Malate dehydrogenase activity in rat heart (hypertensive animal from week 10). The transverse section of a coronary artery is seen (left). The sites of the enzyme activity in medial smooth muscle are shown by a rather poorly defined blue particulate deposit overlying the vessel. The endothelium is not seen. There is no adventitial activity. Activity of the enzyme is also seen in the cardiac muscle.

MDH method, p. 50. x375.

Fig. 31 Lactate dehydrogenase activity in rat heart (normal animal from week 12). A transverse section of a small coronary arteriole is shown. The internal elastic lamina (top left) is recognised as a refractile band. The site of the enzyme activity is shown by a fine blue particulate deposit, which is seen overlying the endothelium (top left, above the elastica) and the media (below the elastica). Activity of the enzyme is also seen in the cardiac muscle.

LDH method, p. 50. x500.

Fig. 32 The appearance of a whole section of mesenteric arterioles cut from the collection of vessels from a rat (uninephrectomised animal from week 12) after they were dissected free of fat and connective tissue. Alkaline phosphatase activity shown. Media stains a homogeneous light orange-brown. Adventitial phosphatase is seen as a dark ring. Coupling azo dye method. ∝-naphthyl phosphate and o-dianisidine (fast blue B), 3 min. at 25° C.

Mayer's carmalum counterstain. x20.

Fig. 33 Higher power view of Fig. 32. Alkaline phosphatase activity in longitudinal section of rat mesenteric arteriole showing (top) a faintly stained layer of endothelial cells and (bottom) the black deposit at the site of phosphatase activity. In the centre can be seen the helicoidal arrangement of the smooth muscle cells.

x450.

Fig. 34 Alkaline phosphatase activity in rat arteriole (uninephrectomised rat from week 12, as in Figs. 32 and 33). Longitudinal section of a mesenteric arteriole showing (bottom) a row of endothelial cell nuclei and (top) the dense brown-black deposit at the site of phosphatase activity. In the centre are the smooth muscle nuclei. Coupling azo dye method. ∝-naphthyl phosphate and fast blue B, 20 min. at 25° C.

x350.

Fig. 35 Lactate dehydrogenase activity in rat arteriole (uninephrectomised animal from week 4). Longitudinal section of a mesenteric arteriole showing a fine blue particulate deposit at the site of the enzyme activity which overlies the medial smooth muscle cells. Endothelial cells are not well shown in this field.

LDH method, p. 50. x500.

Fig. 36 Malate dehydrogenase activity in a small muscular artery of the rat (uninephrectomised animal from week 6). Longitudinal section of a mesenteric vessel. The vessel is collapsed and the two distinct internal elastic laminae are seen as refractile bands to the left of the field. The enzyme activity is recognised by a blue particulate deposit overlying the endothelial cells (between the elastica) and the media.

MDH method p. 50. x425.

Fig. 37 Glucose-6-phosphate dehydrogenase activity in rat renal arteriole (salt-loaded animal from week 3). Longitudinal section of renal arteriole (upper right). The site of glucose-6-phosphate dehydrogenase activity is shown by the fine blue-grey particles which overlie the tissue. Little activity is seen in this vessel.

G-6-PDH method p. 50. x525.

Fig. 38 Lactate dehydrogenase activity in rat renal arteriole (salt-loaded animal from week 4). Longitudinal section of arteriole. The enzyme activity is indicated by the fine particulate deposit which overlies the media of the vessel (centre) and the cells of the convoluted tubules (at each margin).

LDH method p. 50. x525.

Fig. 39 Alkaline phosphatase activity in normal rat heart muscle. Longitudinal section of heart muscle showing branching, intermuscular capillary network. The capillary walls are outlined by a fine deposit of grey-black material at the site of phosphatase activity. Coupling azo dye method. α-naphthyl phosphate and blue BBN, 40 min. at 25° C.

Haematoxylin counterstain. x250.

Fig. 40 Alkaline phosphatase activity in normal rat heart muscle. A longitudinal section of a branching coronary artery showing the arterioles (top and bottom) and the arterial adventitia outlined by a brown-black deposit at the sites of phosphatase activity. No enzyme activity is seen in the arterial intima. Coupling azo dye method. α-naphthyl phosphate and fast blue B. 40 min. at 25° C.

Haematoxylin counterstain. x90.

Fig. 41 Alkaline phosphatase activity in normal rat soleus muscle. In the intermuscular connective tissue is seen a longitudinal section of a dividing arteriole. The vascular branch which is passing toward the muscle tissue (top) is covered in its proximal part by a brown deposit indicating the site of phosphatase activity. It is not possible to be certain whether phosphatase activity is predominantly adventitial, medial or intimal. Coupling azo dye method. α -naphthyl phosphate substrate and fast blue B, 40 min. at 25° C.

Haematoxylin counterstain. x100.

CHAPTER VI

DISCUSSION

The investigations described in this thesis were conducted in an attempt to determine if any alterations occurred in the metabolism of vascular muscle in the prenecrotic phase of experimental hypertension. It had been proposed (Gardner, 1963) that some alteration in the metabolism of arteriolar smooth muscle might precipitate the characteristic focal necrosis of malignant hypertension. The small amounts of arteriolar tissue available limited the study to the measurement of selected enzymes of the major metabolic pathways. Disturbances of the smooth muscle of visceral arterioles and of the striated cardiac muscle are the two principle factors involved in the maintenance of high systemic blood pressure; the enzymes of both forms of tissue were therefore subjected to analysis.

In summary, this work has shown that alk. p'tase* and G-6-PDH are altered in these tissues in rats in the early phase of deoxycortone hypertension. LDH and MDH are apparently unchanged. The variations in the enzyme activities occur

* To make reading easier thefollowing abbreviations are used in this discussion:

alk. p'tase	alkaline phosphatase activity
G-6-PDH	glucose-6-phosphate dehydrogenase activity
LDH	lactate dehydrogenase activity
MDH	malate dehydrogenase activity
DNA	deoxyribonucleic acid

in the period preceding the earliest finding of microscopically visible arterial or arteriolar lesions.

In this discussion the findings in cardiac muscle are first debated. The values of the activities of the enzymes measured in the present experiment in normal animals are correlated with the results of other authors. Each enzyme is then considered individually. Quantitative findings are contrasted with the histochemical observations and the localisation of the enzyme is compared with reports in the literature. The relationship of the measured change in enzyme activity to the blood pressure increase is considered in the light of other quantitative observations on enzyme variation in rat heart in conditions of abnormal endocrine function produced experimentally. The possibility of correlating enzyme variations in other organs with the cardiac enzyme change is then debated. Finally, the possible significance of each enzyme in vascular tissue is discussed. Before correlating the changes in enzymes which are representative of a metabolic pathway with possible functional changes in the heart in experimental hypertension, the general significance of enzyme activity measurements in relation to metabolism is emphasised.

A similar analysis of the results obtained in the study of arteriolar tissue follows. Since other observations in this field are few, comparable aspects of aortic and arterial smooth muscle metabolism are reviewed.

On the basis of the present evidence a possible plan for

future experiments is proposed.

1. Cardiac enzyme changes

A significant decrease in alk. p'tase and a significant increase in G-6-PDH were found in cardiac tissue of those animals which showed a substantial and significant rise in blood pressure after seven days of treatment. A small but statistically significant rise in blood pressure also occurred in control salt-loaded and uninephrectomised rats. It is of interest that a significant change in the activity of the two enzymes was also found in the cardiac tissue of these two groups. The change was of a similar order to that which occurred in deoxycortone-treated animals. The measured LDH and MDH in the hearts of treated animals did not differ from normal.

In a search of the literature, no previous quantitative study of these myocardial enzymes in any form of rat hypertension or in salt-loaded or uninephrectomised animals was found.

(a) Cardiac enzymes in the normal rat

Direct comparison of the values obtained in this work with other measurements of enzyme activities in normal rat hearts is not possible as previous authors have used different substrate concentrations and incubation conditions. The values determined can, however, be compared directly with those for whole rat kidney homogenate measured by Dubach and Recant (1960). Alk. p'tase and G-6-PDH values in kidney are, respectively, twenty times and six times greater than those in cardiac tissue. In contrast cardiac LDH is approximately

three times the renal value and MDH is twice the value found in rat kidney. These relative values of the enzyme activities are in agreement with those obtained by simultaneous measurements on the two tissues of G-6-PDH, (Rudolph and Olsen, 1956) and LDH and MDH (Wenner, Spirtes and Weinhouse, 1952). A low value for rat cardiac alk. p'tase was determined by Albano and Insinna (1960a) but simultaneous measurements in other rat tissues were not recorded.

