THE EFFECTS OF INSULIN AND INSULIN DEFICIENCY ON THE PATTERN OF METABOLISM OF PERFUSED RAT HEART

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ABSTRACT

1. A perfused heart preparation was studied in which oxygenated Krebs-Henseleit buffer introduced into the left atrium was pumped by the heart via the aorta against a pressure of 100 cm H_2O, the heart thereby performing mechanical work. Preparations stable over periods of three hours were obtained. Comparisons with aortic perfused (Langendorff) hearts were made.

2. Working hearts recovered from anoxia provided glucose was present during anoxia and glucose or pyruvate were available during reoxygenation.

3. Increased aortic pressure in Langendorff hearts and performance of work by atrially perfused hearts both increased oxygen consumption, the uptake and oxidation of glucose-U-C^{14} and glycogen turnover, but not lactate production.

4. Glucose oxidation accounted for 40% of respiration of both Langendorff and working hearts initially but rose to 80% after 45 mins of work.

5. Work decreased cardiac G6P, F6P, ATP and phosphocreatine and increased FDP and AMP.

6. Insulin increased glucose uptake and oxidation, lactate production and glycogen formation from glucose in working and Langendorff hearts without affecting oxygen consumption. These effects of insulin could not be reproduced by increasing the extracellular glucose concentration.

7. Glucose metabolism by perfused hearts was reduced by removal of endogenous insulin with anti-insulin serum.

8. A specific insulin deficient hyperglycaemia was observed in rats 7 days after 65 mg/kg i.v. of streptozotocin.

9. Langendorff hearts from streptozotocin diabetic rats and anti-insulin treated rats showed decreased glucose uptake and oxidation which was restored to normal by heart work. Reduced incorporation of glucose into glycogen was observed in both working and Langendorff hearts from streptozotocin and alloxan diabetic rats, but was restored by insulin only in the streptozotocin diabetic hearts. Insulin failed, however, to stimulate glucose oxidation by streptozotocin diabetic working hearts.

10. Cardiac G6P and citrate were reduced in streptozotocin diabetic hearts whilst pyruvate was increased. Even higher pyruvate accumulation was observed in alloxan diabetic hearts.

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Abbreviations

The following abbreviations have been used:

ATP = adenosine-5'-triphosphate;

ADP = adenosine-5'-diphosphate;

AMP = adenosine-5'-monophosphate;

Pi = inorganic phosphate;

CrP = creatine phosphate or phosphocreatine;

NAD & $NADH_2$ = oxidised and reduced forms of nicotinamide-adenine dinucleotide;

NADP & $NADPH_2$ = oxidised and reduced forms of nicotinamide-adenine dinucleotide phosphate;

FFA = free fatty acids or unesterified fatty acids or nonesterified fatty acids (NEFA);

G6P = glucose-6-phosphate;

F6P = fructose-6-phosphate;

FDP = fructose 1,6-diphosphate;

L/P = ratio of concentrations of lactate and pyruvate;

αGP/DHAP = ratio of concentrations of α-glycerophosphate and dihydroxyacetonephosphate.

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The Effects of insulin and insulin deficiency on the pattern of metabolism of perfused rat heart

INTRODUCTION

The importance of insulin in the regulation of glucose uptake in muscle tissue is reflected in the published literature which contains a variety of experimental techniques designed to throw light on the control of glucose uptake and its utilisation. The most direct of these employ isolated preparations of intact muscle such as the rat diaphragm (Gemmill, 1940) and heart (Bleehen and Fisher, 1954), the frog sartorius muscle (Hegnauer and Cori, 1934 or the rat gastrocnemius *in situ* (Lundsgaard, 1939). Isolated preparations have the advantage of allowing close control of experimental variables and can yield relatively precise measurements but the conditions are often unphysiological. The isolated perfused rat heart preparation has several particular advantages for biochemical studies. These are:-

1. The muscle fibres are intact so that it is possible to distinguish between membrane transport processes and diffusion artifacts introduced by cut edges, as found, for example, in the usual diaphragm preparation.
2. The extracellular diffusion of substrates and hormones from the medium to the cells is very rapid since all substrates penetrate the tissue via the extensive capillary network.

3. Uptake of exogenous substrates can be measured directly by the change in concentration of a known volume of medium circulated for a given time.
4. Condition of the preparation can be judged by physiological parameters, i.e. contraction, perfusion pressure, coronary flow, and oxygen consumption.
5. Competition between substrates may be measured without interference from unknown or uncontrolled humoral factors which apply in vivo and to a certain extent in the classical heart-lung preparation.
6. The oxygen tension of the extracellular fluid is higher and more uniform than that of incubated tissues, where fluid of high oxygen tension impinges directly only on the two outermost layers of cells (Creese, Scholes and Whalen, 1958).

A simple, retrograde, aortic perfused isolated heart preparation was described as early as 1895 by Langendorff. Interest in glucose metabolism in the perfused heart dates from at least 1907 when Locke and Rosenheim found glucose uptake in the Langendorff preparation. Many subsequent studies were carried out on the classical heart-lung preparation of the dog as described by Knowlton and Starling (1912a) who established that sugar was used by the dog heart and that pancreatectomy impaired this ability to consume sugar (Knowlton and Starling, 1912b). However, the complex

relationships between mechanical activity, oxygen consumption, substrate utilisation and hormonal control can be studied more readily by using a simpler heart preparation that does not include lungs or other tissues in the circuit and with a synthetic, chemically defined medium as the perfusion fluid in place of blood. Fisher and his associates (Bleehen and Fisher, 1954; Bronk and Fisher, 1957; Fisher and Lindsay, 1956) carried out a systematic investigation into the optimum conditions for isolated heart perfusion using the Langendorff preparation and succeeded in controlling variables which had previously led to doubts as to the stability of this preparation. With their stable preparation, reliable investigations of oxygen consumption, (Fisher and Williamson, 1961 a,b), substrate utilisation and hormonal regulation became possible (Fisher and Zachariah, 1961; Zachariah, 1961). Competition between exogenous and endogenous substrates (Fisher and Williamson, 1961b; Williamson, 1962; Shipp, Matos, Knizley and Crevasse, 1964), and the factors affecting glucose uptake (Morgan, Henderson Regen and Park, 1961b; Opie, Shipp, Evans and Leboeuf, 1962), pyruvate (Evans, Opie and Renold, 1963; Williamson, 1965), lactate (Williamson, 1962), ketone bodies (Williamson and Krebs, 1961), and long chain fatty acid utilisation (Shipp, Opie and Challoner, 1961; Evans, Opie, and Shipp, 1963) have all been extensively studied using such a preparation. The metabolic effects of insulin (Fisher and Zachariah, 1961; Park, Reinwein, Henderson, Cadenas and Morgan, 1959; Morgan et al., 1961b),

adrenaline (Williamson, 1964), glucagon (Cornblath, Randle, Parmeggiani and Morgan, 1963), growth hormone (Regen, Davis, Morgan and Park, 1964), anoxia (Morgan, Randle and Regen, 1959; Morgan et al., 1961b; Newsholme and Randle, 1961) and starvation (Newsholme and Randle, 1964; Randle, Newsholme and Garland, 1964) have all been investigated in order to throw light on the mechanisms of regulation of glucose uptake by heart muscle. However the important interrelationships between mechanical activity, oxygen consumption and substrate utilisation of the isolated perfused heart have received little attention until Opie (1965) pointed out that the perfusion pressure used in the Langendorff preparation was linearly related to the coronary flow and was a major factor influencing the oxidative metabolism.

The relationship between mechanical activity and oxygen consumption has been studied intensively in various types of heart preparation. Factors including heart work (Barcroft and Dixon, 1906; Harrison, Friedman and Resnik, 1936), and contractile element work (Britman and Levine, 1964) end-diastolic volume or fibre length (Decherd and Visscher, 1933; Hemingway and Fee, 1927; Starling and Visscher, 1926; Whalen, 1961) heart rate (Berglund, Borst, Duff and Schreiner, 1958; Cohn and Steele, 1935; Evans, 1917; Feinberg, Katz and Boyd, 1962; Laurent, Bolens-Williams, Williams and Katz, 1956; Van Citters, Ruth, Reissman, 1957; Whalen, 1961),

myocardial tension development (Feinberg et al., 1962; Sarnoff, Braunwald, Welch, Case, Stainsby and Macruz, 1958), coronary flow (Bacaner, Lioy and Visscher, 1965; Gregg, Rayford, Khouri, Kattus and McKeever, 1957; Kahler, Braunwald, Kelminson, Kedes, Chidsey and Segal, 1963; Opie, 1965), and velocity of contraction (Sonnenblick, Ross, Covell, Kaiser and Braunwald, 1965) have all been considered important in the regulation of oxygen consumption and hence presumably of substrate utilisation. It was therefore of considerable interest when Morgan and his colleagues recently described a completely isolated rat heart preparation capable of in vitro mechanical work. (Neely, Liebermeister, Battersby and Morgan, 1967a). In this preparation, perfusate is introduced into the left atrium at filling pressures up to 20 cm of water and pumped by the left ventricle, via the aorta, against a hydrostatic pressure head. Since this preparation, in pumping fluid, carries out the heart's normal physiological role, it was considered of interest to use this "working heart" to compare and contrast the effects of insulin and work load on glucose metabolism by cardiac muscle.

Scope of present investigation

In the study reported here, the stability of the "working heart" preparation, the effect of exogenous and endogenous substrate utilisation on the oxygen consumption and the effects

of insulin and insulin deficiency on the pattern of glucose metabolism of isolated hearts carrying out physiological "work" have been investigated. In addition, for comparison, Langendorff hearts have been perfused at two different aortic perfusion pressures since Neely et al. (1967a) have recently shown that the rat heart perfused by the Langendorff technique develops left ventricular pressure up to the level of the aortic pressure with each systole. This parameter of aortic pressure has not been strictly controlled in many earlier studies of substrate utilisation by the isolated rat heart (Randle, Newsholme and Garland, 1964; Morgan et al., 1961b; Williamson, 1962).

Many of the previous investigations of cardiac metabolism and the effects of insulin and insulin deficiency have relied on glucose uptake from the perfusion medium and total lactate production into the medium as the sole parameters of utilisation, without providing information on the metabolic fate of the glucose taken up by the heart muscle. In order to provide more detailed information, radioactive glucose-U-C^{14} has been used in this study. The $^{14}CO_2$ produced by glucose oxidation (Opie et al., 1962) has been measured and in addition, metabolic intermediates derived from the glucose-U-C^{14} have been revealed and measured by the quantitative radio-chromatographic scanning technique of Beloff-Chain, Catanzaro, Chain, Masi, Pocchiari and Rossi (1955) in a

modified form which is described. In addition, the myocardial content of glycolytic intermediates, adenine nucleotides, phosphocreatine, glycogen and citrate have been measured by enzymatic or chemical methods in both aerobic and anaerobic situations and before and after periods of cardiac work.

A new method has been used for inducing diabetes for the study of effects of insulin deficiency on heart metabolism. Previously reported studies have generally relied on chemically induced diabetes brought about by alloxan (Morgan et al., 1961a; Regen et al., 1964; Greenman and Shipp, 1965; Randle et al., 1964; Garland, Newsholme, Randle, 1964). The acute diabetic rat which is obtained 48 hours after treatment with alloxan has numerous metabolic and toxic abnormalities (Lukens, 1948), in addition to hyperglycaemia and a deficiency of insulin. For this reason a new diabetogenic antibiotic, streptozotocin (Rakieten, Rakieten, Nadkarni, 1963) has been used. With this material it is possible to achieve a type of diabetes which is characterised by an apparent specific insulin deficiency with none of the toxic manifestations of the kidney and liver (Herbut, Watson, Perkins, 1946) associated with the use of alloxan. The metabolic changes occurring during the development of streptozotocin induced diabetes have been investigated.

It has also been shown in the present study, that in the normal rat, glucose metabolism by the isolated perfused Langendorff heart is profoundly influenced by the endogenous insulin present in the muscle. This "bound" insulin although considered by Stadie to be in firm combination with the tissue (Stadie, Haugaard, Hills and Marsh, 1949) can be removed or inactivated by prolonged washing in a pre-perfusion period (Bleehen and Fisher, 1954), but its presence has been overlooked in several studies on the effect of insulin on cardiac metabolism. Morgan et al (1961 b) ↑
A more satisfactory method of obtaining an insulin deficient heart was suggested by the observations of Wright (1961) on the effects of anti-insulin serum (Moloney and Coval, 1955) prepared in guinea pigs (Armin, Grant and Wright, 1960a). Such serum when injected into a number of animal species (Armin, Cunningham, Grant, Lloyd and Wright, 1961) gives rise to hyperglycaemia and rapid loss of both pancreatic insulin and serum insulin (Armin, Grant and Wright, 1960b; Gregor, Martin, Williamson, Lacy and Kipnis, 1963). The use of such specific antiserum therefore provides a method for the production of a reversible experimental diabetes which is thought, at least initially, to be due to uncomplicated insulin deficiency.
In the present study, acutely insulin deficient hearts have been obtained by pretreatment of the donor rats with an anti-insulin serum prepared in guinea pigs by regular treatment with a novel, stable, silicone based emulsion containing

insulin (Mansford, 1967). A comparison has therefore been possible between hearts from these acutely insulin deficient rats and those from rats rendered diabetic with streptozotocin or alloxan.

EXPERIMENTAL

MATERIALS

Rats. All the rats used were male animals from a closed breeding colony of specific pathogen free-derived, Sprague-Dawley strain, originating from Charles River, Inc., U.S.A. They were all within the range 280 - 320 g when used and had unrestricted access to water and stock laboratory diet.

Guinea pigs. The guinea pigs used for preparation of anti-insulin serum were of the Hartley strain obtained from the National Institute for Medical Research, Mill Hill. They were of at least 500 g body weight before use and were maintained on stock laboratory diet supplemented with fresh cabbage twice weekly.

Chemicals and enzymes. ATP, ADP, AMP, glucose-6-phosphate (sodium salt), fructose 1,6-diphosphate (tricyclohexylammonium salt), fructose-6-phosphate (sodium salt) NADH, NAD, NADP, glucose-oxidase, hexokinase, glucose-6-phosphate dehydrogenase, creatine phosphate kinase, phosphoglucose isomerase, triose phosphate isomerase, lactic dehydrogenase (rabbit muscle), pyruvate kinase, citrate lyase, malic dehydrogenase, β-hydroxybutyrate dehydrogenase, aldolase, myokinase, glycerol kinase, glycerol-1-phosphate dehydrogenase were all obtained from Boehringer Corporation. Sodium pyruvate, sodium

lactate, phosphoenolpyruvate (tricyclohexylammonium salt), peroxidase, and tris were obtained from Sigma (London) Ltd., Lettice Street, London. Alloxan, glucose, sorbitol, glycine, hydrazine, EDTA, sodium DL β-hydroxybutyrate and all other chemicals were obtained from British Drug Houses Ltd., (Laboratory Chemicals Division, Poole, Dorset) and were either A.R. grades or the best available. Heparin (as Pularin 1000 u/ml) was purchased from Evans Medical Ltd., Speke, Liverpool. Streptozotocin (batches 4621-HKJ-126D, 4858-THP-106/4) was kindly donated by Messrs Upjohn Ltd., Kalamazoo, Mich., U.S.A. Crystalline insulin (glucagon free) was donated by Burroughs Wellcome Co. Ltd. and was dissolved in 3.3 mM HCl to yield a stock solution of 20 units/ml which was stored at - 15°C in 1 ml samples.

Radioactive chemicals

Chromatographically pure D glucose-U-C^{14} of specific activity 2 to 3 mc/mM was obtained from Radiochemical Centre, Amersham, Bucks. H^3-sorbitol at a specific activity of 200 mc/mM was originally obtained from New England Nuclear-Corporation, Boston, Mass., but more recently was available from the Radiochemical Centre.

METHODS

Preparation of Anti-Insulin serum

Attempts to prepare guinea pig anti-insulin serum using the procedure of Wright (1961) gave rise to high mortalities in the guinea pigs despite the use of a heavy mineral oil (Wright, 1965). These could be overcome by reducing the dosage of emulsion to that corresponding to 0.5 mg of insulin but then at least three injections at intervals of one month were required to produce high enough antibody titres in the serum. A satisfactory method of producing anti-insulin serum of high potency after only two injections and with a mortality of less than 5 per cent was developed using a silicone-based emulsion. The silicone emulsion was produced as follows:

Oil phase:

1 ml mannide mono-oleate emulsifier (Arlacel A, Atlas Powder Co.);

20 ml Isocreme oil base (Croda Ltd);

20 ml Silicone DC 702 (Dow Corning, Midland, Michigan).

Aqueous phase:

150 mg insulin (glucagon-free) (Burroughs Wellcome);

50 ml aqueous 0.3 per cent w/v phenol acidified to pH 2;

Emulsion:

6 ml oil phase, 3 ml aqueous phase, emulsified 30 mins at 22 p.s.i. through an eighteen gauge needle

The final emulsion (containing 1 mg per ml of insulin) was injected through an eighteen gauge needle as a single depot of 1 ml into the backs of guinea pigs weighing at least 500 gm. Injections were carried out at monthly intervals and anti-insulin serum was withdrawn by cardiac puncture two weeks after the injection of insulin emulsion. Only those batches of anti-insulin serum found capable of producing a rise of 180 ± 20 mg per 100 ml in the blood glucose of rats (280-320 gm) one hour after intravenous dosage with 0.5 ml of serum, were used for the metabolic studies (Wright and Rivera-Calimlin, 1965).

Preparation of Diabetic Rats

Alloxan-diabetes was induced by the intravenous administration into the tail vein of alloxan (65 mg/kg) whilst the rats were under light ether anaesthesia. The alloxan solution was made up at 25 mg/ml concentration in isotonic sodium chloride solution. The animals were used 48 hours later without starvation and all had blood glucose concentrations greater than 400 mg %. In addition, alloxan-diabetic animals exhibited distended stomachs and severely infiltrated patchy livers. Moribund animals were not used. The mortality after 48 hours was 20% and after 4 days was virtually 100%.

Streptozotocin-diabetes was induced in a similar manner using 65 mg/kg i.v. of streptozotocin into the

tail vein. In this case the solution of streptozotocin used was kept at pH 4 by a few drops of citric acid buffer because of the instability of streptozotocin solutions at pH7. These animals were used seven days later (unless otherwise indicated) without starvation. All treated animals lost weight during this period, exhibited dystrophic changes in coat and when used had blood glucose concentrations greater than 300 mg/100 ml. The mortality in streptozotocin treated animals was only 5% and no macroscopic evidence of liver damage was observed.

Perfusion apparatus

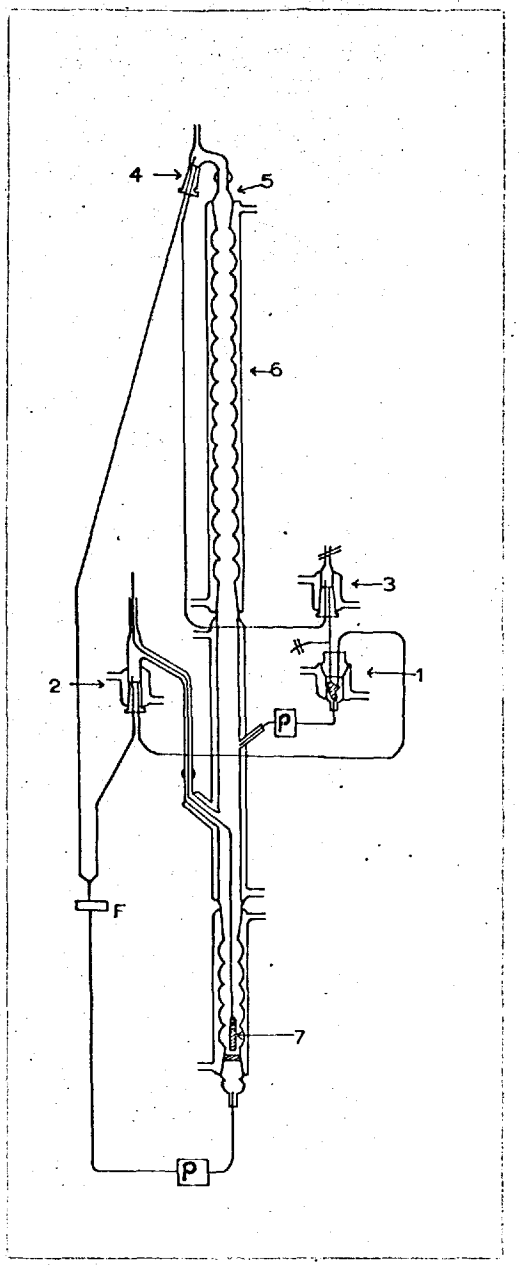
The complete apparatus is shown in Fig 1; an enlargement of the heart chamber, cannula assembly and pressure chamber is shown in Fig 2. The apparatus consisted of the following parts: (1) Heart chamber and cannula assembly. The cannulae (0.134 stainless steel inch o.d.) were held in a silicone rubber bung (Esco Rubber 24 mm) which fitted the top of the jacketed heart chamber. The aortic cannula had a tip of 0.11 inch o.d. tubing sealed into the end and the atrial cannula was bent to an angle of approx. 120° to place it in the correct position with respect to the aortic cannula. Both cannulae were grooved to take the ligatures. The aortic cannula was fitted with a side arm as shown. This was used to deliver perfusion medium during the pre-perfusion period

Fig. 1

Heart perfusion apparatus

for

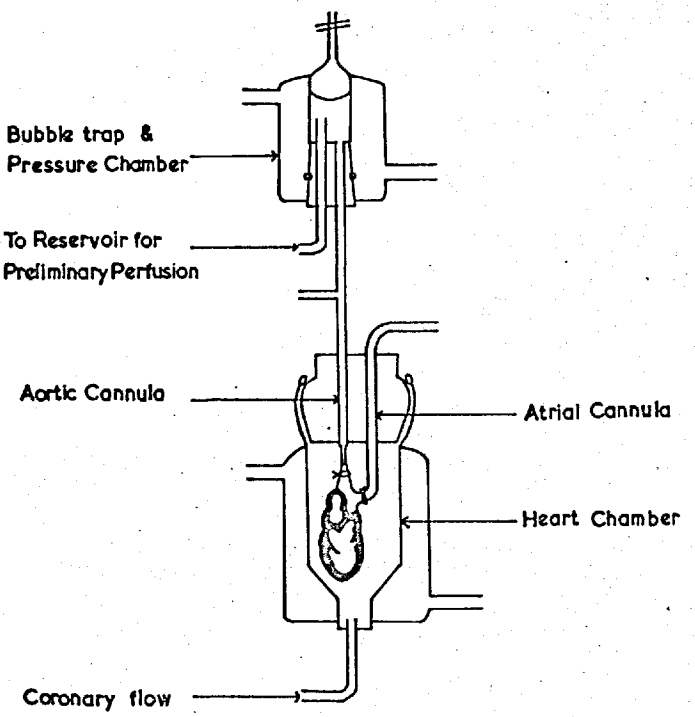
working hearts



Key

1. Perfusion vessel heart chamber
2. Atrial bubble trap
3. Pressure vessel and bubble trap
4. Aortic bubble trap
5. Aortic overflow
6. Oxygenating chamber
7. Gassing tube

F. Millipore filter
P. Peristaltic pump



Heart chamber and cannula assembly

Fig. 2

(see below) and was connected to a transducer during recirculation perfusions to record aortic pressure changes.

(2) Aortic and atrial bubble traps and pressure chamber. These were made from female portions of B10 standard Quickfit joints. Male plugs were made from silicone rubber bungs bored to take delivery and exit tubes (stainless steel tubes 0.11 inch o.d.). The aortic bubble trap (see (4) in Fig.1) was held in the top of the oxygenating chamber by means of a silicone rubber bung which also held a tube for the exit of gas. The atrial bubble trap (see (2) in Fig. 1) was joined to an extension side arm which also delivered the gassing tube into the apparatus. The pressure chamber bubble trap was joined to the aortic cannula by a short length of silicone rubber tubing (2.5 mm I.D.) All other connections were in similar tubing.

(3) Oxygenating Chamber. This was made up of three sections joined by means of standard spherical glass joints (see (6) in Fig. 1). The upper and lower sections were Allihn condensers designed to provide the maximum spread of liquid running down through the atmosphere of 95% O_2/5% CO_2. The central section contained the side arm for the atrial bubble trap.

(4) Peristaltic pumps. Two pumps (see (P) in Fig. 1) were used both of which were Watson-Marlow MHRE Flow Inducers

fitted with silicone rubber tubing (I.D.4.5 mm). One pump was used to control the return of coronary effluent to the oxygenating chamber. The other was used to pump perfusion fluid from the base of the oxygenating chamber through the in-line Millipore (5μ) (xx 30.025.00) filter (see Fig.1) and thence to the aortic and/or atrial bubble traps. Flow in the silicone rubber tubes was controlled by means of plastic roller-clips of the type supplied with blood transfusion drip sets.

Preparation of heart. The rats were anaesthetised in a glass chamber through which was drawn a stream of air saturated with ether. 0.2 ml of heparin (1000 U/ml) was then injected into the femoral vein and, if required, a 0.5-1.0 ml sample of blood removed through the same needle. When free fatty acids were to be estimated in the plasma the blood was withdrawn prior to administration of the heparin. One minute later the abdominal cavity was opened by making a deep transverse incision with scissors. The diaphragm was transected and lateral incisions were made along both sides of the rib cage so that the whole anterior chest wall could then be folded back. The pericardium and other filamentous tissues were then pulled away gently from the heart which was picked up and the lungs and other chest contents pushed towards the back. At this point it was

important to identify the point at which the pulmonary veins joined into the left atrium. A single cut was then made through this point and on through the other vessels arising from the heart, taking care to leave a sufficient length of aorta for easy cannulation. The heart freed in this way was then dropped immediately into a dish containing ice-cold perfusion fluid (see below). Contractions stopped within a few seconds.

Perfusion of heart

Using fine-tipped forceps, the heart was then picked up by the aorta; any connective tissue, thymus or lung adhering was cut away. The aorta was then slipped about 3 mm onto the grooved perfusion cannula (Fig. 2) through which the perfusion fluid was allowed to drip continuously. As soon as the aorta was secured with a ligature (Ethicon Mersilk R 842 s/0) the retrograde perfusion was begun by fully opening the tap supplying the perfusion fluid contained in a reservoir, water jacketed at 37°C, held at either 65 cm or 100 cm above the heart (see below). On supplying this warm oxygenated perfusion medium to the heart, contractions were restored to normal very rapidly. A small snip was then made in the right ventricular outflow tract with a pair of sharp pointed scissors to avoid the development of right ventricular distension. The coronary

output dripping from the heart during this period was collected and not allowed to enter the recirculation part of the perfusion apparatus.

If working rat heart preparations were required, the heart was rotated on the cannula, if necessary, to position the openings into the left atrium to receive the second perfusion cannula. After location of the left atrial opening and suitable trimming if required, the left atrium was slipped on to this second cannula and tied in place. The accuracy and competence of this cannulation were then tested by momentarily unclamping the tube leading from the atrial bubble trap (See 2 in Fig. 1) and observing the filling of the left atrium.

Three types of heart perfusion experiments were carried out.

1. Drip-through perfusions in which the coronary output was allowed to run to waste or was collected for analysis. In this case perfusion fluid was supplied from a large jacketed (37°C) reservoir which had a sintered glass filter sealed into the lower end.

2. Langendorff recirculation perfusions, in which perfusion was solely retrograde via the aorta and the coronary output was returned to the oxygenating chamber and recirculated for a fixed time (usually 30 mins unless otherwise stated).

3. Working heart recirculation perfusions in which after a 5-minute aortic, retrograde, recirculation perfusion, the heart was switched to atrial perfusion as described below.

A preperfusion period of 15 mins drip-through was given to all recirculation hearts both 'working' and Langendorff. This washing (which was done at the same aortic pressure and with the same substrate concentration in the medium that was to be present in the subsequent recirculation perfusion) served to remove all blood, equilibrate the substrate concentrations in the medium with those in the interstitial fluid, and to allow the heart to recover from the period of anoxia associated with excision and cannulation.

When Langendorff recirculation hearts were required, after 15 mins of drip-through preperfusion, the tube leading from the aortic bubble trap (4 in Fig. 1) was unclamped and the tube from the reservoir to the side arm of the aortic cannula was clamped. At the same time the coronary output instead of going to waste was collected from the base of the heart chamber and returned via peristaltic pump (I) to the oxygenating chamber.

When working heart recirculation perfusions were required, 15 mins of drip-through preperfusion were followed by 5 mins of Langendorff recirculation as above. Cardiac work was then begun by switching the output of the peristaltic pump II

(supplying both atrial and aortic bubble traps) solely to the atrial bubble trap and unclamping the tube supplying perfusate to the left atrial cannula from the overflow type bubble trap. This bubble trap design was similar to that described by Neely et al. (1967a) and served to eliminate pressure fluctuations due to the peristaltic action of the pump and ensured a constant hydrostatic pressure (20 cm H_2O) impinging on the heart. Perfusion medium then entered the atrium, passed into the ventricle and ventricular contraction forced the fluid into the pressure chamber attached to the aortic cannula. On the advice of Dr Howard Morgan this chamber was one third filled with air to provide some elasticity to an otherwise rigid system. The volume of air in this chamber had a marked effect on the size and shape of the aortic pressure curve which was registered by means of a pressure transducer (SE4 Medical type S.E. Laboratories Ltd., Feltham, Middlesex) attached to the side arm of the aortic cannula. The output of the transducer was fed via an SE4912 preamplifier and SE4910 amplifier to a UV-light trace recorder (SE 2005 six channel) with power supplies from an SE 4000 unit supply. Contraction of the ventricle then forced fluid from the pressure chamber above the aorta and thence to the aortic bubble trap 100 cm above the heart. The overflow from this aortic bubble trap was returned down

the inside walls of the long oxygenating chamber (6). The aortic output generated by left ventricular contraction was measured by collecting the overflow from the aortic bubble trap with the branch of the tube from pump II to the aortic bubble trap clamped off. Coronary flow was estimated by diverting the effluent from the heart chamber into a graduated cylinder for 30 secs. After measurement this perfusion fluid was returned to the oxygenating chamber.

The perfusate in the recirculation system and in the drip-through reservoir was oxygenated with (95% : 5%), O_2:CO_2 mixture thoroughly equilibrated with water at 37°C by passage through two sintered glass gas washing bottles prior to entry into the perfusion apparatus. Oxygenation was accomplished by bubbling this gas through the perfusate from the sintered plastic tube (7 in Fig. 1) and by exposing a large fluid surface as the medium flowed down the inner walls of the central condensers. In anaerobic experiments, (95%, 5%) N_2:CO_2 replaced the oxygen mixture.

A water jacket, maintained at 37°C by a circulation pump and Grant water bath, was provided for the entire system of bubble traps, heart chamber, oxygenating chamber and the reservoir of buffer used in the preliminary preperfusion and drip-through experiments.

Perfusion medium

Krebs-Henseleit (1932) bicarbonate buffer, pH 7.40, equilibrated with O_2 : CO_2 (95%, 5%) at 37° was used in all experiments. The precautions of Umbreit, Burris and Stauffer (1964) were followed to prevent precipitation of calcium. Additions of glucose (1 mg/ml or 2 mg/ml) and sorbitol (1 mg/ml) were made as indicated in the results. Prior to use, the Krebs-Henseleit perfusion medium was carefully filtered through a Millipore filter (0.8 u porosity Type SMWP) to remove any material not in true solution.

Oxygen consumption

When oxygen consumption was measured, a water jacketed oxygen electrode holder was placed immediately below the heart chamber. By adjusting the speed with which the peristaltic pump (I) removed the coronary effluent, it was possible to pass a continuous stream of the fluid leaving the heart over the oxygen electrode without exposure to air. The Beckman macro oxygen electrode (325814) was connected to a Beckman Physiological Gas Analyser Model 160. Initially, oxygen consumption was estimated from the difference in reading of oxygen tension as registered by this electrode prior to mounting of the heart and during the perfusion. However as the incoming oxygen tension appeared to vary with the rate of flow of perfusion liquid (presumably because of diffusion losses through the

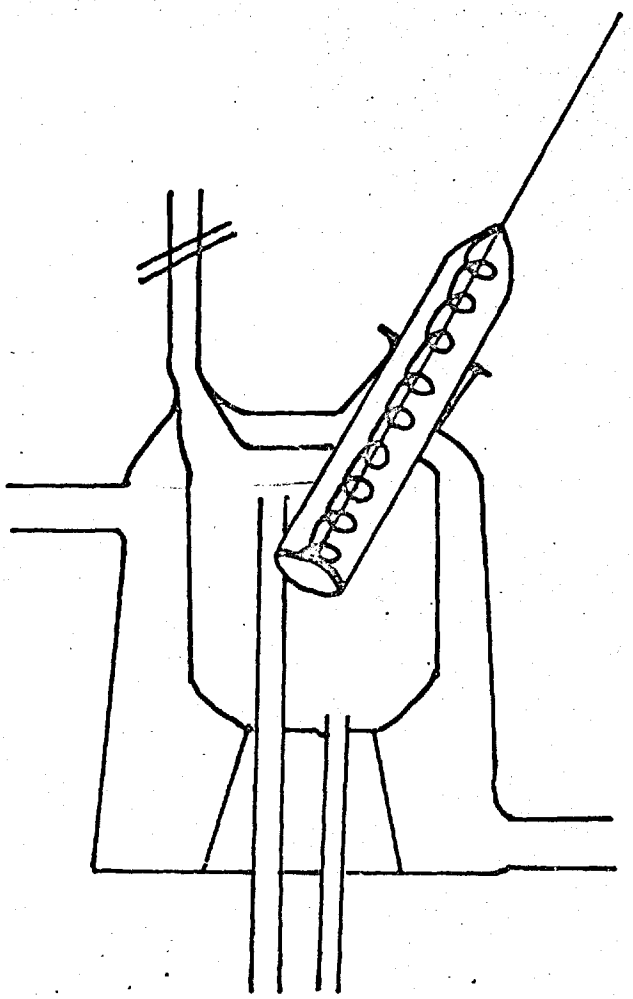


Fig.3 Modified pressure chamber
incorporating oxygen electrode
for measurement of aortic oxygen tension

silicone rubber tubing) a second oxygen electrode was introduced into a modified form of the pressure chamber mounted immediately above the aorta (see Fig. 3). Oxygen consumption was calculated using the following formula:-

O_2 consumption (qO_2 in µl/min/g dry)

$$= \frac{\text{arterial (incoming) -venous (outgoing) } O_2 \text{ tension (mmHg)}}{760 \text{ (mmHg)}}$$

$$\times \text{ solubility of } O_2 \text{ at } 37^{\circ}C \text{ (µl/ml } H_2O)$$

$$\times \frac{\text{coronary flow (ml/min)}}{\text{dry weight of heart (g)}}$$

Collection of samples

In recirculation perfusions, samples of medium were taken 2 mins after the addition of C^{14} glucose and/or H^3-sorbitol. This was taken as the time necessary for equilibration with the extracellular space of the heart, (Morgan et al., 1961b). The dilution of the H^3-sorbitol was used as a measure of the circulating volume. Mock perfusions carried out without hearts to check on possible losses of fluid by evaporation gave no indication of increase in concentration of the H^3-sorbitol. Samples of medium were also taken immediately prior to termination of the perfusion (normally after 30 mins). In experiments on the fate of C^{14}-glucose, the $^{14}CO_2$ produced by oxidative metabolism

was trapped by bubbling the effluent gas from the top of the oxygenating chamber through ethanolamine-methanol (1:1) mixture. The $^{14}CO_2$ in solution as bicarbonate at the end of the perfusion was measured by rapid transfer of cooled 1 ml samples of the final medium into scintillating vials. These vials were then quickly closed with skirted rubber caps which also held a wire support for a small glass centre well containing 0.5 ml of ethanolamine-methanol (3:2) mixture. 0.5 ml of 10 $H\text{-}H_2SO_4$ to liberate the $^{14}CO_2$ was then carefully injected through the rubber caps into the medium sample with care being taken not to disturb or contaminate the centre well. The vials were then allowed to stand at least 3 hrs before the caps were removed and the small glass wells containing the trapped $^{14}CO_2$ removed with forceps and placed bodily into new vials containing 10 mls of a modified Brays (1960) scintillant. Recovery of $H^{14}CO_3^-$ was found to be 98-106% by this method. Initial and final perfusate samples were assayed for radioactivity by mixing 0.1 ml with 10 mls of the same scintillant mixture. All samples were counted in a Packard Tricarb Liquid Scintillation Spectrometer (Model 3003) and corrected for quenching by the channels ratio method of Baillie (1960). The samples of medium obtained at the beginning and end of the perfusion were immediately deproteinised either with 5% $ZnSO_4$ and 0.3N $Ba(OH)_2$

for subsequent glucose assay or by means of addition to an equal volume of ice-cold 6% W/V perchloric acid (for lactate, pyruvate, glycerol assays).

Extraction of heart

Perfusions were terminated by freezing the heart between aluminium clamping blocks cooled in liquid nitrogen. (Wollenberger, Ristau and Schoffa, 1960). The frozen muscle was then placed in liquid nitrogen, removed onto a block of solid carbon-dioxide and quickly trimmed of frozen atrium. The ventricle was then powdered in a percussion mortar held at liquid nitrogen temperature. Two hammering periods each of twelve strokes, with the pestle being rotated through 180° between each period, was sufficient to produce a fine powder suitable for extraction. Powder was removed from the mortar with a spatula chilled in liquid nitrogen. An aliquot was transferred to a weighed container for determination of dry weight. A second aliquot was added to a weighed glass centrifuge tube containing 1 ml 30% KOH. After addition of the powder this tube was reweighed and then placed in a boiling water bath for digestion (30 min) prior to isolation of glycogen by the method of Good, Kramer and Somogi, (1933).

A third aliquot (the remaining powdered frozen tissue) was placed in a weighed tube containing either 20 ml ice-cold 60% aqueous ethanol (if chromatographic assay was required),

or 4 ml ice-cold 6% perchloric acid (if glycolytic intermediates, nucleotides etc. were to be determined enzymatically). After reweighing, the contents of the tube were thoroughly mixed by homogenising on a Vortex Mixer. Subsequent treatment depended on the assay requirement.

Heart analysis

(a) Experiments requiring chromatographic assay

In this case, the suspension of tissue in 60% ethanol was centrifuged and the residue resuspended in a further 20 ml of cold 60% ethanol and homogenised, and recentrifuged. The combined ethanolic supernatants were then evaporated to 1 ml on a Buchler Evapo-Mix under water-pump vacuum and at room temperature. The concentrated extract was then loaded into an Agla microsyringe and both mono- and bi-dimensional chromatograms prepared by spotting 20 µl (mono) or 50 µl (bi-) in 2 µl aliquots. Evaporation of the ethanol during spotting of the extract was assisted by cold air from a hair dryer held below the chromatography paper (Whatman No.1 chromatography grade). Medium samples (1 ml) were evaporated to dryness and taken up in 0.2 ml H_2O before chromatography in a similar way. The solvents employed were as follows:-

monodimensional:-

n. butanol/acetic acid/H_2O (40:11:25).

Run for 16 hours.

bidimensional:-

solvent (1) as above

solvent (2) picric acid/tert.butanol/H_2O (4 g: 80:20).

Run for 72 hours (Hanes and Isherwood, 1949),

or

solvent (1) sec.butanol/formic acid/H_2O (75:15:15).

Run for 16 hours.

Solvent (2) phenol/NH_4OH/H_2O (80:1:20).

Run for 24 hours.

or

solvent (1) as for monodimensional

solvent (2)

n propanol	350 ml
iso propanol	75 ml
n butanol	75 ml
iso-butyric acid	2500 ml
EDTA	1.2 g
H_2O	950 ml

Run for 48 hours (Crowley, Moses and Ullrich, 1963).

The chromatograms were developed by descending solvent run in all-glass tanks at 20°C for the times indicated. The monodimensional chromatograms were placed in a tank containing

ammonia vapour for ½ hour as soon as they had been taken from the solvent tank, in order to "fix" the volatile free lactic acid as its less volatile ammonia salt.

After allowing the chromatogram to dry in air, the paper was placed in contact with a radiographic film plate (Kodak, Kodirex X ray) for a period of approximately 7 days. The autoradiographs were then developed for 3 minutes at 20°C with D 19B (Kodak), washed in 2% v/v acetic acid and fixed for 15 minutes in Kodak acid fixing salt solution before washing in running water for 30 minutes. The radioactive spots on monodimensional chromatograms were also detected by the spark chamber method of Pullan, Howard and Perry (1966) (see below).

The radioactivity of all the substances separated on the chromatograms of the heart extracts and perfusion media was quantitatively measured using a computerised form of the automatic radioactive chromatogram scanner originally developed in the Istituto Superiore di Sanita, Rome (Frank, Chain, Pocchiari and Rossi, 1959; for details of this modified scanner see below).