(b) The changes in cardiac alkaline phosphatase activity

The alk. p'tase per unit of dry heart weight was significantly decreased by approximately 20 per cent in the cardiac tissue of the three treated groups of rats. By the histochemical method of Gomori, with a five minute incubation period, the enzyme, demonstrated clearly in the sites of maximum activity, was seen in the adventitia of coronary arteries. However, in contrast with the chemical measurements no difference was detected between the activity in the hearts of the four groups of animals. In an additional experiment it was shown that the presence of alk. p'tase could also be demonstrated in the capillaries of the normal rat heart after longer incubation periods. Activity in intercalated discs has not yet been recognised in our preparations. Although it was confirmed that high activity of this enzyme was found in small branching arteries and arterioles at their point of origin from a larger vessel it was not shown that the enzyme was localised in the endothelium (Romanul and Bannister, 1962). It appeared more probable that this high focal activity was adventitial. The alk. p'tase in arterioles at

their origin from the larger vessel must, however, represent only a very small proportion of the total measured activity in a sample of heart muscle.

There is now an increasing volume of evidence to show that there is more than one enzyme which acts as an "alkaline phosphatase" and that their pattern of activity in pathological conditions varies. In normal rat heart the alk. p'tase which Bourne (1953) found in capillaries and that detected in intercalated discs did not react with the same range of substrates. He also noted that adrenalectomy caused a reduction of the enzyme reaction in the intercalated discs but not in the capillaries, indicating a different susceptibility to endocrine control.

In spite of the numerous studies in other organs of the distribution and specificity of alk. p'tase in various conditions no qualitative observations were found in the literature on the effect of experimental hypertension on this enzyme in rat heart.

No difference was seen in adventitial alk. p'tase of coronary arteries in the different groups of rats. Nevertheless it cannot be assumed that the measured decrease occurred at a site which was not illustrated by the qualitative technique used. The measured change in activity was of the order of 20 per cent and it is unlikely that a difference of this magnitude could be seen microscopically. It is, however, possible that the qualitative and quantitative methods, which used different substrates, were delineating different enzymes.

Interpretation of the changes in cardiac alk. p'tase was complicated by the finding of different relationships in the treated groups of animals between enzyme activity and the final blood pressure of individual animals. With the administration of salt to intact rats a decrease in alk. p'tase occurred which was greater in animals with higher blood pressure. In uninephrectomised animals a different factor appeared to affect alk. p'tase. Thus although the mean value of the phosphatase activity of this group was still decreased, a trend appeared for enzyme activity values for the individual animals to be greater in those with higher blood pressures. This positive regression of alk. p'tase on the final blood pressure of individual animals became statistically significant in the hypertensive group although the mean value for these rats was lower than normal. The decreased cardiac alk. p'tase therefore showed a tendency to return to normal only in groups where a reduction in renal mass formed part of the treatment. These observations have certain implications which require consideration.

The compensatory hyperplasia of the remaining kidney which occurs in uninephrectomised animals (Rollason, 1949) has been shown to be under the control of the anterior pituitary (Astarabadi, 1962). It is possible that this effect is mediated by growth, thyrotrophic, gonadotrophic and adrenotrophic hormones (Selye, 1941) as well as by the postulated renotrophic hormone. It seems probable therefore that any of the hormones concerned with renal hyperplasia may, in the hypertensive group, superimpose their influence on an

original decrease in alk. p'tase initiated by the salt-loading, causing, directly, or indirectly, a return of cardiac alk. p'tase to normal.

In their quantitative studies of cardiac alk. p'tase Albano and Insinna studied the effect of hypophysectomy on this enzyme in female rats. They found that the decrease in activity caused by administration of pituitary extract to the normal rat, and the return to normal values of the decreased activity in hypophysectomised rats effected by this treatment (Albano and Insinna, 1960a) were partially reproduced by administration of vasopressin (Albano and Insinna, 1960b). The implications of these observations are to suggest that in deoxycortone hypertension there is an alteration in pituitary secretory activity which influences the cardiac alk. p'tase activity.

(c) Alkaline phosphatase in other organs

Finegan (1963) used qualitative methods to show that more than one alk. p'tase could be demonstrated in an organ and that there was a variation in their behaviour in pathological conditions. He observed that the kidney phosphatase demonstrated by Gomori's method, using glycerophosphate as a substrate, was lacking or decreased in biopsy material from patients with benign essential hypertension whereas the enzyme demonstrated by using sodium β-naphthyl phosphate was normal or only slightly reduced. He noted a similar decrease in patients with diabetes mellitus, a condition in which renal damage and hypertension frequently develop. Holmgård (1962) found that, in a few cases examined in a preliminary study, the value of

alk. p'tase measured in renal biopsy material was decreased in patients with glomerulonephritis and pyelonephritis when arterial hypertension was not present. Observations on hypertensive patients were not made in this series.

The evidence there is suggests that renal damage, in conditions in man which can lead to arterial hypertension, is associated with a decrease in alk. p'tase activity. In man however, it is almost impossible to obtain a series of observations indicating whether such an alteration is a cause or an effect of the condition present.

Pasqualino and Bourne (1958) observed a decrease in renal alk. p'tase activity which was greatest in the unclipped kidney in rats with renal hypertension within two days after constricting a renal artery; this change preceded the pathological changes and the hypertension. Whereas the activity in the clipped kidney returned to normal within three weeks, the enzyme activity remained low in the contra-lateral kidney and it was suggested that this defect played a part in the development of the pathological changes. Unfortunately, the method used for the enzyme demonstration was not specified. With Gomori's qualitative methods Eränkö and Niemi (1955) did not find an alteration in the renal alk. p'tase of rats treated with deoxycortone acetate and salt but the animals used showed a very slow elevation of blood pressure although the difference was statistically significant. Dubach and Recant (1960) also measured a striking decrease in alk. p'tase in the rat kidney in experimental nephrosis.

Although the present evidence in man is insufficient to determine whether a decreasedrenal alk. p'tase is a sign associated with the imminent development of hypertension the results in rats with developing renal hypertension do suggest that such a relationship can exist.

There is little information available on the effect of hormones on alk. p'tase activity of rat heart. Several studies have, however, been made of their influence on the activity of this enzyme in other organs of the rat. No clear pattern emerges.

Hypophysectomy and adrenalectomy caused a decrease in the values of renal alk. p'tase of the rat. The enzyme activity was restored to normal in hypophysectomised rats by administration of testosterone (Kochakian and Robertson, 1950), and of growth hormone (Mathies, Gaebler and Palm, 1949). In adrenalectomised rats the decrease, detected by histochemical methods, was prevented by deoxycortone acetate, one per cent sodium chloride or by testosterone propionate (Vail and Kochakian, 1947). However, hypophysectomy and adrenalectomy caused an <u>increase</u> in the activity of this enzyme in the liver of comparable animals (Kochakian and Robertson, 1950; Vail and Kochakian, 1947). The effects of hormones on the activity of alk. p'tase therefore differed depending on the organ studied. Thus although cardiac and renal alk. p'tase seem to respond in a similar manner to adrenalectomy and hypophysectomy it is inadvisable to extend this comparison to the variations produced in the renal enzyme by administered hormones. The alk. p'tase has not been shown to be identical

in the two organs and the enzynes probably have different functions.

The significance of cardiac alkaline phosphatase

The function of alk. p'tase in heart and arterial tissue is not clearly understood. In rat heart the majority of the activity is associated with arteries and capillaries. When the muscle mass increases in cardiac hypertrophy if the amount of capillary and arterial tissue does not increase the measured decrease in cardiac alk. p'tase could be due to the smaller proportion of arteries and capillaries present per unit weight of tissue. However significant hypertrophy is not yet present in the treated animals. It is therefore improbable that this is the whole of the explanation in the present experiment. Until the precise site at which the decrease in activity takes place is identified, it is only possible to speculate on the effects of the observed change.

The function of the enzyme in heart tissue probably differs in the different sites. In the adventitia of coronary arteries the enzyme is probably associated with fibroblasts and the formation of collagen fibres. In the endothelium of arteries and arterioles Romanul and Bannister (1962) have suggested that the enzyme takes part in local active transport mechanisms. The possibility that this is a function of alk. p'tase in arteriolar endothelium and in capillaries is supported by observations made with the electron microscope (Clark, 1961) that in sites of high activity the enzyme is localised in the cell membrane. Decreased alk. p'tase may, therefore effect a decrease in the supply of carbohydrates

and phosphate ions to muscle cells.

(d) The change in cardiac glucose-6-phosphate dehydrogenase activity

G-6-PDH activity was increased by approximately 18 per cent in cardiac tissue of the hypertensive rats and in control salt-loaded and uninephrectomised animals. The low activity of this enzyme was seen by qualitative methods to be distributed throughout heart muscle and coronary arteries. No difference in activity was recognised in the hearts of the different groups of rats by qualitative methods.