(b) Experiments requiring enzymatic or chemical assay

In this case, the tissue and cold perchloric acid were rapidly filtered through a Millipore filter (pore size 0.8 mμ). The volume of clear filtrate obtained was noted and 0.5 ml of a mixture of 30% KOH saturated with KCl (6 parts) and 0.2 M Tris, pH 7.5 (4 parts) was added. Neutralisation (as judged by universal indicator solution, B.D.H.) to pH 7.0 was then completed by dropwise addition of 2.5% KOH saturated with KCl. All of these operations were carried out in the cold room (+ 4°C). The volume of the neutralised extract was again noted and the cold extract centrifuged at + 2°C for 10 minutes at 3000 r.p.m. The neutralised extract freed from perchlorate was then used for assays of the metabolic intermediates shown below:

(1) ATP, ADP, AMP and phosphocreatine

$$\text{ATP} + \text{glucose} \xrightarrow{\text{hexokinase}} \text{ADP} + \text{G6P}$$

$$\text{G6P} + \text{NADP} \longrightarrow \text{6PGA} + \text{NADPH} + \text{H}^+$$

ATP was assayed by the change in extinction at 340 mμ in a Unicam SP 800 spectrophotometer (fitted with external recorder and range expansion) on the addition of hexokinase (5 μl of 10 mg/ml) to a mixture of 0.1 ml of heart extract (or standard or blank) and 2.9 ml of assay medium, (Lamprecht and Trautschold, 1963). The assay medium contained the following:-

0.2 M Tris buffer pH 7.5	0.1 ml
NADP 1% w/v	0.1 ml
Glucose 100 mM	0.05 ml
Glucose-6-phosphate dehydrogenase (10 mg/ml)	5 μl

When the change in extinction had stabilised, 0.05 ml of 10 mM ADP were added (and any further change in extinction noted) to provide excess ADP for the subsequent assay of phosphocreatine. Standards of 0·2ml of 0·5mM ATP and of CrP were run on each occasion. Phosphocreatine was then assayed by the further change in extinction at 340 mμ on addition of 10 μl creatine phosphate kinase (5 mg/ml made up in 0.1% bovine albumin) to the reaction mixture in which the ATP assay had been completed.

$$\text{ADP} + \text{phosphocreatine} \xrightleftharpoons{Mg^{++}} \text{creatine} + \text{ATP}$$

ADP was assayed by the change in extinction at 340 mμ on adding 5 μl of pyruvic kinase (10 mg/ml) to a mixture of 0.2 ml of heart extract, 2.8 ml of assay medium and 5 μl lactic dehydrogenase (25 mg/ml).

$$ADP + 2\ PEP \xrightleftharpoons{PK} 2\ ATP + 2\ pyruvate$$

$$2\ pyruvate + 2\ NADH + 2H^{+} \xrightleftharpoons{LDH} 2\ lactate + 2\ NAD^{+}$$

In this case the assay medium contained the following:

EDTA - triethanolamine buffer pH 7.5	0.4 M	0.6 ml
KCl	0.5 M	0.5 ml
$MgCl_2$	1.0 M	0.1 ml
$NADH_2$	0.125%	0.1 ml
Phosphoenolpyruvate (tricyclohexylammonium salt),	10 mM	0.2 ml
ATP	10 mM	0.01 ml

Standards of 0.2 ml of ADP 0.5 mM were run on each occasion.

AMP $\qquad AMP + ATP \xrightleftharpoons{MK} 2\ ADP$

was assayed by the further change in extinction on the addition to the mixture in which the ADP assay had been completed, of 5 µl of myokinase (10 mg/ml). This change in extinction required correction for a blank value obtained when water was substituted for heart extract, probably because of contamination of $NADH_2$ with AMP (Williamson, 1966). Standards of 0.2 ml of AMP (0.1 mM) were run on each occasion and in addition 10 µl of 1.0 mM AMP was added to the cuvettes containing the blank, the standard and several heart extract samples, in each run, to check on the additional change in extinction brought about and thereby test for the possible presence of myokinase inhibitors in the heart extracts.

(2) Dihydroxyacetone phosphate, fructose-1,6-diphosphate, pyruvate

These were assayed by an $NADH_2$ coupled system based on the following reactions (Bucher and Hohorst, 1963)

(a) dihydroxyacetone phosphate + NADH + H^+ $\xrightarrow{GDH}$ glycerol - 1 phosphate + NAD^+

(b) fructose-1,6-diphosphate $\xrightarrow{aldolase}$ glyceraldehyde-3-phosphate+dihydroxyacetone phosphate

(c) glyceraldehyde-3-phosphate $\xrightarrow{triosephosphate\ isomerase}$ dihydroxyacetone phosphate

(d) fructose-1,6-diphosphate + 2 NADH + $2H^+$ $\longrightarrow$ 2 glycerol-1-phosphate + $2NAD^+$

For this sequence 1.0 ml of each heart extract was added to cuvettes containing:-

$NADH_2$ (0.125%)	0.1 ml
0.2M Tris buffer pH 7.5	1.0 ml
H_2O	0.9 ml

Standards of 0.2 ml of 0.1 mM fructose-1,6-diphosphate plus 0.8 ml of water (in place of the heart extract) were run on each occasion together with similar standards of 0.1 mM pyruvate and distilled water blanks.

The sequential changes in extinction at 340 mμ were followed after the following additions:

(a) 5 μl glycerol-1-phosphate dehydrogenase

(b) 5 μl triosephosphate isomerase (10 mg/ml)

(c) 5 μl aldolase (10 mg/ml)

(d) 5 μl lactic dehydrogenase (10 mg/ml.)

As internal standards, 10 μl of FDP (1.0 mM) was added to the cuvettes after the addition of aldolase and 10 μl of pyruvate (1.0 mM) subsquent to the addition of lactic dehydrogenase.

(3) G6P, F6P and glucose (Hohorst, 1963)

These were assayed by an NADP coupled system based on the following reactions:-

(1) G6P + $NADP^+$ $\xrightleftharpoons{G6PDH}$ 6-phosphogluconolactone + NADPH + H^+

(2) F6P $\xrightleftharpoons{\text{phosphoglucose isomerase}}$ G6P

(3) Glucose + ATP $\xrightleftharpoons{\text{hexokinase}}$ G6P + ADP

For this reaction sequence 0.5 ml of each heart extract was added to cuvettes containing:

0.2 M Tris buffer pH 7.5	1.0 ml
1% NADP	0.1 ml
20 mM ATP	0.1 ml
1 M Mg Cl_2	0.1 ml
Distilled water	1.2 ml

The glucose-6-phosphate was then assayed by the change in extinction at 340 mμ brought about by the addition of 5 μl of glucose-6-phosphate dehydrogenase (10 mg/ml).

Fructose-6-phosphate was then assayed by the further change in extinction at 340 mμ on the addition to the mixture in which the G6P assay had been completed, of 5 μl of the phospho-glucose isomerase (10 mg/ml).

Glucose in the heart extracts, was then assayed (again in the same mixture in which the G6P and F6P assays had been completed) by the further addition of 5 µl of hexokinase (10 mg/ml). Standards of 0.2 ml of 0.1 mM G6P and F6P with 0.3 ml of distilled water in place of the 0.5 ml of heart extract were assayed in each run.

Glucose in samples of blood, and perfusion medium were analysed by a modification of the method of Huggett and Nixon (1957) in which the glucose oxidase reaction,

$$\text{D-glucose} + H_2O + O_2 \xrightarrow{\text{glucose oxidase}} \text{D-gluconic acid} + H_2O_2$$

was coupled to an indicator reaction in which the hydrogen peroxide was decomposed by peroxidase. The oxygen liberated then oxidised a hydrogen donor to form an oxidised chromogen

$$H_2O_2 + \text{donor } (H_2) \xrightarrow{\text{peroxidase}} 2\ H_2O + \text{chromogen}$$

The chromogen was that formed from O - dianisidine dihydrochloride. Because of the contamination of commercial glucose oxidase preparations with amylase and maltase (Crowne and Mansford, 1964) the enzyme-dye reagent containing glucose oxidase, peroxidase, O-dianisidine dihydrochloride was made up in Tris-glycerol buffer pH 7.0 (Mansford and Opie, 1963). The reaction was carried out with 0.5 ml of a neutral deproteinised filtrate (prepared from blood (0.1 ml) or perfusion medium samples by the addition of 1 ml of $ZnSO_4$ (5%) and 1 ml of $Ba(OH)_2$ (0.3N) solutions) with 2 ml

of the enzyme-dye reagent. Incubation at 37°C for 30 mins was then carried out and the reaction terminated by rapid addition of 4 ml of N H_2SO_4 which also served to stabilise the colour of the oxidised chromogen (Fleming and Pegler, 1963). Measurements of extinction were then carried out at 540 mμ on a SP600 spectrophotometer equipped with a flow cell.

(4) α-glycerol-phosphate and lactate (Hohorst, 1963 b).

In the reactions in which α-glycerol-phosphate and L (+) lactate are oxidised by NAD,

(1) glycerol-1-phosphate + NAD^+ $\xrightleftharpoons{\text{glycerol-1-phosphate dehydrogenase}}$ dihydroxyacetone phosphate + NADH + H^+

(2) L-lactate + NAD^+ $\xrightleftharpoons{LDH}$ pyruvate + NADH + H^+

the equilibrium in both cases lies far to the left. The oxidations are, however, virtually quantitative if the reaction products are removed from the mixture. In both cases this was conveniently brought about by the use of an alkaline reaction mixture (to bind the protons) and the use of hydrazine to trap the dihydroxyacetone phosphate and pyruvate as their respective hydrazones.

The buffer used contained 30 g glycine, 20.8 g hydrazine sulphate and 0.8 g EDTA in 200 ml distilled water to which was added

immediately before use 12.75 ml of 2 N NaOH to give a pH of 9.5 (measured by pH meter).

For the assay of α-glycerol phosphate, 0.5 ml of the heart extracts were placed in cuvettes containing 1.5 ml of the hydrazine-glycine buffer, 0.2 ml of 1% NAD and 0.8 ml of distilled water.

The change in extinction at 340 mμ brought about as a result of the addition of 20 μl of glycerol-1-phosphate dehydrogenase (10 mg/ml) was then measured.

Lactate was assayed by the further change in extinction on the addition, to the mixture in which the assay of glycerol-1-phosphate had been completed, of 20 μl of lactic dehydrogenase (25 mg/ml). 30 mins was allowed for this reaction to come to completion. To check that the assay was functioning correctly, 10 μl of a 2.5 mM lactate standard solution was added to all the cuvettes and the further change in extinction measured after 30 minutes. A 1 M standard solution of L (+) lactate was obtained from Boehringer Corporation.

5) Citrate. Citrate was assayed by the citrate lyase method of Moellering and Gruber (1966) using the reactions:-

$$\text{Citrate} \xrightleftharpoons{\text{citrate lyase}} \text{oxaloacetate} + \text{acetate}$$

$$\text{oxaloacetate} + \text{NADH} + \text{H}^+ \xrightleftharpoons{\text{malic dehydrogenase}} \text{malate} + \text{NAD}^+$$

Both the assay mixture and the enzyme (citrate lyase) solution contained Zn^{++} to stabilise the enzyme activity. In the original procedure of Dagley (1963) oxaloacetate formed from citrate was decomposed to pyruvate by oxaloacetate decarboxylase present in cell-free extracts. The Moellering and Gruber (1966) procedure was found to be preferable since it eliminated the uncertainty that sufficient amounts of the decarboxylase might not be present for this conversion, by using a mixture of lactate dehydrogenase and malate dehydrogenase. Any pyruvate that was formed from oxaloacetate non-enzymically was assayed by lactate dehydrogenase whilst unchanged oxaloacetate was assayed by the malate dehydrogenase. By coupling with the dehydrogenase systems the equilibrium of the citrate lyase reaction was shifted to the direction of complete splitting of citrate.

For the assay, the cuvettes (10 mm) contained the following reaction mixture

0.5ml heart extract

2.0ml triethanolamine buffer (50 mM) + 0.2mM $ZnCl_2$ (pH 7.6)

0.1ml NADH 0.125% w/v

0.4ml distilled water

5 µl of lactic dehydrogenase (5 mg/ml) and 5 µl of malate dehydrogenase (5 mg/ml) were then added to each cuvette and the extinction recorded at 340 mµ after a stable reading had

been obtained (approx. 10 mins). The change in extinction which was brought about by the addition of 10 µl of a solution of citrate lyase (10 mg/ml) (made up in triethanolamine 10 mM + 0.3 mM $ZnCl_2$ pH 7.6) was then used as a measure of citrate content. Stable readings of this change in extinction were obtained after 5 - 10 min. After this time a steady creep in extinction was observed in all cells both containing standards and heart extracts. Standards (0.5 ml of 0.05 mM citrate) were run on all occasions.

(6) Glycerol. The assay procedure of Garland and Randle (1962) was used. This was based on the following reactions

$$\text{glycerol} + \text{ATP} \xrightleftharpoons{\text{glycerol kinase}} \text{L-glycerol-1-phosphate} + \text{ADP}$$

$$\text{ADP} + \text{phosphoenol pyruvate} \xrightleftharpoons{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate}$$

$$\text{Pyruvate} + \text{NADH} \xrightleftharpoons{\text{lactate dehydrogenase}} \text{lactate} + \text{NAD}^+$$

The phosphorylation of glycerol was therefore coupled with the oxidation of $NADH_2$. The forward reaction was favoured in each case by the use of a buffer at pH 7.6.

The assay mixture was prepared as follows:-

0.1 M triethanolamine hydrochloride (adjusted to pH 7.6 with saturated NaOH) containing 6 mM magnesium chloride 7 mM phosphoenolpyruvate (tricyclohexylammonium salt), and 2 mM KCl	10 ml
75 mM ATP disodium salt	0.25 ml
3 mM NADH	1 ml

For glycerol assay, cuvettes (1 cm) were set up containing 2 ml of this assay mixture together with 1 ml of the heart extract or perfusion medium sample. 10 µl of pyruvate kinase and 10 µl of lactic dehydrogenase were then added to each cuvette and the change in extinction due to reaction of any ADP and pyruvate in the sample allowed to stabilise. The change in extinction due to glycerol was then measured following the addition of 10 µl of glycerol kinase. Because of the small but steady drift in extinction during prolonged readings, all samples were read at 5 minutes after the addition of glycerol kinase. This time was found to give satisfactory results with standards (1 ml of 0.05 mM glycerol) and when 10 µl of 2.5 mM glycerol was added to the cuvettes in which the glycerol assay of hearts extracts had been completed.

(7) β-hydroxybutyrate

For the determination of the concentration of β-hydroxybutyrate in blood, the enzymic method of Williamson et al., (1962) was used. This involved the use of β-hydroxybutyrate dehydrogenase in the reaction:

$$\beta\text{-hydroxybutyrate} + NAD^+ \rightleftharpoons \text{acetoacetate} + NAD + H^+$$

For reasons similar to those mentioned in connection with the lactate assay, hydrazine was incorporated into the assay buffer (pH 8.5) in order to form the hydrazone of acetoacetate and force the reaction to completion. In this case the reaction mixture in the cuvette contained:-

0.8 ml buffer pH 8.5 (12.5 ml of 15% w/v glycine, 10.4% w/v hydrazine sulphate, 0.4% w/v EDTA, + 7.5 ml 2N NaOH)

0.2 ml NAD (1.5% w/v)

2.0 ml neutralised supernatant from blood sample.

The change in extinction at 340 mμ, 30 min after addition of 20 μl of β-hydroxybutyrate dehydrogenase (5 mg/ml) was measured. The blood supernatant was prepared by mixing 1 ml of blood and 1 ml of 0.7 M perchloric acid solution and then centrifuging at 3000 r.p.m. for 10 minutes. To a measured aliquot of the supernatant, 0.3 ml of 2 M KOH/0.65 M K_2CO_3 mixture was added. This raised the pH of the supernatant to approximately 9-10. The supernatant was then allowed to

stand in an ice bath for 30 minutes and the potassium perchlorate which precipitated was filtered off.

(8) Plasma free fatty acids

Plasma free fatty acids were determined by a titrimetric procedure devised by Chlouverakis (1963) and based on the method of Dole (1956). Following extraction of 0.6 ml of plasma with 3 ml of Dole's extraction mixture (isopropyl alcohol 40 parts, heptane 10 parts, 1N H_2SO_4 1 part) the system was divided into two phases by the addition of 1.8 ml of heptane and 1.2 ml of water. At least 10 mins after the two phases had separated 2 ml of the upper phase were pipetted into conical tubes to which 2 ml of nile blue indicator (0.02% w/v in 90% v/v ethanol) were added. Titration was then carried out against standardised 0.02N NaOH with an Agla micrometer syringe burette under a stream of nitrogen.

(9) Tissue Sorbitol and Sorbitol space: Intracellular glucose

Two 0.1 ml aliquots of the neutralised tissue extract were counted for H^3-sorbitol content by addition to 10 ml of scintillant containing 0.5 ml of water to prevent salt precipitation. Samples (0.1 ml) of the perfusion medium collected at the end of the experiment were counted at the same time. In this way the extracellular space was determined, on the assumption that sorbitol does not penetrate the muscle cell, (Morgan et al., 1961b), from the following formula:

Sorbitol space µl/g

$$= \frac{\text{(muscle sorbitol µg per g of wet muscle) 1000}}{\text{medium sorbitol µg per ml}}$$

Glucose space was calculated in a similar manner using the results of the hexokinase assay of tissue extracts and the glucose oxidase method on medium samples.

From the results of these calculations of sorbitol space and glucose space it was possible to calculate in turn whether any intracellular glucose was evident under the conditions of the perfusion, since as Morgan et al. (1961b) have shown,

intracellular glucose(mg/100 ml)

$$= \frac{\text{(glucose space - sorbitol space µl/g)(perfusate glucose conc.mg/100ml)}}{\text{(Total water - sorbitol space µl/g)(0.75)}}$$

The factor 0.75 was derived by Morgan on the basis of studies with non-metabolised sugars which showed that only 75% of the intracellular water was available for sugar distribution. Total water was obtained from the dry weight/wet weight ratio determined as described above.

Detection of radioactive materials on chromatograms by means of a crossed-wire spark chamber

A number of workers have published details of spark chambers with electrodes which consist of sets of wires arranged so that electrical signals corresponding to spark positions can be extracted (Pullan, Howard and Perry, 1966). The chamber used in this investigation was similar to that shown in Fig.4. The two electrodes consisted of sets of stretched wires mounted on perspex frames. These frames had removable sections so that the chromatograms could be inserted into the chamber. The positive electrode consisted of a set of 0.005 in diameter stainless steel wires spaced 1/8 inch apart over the sensitive region of the chamber. At the edges of the sensitive region these wires were replaced by 24 gauge tinned copper wires. Both sets were stretched over two 1/4 inch diameter brass bars that were placed 20 cm apart on a flat perspex surface so that the separation between the electrodes was gradually increased outside the sensitive region to reduce spurious sparking. The negative or ground electrode consisted of 24 gauge tinned copper wires placed 1/8 inch apart and stretched over two bars in the same way as the positive electrode. These thick wires were used for the negative electrode to avoid corona discharge into the sensitive region of the chamber. The two halves of the

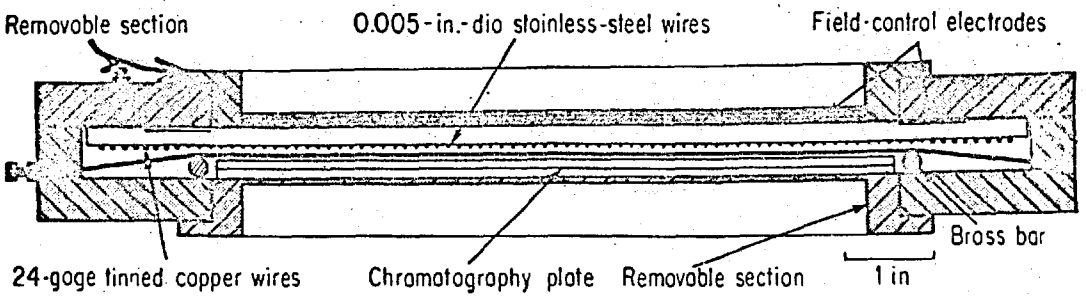


Fig. 4 Diagram of spark chamber

chamber were assembled so that the wires on the two electrodes were at right angles to each other and were separated by 25 mm. The technique of separating the electrodes away from the sensitive region and using the thick wires at the edges was not sufficiently successful to stop spurious sparking at the edges when the chamber operated at only 100-200 volts above the spark threshold. Pullan et al., (1966), therefore introduced two field control electrodes to reduce gradually the field around the fine wires at the edges of the sensitive region.

The chamber was operated with a continuous flow of 90% argon and 10% methane gas and the electrodes were connected through a high resistance to a 5000 volt power supply. β particles from the radioactive spots on the chromatogram, entering the field between the two electrodes, produced ionisation which resulted in a spark between the nearest pair of electrode wires (one in each plane). Each spark discharged the capacity of the chamber and the voltage on the chamber then recovered according to the time constant of the series resistance and the capacity of the chamber. With a resistance of 250 Megohms and a chamber capacity of 150 F the chamber operated stably for 1300 volts above the spark threshold. With the subsequent time constant and the large chamber area, the dead time correction was considerable.

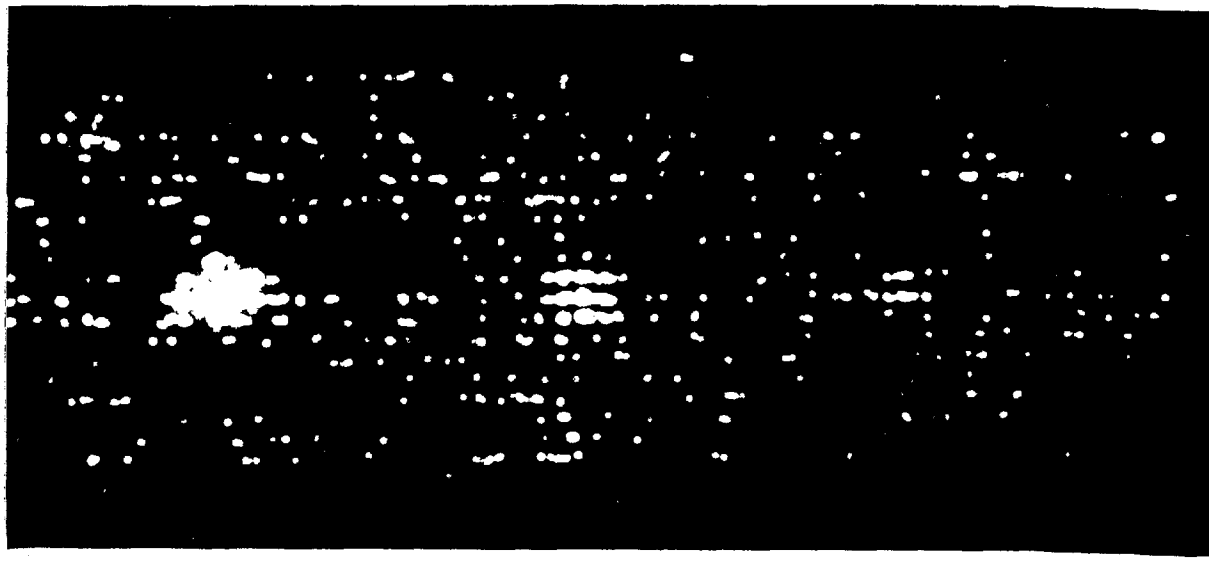


Fig. 5 Typical photograph of spark chamber detection of radioactivity on chromatograms.

Because of this disadvantage quantitative measurements by the spark chamber were not utilised in these experiments. The presence of radioactive spots was revealed simply by mounting a Polaroid camera over the top of the chamber and taking a 5 min. time exposure to integrate the flashes produced by the sparks. In this simple manner it was found possible to reveal the presence on paper chromatograms of radioactive spots which normally required 3-7 days of exposure on autoradiographs. A typical spark chamber photograph is shown in Fig. 5.

Quantitative bidimensional paper chromatography

An automatic scanning machine for the quantitative evaluation of paper chromatograms of radioactive substances was developed in the Istituto Superiore di Sanita, Rome (Chain, Frank, Pocchiari, Rossi, Ugolini, 1956; Frank, Chain, Pocchiari and Rossi, 1959). This machine has been used extensively for studying the metabolic fate of radioactive substrates in isolated tissues (e.g. Chain, Mansford and Pocchiari, 1960). This apparatus consisted of an electric typewriter, the number keys of which had a series of solenoids mounted over them; the number keys were therefore depressed when the solenoids were energised. The chromatogram was fastened by sellotape to a sheet of blank paper inserted into the carriage of the typewriter. The chromatogram was thereby

drawn by the movements of the typewriter carriage between two end-window Geiger counters attached to the back of the typewriter carriage, thus exposing a unit square area of the radiochromatogram for counting for a period which depended on the intensity of the radiations, i.e. for short periods in the case of the background radiation, and for longer periods if the number of counts per unit time exceeded the preset background value. The number of counts, detected by the Geiger counters and counted in the scaler unit, was then read out to the solenoids over the corresponding number keys of the typewriter, so that at the end of the counting period, determined by an electronic timer, the number of counts per time unit was printed on the blank sheet in the typewriter carriage in a position corresponding to the position of the radioactive spot on the chromatogram; in printing out this information the typewriter carriage moved to the next adjacent position. When the whole line had been explored, additional solenoids actuated the carriage return key and thus returned the carriage to its original position, simultaneously turning the roll so that the blank paper sheet, and with it the chromatogram, was moved to the next line. This process was repeated until all the chromatogram had been scanned and a number-map indicating the distribution and intensity of radioactivity was thereby obtained.

In the present work a computerised modification of this scanner has been used. As development of this modified scanner and the evaluation of its performance were a necessary preliminary to the present metabolic study, details are given of this new machine.

General Principle of operation

The electric typewriter used in the old model had the dual function of:-

(1) moving the radiochromatograms through the Geiger counters and

(2) recording the distribution of radioactivity. For computerisation these two functions had to be separated; hence the idea of using the electric typewriter in which both functions were combined, had to be, regretfully, abandoned.

In the new, computerised model, the scanning function was carried out by specially constructed stands (Fig. 6) in which the radiochromatograms were held in frames (1, Fig. 6), which could be moved vertically between the two Geiger counters (2, Fig. 6). The counters were moved horizontally across the radiochromatogram discontinuously exploring unit areas. After the end of one line had been reached, the counters were made to return to their original position, and simultaneously the frames were moved upwards so that the next line could be explored by the counters. Both the horizontal movement of

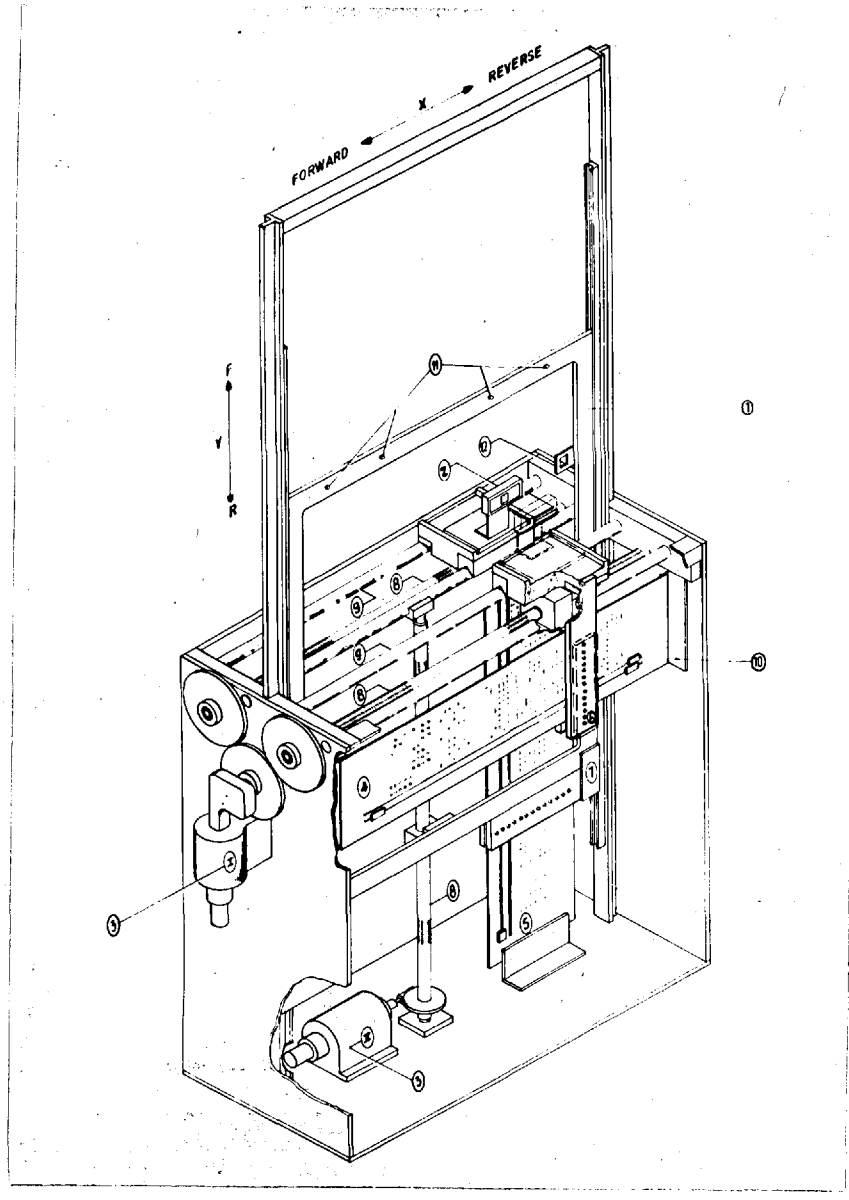


Fig. 6 Diagram of scanner for quantitative determination of radioactivity on bidimensional paper chromatograms.

the counter and the vertical movement of the frames were effected by two servo-control motors I and II (3, Fig. 6). The horizontal movement of the Geiger counters effected by motor I was started by a timing device which also controlled the counting period. This was set at two values; a short one for counting the background activity and a multiple of this value for counting intensities of radioactivity exceeding the set threshold for background activity.

Programming, indexing of the positions to be explored (both in horizontal and vertical directions), and identification of the scanning machines was effected by a series of parallel rows of perforations and three slots, drilled or cut, respectively, in two aluminium plates (4 and 5), one (4) for the horizontal movement of the Geiger counters, the other (5) for the vertical movement of the radiochromatogram.

The sensing device for detecting the scanning positions and the other parameters to be recorded consisted of a series of pairs of small photocells and lamps mounted opposite to each other in perforations drilled into each of two strips, forming the longitudinal sides of a rectangular housing carrier (6 and 7), with the perforated aluminium programming plate located between them; one photocell-lamp pair was mounted at a height corresponding exactly to the position of each line of perforations or each slot in the aluminium

programming plates. The aluminium plate thus separated the photocells from their respective light sources, except at the positions of the perforations or the slots through which the light beams were able to penetrate and impinge on the photocells.

Two sensing devices were mounted, one attached to one of the saddles of the Geiger counters for horizontal exploration and indexing (6), the other attached to the lower bar of the radiochromatogram frame, for vertical exploration and indexing (7).

The positions of each of the small perforations on the first horizontal line on the programming plate (4) determined the positions where the Geiger counters stopped for horizontal exploration. This occurred when motor I was stopped by a signal from the topmost photocell, located at the height of the first row of perforations, as soon as the Geiger counters (and the sensing device attached to the saddle of one of them) had reached the position of the first of the perforations and the light beam from the opposite lamp had impinged through it onto the photocell.

After the completion of the exploration period, the timer set motor I in motion again, and the motor ran until the Geiger counters were moved by means of the threaded drives (8) to the position of the next perforation of the top row of

perforations drilled into the programming plate when it was stopped by the same photocell device as described. These periodic horizontal movements of the Geiger counters were repeated until the end of the line was reached, at which point, motor I was caused to reverse.

The two ends of the required exploration line were marked by two small aluminium stops (10), which could be moved in two of the slots to any desired position. When the sensing device had been transported to the position of the terminal aluminium stop, the light beam emanating from the source located at the height of the first slot and reaching the corresponding photocell through the whole length of the slot, was obstructed by the aluminium stop. This caused the photocell to emit a signal causing motor I to reverse and transport the Geiger counters and the sensing system to the point of origin. This position was marked by an appropriately placed aluminium stop in the next lower slot II. Once this was reached by the sensing system, motor I was again reversed by a signal from the appropriate photocell, located at the height of slot II, (on obstruction of the light beam from its opposite lamp by the aluminium stop), and the exploration of the next horizontal line would begin. This was reached by an upward movement of the radiochromatogram frame, effected by motor II on receiving a signal from motor I during its reverse excursion.

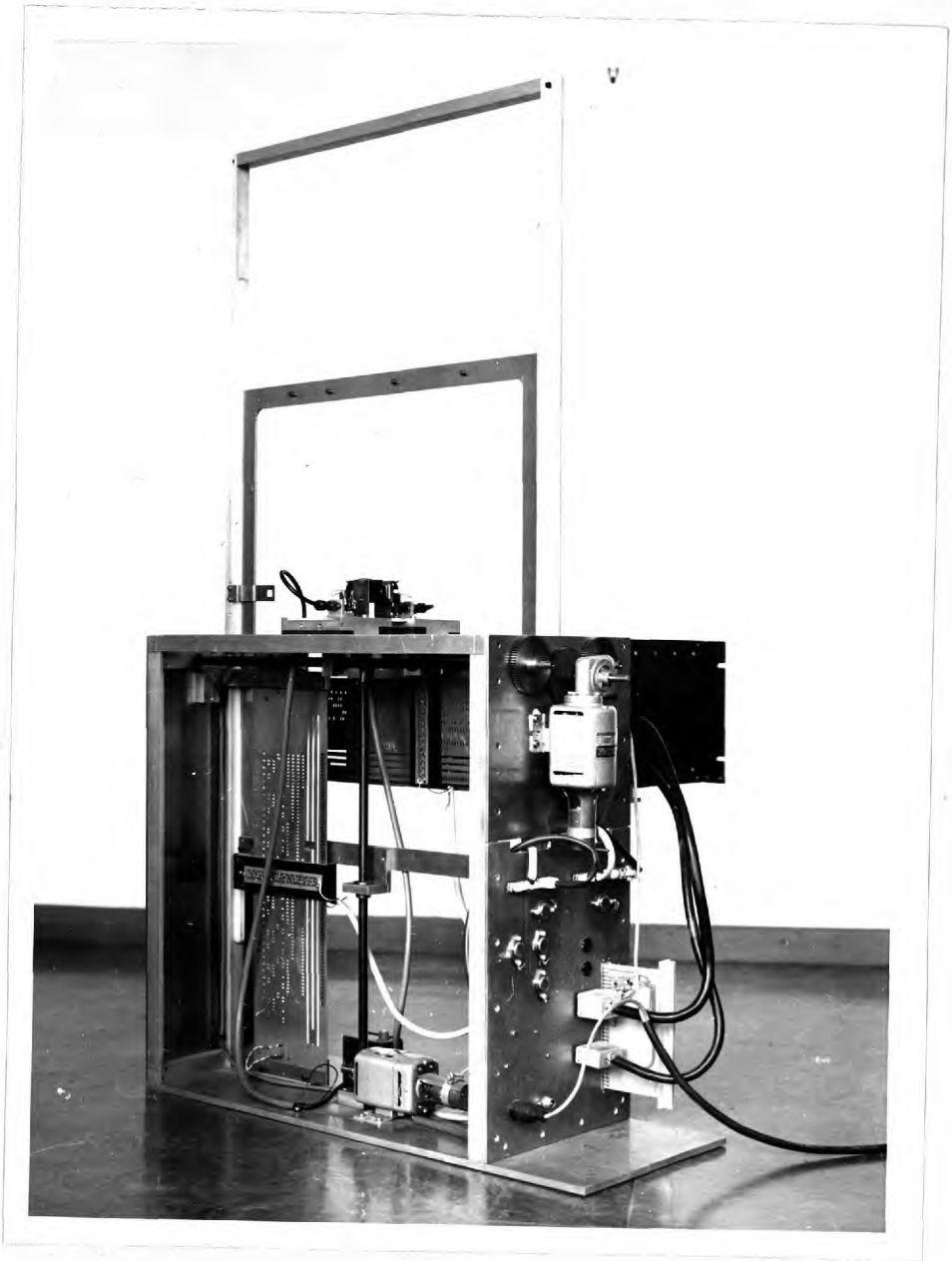


Fig. 7

Scanner showing frame for chromatograms and gas flow counters.

The vertical positions of the frame determining the positions of each of the horizontal exploration lines were programmed by the first vertical row of perforations drilled into the second, vertically placed, aluminium programming plate. Motor II was started and stopped by signals from the timer and the first photocell of the second sensing device (7), in the same manner as described for the control of the movements of motor I. On arriving at the last vertical position, motor II was stopped permanently by the same light beam obstruction mechanism, by a suitably placed aluminium stop in the first of the three slots of the vertical programming plate. On operating a switch, motor II was made to return the frame to its position of origin, indicated by an aluminium stop suitably placed in the second slot of the vertical programming plate. The complete apparatus is shown in Fig. 7.

The recording of the results was done by a data punch (Fig. 8). On termination of the exploration period the following parameters were recorded on 7 hole paper tape in Atlas-Orion code:-

(1) A code identifying the scanning machine (two digits)

(2) The exact position of the explored area, i.e. its x and y co-ordinates (four digits)

(3) The number of counts; only activities exceeding the background threshold value were recorded (four digits)

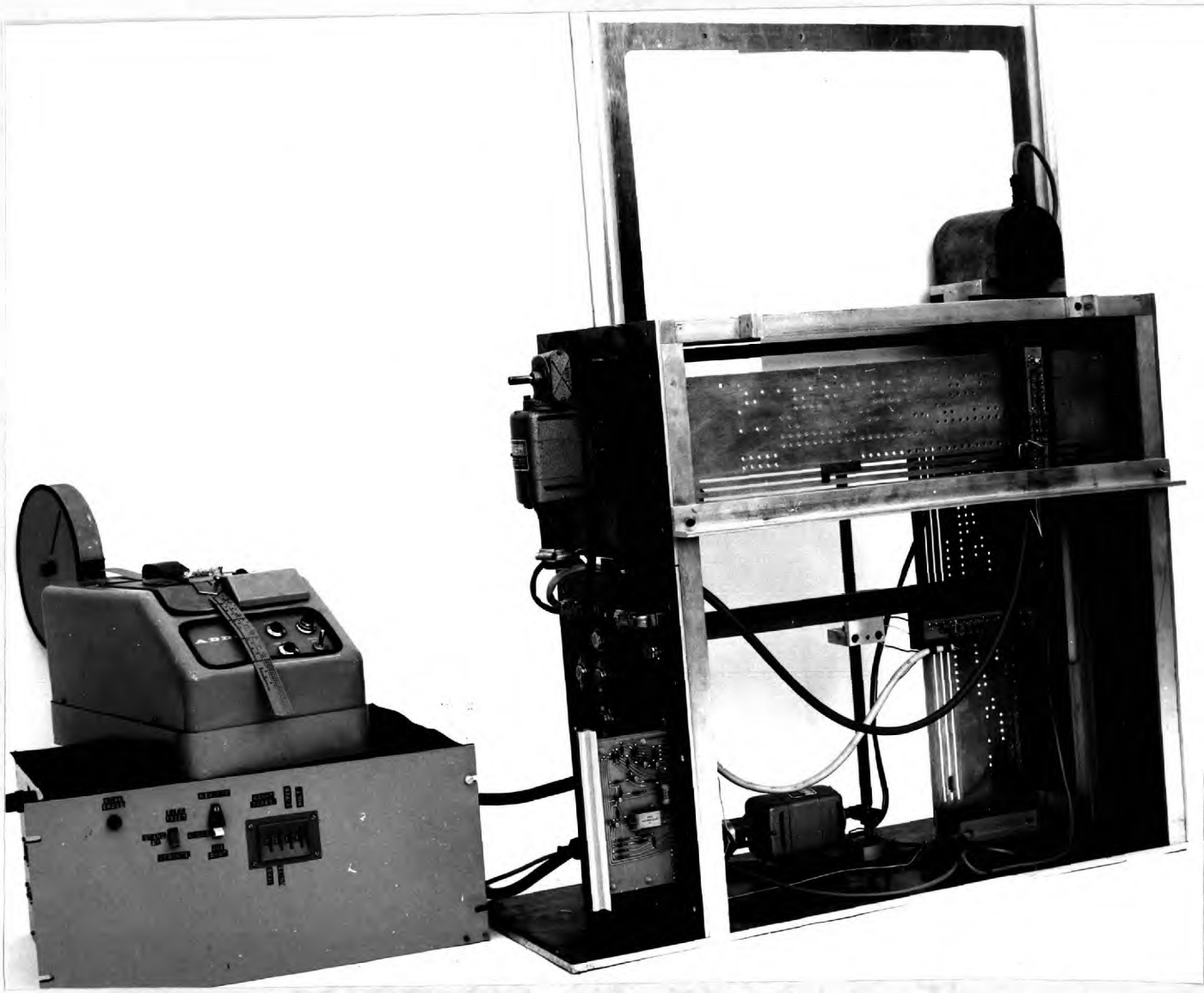


Fig. 8 Scanner showing connection to programmer and timer unit and to data punch.