It is difficult to account for these changes in G-6-PDH activity. The activity of this enzyme in rat heart shows a positive correlation with phosphorylase activity and a negative correlation with glucokinase activity (Hannon and Vaughan, 1961). Thus it is possible that the factor in deoxycortone hypertension and in the treated control groups responsible for the raised G-6-PDH operates indirectly by altering the activity of either glucokinase or phosphorylase. Catecholamines could effect this result. It is known that cardiac phosphorylase is activated by adrenaline in the rat (Hess and Haugaard, 1958). There is, however, no conclusive evidence of increased secretion of catecholamines in deoxycortone hypertension although Masse and Chollot (1962) have shown that their excretion is greater in the first week of treatment than at later stages when hypertension is established. The pituitary hormones, which can cause hypertension in "sensitised" rats (Masson et al., 1950) are unlikely to affect the G-6-PDH in cardiac tissue as the enzyme activity

is not altered by hypophysectomy (Rudolph and Olsen, 1961). In a search of the literature no other relevant information on the effect of hormones on the activity of this enzyme in cardiac tissue has been found.

(e) Cardiac lactate dehydrogenase and malate dehydrogenase

The LDH and MDH in the hearts of treated animals were not different from normal. High activity of these enzymes was seen by qualitative methods to be distributed throughout heart muscle and coronary arteries. Gollnick and Hearn (1961) studied LDH in the hearts of rats exercised to produce cardiac hypertrophy. They found that when the hearts enlarged, presumably in the presence of normal blood pressure although this was unrecorded, the LDH increased. In the present experiment the cardiac LDH increased with increasing heart weight. At this early stage of hypertensive disease, however, significant differences had not yet appeared in the heart weights in the different groups.

(f) The significance of cardiac enzyme changes

The enzymes measured were chosen in an attempt to demonstrate possible changes in the various metabolic pathways. Measurements of individual enzyme activities cannot however be assumed to demonstrate the relative importance of the metabolic pathways in tissue: the values are determined under optimal enzyme kinetic conditions which have little relation to conditions in the living cell. A change in enzyme activity measured *in vitro* may however reflect a change in the enzyme population present *in vivo* and it seems reasonable to suppose that this is related to an altered metabolism by the pathway

to which the enzyme belongs. The finding of a normal value for an enzyme activity *in vitro* does indicate that the tissue has the potential for normal activity of the enzyme *in vivo* although the actual activity *in vivo* may be altered by a variation in other factors such as available substrate concentration (Weber, 1959).

Although the factor causing the increase in G-6-PDH is obscure, an increase in metabolism of glucose by the pentose phosphate pathway can be related to some of the changes which occur in hearts of hypertensive animals. In the first step in the pentose phosphate pathway, catalysed by this enzyme, and in the subsequent oxidative decarboxylation of 6-phosphogluconic acid, reduced nicotinamide-adenine dinucleotide phosphate is formed. The reduced form of this coenzyme stimulates fatty acid and cholesterol synthesis in rat liver (Siperstein and Fagan, 1958) and it has become clear that the pentose phosphate pathway and that of lipid synthesis are closely associated (McLean, 1962). It seems possible that a similar stimulus may operate in myocardial tissue and that the observed increase in G-6-PDH in the salt-loaded group may be related to the disturbance of lipid metabolism associated with advanced stages of sodium hypertension (Meneely, Tucker, Darby, Ball, Kory and Auerbach, 1954).

The observed rise in G-6-PDH may also be related to the cardiac hypertrophy associated with hypertension. A high activity of the pentose phosphate pathway has been observed in embryonic and proliferating tissues (Glock and McLean, 1954) and the ribose-5-phosphate produced by the operation of the pathway may be incorporated into ribonucleic acids.

Increased ribonucleic acid synthesis during hypertensive cardiac hypertrophy may consequently be accompanied by increased utilisation of this pathway.

The energy requirements of the hearts of the animals in the treated groups are increased at this early stage of treatment: they will continue to increase in hypertensive animals. The mechanical work of the hearts is increased by the existence of increased blood pressure and the extent of the increase is greater in the hypertensive group. Energy requirements for syntheses also continue to increase as cardiac hypertrophy develops.

The evidence in this experiment suggests that in the early stage of rat hypertension there is no quantitative alteration in the heart in metabolism by glycolysis or the citric acid cycle, the main pathways of energy liberation. It is known that the rat heart has the capacity to increase the content of enzymes such as aldolase of the glycolytic pathway, (Hearn and Wainio, 1957) and lactate dehydrogenase (Gollnick and Hearn, 1961) when hypertrophy occurs as a result of moderate exercise. In similar circumstances no increase in succinic dehydrogenase of the citric acid cycle is observed (Hearn and Wainio, 1956). It has been suggested that the succinic dehydrogenase of the heart ventricle is present in amounts greater than that required to supply the energy for the stress of moderate intermittent exercise. The lack of a significant increase in LDH and MDH in the hypertensive animals suggests that the rat heart has sufficient excess of the enzymes of the

main pathways of energy liberation to accommodate the increase in energy demand occurring at this early stage in the development of hypertension.

The study of heart tissue: a summary

When deoxycortone hypertension is induced in the rat, significant changes occur in the enzyme content of heart muscle within one week. The changes are not in the enzymes studied as representative of the main pathways of energy liberation but in alk. p'tase and G-6-PDH. Changes of a similar size occur in the cardiac content of these two enzymes in control salt-loaded rats and in uninephrectomised animals. The effects on the cardiac enzymes of the procedures used to predispose the animals to the hypertensive effects of deoxycortone do not therefore appear to be additive. Small, but statistically significant increases in blood pressure can occur in these two control groups at this early stage of treatment.

Pituitary hormones can alter cardiac alk. p'tase. Indirect evidence suggests that adrenal hormones influence cardiac G-6-PDH. The different relationships between the blood pressure level and the cardiac alk. p'tase within the different groups indicate that in the hypertensive animals a hormonal factor associated with the occurrence of renal hyperplasia superimposes an influence on an original decrease in enzyme activity caused by sodium-loading.

The observation that there are no changes in the enzymes of the glycolytic pathway and the citric acid cycle suggests that cardiac tissue has an excess of these enzymes adequate to allow for considerable variation in energy production.

2. Arteriolar Enzyme Changes

The results show that significant increases occurred in arteriolar alk. p'tase and G-6-PDH only in the deoxycortone-treated group of animals. In this group there was a significant and substantial increase in blood pressure. The changes were found after one week of treatment known, from previous work, to lead within three weeks to pronounced hypertension and fibrinoid arteriolar necrosis. Enzyme activities measured in arteriolar tissue of control uninephrectomised rats were indistinguishable from normal. The mean values of alk. p'tase and G-6-PDH in salt-loaded animals showed a slight increase which was not statistically significant but which should not therefore be totally disregarded.

(a) Arteriolar enzymes in the normal rat

The values for alk. p'tase, G-6-PDH, LDH and MDH in normal rat mesenteric arteriole, expressed as mole/kg. dry weight/hr. are 5.50, 0.777, 18.72 and 11.88 respectively. In renal arterioles of less than 100 μ diameter from normal rats, Dubach and Recant (1960) determined the values 19.62, 0.845, 33.3 and 30.4 mole/kg. dry weight/hr. respectively for the activity of these enzymes. The enzyme determinations were made under directly comparable assay conditions. They were however made on small numbers (3 to 5) and may not be truly representative. It was noted that the renal arterioles were obtained from younger animals of the Sprague-Dawley strain. The higher values of alk. p'tase measured in renal arterioles may partly be explained by the fact that the rats were of an average weight of 105 g. and were thus growing quickly. The

normal rats used in the study of mesenteric vessels had an average weight of 223 g. and although not mature, were growing less quickly. A negative relationship between alk. p'tase in the arterioles and body weight in the range 198 to 246 g. was observed in our experiments. The G-6-PDH value in mesenteric vessels was very similar to that measured in renal arterioles; the LDH and MDH measured in mesenteric arterioles were lower than in renal vessels. The ratio of $\frac{MDH}{LDH}$ was 0.91 in 100 μ diameter renal arterioles and 0.63 in 200 μ mesenteric arterioles. This suggests that the citric acid cycle could be relatively the more important route of metabolism in smaller than in larger vessels.

A number of factors can be suggested to account for the differences in the measured enzyme activities. The difference in body weight, indicative of a difference in growth phase of the animal may be partly responsible for the differences. It is also possible that these differences are caused by a variation in the proportion of smooth muscle in arterioles from different sites and of different diameters. The larger mesenteric arterioles may contain a greater proportion of adventitial fibroblasts and inert collagen and elastin material per unit dry weight. Dubach and Recant (1960) do not describe the smooth muscle content of the vessels they dissected. Experience with freeze-dried sections suggests that their technique of dissecting the vessels from dry kidney sections would result in material with a smaller proportion of adventitia and therefore with more smooth muscle present per unit weight.

A comparison of the methods of preparation of the arterioles suggests the possibility that better preservation of the enzymes is obtained with their technique.