(4) The time period of counting (three digits)

The data on the paper tape, suitably programmed, was then processed by means of the Atlas computer. The programme involved:-

1. sorting of the data from each scanning machine;
2. presenting the figures as "number maps" of the distribution of radioactivity, the display corresponding exactly to the positions of the radioactive spots on the radiochromatograms (see Fig. 9). The figures were expressed as counts per 10 seconds per unit area scanned;
3. summing the total radioactivity of each spot with appropriate corrections for any overlapping of neighbouring spots, correcting at the same time for background, counting efficiency, decay time if appropriate and daily instrument variations; the latter being assessed by means of a fixed radioactive standard (12) which was counted at the beginning of each line.
4. Expressing the final data as micro-microcuries for each spot.

In order to test the accuracy and reproducibility of this method of assaying radioactive substances on paper chromatograms, 5 µl, 10 µl and 20 µl spots of a solution of glucose-U-C^{14} containing 200 dpm/µl were spotted in triplicate on six chromatograms run overnight in BuOH/acetic acid/H_2O (40::11:25).

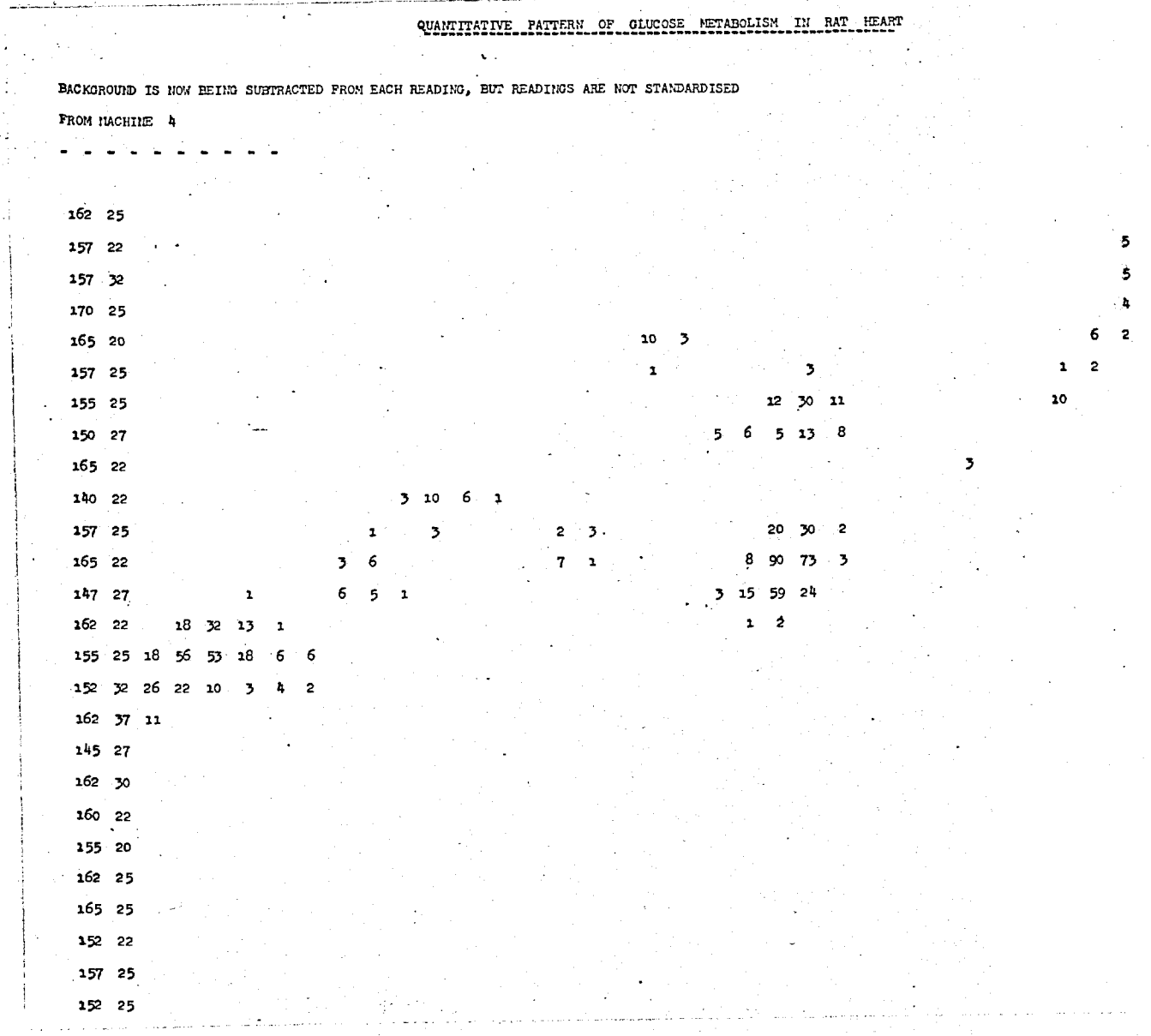


Fig. 9 Typical number map output from scanner showing quantitative distribution of radioactivity on paper chromatogram of heart tissue extract after perfusion with glucose-U-C^{14}.

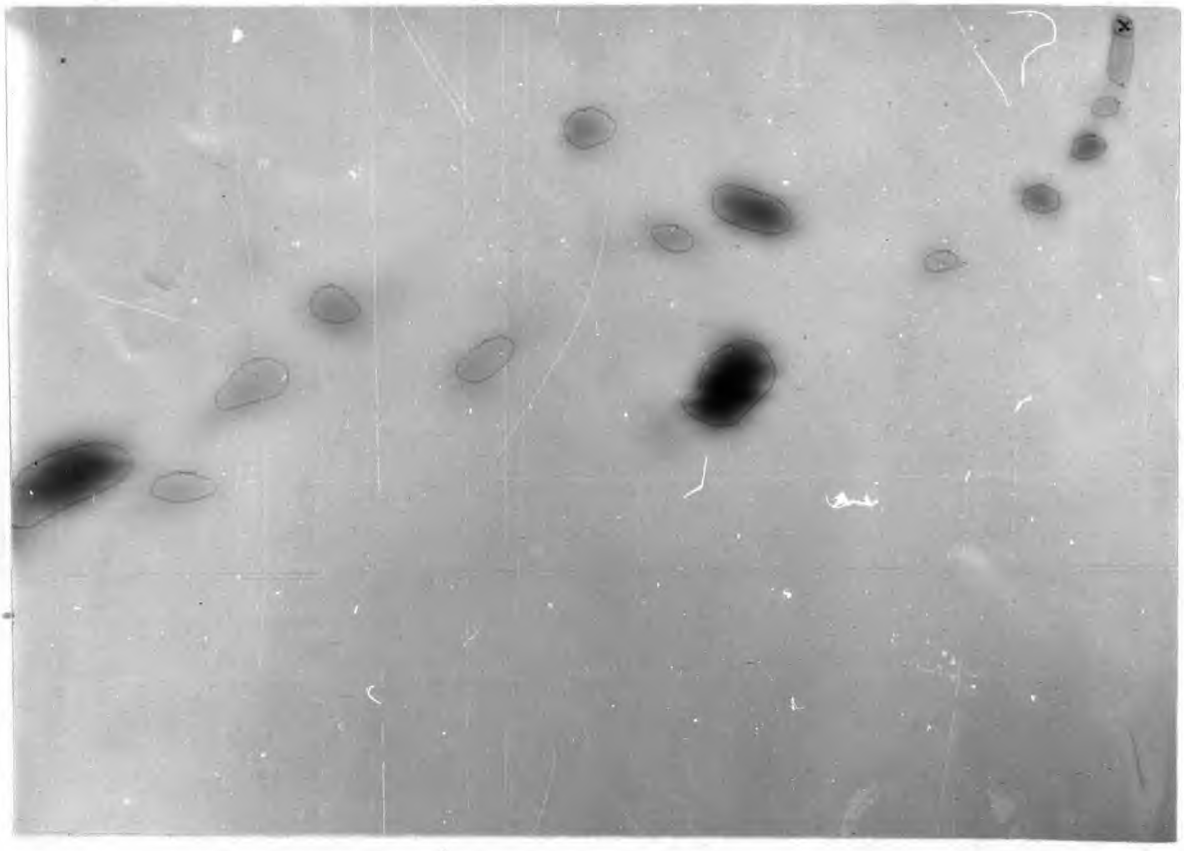


Fig. 10 Corresponding autoradiograph of chromatogram shown in Fig. 9.

Each chromatogram after drying was then scanned on four separate occasions on each of the six scanners. In this way variations between chromatograms could be assessed as well as variations between machines and the reproducibility of a given machine. Thus in Table I the mean ± s.e.m. value (dpm/10 µl) given for each scanner represents the reproducibility of a given machine whilst the vertical means ± s.e.m. indicate the reproducibility of the spotting and chromatography method.

TABLE 1

Reproducibility of the scanning of a radiochromatogram containing spots of different intensities

SPOT	5 µl	10 µl	20 µl	Mean dpm/10µl
Actual dpm	1000 dpm	2000 dpm	4000 dpm	Calc:- 2000 dpm
Scanner 00	970 ± 120	2090 ± 245	4160 ± 390	2063
" 01	962 ± 128	2120 ± 238	4092 ± 372	2050
" 02	1071 ± 150	2006 ± 211	4185 ± 450	2075
" 03	922 ± 108	1996 ± 201	3902 ± 386	1948
" 04	1054 ± 175	1896 ± 260	3858 ± 410	1939
" 05	986 ± 145	1910 ± 198	4011 ± 401	1973
Mean value ± s.e.m.	994 ± 137	2003 ± 225	4034 ± 401	2008

Chromatograms prepared from glucose-U-C^{14} (200 dpm/µl and run overnight in BuOH/acetic acid/H_2O (40:11:25).
Each chromatogram scanned on four separate occasions on each scanning machine. Data from computer output is already corrected in the programme for efficiency of counter and variations in counting a fixed radioactive standard of polymethylmethacrylate-C^{14}.

RESULTS (1) Oxygen consumption, availability and requirement in the perfused rat heart.

The effects of increased contractility* (resulting from increased aortic perfusion pressure) and of external work during atrial perfusion, on the oxygen consumption of the isolated perfused rat heart is shown in Fig 11. In the non-contracting heart, arrested by increased K^+ concentration in the perfusion fluid, the oxygen consumption was only 65% of that of the normal contracting aortic perfused (65 cm) heart. An increase in perfusion pressure to 100 cm brought about a further 50% increase in oxygen consumption. The most dramatic increase in oxygen consumption however was observed during external work at 20 cm atrial pressure, where the qO_2 rose to over three times that observed in a Langendorff perfusion at 65 cm H_2O pressure.

The time course of oxygen uptake by the perfused rat heart is shown in Fig. 12 both in the presence and absence of exogenous substrate. In the initial period after cannulation and commencement of perfusion by the aortic route (100 cm) there was a drop in the qO_2 which became stabilised however within 5-10 minutes. This period of stabilisation and the level of oxygen uptake was independent of the presence or absence of exogenous substrate. After 15 mins of preperfusion

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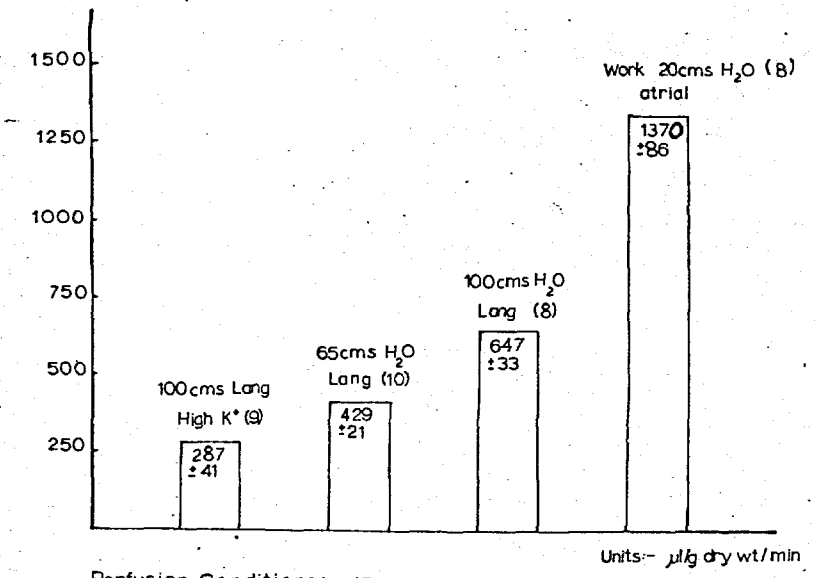
* The term 'contractility' (Zachariah, 1961; Opie, 1965) is used to indicate the force of contraction of the left ventricle in expelling its contents against the hydrostatic pressure on the aortic valve.

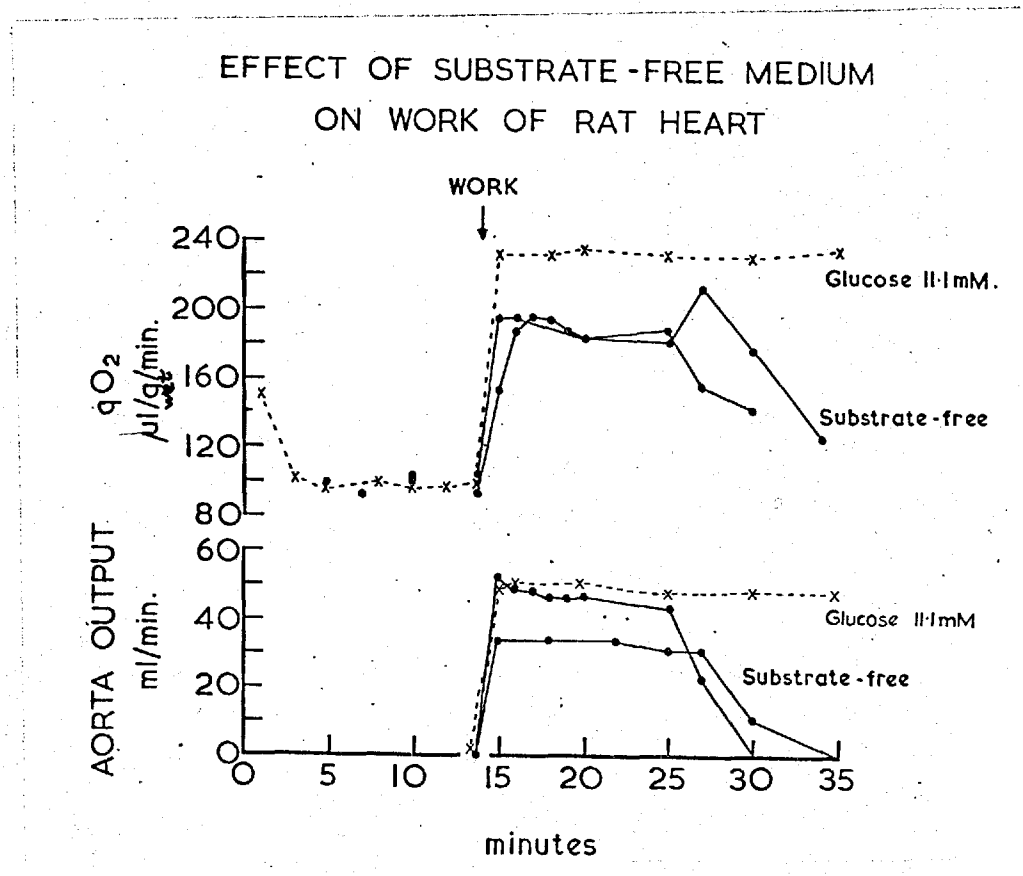
EFFECTS OF INCREASING CONTRACTILITY AND HEART WORK ON OXYGEN CONSUMPTION



Perfusion Conditions:- 15 mins, drip through with glucose 5·5 mM.
No. of hearts under each condition given in brackets.

High K^+ = 14.2 mM

Fig. 11



qO_2 as µl./g wet/min.

All three hearts shown pre-perfused for 15 mins. by aortic route (100 cms. H_2O pressure) then switched to atrial perfusion (20 cms. H_2O pressure).

Perfusion medium:- Krebs-Henseleit bicarbonate buffer

---x---- medium contains glucose 11·1 mM.

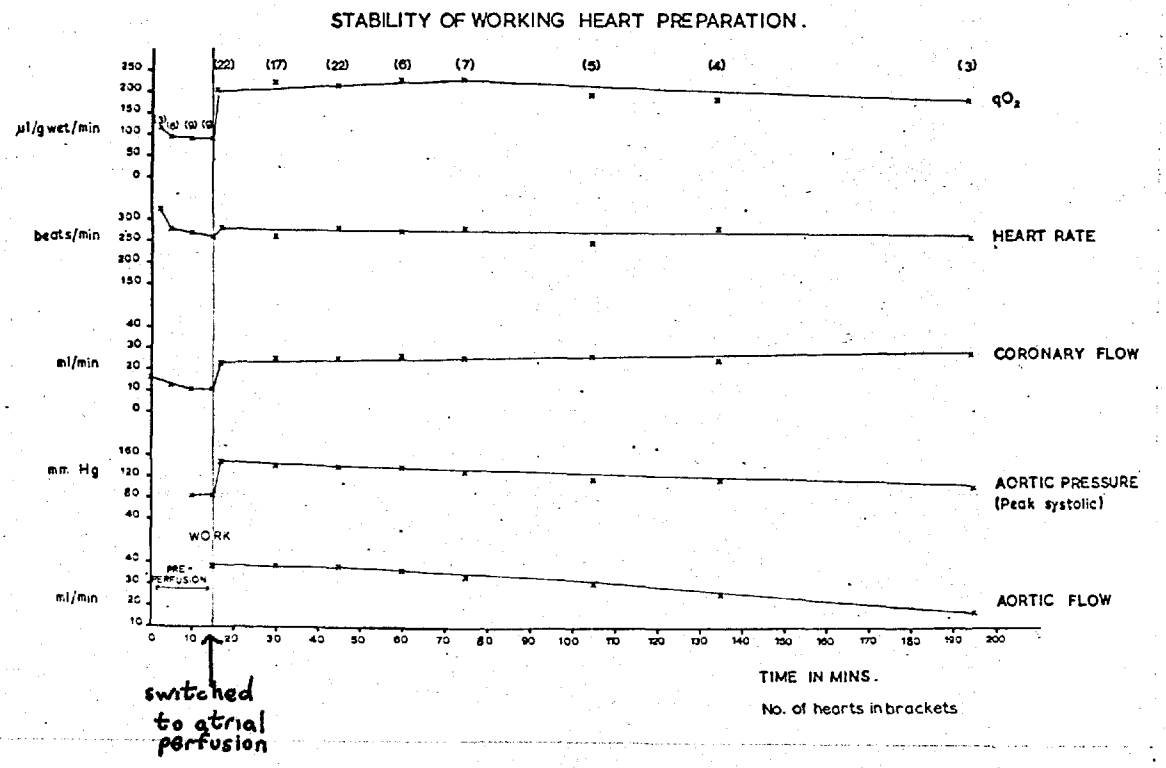
—•— medium contains no added substrate

Fig. 12

the switching of perfusion from the aortic route to the left atrial cannula with resulting performance of external work, brought about an immediate steep rise in oxygen consumption which rapidly stabilised at a value at least double that during the Langendorff "preperfusion" period. In the presence of glucose as exogenous substrate this high level of oxygen consumption was maintained. In the absence of exogenous substrate however there was a marked decline in mechanical performance so that after 15-20 mins no external work was being performed. At this point the oxygen consumption also declined rapidly. Hearts showing this failure because of lack of substrate, recovered the ability to perform external work when provided with glucose, (5.5 or 11mM), or pyruvate, (5mM).

Hearts perfused for 30 minutes (20 cm atrial pressure) with 5 mM pyruvate as substrate gave oxygen consumption (1929 ± 93 µl/g dry/min) and aortic flow rates (39 ± 4 mls/min) not significantly different from the corresponding values obtained with glucose 11.1 mM as substrate.