In a search of the literature no other reports of quantitative studies of the enzyme content or of the metabolism of arterioles was discovered and this imposes a limitation on the interpretation of present results.

(b) The change in arteriolar alkaline phosphatase activity

There was a 65 per cent increase in the mean alk. p'tase in arteriolar tissue of hypertensive animals. The 13 per cent increase in the salt-loaded group was not statistically significant but should not for this reason be entirely disregarded.

By Gomori's method alk. p'tase was seen to be localised in the adventitia of the vessels of rat mesentery and no difference was recognised in this activity in arterioles from the four groups of animals. This enzyme has also been demonstrated in the endothelium of arterioles with luminal diameters of less than 25 μ in rat mesentery where they branch from larger arteries (Romanul and Bannister, 1962). This finding has not yet been confirmed in this laboratory. Activity of the enzyme in this site would however represent only a very small proportion of the total value determined for these vessels. It is therefore unlikely that changes in endothelial activity account for the differences measured.

As the site at which the increase in alk. p'tase occurred was not recognised by histochemical methods it cannot be assumed to have taken place wholly in the adventitia of the vessels. Nevertheless the suggestion that the enzyme activity which was

measured by quantitative methods is at least partly that at this site is supported by observations made during the development of the method. It was noted at that time that when individual sections of the tissue from one animal contained excessive amounts of adventitia (recognised by its different light density) high values of alk. p'tase were measured.

No relationship was demonstrated between arteriolar alk. p'tase of the rats and blood pressure levels. A relationship between arteriolar alk. p'tase and body weight was, however, evident. Although the four animals studied in each week were closely matched for weight it is possible that the variation in weight of the twelve animals in any one treated group may have obscured a relationship between enzyme activity and blood pressure with this small number.

It is difficult to determine the possible significance of the measured increase in arteriolar alk. p'tase. The function of the enzyme in the adventitia of vessels is not yet clarified. This area in these mesenteric resistance vessels is composed of fibroblasts, mucopolysaccharide ground substance, mast cells, and collagenous fibrils. There is evidence to suggest that the enzyme is associated with the formation of collagen fibres by fibroblasts (Fell and Danielli, 1943; Gould, 1960). The increase in the activity of arteriolar alk. p'tase may be associated with an increase in the collagen content of the adventitia or in the fine collagenous and reticular fibrils in the media.

Electron microscope studies of these mesenteric vessels would assist in determining if any such structural change

occurred in the media or adventitia of the mesenteric vessels of deoxycortone-treated animals. With the co-operation of Professor A. Wynn Williams vessels were fixed for such a study but unfortunately his departure precluded the completion of this investigation. Studies of the distribution of alk. p'tase in the walls of these small vessels with the electron microscope would also assist in clarifying the exact location of the enzyme.

It remains possible despite the lack of evidence from qualitative methods, that alk. p'tase is also present in the smooth muscle of the arteriole. Some of the alteration detected quantitatively in the activity of this enzyme may have occurred in this area. It is probable that it is associated there with some alternative function such as the supply or transfer of available phosphate ions.

(c) Alkaline phosphatase in the aorta

A study of the reports of measurements of rat aortic alk. p'tase was made in an attempt to illuminate the possible causes of the change observed in this enzyme in the present experiment. It was appreciated that the aorta has a low smooth muscle content per unit mass and a high proportion of elastic and collagen and connective tissue cells compared with small resistance vessels. It has however, been shown by histochemical methods that whereas in rabbit aorta the alk. p'tase was associated with the vasa vasorum, the higher value of the enzyme activity determined for the rat aorta was associated with collagen fibres (Lojda and Zemplényi, 1961). There may, therefore, be some basis for drawing a comparison between the behaviour of the enzyme

activity in the rat aorta and that which is found in the smaller vessels. The relative proportions of fibroblasts, ground substance and collagen differ in the adventitia of the vessels but it is conceivable that the enzyme in the two sites has a similar function.

Alk. p'tase in homogenates of whole rat aorta, has been shown to be dependent on body weight (Albrecht, 1960). A maximum value was determined for male rats of 170 to 190 grams; the alk. p'tase decreased with increasing animal weight. Aortic alk. p'tase was also dependent on the sex of the rat and on the season of the year. These observations suggested that the level of activity of this enzyme was determined by the interplay of endocrine factors. Hypophysectomy decreased aortic alk. p'tase in males but not in females; gonadectomy had no effect on activity in males but resulted in an increased activity in females (Albrecht, 1963). The explanation suggested for these observations was that a pituitary factor stimulated alk. p'tase activity in both sexes, the activity being decreased in the female by the influence of the ovaries. After adrenalectomy the decrease in aortic alk. p'tase was not significant in either sex, although the short period of the rats' survival on one per cent sodium chloride solution made this evidence inconclusive.

The effect on the aortic alk. p'tase values of various factors influencing blood pressure were also studied. Significantly reduced values of aortic alk. p'tase were determined after daily injections of reserpine or guanethidine

into normal rats for four days (Albrecht, 1960). The 23 per cent decrease in alk. p'tase in the aorta after daily hydralazine injections was not statistically significant. In contrast, when female rats were killed one hour after the last of four daily injections of adrenaline, an increase of 125 per cent in the activity of the aortic enzyme was found. On the basis of these observations it was suggested by Albrecht that the variation in the aortic alk. p'tase parallelled the adrenaline content of the tissues rather than the level of the blood pressure. However, as they did not report the daily variation in blood pressure or the level immediately before death this dissociation of the enzyme value from the blood pressure level is not proven.

Albrecht (1963) also studied aortic alk. p'tase in rats with experimental renal hypertension. The hypertension was induced by clamping the renal artery of normal and uninephrectomised animals. Significantly increased values of aortic alk. p'tase were found in animals which developed elevated blood pressure values after one and six months. The aortic enzyme was unaffected in operated animals which did not develop elevated blood pressure. No relationship was observed between the blood pressure values in hypertensive animals and the alk. p'tase values in the aortae. It seems possible that after this time a number of secondary factors such as the extent of arteriolar necrosis may have superimposed their effect on the mechanism responsible for initiating the elevated blood pressure. A relationship between blood pressure and a factor associated with its initial elevation would be obscured at this late stage.

The observations on aortic alk. p'tase give support to our finding that a relationship exists between the activity of this enzyme in vascular tissue and body weight. The analysis made of the hormonal factors which affect the aortic enzyme suggest that the endocrine control of vascular alk. p'tase activity is complex. The occurrence and direction of changes in aortic alk. p'tase can parallel the effect of drugs, or operational treatment, on the blood pressure. This evidence gives support to the idea that the changes in arteriolar alk. p'tase are not an incidental result of some hormonal imbalance in the animals but are associated with the effect of the treatment on the blood pressure.

(d) <u>The change in arteriolar glucose-6-phosphate dehydrogenase activity</u>

The increase in the G-6-PDH in the arteriolar tissue of the hypertensive rats was 19 per cent which is close to the value of 17 per cent found in cardiac tissue. This enzyme was demonstrated by qualitative methods to be distributed throughout the endothelium and media of arterioles but no difference between the activity in the arterioles of the four groups of animals was recognised. It is unlikely that a change in activity of this size would be detected by a subjective method.

The observation in the present experiment that there was a trend for the increase in G-6-PDH to be higher in animals showing the greatest elevation of blood pressure in response to treatment with deoxycortone acetate suggested that the activity of this enzyme in arterioles might be expected to increase

throughout the development of hypertension. This suggestion is given support by the observations of Hess and Pearse (1959) who detected, with qualitative histochemical methods, an increase in the activity of the enzyme in the endothelium and smooth muscle cells of hypertrophic interlobular arteries and glomerular arterioles of rats in the later stages of renal hypertension. Dubach and Recant (1960) however, measured an increase in this enzyme in the 100 μ diameter renal arterioles of a small number of rats with aminonucleoside nephrosis; the number of animals used was too small for statistical analysis. Renal arteriolar alk. p'tase in this condition appeared to be decreased. The evidence suggests that the increase in renal arteriolar G-6-PDH may not be specific to hypertension but in the absence of blood pressure measurements on the nephrotic rats this cannot be confirmed.

(e) Arteriolar lactate and malate dehydrogenase

LDH and MDH per unit of dry weight were unchanged in the mesenteric arterioles of treated animals. These enzymes were demonstrated by qualitative methods to be distributed throughout the endothelium and media of the resistance vessels. Although the statistical significance of the differences between the values found for these enzymes in the afferent glomerular arterioles of a few normal and nephrotic rats were not evaluated by Dubach and Recant, the differences found in such circumstances were very slight.

(f) A comparison of cardiac and arteriolar metabolism

Alk. p'tase and the G-6-PDH in arteriolar tissue of normal rats are approximately four times greater per unit dry weight

than in normal rat cardiac muscle. This greater value measured per unit dry weight is no criteria of functional importance in the case of alk. p'tase where the majority of the enzyme in the cardiac tissue appeared to be localised in arteries and capillaries. G-6-PDH however is distributed throughout the heart muscle and throughout arterial and arteriolar tissue and the greater value measured in the resistance vessels indicates that metabolism via the pentose phosphate pathway is likely to be more important in the smooth muscle cells of arterioles. Arteriolar LDH and MDH are 8 per cent of the values measured per unit dry weight for cardiac tissue. It seems possible that the capacity of arteriolar smooth muscle to metabolise substrates by the glycolytic and citric acid cycle is much less than that of cardiac muscle. To clarify this it is necessary to perform enzyme assays on material composed entirely of each type of muscle cell. The determinations of enzyme activity require to be made related to DNA and total enzyme protein measurements: the content of the enzymes can then be assessed as proportions of the total enzyme content per cell.

(g) <u>The significance of arteriolar enzyme changes</u>

It is not possible to deduce from the limited experiments reported here whether the observed changes in enzyme content did, in fact, occur in the smooth muscle cells or whether they could effectively predispose the arteriolar smooth muscle cell to focal hypercontractility. The histochemical methods employed to determine the localisation of the enzymes measured quantitatively were insufficently sensitive to show where the observed

changes occurred. These methods suggested that at least part of the change in alk. p'tase activity occurred in the adventitia of the mesenteric vessels but the presence of this enzyme in smooth muscle cells is not precluded. A change in adventitial alk. p'tase could be associated with increased synthesis of extracellular material.

The observed increase in G-6-PDH may be associated with increased synthetic activity in arteriolar cells. The energy required for synthesis may be increased and the energy required to maintain the increased peripheral resistance is increased. The situation may therefore arise where smooth muscle cells cannot continuously maintain this required increase in energy production; they appear to possess a limited capacity to metabolise substrates by glycolysis and by the citric acid cycle. The pentose phosphate pathway in arteriolar tissue may however, have some significance not yet clarified.

(h) <u>The metabolism of aortic and arterial tissue</u>

It was appreciated that the larger arteries have a much higher proportion of elastic and collagen and connective tissue cells than the small resistance vessels and consequently a low smooth muscle content per unit mass. A study of the reports of measurements of metabolism of aortic and arterial tissue was, however, made in an attempt to elucidate the metabolism of vascular smooth muscle. The majority of reports were on the metabolism of vessel walls in larger species than the rat.

It has been suggested (Mandel and Kempf, 1963) that the pentose phosphate pathway is, relatively, a more important route of glucose metabolism in other tissue containing vascular

smooth muscle, such as the aorta of adult cattle, than in such tissues as heart, liver and kidney. The methods used to demonstrate the proportion of glucose metabolised by the pentose phosphate pathway are, however, still under question.

One aspect of arterial and aortic metabolism which may have some as yet undetermined significance is the high rate of aerobic glycolysis observed in this tissue *in vitro*. As far as could be determined this has not been confirmed for the rat but it has been shown in fresh aortic tissue of the dog and human (Kirk, Effersøe and Chiang, 1954). It was calculated that in the dog aorta aerobic glycolysis accounted for as much as 39 per cent of the total energy produced *in vitro*. Südhof (1950) observed that if the tone of a beef carotid artery ring was increased by addition of adrenaline, the addition of glucose further increased both tension and lactic acid formation under aerobic conditions, suggesting that the smooth muscle cells of the media were partly responsible for aerobic glycolysis.

The aerobic production of lactic acid which occurred with contraction in vascular smooth muscle has been studied in bovine mesenteric arteries. When contraction occurs in striated muscle the glycogen content decreases (Cori, 1956): vascular smooth muscle contraction was not consistently associated with glycogenolysis but was associated with increased lactic acid production. Aerobically, electrical stimulation resulted in contraction and glycogenolysis. Anaerobically the contraction of mesenteric vessels was accompanied by increased lactic acid production but not be increased glycogenolysis (Lundholm and

Mohme-Lundholm, 1963). When adrenaline induced a contraction in the mesenteric vessel spontaneous aerobic glycogenolysis was inhibited but lactic acid production was stimulated.

The effect of adrenaline on carbohydrate metabolism in striated muscle is to accelerate the formation of the active form of the enzyme phosphorylase which catalyses the conversion of glycogen to glucose-1-phosphate(Cori, 1956). However the behaviour of phosphorylase activity on addition of adrenaline to smooth muscle varied. In tracheal muscle, where adrenaline produced glycogenolysis and relaxation, the activity of this enzyme increased whereas in mesenteric artery, contracted by adrenaline with inhibition of glycogenolysis, the phosphorylase activity was not demonstrably affected (Mohme-Lundholm, 1962).

Great caution must be observed in attempting to relate results obtained with vascular muscle in vitro to the situation in vivo or to vascular tissue in different sites in different species. Nevertheless the evidence on the behaviour of vascular smooth muscle in vitro does suggest that it may, in certain circumstances, utilise substrates on the glycolytic pathway other than glycogen for the production of energy for contraction. No information is available on the content of the intermediates in the glycolytic pathway in arterial or arteriolar smooth muscle. The relatively high activity of the enzyme of the pentose phosphate pathway in arterioles suggests that the tissue may utilise relatively high amounts of hexose phosphates.

It would therefore be of interest to determine the activity of other enzymes such as hexokinase and phosphofructokinase

concerned with hexose phosphate formation and utilisation in the arterioles of hypertensive animals.

The study of arteriolar tissue: a summary

In the prenecrotic phase of deoxycortone hypertension in the rat significant changes occur in the enzyme activity of visceral arterioles. The increases do not occur in enzymes studied as representative of the main pathways of energy liberation but in alk. p'tase and G-6-PDH. In contrast to findings in heart tissue the small changes in the enzymes in control salt-loaded animals are not statistically significant and none occur in uninephrectomised animals. This suggests that in comparison with cardiac enzyme changes the changes in arteriolar enzymes are more closely related to the capacity of the treatment for influencing blood pressure and for producing arteriolar damage.

By analogy with observations reported on larger vessels it is considered that the level of alk. p'tase in arterioles may be determined by the balance of pituitary, sex, and possibly adrenal hormones. The increase in G-6-PDH in arterioles of hypertensive animals appears to be more directly associated with the increase in blood pressure level than is the change in alk. p'tase. This evidence, viewed in the light of events in the contraction of smooth muscle in larger vessels, suggests that other enzymes directly concerned with the production and utilisation of hexose phosphates in arteriolar smooth muscle of hypertensive animals require investigation.

3. Possible future work

The problem of a more suitable method of expressing enzyme

activity measurements remains. It would be advantageous to be able to relate them to the alkali-soluble protein determined per unit of DNA in the tissue. This would provide an estimate of the proportion of enzyme present in relation to the total enzyme protein present per cell.

Attempts have been made by the present author to determine the DNA content of rat heart tissue with the ultramicro method of Keck (1956). The estimation of DNA was based on the reaction of the purine-bound deoxyribose with indole. The colorimetric estimation of DNA in standard solutions was satisfactory. With the microcell accessory for the Unicam SP.600 and cells with a 450 µl. capacity the DNA determination was made with 100 to 200 µg. dry weight of heart tissue. The extraction of DNA from the tissue on this small scale was, however, technically difficult and time-consuming.

The fluorimetric method for DNA estimation (Kissane and Robins, 1958) based on the reaction with the dihydrochloride of 3,5-diaminobenzoic acid is applicable to 2-25 µg. dry weight of tissue and is therefore preferable for the estimation of DNA in arterioles. This method has yet to be assessed. As the reagents have to be freshly prepared for each analysis this method is possibly more time-consuming.

In future experiments an improvement in the technique for the preparation of mesenteric arterioles may be possible. Dissecting the vessels free of fat and connective tissue before freeze-drying is not the ideal method of preparation for tissue to be used for enzyme determinations. There is a possibility that the enzyme contents of the tissue after the period of dissection were not identical with those in the living animal.

A practical method for freeze-drying the mesenteric vessels immediately after removal from the animal might be found if the thermo-electric freeze-dryer invented by Dr. A.G.E. Pearse and not obtainable from Edwards High Vacuum Ltd. were available. It is claimed that this equipment dries blocks of tissue 2 mm. thick at -40°C in less than 4 hr. It might, therefore be possible to dry mesenteric arterioles, freed from the excess fat, in a reasonable time. It would have to be determined whether the final careful removal of the remainder of the fat and connective tissue from a whole, freeze-dried vessel is possible.

If this technique were successful it might subsequently provide the answer to the problem of obtaining smooth muscle, free from adventita, from these vessels. To obtain a quantity of muscle of the order of 1 µg. from the sections prepared from arterioles it is necessary to collect a number of separate pieces. In the subsequent enzyme assay it is difficult to ensure that all the pieces of tissue remain in solution. Larger portions of smooth muscle obtained in one piece from a freeze-dried mesenteric arteriole would eliminate this problem.