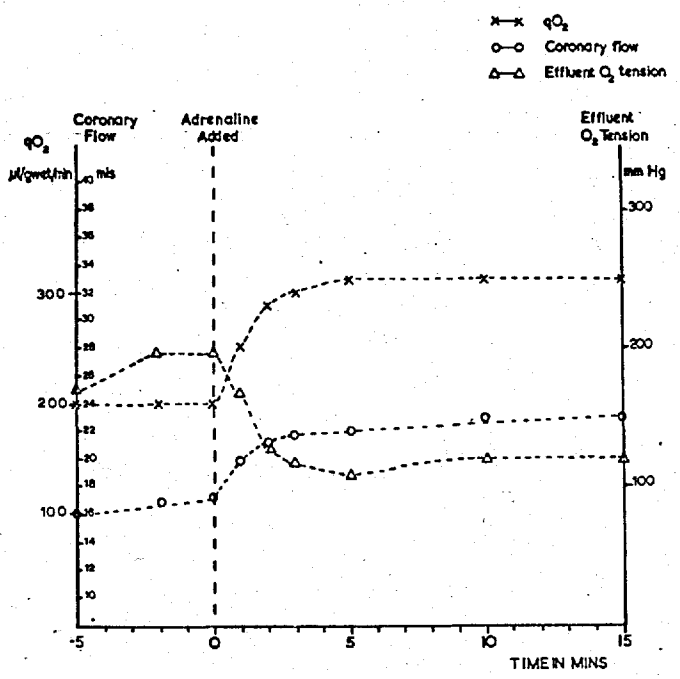
Evidence of the stability of the working heart preparation as regards oxygen consumption and mechanical performance is given in Fig. 13. Over periods of nearly three hours the oxygen consumption, coronary flow rate, and rate of contraction remained essentially stable. After one hour of atrial perfusion, working hearts showed a decline in the aortic peak systolic



Hearts perfused for 15 mins. at 100 cms. H_2O aortic pressure then switched to atrial perfusion at 20 cms. H_2O pressure.

Fig. 13

EFFECT OF ADRENALINE ($1{\cdot}2 \times 10^{-6}$M) ON OXYGEN UPTAKE, CORONARY FLOW AND EFFLUENT OXYGEN TENSION IN THE PERFUSED WORKING RAT HEART.



Values shown are mean of three hearts.

Fig. 14

pressure and a corresponding decline in the aortic flow rate. These results (together with the detailed physiological investigations of Neeley et al., 1967 a, b) were considered sufficient evidence for mechanical stability of the preparation over the period (30-45 mins) of the subsequent metabolic investigati

Although the fact that there was regularly an oxygen tension in the coronary effluent of similar magnitude, (>150 mm Hg) to that found in arterial blood was some indication of adequacy of oxygen supply, the possibility still had to be considered that the working heart could not extract oxygen efficiently below a certain oxygen tension in the fluid supplying it. In order to test this possibility, the oxygen consumption, coronary flow and effluent pO_2 of hearts performing external work were measured before and after addition of adrenaline (1.2×10^{-6} M). As can be seen from Fig. 14, this concentration of adrenaline brought about a rapid rise in the coronary flow, and the oxygen consumption whilst the effluent pO_2 fell by over 60 mm Hg. Consequently the 'working' heart was capable of extracting more oxygen even from an increased coronary flow.

II Effects of increased contractility and external work on the pattern of glucose metabolism

The uptake of glucose by the isolated perfused rat heart is shown in Table 2 for both working and Langendorff conditions, and in the latter case at both 65 cm and 100 cm aortic perfusion pressure. Increasing heart contractility by increasing the perfusion pressure from 65 to 100 cm brought

TABLE 2

METABOLIC EFFECTS OF INCREASED CONTRACTILITY AND THE PERFORMANCE OF EXTERNAL WORK BY THE PERFUSED RAT HEART

	Glucose uptake	Lactate production	Pyruvate production	Medium L/P	Glycogen Initial	Glycogen Final	qO_2
Langendorff (65 cm)	67.2 ± 2.6 (16)	17.4 ± 3.1 (12) (n.s.)	2.03 ± 0.72 (7) (n.s.)	9	105 ± 6 (15)	89 ± 6 (13)	362 ± 82 (7)
Langendorff (100 cm)	110.5 ± 4.3 (19)	22.3 ± 3.4 (23)	2.16 ± 0.29 (7)	10	96.3±3.6 (35)	88.1±4.9 (7)	783 ± 59 (7)
Work (20 cm atrial)	269.7 ± 8.6 (18)	27.5 ± 8.7 (15) (n.s.)	1.88 ± 0.25 (4) (n.s.)	14	(96.3)	62.5±3.4 (8)	1911±79 (30)

All hearts preperfused for 15 mins then perfused under conditions specified by recirculation for 30 mins with glucose (11.1 mM) as substrate.

Units = μmoles glucose equivalent/g dry wt/30 mins ± s.e.m. (no. observations)

qO_2 as μl/g dry wt/min. (n.s.) indicates difference (with corresponding result of Langendorff (100 cm) experiments) not significant ($p > 0.05$).

L/P denotes ratio of lactate and pyruvate concentrations in perfusion medium.

about an increase (65%) in the glucose uptake without corresponding increases in lactate or pyruvate output. The mobilisation of glycogen in the two situations was also not markedly different.

Causing the perfused heart to perform mechanical work at 20 cm atrial pressure brought about a further pronounced increase (145%) in the glucose uptake with only small changes in lactate and pyruvate output (not significant at 5% level). In this case however there was a significant reduction in the glycogen remaining in the heart after 30 min of perfusion.

Further information on the effects of heart work on the pattern of glucose metabolism is provided in Table 3 where the overall fate of radioactive glucose-U-C^{14} is shown under both 'working' and 'non-working' conditions. The pattern of glucose metabolism revealed shows a doubling of the amount of glucose oxidised to $^{14}CO_2$ when the perfusion pressure was increased from 65 cm to 100 cm. The performance of external work brought about a further increase of 200% in $^{14}CO_2$ production but no increase in the formation of C^{14} lactate from glucose. Despite the increased mobilisation of glycogen shown in Table 2 the amount of C^{14} label found in heart glycogen after perfusion with C^{14}-glucose rose with cardiac work (Table 3).

TABLE 3

FATE OF GLUCOSE-U-C^{14} IN THE PERFUSED RAT HEART

EFFECTS OF INCREASED CONTRACTILITY AND THE PERFORMANCE OF EXTERNAL WORK

	Glucose uptake	C^{14} lactate output	C^{14} intermediates in tissue	C^{14} as glycogen	$^{14}CO_2$	% respiration accounted for by glucose oxidation
Langendorff (65 cm)	67.2	-	11.2 ± 1.5 (7)	12.6 ± 1.5 (7)	33.0 ± 2.7 (10)	41% ± 4 (n.s.)
Langendorff (100 cm)	110.5	25.2 ± 2.8 (9)	19.0 ± 3.2 (10)	3.2 ± 0.5 (7)	65.4 ± 6.6 (7)	38% ± 4
Work (20 cm atrial)	269.7	19.3 ± 2.6 (4) (n.s.)	37.2 ± 3.6 (7)	7.4 ± 1.4 (7)	196.5 ± 8.6 (24)	46% ± 3 (n.s.)

All perfusions by recirculation for 30 mins with glucose (11.1 mM) as substrate.
Units = umoles glucose equivalent/g dry wt/30 mins ± s.e.m.
n.s. indicates difference with Langendorff (100 cm) heart not significant ($p > 0.05$)

TABLE 4

EFFECTS OF PERFORMANCE OF HEART WORK AND OF INSULIN ON RADIOACTIVE INCORPORATION OF C^{14} FROM GLUCOSE-U-C^{14} INTO METABOLIC INTERMEDIATES IN PERFUSED RAT HEART MUSCLE

	LANGENDORFF 100cm aortic pressure (4)	WORK 20cm atrial pressure (4)	WORK & INSULIN 20cm atrial pressure (4)
Oligosaccharides	1.0 ± 0.2	3.7 ± 0.5*	17.3 ± 3.5†
Hexose phosphates	0.8 ± 0.2	0.4 ± 0.1	2.9 ± 0.4†
Glucose	8.0 ± 3.0	11.1 ± 3.1	10.8 ± 3.2
Aspartic acid	1.5 ± 0.5	4.4 ± 0.9	6.5 ± 1.2
Glutamic acid	2.3 ± 1.0	8.1 ± 2.1*	15.1 ± 2.4†
Glutamine	0.6 ± 0.2	1.9 ± 0.5*	2.2 ± 0.7
Alanine	0.8 ± 0.2	2.6 ± 0.5*	2.2 ± 0.5
Citrate cycle acids	0.4 ± 0.1	1.5 ± 0.2*	4.3 ± 0.4†
Lactate	3.6 ± 0.8	2.6 ± 0.7	8.6 ± 1.4†
Unknowns	0.2 ± 0.1	0.7 ± 0.2	1.4 ± 0.2†

Results as μmoles glucose equivalent incorporated/g dry wt/30 mins (mean ± s.e.m.).

* denotes significant difference ($p \leqslant 0.05$) between work and Langendorff results.

† denotes significant difference ($p \leqslant 0.05$) between work + insulin and work - insulin results.

Substrate:- glucose 11.1mM ± insulin (2mU/ml).

Further details of the heart C^{14} intermediates is given in Table 4. This data has been obtained by the quantitative radiochromatography scanning technique using computerised number 'maps' similar to those shown in Fig.9. The effect of heart work was to bring about an overall increase in the radioactivity associated with hexose phosphates, citric acid cycle intermediates, oligosaccharides and amino acids.

Despite the marked increase in glucose uptake and glucose oxidation associated with increased contractility and the performance of external work for 30 minutes, there was no corresponding increase initially in the percentage of the total respiration which could be accounted for by glucose oxidation. In both working and non-working hearts oxidation of exogenous glucose accounted for approximately 40% of the respiration during this period. Consequently increasing heart work stimulated the oxidation of both exogenous and endogenous fuel. This was confirmed by experiments in which the time course of glucose uptake and oxidation was followed for perfusion periods of 15, 30 and 45 minutes. As the results of Table 5 show, in longer perfusion periods there was a pronounced increase in the rate of glucose uptake and of $^{14}CO_2$ production from this exogenous substrate, the proportion of glucose used for respiration rising until

TABLE 5

EFFECT OF WORKING TIME ON RATE OF GLUCOSE UPTAKE AND METABOLISM IN PERFUSED RAT HEART

Perfusion time	Rate of Glucose uptake	Rate of Lactate output	Rate of $^{14}CO_2$ formation	% respiration accounted for by glucose oxidation
0-15 mins (20)	180 ± 7	36.3 ± 7.7	127 ± 8	30%
15-30 mins (12)	264 ± 8	17.8 ± 3.6	214 ± 9	50%
30-45 mins (12)	450 ± 10	30.6 ± 4.7	382 ± 12	79%

All hearts preperfused for 15 mins at 100 cm aortic pressure, then switched to atrial perfusion at 20 cm H_2O pressure for times indicated.

Glucose 11.1 mM (without insulin) as substrate.

qO_2 values used for calculation of contribution of glucose to respiration taken from data of Fig. 13.

Units = μmoles glucose equivalent/g dry wt/30 mins ± s.e.m.

nearly 80% of the respiration could be accounted for by glucose oxidation.

Glycerol output. In view of the results indicating increased utilisation of endogenous substrate by the perfused rat heart performing mechanical work, determinations of glycerol released into the perfusion medium were carried out. The results were surprising in that they revealed that the glycerol concentration in the perfusion medium after 2 mins of recirculation perfusion was as high (approx. 30-40 μM) as that obtained after 30 mins of recirculation irrespective of whether Langendorff or working hearts were being used. It was concluded that glycerol release was not a reliable index of utilisation of heart glycerides.

III Effects of insulin and cardiac work on glucose metabolism

The effects of insulin on glucose uptake, lactate production and nett heart glycogen content in both Langendorff (100 cm) and 'working' perfused hearts are given in Table 6. In Langendorff hearts, insulin increased glucose uptake by 106%, lactate production by 152% and increased cardiac glycogen. In working hearts the effects were similar (glucose uptake + 100%, lactate production + 190%) and the loss of glycogen normally observed in working hearts was prevented.

The fate of radioactive glucose-U-C^{14} in the presence of insulin is summarised in Table 7. Apart from the effects already described on lactate production and glucose uptake,

TABLE 6

METABOLIC EFFECTS OF INSULIN IN THE PERFUSED RAT HEART

	Glucose uptake	Lactate production	Pyruvate production	Medium L/P	Glycogen Initial	Glycogen Final	qO_2
Langendorff (100 cm) + insulin	228 ± 9 (6)	56.3 ± 4.4 (13)	6.3 ± 0.8 (6)	11	96 ± 4 (35)	136 ± 10 (6)	731 ± 24 (6)
% increase over control (- insulin)	+ 106%	+ 152%	+ 192%	n.s.	-	+ 53%	n.s.
Work (20 cm atrial) + insulin	538 ± 17 (15)	79.9 ± 10.8 (19)	7.5 ± 1.6 (8)	15	96 ± 4 (35)	108 ± 3 (13)	2024 ± 101 (7)
% increase over control (- insulin)	+ 100%	+ 190%	+ 300%	n.s.	-	+ 73%	n.s.

All perfusions by recirculation for 30 mins with glucose (11.1 mM) as substrate.
Units = umoles glucose equivalent/g dry wt/30 mins ± s.e.m.(no.of observations)
Insulin used at 2 mU/ml during recirculation period only.
L/P denotes ratio of concentrations of lactate and pyruvate in perfusion medium.
qO_2 as µl/g dry wt/min. n.s. = changes not significant. ($p > 0.05$).

TABLE 7

FATE OF GLUCOSE-U-C^{14} IN THE PERFUSED RAT HEART

EFFECTS OF INCREASED CONTRACTILITY AND WORK IN PRESENCE OF INSULIN

	Glucose uptake	C^{14} lactate output	C^{14} intermediates in tissue	C^{14} as glycogen	$^{14}CO_2$
Langendorff (100 cm) + insulin	228	57.6 ± 6.1 (16)	38.8 ± 3.2 (6)	21.2 ± 4.4 (6)	112.7 ± 6.8 (6)
% increase over control (- insulin)	+ 106%	+ 128%	+ 104%	+ 562%	+ 73%
Work (20cm atrial) (+ insulin)	538	83.8 ± 17.3 (7)	72.1 ± 2.8 (7)	26.0 ± 3.6 (16)	333.6 ± 18 (12)
% increase over control (- insulin)	+ 100%	+ 334%	+ 94%	+ 513%	+ 70%

Conditions as for Table 6

Units: µmoles glucose equivalent/g dry wt/30 mins ± s.e.m.

this reveals striking increases in the $^{14}CO_2$ formation from glucose (+ 70 to 73%) and in the C^{14} label in heart glycogen (+ 513 to 562%) and tissue intermediates (+ 94 to 104%) in both Langendorff and working hearts. The results obtained by application of the radioactive chromatographic scanning method to hearts perfused in the presence of insulin (2 mU/ml) are included in Table 4. The increases brought about by insulin were mainly in the incorporation of C^{14} label into oligosaccharides, lactate, hexose phosphates, and amino acids.

In view of the fact that the action of insulin has been suggested as bringing about an increase in cell permeability (Levine and Goldstein, 1955) the effects of increasing glucose concentration on $^{14}CO_2$ production, lactate formation and glucose uptake in the Langendorff heart were examined. There was a rise in glucose uptake as the substrate concentration was raised from 1 to 10 mM but beyond this, further increases in external glucose concentration were without significant effect on glucose uptake and oxidation (Table 8). The high levels of glucose uptake and oxidation, of lactate formation and incorporation of C^{14} into glycogen, achieved in the presence of insulin could not be reproduced by increasing glucose concentration.

Measurements of the sorbitol and glucose spaces in working hearts (as described under "experimental") gave values of

TABLE 8

EFFECT OF GLUCOSE CONCENTRATION ON $C^{14}O_2$ PRODUCTION, C^{14}LACTATE FORMATION AND GLUCOSE UPTAKE IN LANGENDORFF PERFUSED HEART

Glucose Concentration	Glucose Uptake	$^{14}CO_2$	^{14}C-Lactate	^{14}C-Glycogen
1 mM (3)	35 ± 3.7	22.4 ± 4.1	13.3 ± 2.2	2.1 ± 0.3
3 mM (3)	56 ± 4.2	35.7 ± 4.4	23.1 ± 4.2	2.1 ± 0.6
5 mM (6)	119 ± 7.6	60.2 ± 3.6	22.9 ± 2.1	2.8 ± 0.4
10 mM (6)	140 ± 12	74.9 ± 3.6	28.7 ± 3.5	4.2 ± 1.2
20 mM (3)	154 ± 17	79.8 ± 4.8	28.0 ± 3.2	4.2 ± 0.8
10mM + Insulin (6)	245 ± 9	112.7 ± 10.2	74.2 ± 8.5	14.7 ± 1.2

All hearts perfused for 30 mins by recirculation.
Insulin, when present, used at 2 mU/ml.

Units: μ moles glucose equivalent/g dry wt/30 mins (mean ± s.e.m.)

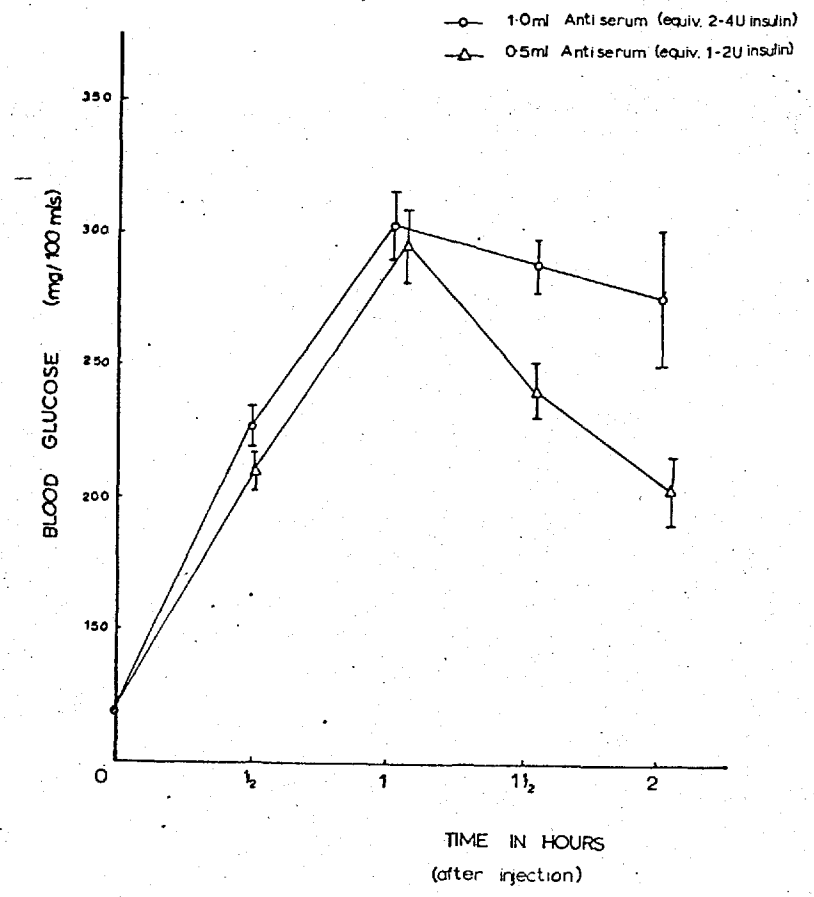
No. of hearts at each concentration in brackets.

502 ± 16 µl/g for the sorbitol space which was unaffected by the presence of insulin, whilst the glucose spaces were 444 ± 27 µl/g (without added insulin) and 463 ± 22 µl/g (in the presence of 2 mU/ml insulin). Thus since the glucose space remained less than the sorbitol space (unlike in the Langendorff heart perfused in the presence of insulin) no intracellular accumulation of glucose was demonstrated.

IV Production of insulin deficiency by means of anti-insulin serum.

In considering the results given above, it should be borne in mind that the control hearts (i.e. perfused without added insulin) still contained endogenous tissue insulin. For this reason, insulin deficient hearts were of interest and were obtained by pretreatment of donor rats with anti-insulin serum. The efficacy of this anti-insulin serum prepared in guinea pigs as described above, in producing hyperglycaemia in donor rats is shown in Fig. 15. Increasing the amount of anti-insulin antibody injected had little effect on the degree of hyperglycaemia, but influenced the time taken for the blood glucose level to return to normal. Hearts were therefore removed from rats rendered insulin deficient, 1 hour after injection of the anti-insulin serum.