Estimations of other enzymes such as hexokinase and phosphofructokinase, in arterioles would be of interest. Firstly, however it would be essential to establish if changes similar to those found in deoxycortone-treated animals occurred in some other form of hypertension. In steroid hypertension and in adrenal-regeneration hypertension the number of control groups of animals required becomes excessive. The simplicity

of the treatment of the animals required to produce renal hypertension recommends this form for further study.

Hypertension can be induced in rats by applying a clip to one renal artery (Wilson and Byrom, 1939). The animals are allowed to drink tap water. The use of this form of hypertension would therefore provide an experiment where only two control groups are necessary. Normal animals are required as one group of controls and a group of sham-operated animals is also necessary. It would be of great interest to study the values of arteriolar enzymes in animals with a constricted renal artery given daily injections of an anti-pressor drug in amounts known to lower blood pressure and prevent development of necrosis in these vessels. It would be desirable to determine the effect of treatment with the drug on arteriolar enzymes of normal animals.

The proportion of animals which develop renal hypertension is less than that after treatment with deoxycortone and the elevation in blood pressure is more variable. In order to have the best basis for comparing the results with those found in deoxycortone hypertension, it would be desirable to select those animals showing comparable increases in blood pressure within one week. The difference from normal of this rate of increase of the blood pressure would be more definite after two weeks. Estimations on the tissues of such animals would assist in establishing if any enzyme changes which occurred were increased with time of treatment and with the increasing

blood pressure. A detailed histological examination of the mesenteric vessels would be required to ascertain that necrosis was not evident.

It is known that a comparable study with four groups of animals is possible. Six groups would be the ideal number in this experiment. The successful execution of an experiment with the additional numbers of animals would depend on the amount and quality of technical assistance available.

The present results suggest that the value of qualitative histochemical methods in the study of vascular tissues from animals in the early stage of hypertension under investigation is doubtful. The application of these methods to the kidneys of renal hypertensive animals might, however, assist in assessing if any gross changes occurred in the enzymes in this organ during the development of pathological changes.

A subsequent study of the enzymes in the cardiac tissue of rats in the prenecrotic phase of renal hypertension would be valuable. It has been shown in unilaterally nephrectomised rats with a clip on the remaining renal artery that the increase in cardiac output which occurred after five and ten days was no longer found after fifteen days (Ledingham and Cohen, 1963). It would be desirable to determine whether any cardiac enzyme changes were reversed at the time when cardiac output returned to normal. The results obtained with deoxycortone hypertension however, show that unilateral nephrectomy complicates the interpretation of cardiac enzyme measurements. It would, therefore, be preferable to study the cardiac enzymes in groups

of animals similar to those suggested for further study of arteriolar enzymes in hypertension.

The quantitative techniques of enzyme assay in freeze-dried sections developed in this work have many possible future applications in the study of vascular tissue in experimental hypertension. Although it would be premature to claim that the enzyme content of the arterioles of hypertensive patients is altered, a study of such vessels dissected from human biopsy material would be of great interest.

The useful application of these methods of analysis extends beyond the problem of hypertension to the general study of the events resulting in pathological changes in cells and tissues which are available only in limited quantities. The techniques evolved in the work reported in this thesis may be regarded as potential tools of experimental pathology.

SUMMARY

1. The acute fibrinoid necrosis of visceral arterioles which occurs in the malignant phase of hypertension is generally attributed to the critical rise in blood pressure which takes place in this condition. Experimental evidence, obtained in the rat with deoxycortone hypertension, led to the hypothesis that early in the development of the hypertension a change occurred in the internal environment of the smooth muscle cell which might predispose the cell to necrosis.

2. It was suggested that this alteration might be, or could be attributable to, a disturbance of the energy production by metabolism in the cell. A study of selected enzymes in the arterioles of rats in the early phase of deoxycortone hypertension was therefore undertaken. As energy production in cardiac muscle may also be disturbed when raised blood pressure is maintained, enzymes in this muscle were also studied.

3. The techniques chosen for enzyme assays were those which could subsequently be adapted to the study of isolated arteriolar smooth muscle cells. Methods were developed for the preparation of freeze-dried vascular tissue and for the assay of alkaline phosphatase, glucose-6-phosphate dehydrogenase, lactate dehydrogenase and malate dehydrogenase in microgram quantities of this material. The localisation of the enzymes was also studied by qualitative histochemical methods.

4. Hypertension was initiated in male, albino, Wistar rats by implanting deoxycortone acetate subcutaneously in uninephrectomised, salt-loaded animals. Control groups of

uninephrectomised rats, salt-loaded rats and normal animals were maintained under identical conditions.

5. A significant decrease in alkaline phosphatase activity and a significant increase in glucose-6-phosphate dehydrogenase activity were found in the cardiac tissue of deoxycortone-treated rats after one week: the systolic blood pressure had risen significantly in these animals. Changes of a similar size were found in the activity of these two enzymes in the cardiac tissue of control uninephrectomised and salt-loaded animals: a slight but statistically significant increase in blood pressure occurred in these groups.

No differences were found in the mean values of the activities of lactate dehydrogenase or malate dehydrogenase in heart tissue from the four groups of rats.

6. The different relationships observed between cardiac alkaline phosphatase activity and the blood pressure in the individual animals of the treated groups suggested that the enzyme value showed a trend of returning to normal in these animals in which raised blood pressure was accompanied by compensatory renal hyperplasia.

7. In the second experiment the enzymes were studied in mesenteric arterioles. A significant increase in blood pressure occurred only in the deoxycortone-treated rats. A significant increase in alkaline phosphatase and in glucose-6-phosphate dehydrogenase activity in mesenteric arterioles occurred only in this group. There was a trend for arteriolar alkaline phosphatase activity to decrease with increasing weight of the animals. This was not significant in normal

animals but was so in uninephrectomised rats. The trend for the increase in arteriolar glucose-6-phosphate dehydrogenase activity to be greater in animals with the greater increase in blood pressure failed to reach conventional levels of significance.

No differences were observed in the mean values of the activities of lactate dehydrogenase or malate dehydrogenase in the arterioles of the four groups of rats.

8. A comparison of the enzyme activities measured suggested that whereas cardiac muscle has a greater capacity for metabolism by glycolysis and the citric acid cycle the pentose phosphate pathway is more important in arteriolar tissue.

9. Neither in cardiac tissue nor in arteriolar tissue of hypertensive animals were there alterations in the enzymes of the glycolytic pathway or the citric acid cycle, the main energy-supplying routes of metabolism. The activities of the enzymes altered are known to be under hormonal control. The small amount of evidence there is on vascular tissue enzymes suggests that the variation in the enzyme activities in the early stage of experimental hypertension is possibly determined by a pituitary or adrenal dysfunction initiated by the sodium load.

10. The enzyme changes which occurred in the different groups of animals in arteriolar tissue showed a closer relationship to the capacity of the given treatment for increasing blood pressure and producing arteriolar damage than those which occurred in cardiac tissue.

11. Further studies are necessary to determine if the observed changes in the vascular enzymes are also found at an

early stage in other forms of experimental hypertension. A study of the enzymes in arterioles from human biopsy material from hypertensive patients would establish the relationship of these observations in experimental disease to the human condition.

ACKNOWLEDGMENTS

The work described in this thesis was carried out while the author was employed as a Research Assistant by the Hypertension Research Unit in the Department of Pathology of the University of Edinburgh under the direction of Dr. D.L. Gardner. I am indebted to him for his advice and co-operation and for his generous provision of facilities. The interest taken in this work and the advice given by Professor G.L. Montgomery is gratefully acknowledged.

Dr. C.P. Stewart and subsequently Dr. J.A. Owen and Professor L.G. Whitby of the Department of Clinical Chemistry of the Royal Infirmary kindly made the facilities of their department available. The co-operation of their Staff was appreciated.

My thanks are due to Professor R.B. Fisher for instruction in statistical methods. With his co-operation the visit to the Chemical Defence Experimental Establishment was made possible.

The animal operations were carried out by Dr. D.L. Gardner. I am also indebted to him for the assessment of the histochemical preparations.

The practical help and advice of Dr. M.J.W. Faed was generously given. My thanks are also due to Miss Elizabeth Ramage for indispensable technical assistance. Mr. Robin Ewart, B.Sc. performed the malate dehydrogenase assays in cardiac tissue.

The graphs were photographed by Mr. G. Renwick and the

microphotography was done by the Medical Photography Unit of the University. I wish to express my appreciation of this service.

The assistance given in the preparation of this thesis by the typist, Mrs. C. Graham is gratefully acknowledged.

This work was made possible by the grant to the Hypertension Research Unit from the National Institutes of Health, Washington (HE-05173).

PUBLICATION

A report of part of the work described in this thesis has been accepted for publication by the British Journal of Experimental Pathology under the title "Cardiac metabolism in early rat hypertension: quantitative enzyme histochemistry in the prenecrotic phase".

The results of the investigations described in this paper were presented to the Edinburgh Pathological Club and subsequently to the Pathological Society of Great Britain and Ireland in January, 1963.

APPENDICES

1. Measurements made on the individual animals used in the study of heart tissue.
   Tables XXIV to XXVI; XXIX to XXXII.

2. Measurements made on the individual animals used in the study of arteriolar tissue.
   Tables XXXV and XXXVI; Tables XXXIX to XLII.

APPENDIX I

TABLE XXIV

Systolic blood pressure of rats before treatment and six days after commencing treatment

N = normal, S = salt-loaded, U = uninephrectomised
H = DOCA hypertensive

Rats used on week no.	Blood pressure mm.Hg							
	Group N		Group S		Group U		Group H	
	Before	After	Before	After	Before	After	Before	After
1	100	109	98	105	104	118	98	146
2	117	116	118	124	100	121	103	128
3	126	128	90	115	108	120	103	141
4	115	113	125	127	110	120	103	127
5	109	111	105	110	113	115	101	127
6	102	103	112	119	107	109	115	134
7	111	113	119	120	111	118	122	137
8	107	110	110	125	125	130	108	144
9	130	132	115	120	115	121	124	139
10	110	112	110	112	110	115	116	130
11	120	123	106	120	107	109	106	139
12	103	104	120	129	124	125	110	121
Mean	113	115	111	119	111	118	109	134
S.E.M.	±2.6	±2.6	±2.9	±2.0	±2.0	±1.7	±2.3	±2.3
p*	n.s.		< 0.05		< 0.02		<< 0.001	

* Significance of the increase in the mean.

TABLE XXV

Body weight of rats before treatment and seven days after commencing treatment

N = normal, S = salt-loaded, U = uninephrectomised
H = DOCA hypertensive

Rats used on week no.	Body weight g.							
	Group N		Group S		Group U		Group H	
	Before	After	Before	After	Before	After	Before	After
1	209	202	235	201	198	219	233	189
2	141	170	182	201	158	193	236	162
3	257	250	223	220	164	158	202	201
4	229	235	268	272	257	255	205	210
5	250	263	234	249	266	269	257	261
6	266	272	274	283	245	263	256	260
7	201	221	267	275	280	289	231	242
8	203	215	192	207	210	206	235	246
9	257	263	203	211	201	213	195	196
10	202	209	151	160	190	199	195	200
11	180	200	198	220	175	189	182	191
12	206	215	207	219	227	234	215	220
Mean	217	226	220	227	214	224	220	215
S.E.M.	±10.7	±8.95	±11.0	±10.4	±11.8	±11.3	±7.22	±8.95
p*	n.s.		n.s.		n.s.		n.s.	

* Significance of the change in the mean.

TABLE XXVI

Heart weight of rats seven days after commencing treatment

N = normal, S = salt-loaded, U = uninephrectomised
H = DOCA hypertensive

Rats used on week no.	Heart weight g.			
	Group N	Group S	Group U	Group H
1	0.630	0.594	0.717	0.661
2	0.694	0.679	0.596	0.573
3	0.731	0.744	0.530	0.645
4	0.711	0.863	0.751	0.682
5	0.628	0.694	0.756	0.896
6	0.783	0.776	0.698	0.771
7	0.675	0.742	0.634	0.670
8	0.575	0.590	0.549	0.658
9	0.665	0.730	0.618	0.624
10	0.662	0.713	0.568	0.673
11	0.616	0.697	0.523	0.598
12	0.716	0.715	0.672	0.695
Mean	0.674	0.711	0.634	0.679
S.E.M.	±0.016	±0.021	±0.024	±0.025
p*		n.s.	n.s.	n.s.

* Significance of the difference of the mean from that of group N.

TABLE XXIX

Alkaline phosphatase activity
in rat left ventricle apex

N = normal, S = salt-loaded, U = uninephrectomised
H = DOCA hypertensive

Rats used on week no.	Alkaline phosphatase activity mole/kg./hr.*			
	Group N	Group S	Group U	Group H
1	1.535	1.246	0.897	0.780
2	1.068	0.967	0.721	0.833
3	1.447	1.102	1.082	1.046
4	1.469	1.006	1.579	0.936
5	1.052	1.385	0.763	0.662
6	1.212	1.224	0.834	1.059
7	1.413	1.176	0.896	1.464
8	1.766	0.895	1.642	1.626
9	1.035	1.042	1.245	1.188
10	1.479	1.268	1.133	1.250
11	1.184	1.267	0.478	1.238
12	1.357	0.654	0.634	0.441
Mean	1.335	1.103	0.992	1.044
S.E.M.	±0.065	±0.058	±0.104	±0.097
p+		<0.02	<0.02	<0.05
Mean as Units ## /mg.	0.0223	0.0184	0.0165	0.0174

* Activity expressed as moles of substrate transformed by one kilogram of dry tissue weight per hr.

\+ Significance of the difference of the mean from that for group N.

The standard unit as recommended by the "Report of the Commission on Enzymes of the I.U.B."1961, i.e. that amount of enzyme which will catalyse the transformation of 1 μ-mole of substrate per min. under defined conditions per mg. dry weight of tissue.

TABLE XXX

Glucose-6-phosphate dehydrogenase activity
in rat left ventricle apex

N = normal, S = salt-loaded, U = uninephrectomised
H = DOCA hypertensive

Rats used on week no.	Glucose-6-phosphate dehydrogenase activity mole/kg./hr.*			
	Group N	Group S	Group U	Group H
1	0.185	0.193	0.186	0.202
2	0.260	0.278	0.202	0.278
3	0.199	0.248	0.290	0.238
4	0.182	0.186	0.292	0.191
5	0.256	0.268	0.220	0.318
6	0.177	0.230	0.226	0.270
7	0.258	0.362	0.212	0.262
8	0.222	0.318	0.250	0.242
9	0.183	0.254	0.354	0.260
10	0.242	0.234	0.310	0.258
11	0.220	0.240	0.250	0.242
12	0.180	0.268	0.244	0.250
Mean	0.214	0.257	0.253	0.251
S.E.M.	±0.010	±0.014	±0.014	±0.010
p+		<0.02	<0.05	<0.02
Mean as Units #/mg.	0.00357	0.00428	0.00422	0.00418

* Activity expressed as moles of substrate transformed by one kilogram of dry tissue weight per hr.

+ Significance of the difference of the mean from that for group N.

\# The standard unit as recommended by the "Report of the Commission on Enzymes of the I.U.B." 1961, i.e. that amount of enzyme which will catalyse the transformation of 1 µ-mole of substrate per min. under defined conditions per mg. dry weight of tissue.

TABLE XXXI

Lactate dehydrogenase activity
in rat left ventricle apex

N = normal, S = salt-loaded, U = uninephrectomised
H = DOCA hypertensive

Rats used on week no.	Lactate dehydrogenase activity mole/kg./min.*			
	Group N	Group S	Group U	Group H
1	4.226	4.658	4.390	4.360
2	4.616	4.930	4.248	4.298
3	4.308	4.250	3.916	3.888
4	4.332	4.452	4.900	4.384
5	4.270	4.204	4.198	4.132
6	4.262	4.046	4.638	4.818
7	4.154	4.196	3.762	4.286
8	3.750	3.926	3.868	3.730
9	3.604	3.478	3.796	3.708
10	3.890	3.322	3.484	3.640
11	3.620	3.638	3.310	3.618
12	3.722	3.948	3.788	3.946
Mean	4.06	4.09	4.03	4.07
S.E.M.	±0.10	±0.14	±0.13	±0.11
p+		n.s.	n.s.	n.s.
Mean as Units// /mg.	4.06	4.09	4.03	4.07

* Activity expressed as moles of substrate transformed by one kilogram of dry tissue weight per min.

\+ Significance of the difference of the mean from that for group N.

// The standard unit is that amount of enzyme which will catalyse the transformation of 1 μ-mole of substrate per min. under defined conditions, per mg. of dry weight.

TABLE XXXII

Malate dehydrogenase activity
in rat left ventricle apex

N = normal, S = salt-loaded, U = uninephrectomised
H = DOCA hypertensive

Rats used on week no.	Malate dehydrogenase activity # mole/kg./min.*			
	Group N	Group S	Group U	Group H
1	2.434	-	-	2.547
2	2.411	-	-	2.334
3	2.462	2.515	2.472	2.550
4	2.291	2.615	2.592	2.604
5	2.327	2.512	2.402	2.572
6	2.346	2.467	2.486	2.558
7	2.413	2.452	-	2.517
8	2.465	2.379	-	2.257
9	2.365	-	2.363	-
10	-	-	-	2.187
11	2.306	2.283	-	2.246
12	2.193	2.273	2.214	2.199
Mean	2.37	2.44	2.42	2.42
S.E.M.	±0.025	±0.042	±0.052	±0.051
p+		n.s.	n.s.	n.s.
Mean as Units #/mg.	2.37	2.44	2.42	2.42

* Activity expressed as moles of substrate transformed by one kilogram of dry tissue weight per min.