EFFECT OF ADMINISTRATION OF GUINEA PIG ANTI-INSULIN SERUM ON BLOOD GLUCOSE LEVEL IN RATS.



Vertical bars indicate mean ± s.e.m (4 rats)

Fig. 15

V Effects of insulin deficiency on the pattern of glucose metabolism.

The striking effect on cardiac glucose metabolism of pre-treatment of the donor rat with this potent anti-insulin serum is shown in Table 9 which summarises the fate of C^{14} glucose in these hearts. Figs. 16 and 17 show autoradiographs and scanning maps of chromatograms of heart extracts in the Langendorff experiments. In the Langendorff hearts, pre-treatment of the donor rat to deplete cardiac insulin resulted in a lowering in glucose uptake (- 55%), glucose metabolism to C^{14} lactate (- 50%), and glucose oxidation to $^{14}CO_2$ (- 55%) as well as in the incorporation of ^{14}C-glucose into glycogen (- 75%). The number maps and autoradiographs show a striking effect of insulin deficiency on the incorporation of ^{14}C from glucose into oligosaccharides and hexose phosphates.

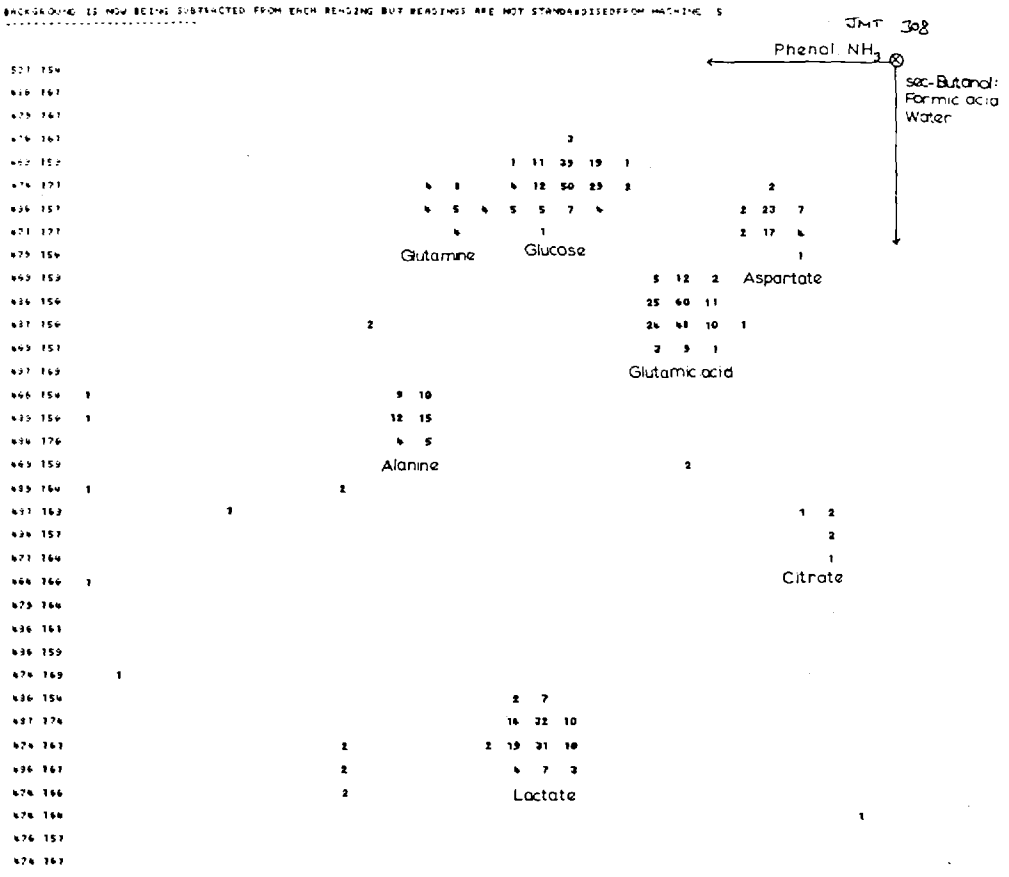
In contrast to these results, in the working heart preparation there was no significant difference in the amount of either glucose uptake or glucose oxidation to $^{14}CO_2$ between normal hearts and insulin depleted hearts. The difference in incorporation of C^{14} into glycogen however was still apparent (- 170%). The addition of insulin to the perfused hearts from anti-insulin treated rats, restored glucose uptake, C^{14}-lactate, $^{14}CO_2$ and C^{14} incorporation into

TABLE 9

FATE OF GLUCOSE-U-C^{14} IN THE PERFUSED HEARTS OF INSULIN DEFICIENT RATS
EFFECTS OF CARDIAC WORK AND OF INSULIN (IN VITRO)

	^{14}C Glucose uptake	^{14}C Glycogen	^{14}C Lactate	^{14}C-tissue Intermediates	$^{14}CO_2$
(a) Langendorff Hearts (Aortic Pressure 100 cms)					
Control	142 ± 20 (9)	4.2 ± 0.8	28.7 ± 3.3	21.2 ± 4.1	74.9 ± 9.1
*Control + insulin (in vitro)	228 ± 9 (6)	21.2 ± 4.4	57.6 ± 6.1	38.8 ± 3.2	112.7 ± 6.8
Anti-insulin treated	63 ± 8 (6)	1.1 ± 0.6	18.2 ± 3.3	11.7 ± 1.3	33.6 ± 4.6
Anti-insulin treated + insulin (in vitro)	247 ± 24 (6)	35.2 ± 5.6	38.2 ± 7.4	46.4 ± 8.2	100.8 ± 9.6
(b) Working Hearts (Atrial Pressure 20 cms)					
Control	249 ± 21 (6)	5.9 ± 1.5	23.5 ± 2.9	41.6 ± 5.2	189 ± 21
Anti-insulin treated	289 ± 19 (6)	2.2 ± 1.2	11.4 ± 1.2	24.2 ± 6.6	233 ± 28

All perfusions carried out by recirculation for 30 mins.
Insulin, where present, used at 2 mU/ml in perfusion fluid.
Substrate:- Glucose 11.1 mM.
Units:- µmoles glucose equivalent/g dry wt/30 mins.
Mean ± s.e.m. (Number of experiments)
* indicates data from Table 7.



CHROMATOGRAM OF METABOLITES OF GLUCOSE-U-C^{14} IN HEART FROM RAT TREATED WITH ANTI-INSULIN SERUM

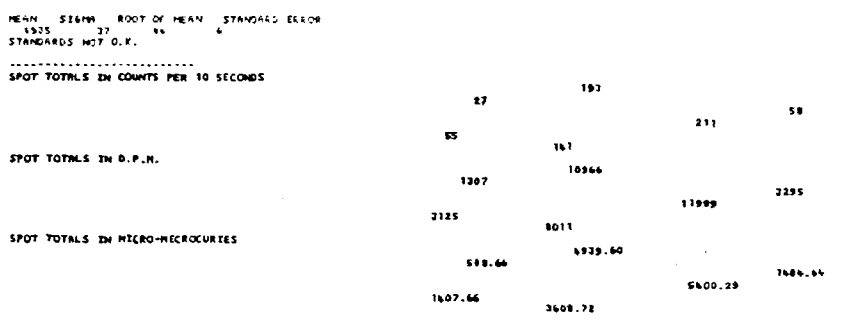
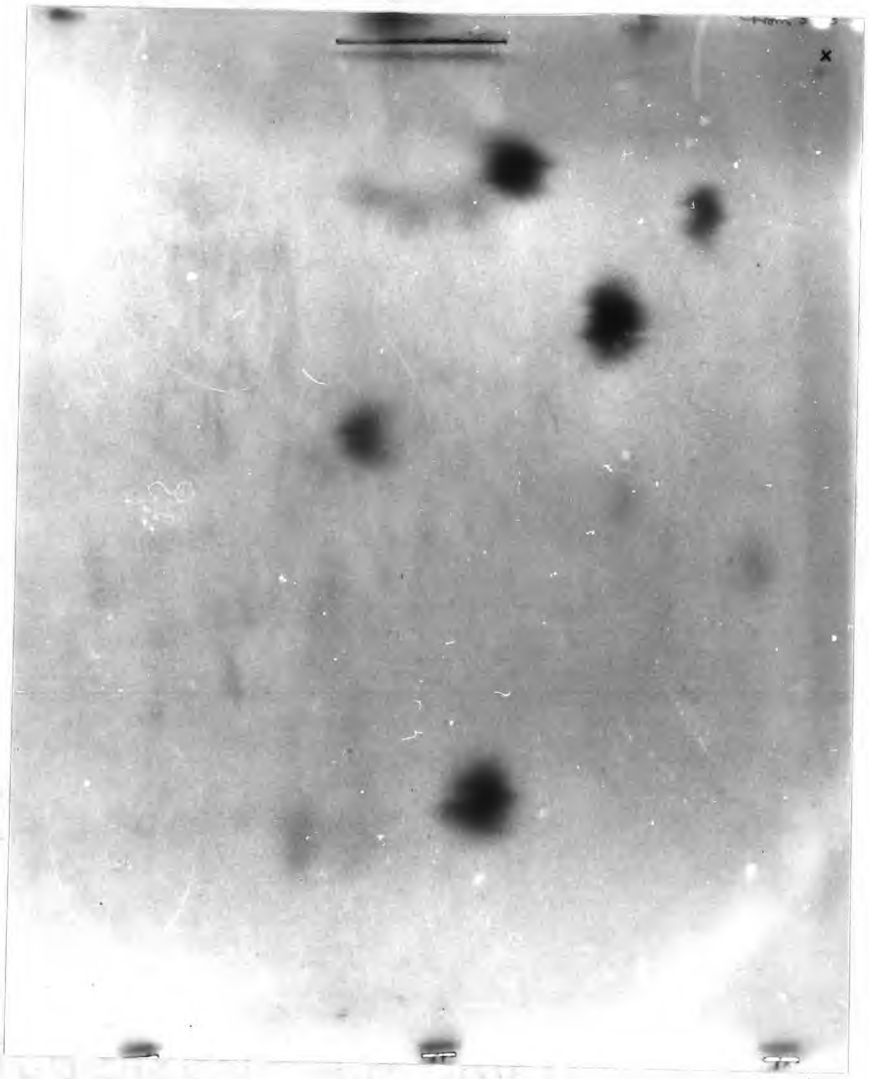
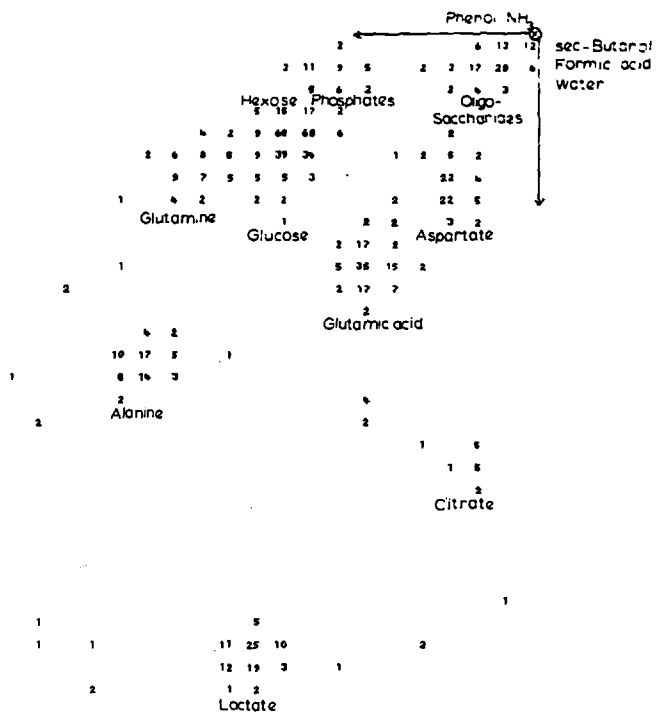


Fig. 16a



Corresponding autoradiograph for chromatogram of Fig. 16a

Fig. 16b



CHROMATOGRAM OF METABOLITES OF GLUCOSE-U-C^{14}
IN HEART FROM CONTROL RAT

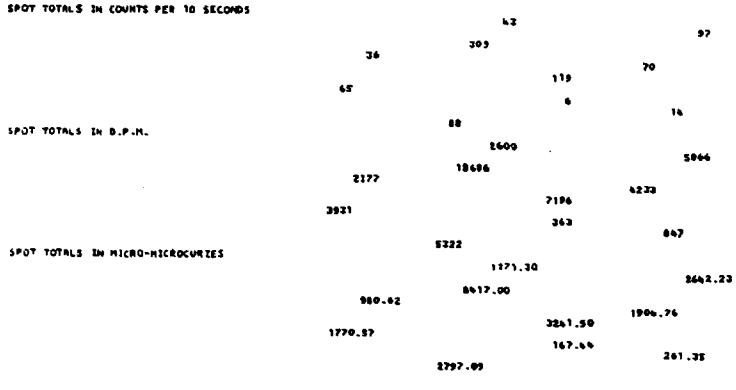


Fig. 17a

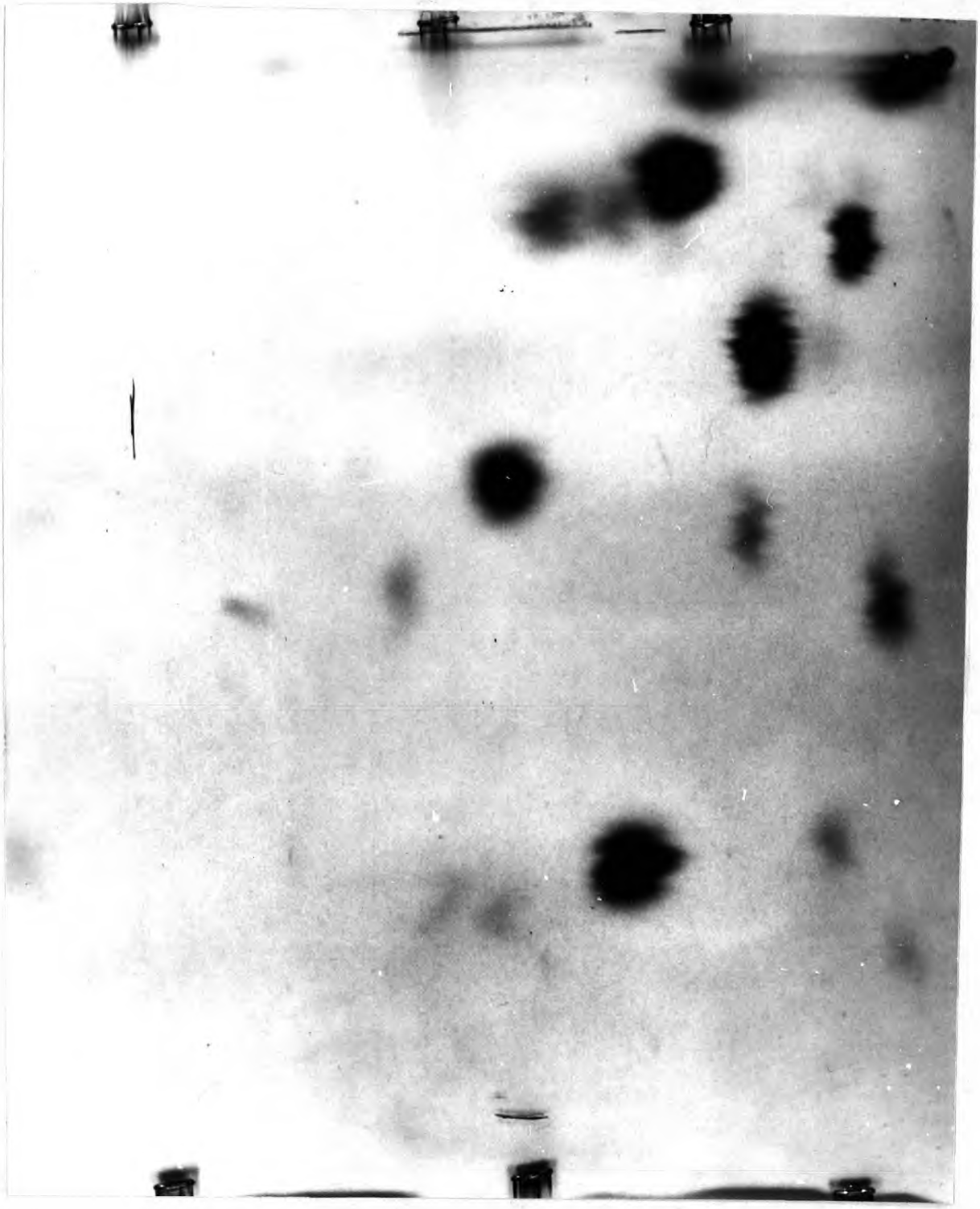


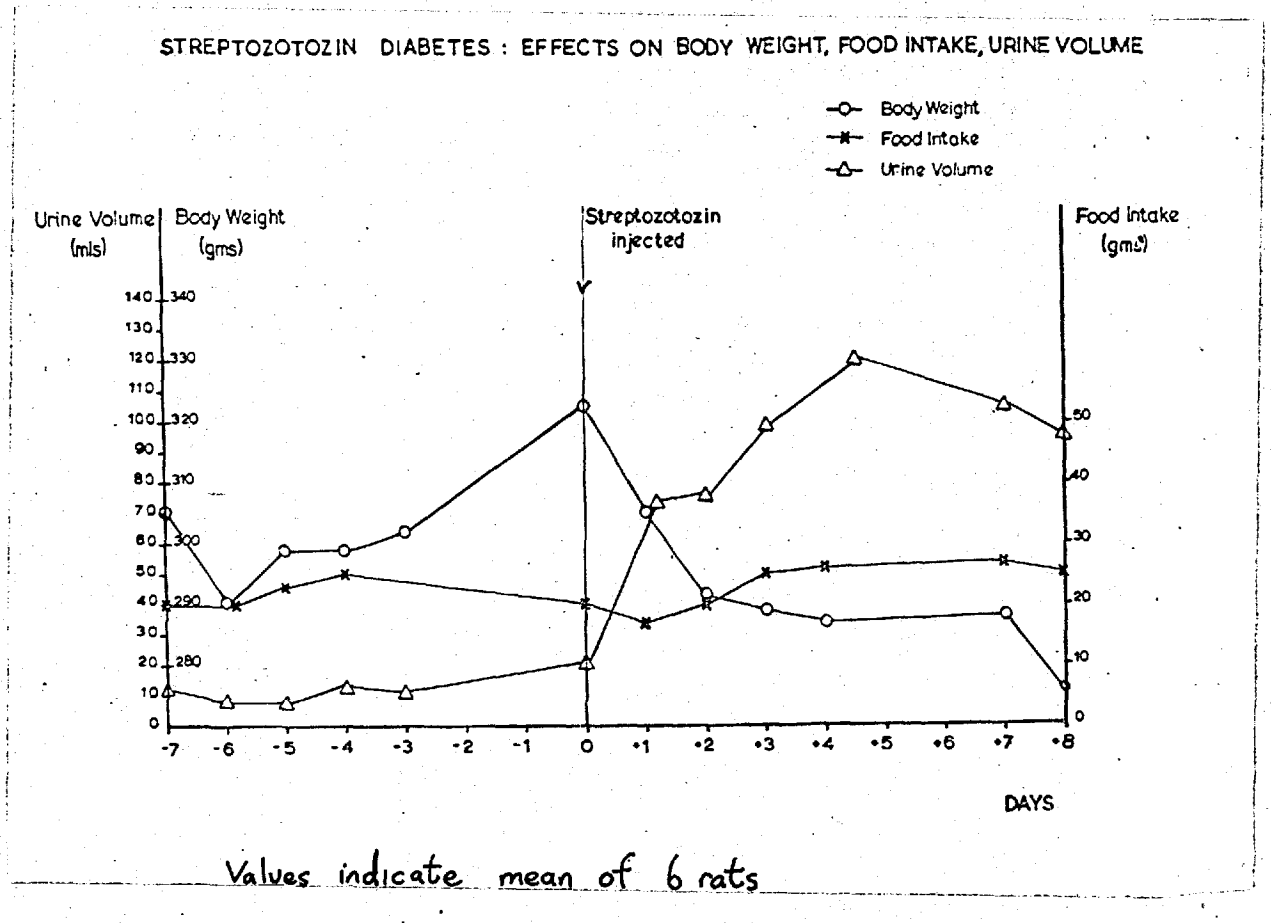
Fig. 17b Corresponding autoradiograph for chromatogram of Fig. 17a

glycogen to normal (+ insulin) levels, so that the effects of insulin deficiency induced by anti-insulin serum were completely reversible. The striking effects of anti-insulin treatment on cardiac glucose metabolism and the interesting differences between working and non-working hearts under these conditions suggested the investigations which followed on other methods of producing insulin deficiency.