\+ Significance of the difference of the mean from that for group N.

\# The standard unit is that amount of enzyme which will catalyse the transformation of 1 µ-mole of substrate per min. under defined conditions, per mg. of dry weight.

Estimated in freeze-dried tissue stored for 1 year at -20° C.

APPENDIX II

TABLE XXXV

Systolic blood pressure of rats before treatment and seven days after commencing treatment

N = normal, S = salt-loaded, U = uninephrectomised
H = DOCA hypertensive

Rats used on week no.	Blood pressure mm.Hg							
	Group N		Group S		Group U		Group H	
	Before	After	Before	After	Before	After	Before	After
1	109	110	101	103	118	120	102	114
2	120	122	100	106	104	108	121	129
3	110	111	98	106	115	120	120	130
4	106	110	109	112	119	124	121	138
5	104	107	111	117	114	120	113	124
6	117	119	117	121	109	111	117	129
7	120	123	100	106	114	117	110	120
8	121	121	104	105	104	110	111	121
9	109	110	105	107	121	124	124	129
10	119	123	119	121	115	118	101	118
11	112	114	106	111	104	112	115	124
12	107	109	109	120	119	125	111	129
Mean	113	115	107	111	113	117	114	125
S.E.M.	±1.8	±1.8	±1.9	±2.0	±1.7	±1.7	±2.1	±1.9
p*	n.s.		n.s.		n.s.		<0.001	

* Significance of the increase in the mean.

TABLE XXXVI

Body weight of rats before treatment and seven days after commencing treatment

N = normal, S = salt-loaded, U = uninephrectomised
H = DOCA hypertensive

Rats used on week no.	Body weight g.							
	Group N		Group S		Group U		Group H	
	Before	After	Before	After	Before	After	Before	After
1	198	211	198	207	202	203	208	202
2	221	240	228	239	228	235	223	232
3	224	234	237	245	230	233	230	234
4	238	246	233	239	236	247	232	240
5	194	209	190	205	200	204	206	205
6	206	218	206	220	227	230	216	215
7	216	225	200	210	218	224	223	221
8	218	228	224	234	228	227	224	226
9	209	218	213	222	210	225	235	231
10	218	229	206	221	213	220	214	222
11	182	198	182	194	200	212	193	204
12	215	224	223	227	219	226	226	229
Mean	212	223	212	222	218	224	219	222
S.E.M.	±4.3	±3.9	±5.1	±4.5	±3.6	±3.7	±3.5	±3.7
p*	n.s.		n.s.		n.s.		n.s.	

* Significance of the change in the mean.

TABLE XXXIX

Alkaline phosphatase activity
in rat mesenteric arterioles

N = normal, S = salt-loaded, U = uninephrectomised
H = DOCA hypertensive

Rats used on week no.	Alkaline phosphatase activity mole/kg./hr.*			
	Group N	Group S	Group U	Group H
1	7.261	6.785	8.263	7.559
2	4.202	4.882	6.029	8.203
3	4.993	5.155	3.010	6.389
4	2.889	3.093	3.904	10.086
5	8.209	5.247	9.273	9.823
6	7.003	5.899	4.095	7.436
7	5.222	8.083	5.141	6.618
8	4.172	7.840	4.418	16.089
9	4.027	7.615	4.356	4.143
10	4.014	9.035	2.686	8.011
11	5.386	3.694	5.579	16.164
12	8.619	7.059	8.046	8.043
Mean	5.50	6.20	5.40	9.05
S.E.M.	±0.53	±0.53	±0.61	±1.05
p^{+}		n.s.	n.s.	< 0.01
$p^{\#\#\#}$		< 0.05	< 0.01	
Mean as Units ##/mg.	0.0917	0.1033	0.0900	0.1508

* Activity expressed as moles of substrate transformed by one kilogram of dry weight of tissue per hr.

\+ Significance of the difference of the mean from that for group N.

The standard unit is that amount of enzyme which will catalyse the transformation of 1 μ-mole of substrate per min. under defined conditions per mg. dry weight.

Significance of the difference of the mean from that for group H.

TABLE XL

Glucose-6-phosphate dehydrogenase activity
in rat mesenteric arterioles

N = normal, S = salt-loaded, U = uninephrectomised
H = DOCA hypertensive

Rats used on week no.	Glucose-6-phosphate dehydrogenase activity mole/kg./hr.*			
	Group N	Group S	Group U	Group H
1	0.6867	0.7597	0.8208	0.8504
2	0.8345	0.8094	0.7426	0.9162
3	0.7748	0.8459	0.6585	0.8672
4	0.6671	0.8065	0.9812	1.0316
5	0.8092	0.7052	0.7729	0.9959
6	0.7304	0.6887	0.7588	0.7722
7	0.6829	0.8094	0.9222	0.7619
8	0.7498	0.9461	0.7410	0.9184
9	0.9200	1.0571	0.8454	1.0915
10	0.7334	1.0021	0.8099	0.9495
11	0.9452	0.7102	0.7968	0.9443
12	0.7837	0.8040	0.7134	0.9659
Mean	0.777	0.829	0.797	0.922
S.E.M.	±0.026	±0.034	±0.026	±0.028
p+		n.s.	n.s.	<0.001
p###		<0.05	<0.01	
Mean as Units ##/mg.	0.0130	0.0138	0.0133	0.0154

* Activity expressed as moles of substrate transformed by one kilogram of dry weight of tissue per hr.

\+ Significance of the difference of the mean from that for group N.

The standard unit is that amount of enzyme which will catalyse the transformation of 1 μ-mole of substrate per min. under defined conditions per mg. dry weight.

Significance of the difference of the mean from that for group H.

TABLE XLI

Lactate dehydrogenase activity
in rat mesenteric arterioles

N = normal, S = salt-loaded, U = uninephrectomised
H = DOCA hypertensive

Rats used on week no.	Lactate dehydrogenase activity mole/kg./min.*			
	Group N	Group S	Group U	Group H
1	0.3020	0.3183	0.3493	0.3225
2	0.3325	0.2853	0.2986	0.3456
3	0.3425	0.3476	0.3126	0.3592
4	0.2822	0.2675	0.3529	0.3428
5	0.3078	0.2937	0.3766	0.3289
6	0.2902	0.3235	0.2800	0.3132
7	0.2855	0.2871	0.3381	0.3137
8	0.3516	0.3964	0.3089	0.3146
9	0.3110	0.2990	0.2930	0.2716
10	0.2852	0.3252	0.2821	0.2422
11	0.3236	0.2482	0.3180	0.2432
12	0.3325	0.2824	0.2999	0.3958
Mean	0.312	0.306	0.318	0.318
S.E.M.	±0.007	±0.011	±0.009	±0.013
p+		n.s.	n.s.	n.s.
Mean as Units #/mg.	0.312	0.306	0.318	0.318

* Activity expressed as moles of substrate transformed by one kilogram of dry weight of tissue per min.

+ Significance of the difference of the mean from that for group N.

\# The standard unit is that amount of enzyme which will catalyse the transformation of 1 µ-mole of substrate per min. under defined conditions per mg. dry weight.

TABLE XLII

Malate dehydrogenase activity
in rat mesenteric arterioles

N = normal, S = salt-loaded, U = uninephrectomised
H = DOCA hypertensive

Rats used on week no.	Malate dehydrogenase activity mole/kg./min.*			
	Group N	Group S	Group U	Group H
1	0.1833	0.1771	0.1795	0.1834
2	0.1854	0.1659	0.1647	0.2051
3	0.2786	0.2878	0.2114	0.2708
4	0.1628	0.1653	0.1948	0.2105
5	0.1877	0.1793	0.2016	0.1808
6	0.1711	0.1697	0.1517	0.1752
7	0.1980	0.1496	0.1877	0.2103
8	0.2105	0.2195	0.1908	0.2052
9	0.2128	0.1706	0.2119	0.1802
10	0.1864	0.2231	0.2041	0.1831
11	0.1847	0.1538	0.1648	0.1521
12	0.2186	0.1626	0.1843	0.2954
Mean	0.198	0.185	0.187	0.204
S.E.M.	±0.009	±0.011	±0,006	±0.012
p+		n.s.	n.s.	n.s.
Mean as Units ##/mg.	0.198	0.185	0.187	0.204

* Activity expressed as moles of substrate transformed by one kilogram of dry weight of tissue per min.

\+ Significance of the difference of the mean from that for group N.

The standard unit is that amount of enzyme which will catalyse the transformation of 1 µ-mole of substrate per min. under defined conditions per mg. dry weight.

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