VI Metabolic comparison of alloxan and streptozotocin induced diabetes

The known disadvantages associated with the use of alloxan (see below) made it of interest to study the characteristics of the chemically induced diabetes resulting from the administration of the antibiotic streptozotocin. Rats given a single injection 65 mg/kg i.v. of streptozotocin developed hyperglycaemia (Fig. 18a) glucosuria and polyuria (Fig. 18b) within 24 hours of dosage. Although appetite was normal there was rapid loss of body weight (Table 10). At the same time there was generalised lipolysis which resulted in a transient increase in serum free fatty acids (Fig. 19a) and blood ketones (Fig. 19b). Whereas these changes were only observable for the first 1 - 5 days after treatment, the hyperglycaemia and glucosuria persisted for at least 4 months.

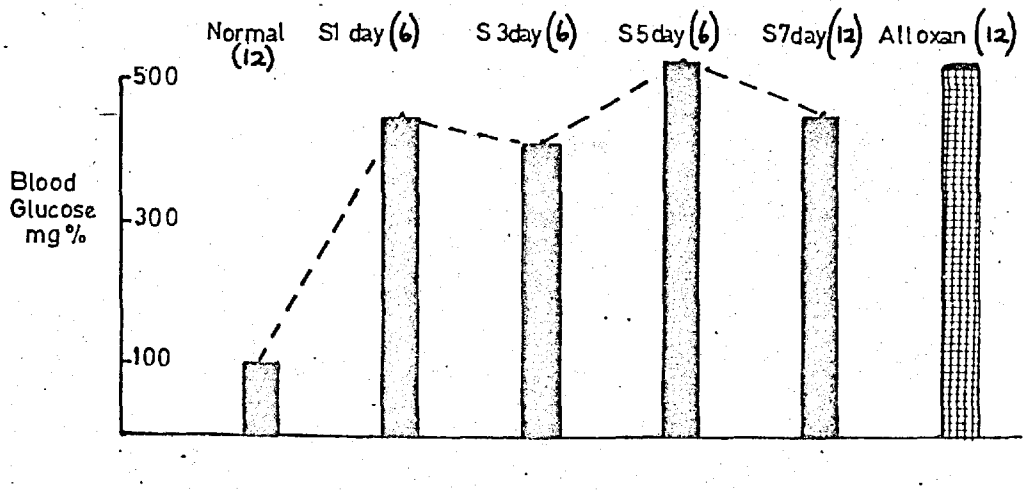
In the case of alloxan diabetes, the results confirm



Values indicate mean of 6 rats

Fig. 18a

Fig. 18 (b) Comparison of blood glucose concentration following injection (65 mg/kg i.v.) of streptozotocin or alloxan.



S1, S3, S5, S7 indicates days after dosage with streptozotocin.

Alloxan animals used 48 hours after dosage.

No of rats in brackets.

TABLE 10

STREPTOZOTOCIN-INDUCED DIABETES

EFFECTS ON BLOOD GLUCOSE, URINE GLUCOSE, BODY WEIGHT

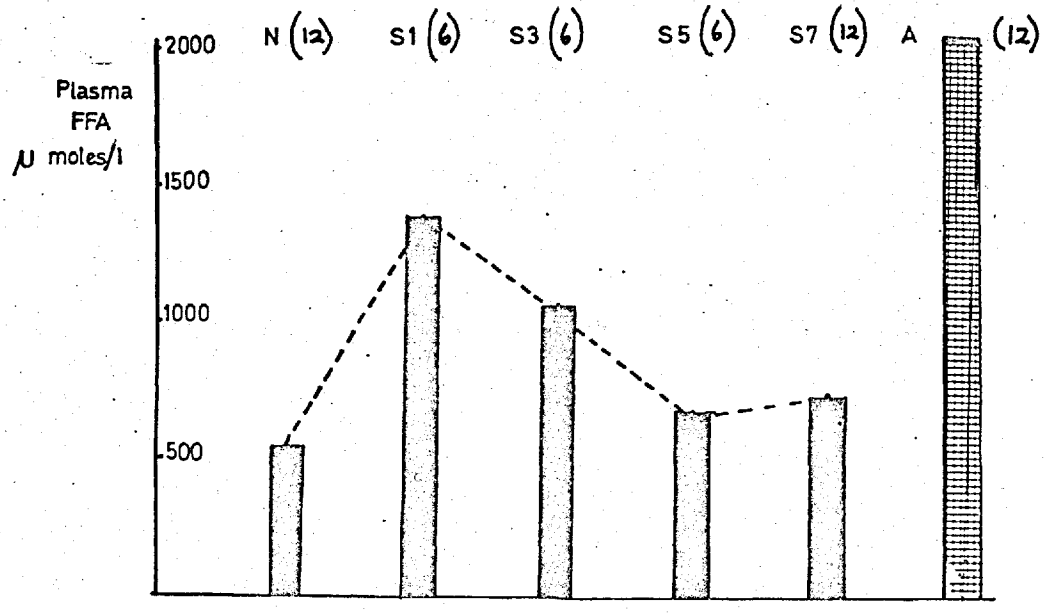
	Day 0	1 day	3 days	5 days	7 days	96 days
Blood glucose mg/100 mls	115 ± 5 (5)	411 ± 28 (11)	352 ± 22 (9)	533 ± 18 (6)	501 ± 16 (5)	480 ± 42 (3)
Loss of body wt g.	-	22 ± 3 (11)	34 ± 3 (10)	36 ± 4 (10)	51 ± 6 (17)	72 ± 3 (3)
Urine glucose mg/ml	-	24 ± 4 (6)	75 ± 8 (6)	92 ± 6 (6)	90 ± 3 (6)	93 ± 6 (3)

No. of animals given in brackets.

All animals received a single injection of 65 mg/kg intravenously of streptozotocin (25 mg/ml in aqueous saline at pH 4). Initial wt 280-320 g.

Access to water and food was unrestricted.

Fig. 19 (a) Comparison of plasma FFA concentration following injection (65mg/kg i.v.) of streptozotocin or alloxan.

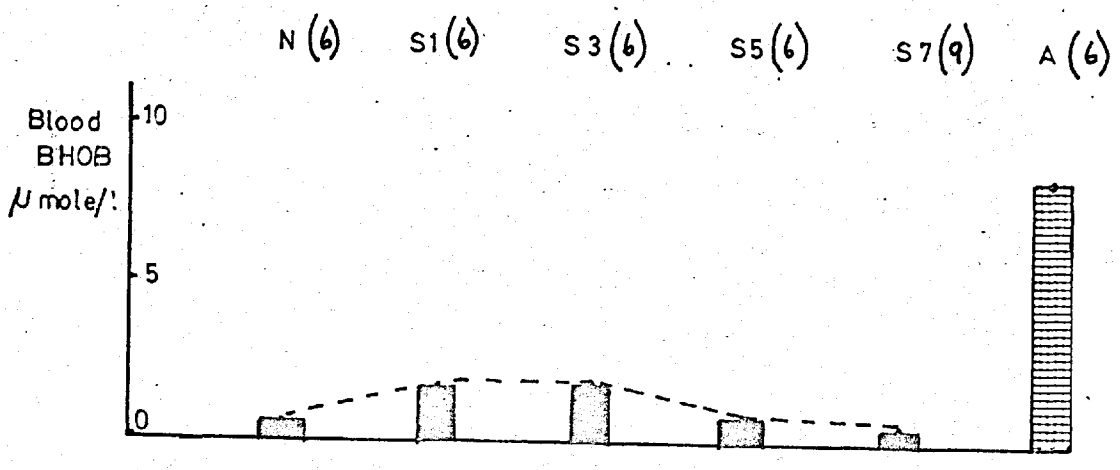


S1, S3, S5, S7 indicates days after dosage with streptozotocin.

A indicates alloxan animals, 48 hours after dosage.

N indicates normal animals. No. of rats in brackets.

Fig. 19 (b) Comparison of blood βhydroxybutyrate concentration following injection with streptozotocin or alloxan.



Conditions as specified in Fig 19(a)

TABLE 11

COMPARISON OF IN-VIVO EFFECTS OF STREPTOZOTOCIN AND ALLOXAN DIABETES ON RATS

Type of animal	Normal	Strepto-zotocin diabetes	Alloxan diabetes
Blood-glucose (mg/100 ml)	109 ± 6 (9)	445 ± 25* (8)	527 ± 16* (14)
Plasma-F.F.A. (nmole/l.)	0.574 ± 0.038 (9)	0.667 ± 0.083 (8)	2.076 ± 0.307* (9)
Blood-ketones (µmole/l.)	0.19 ± 0.09 (6)	0.49 ± 0.19 (6)	7.62 ± 0.63* (15)
Heart glycogen (*in situ*) (µmole/g)+	20.7 ± 1.6 (4)	17.8 ± 1.3 (14)	41.8 ± 3.7* (4)

Streptozotocin animals used 7 days after 65 mg/kg i.v.
Alloxan animals used 48 hours after 65 mg/kg i.v.

All values per g wet weight and expressed as means ± standard error of mean (number of hearts analysed in brackets)

* Denotes p values $\leqslant 0.05$ compared with normal.

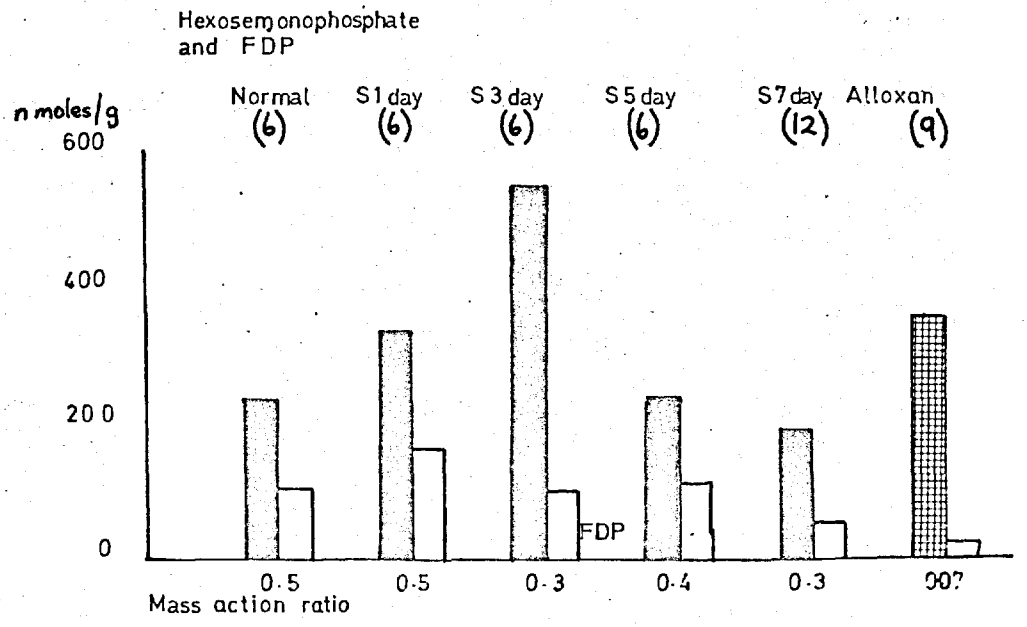
+ Expressed as glucose equivalents.

that 48 hours after a single administration of 65mg/kg alloxan i.v. the hyperglycaemia was accompanied by very high circulating free fatty acids and ketone bodies and a significantly elevated cardiac glycogen content *in situ*. This was in marked contrast with the normal levels for these parameters seen in the streptozotocin treated rat 7 days after treatment (see Table 11).

In view of these interesting differences, the effects of streptozotocin and alloxan diabetes on the levels of hexose phosphates, citrate and glycogen in perfused Langendorff hearts were compared. In view of the earlier studies of Randle et al. 1963, 1964, on alloxan diabetic animals a similar experimental design was used, i.e. a drip-through non-recirculation system with Langendorff perfusion at 65 cm H_2O pressure using glucose (5.5 mM) as substrate in the presence of 0.1 unit/ml of insulin. The results of this comparison of streptozotocin diabetic rats (1, 3, 5 and 7 days after dosage) with alloxan diabetic rats (48 hours after dosage) are shown in Fig. 20 (hexose phosphates and citrate) and Fig. 21 (glycogen).

In alloxan diabetes the perfused heart showed increases in cardiac glycogen content, G6P, F6P and citrate and a decrease of FDP, thus confirming the results of Randle et al. (1964), Regen et al. (1964) and Bowman (1965, 1966). In

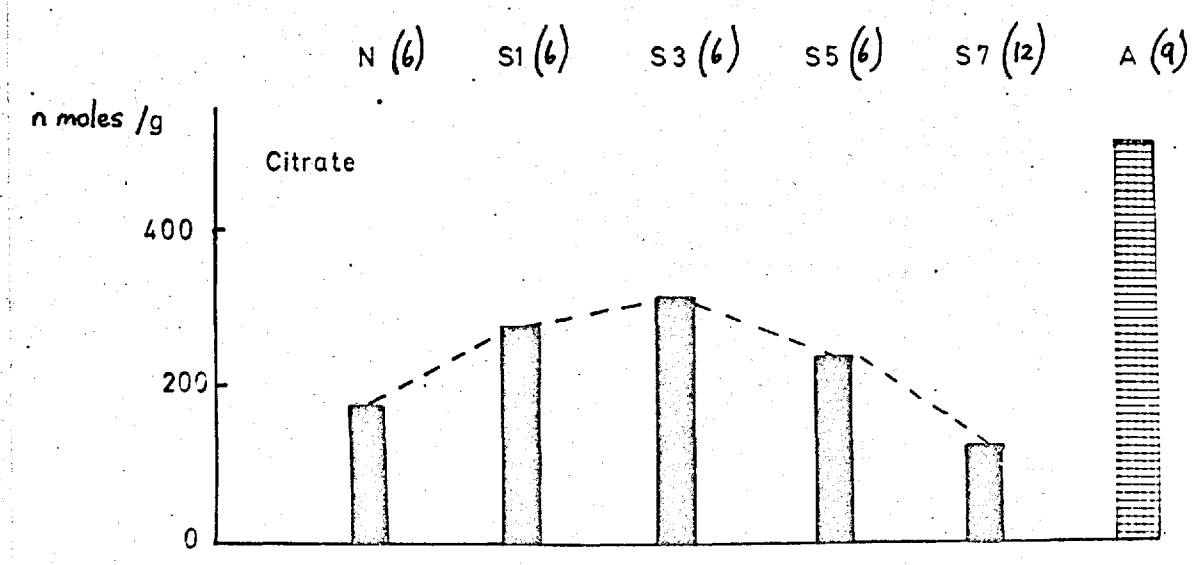
Fig. 20(a) Cardiac content of hexose phosphates after perfusion (15 mins at 65 cm aortic pressure) with glucose (5.5mM) and insulin (100mU/ml).
Comparison of streptozotocin and alloxan diabetes.



No. of hearts given in brackets
shaded areas = G6P + F6P content
unshaded areas = FDP content
mass action ratio = FDP / G6P + F6P

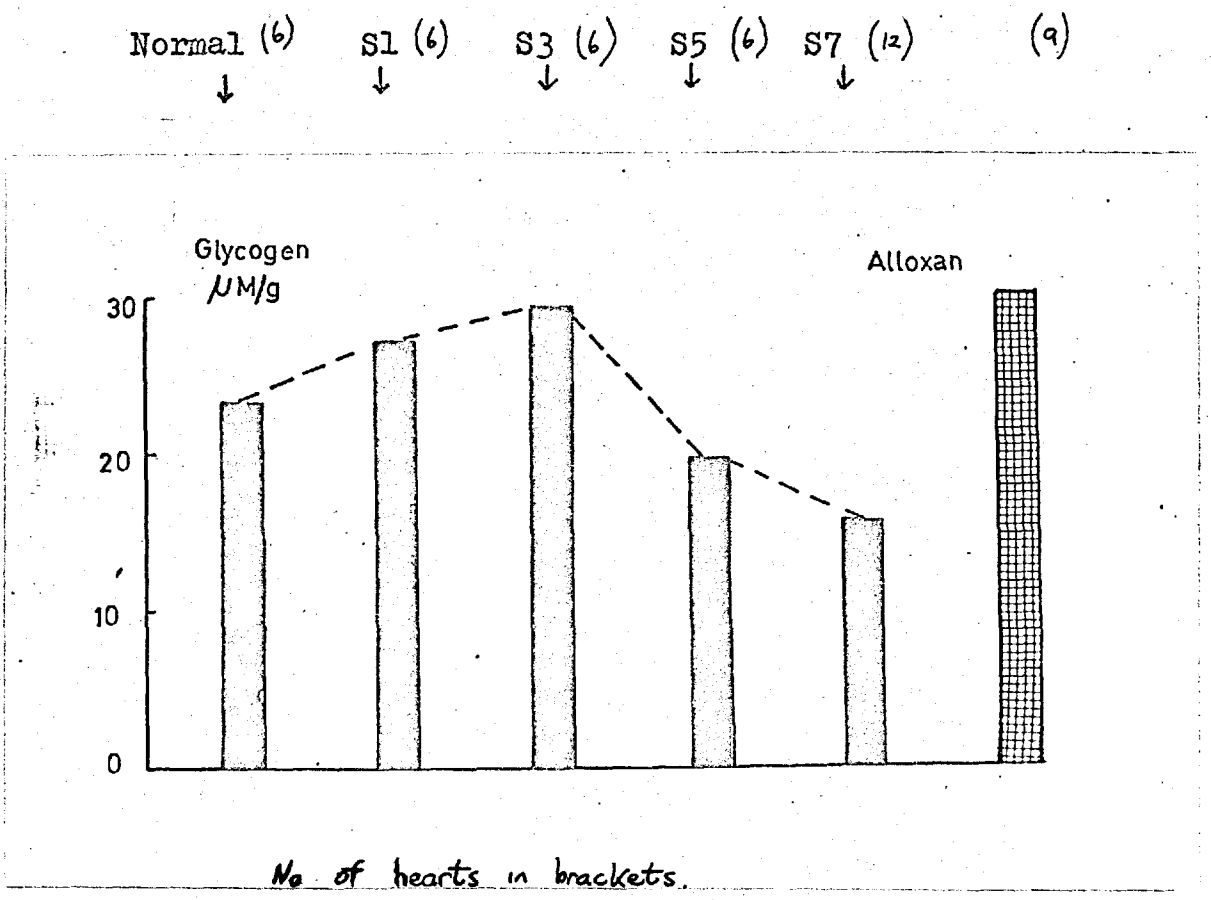
Fig. 20 (b) Comparison of streptozotocin and alloxan diabetes. Cardiac content of citrate.

(Conditions as in Fig. 20a)



No of hearts in brackets.

Fig. 21 Comparison of streptozotocin and alloxan diabetes. Cardiac content of glycogen. (Conditions as in Fig. 20a)



Glycogen as µmoles glucose equivalent/g wet wt.

TABLE 12

EFFECTS OF STREPTOZOTOCIN AND ALLOXAN DIABETES ON CONTENTS OF GLYCOGEN, HEXOSE PHOSPHATES AND CITRATE IN PERFUSED RAT HEART

-	Normal	Strepto-zotocin diabetes	Alloxan diabetes
Glycogen (μmole/g)	22.2 ± 2.3 (8)	15.2 ± 1.1* (8)	30.0 ± 1.2* (8)
Glucose-6-phosphate (nmole/g)	167 ± 10 (13)	147 ± 16 (7)	298 ± 16* (15)
Fructose-6-phosphate (nmole/g)	36 ± 3 (12)	34 ± 3 (7)	66 ± 3* (15)
Fructose-1,6-diphosphate (nmole/g)	69 ± 9 (13)	57 ± 10 (17)	23 ± 3* (14)
Citrate (nmole/g)	182 ± 9 (8)	139 ± 11* (7)	464 ± 38* (14)

* Denotes p values $\leq$ 0.05 compared with normal.
Streptozotocin animals used 7 days after 65 mg/kg i.v.
Alloxan animals used 48 hours after 65 mg/kg i.v.
All values per g wet weight and expressed as means ± standard error of mean (No. of hearts analysed in brackets)

contrast, streptozotocin showed similar changes to alloxan at 1 and 3 days but at 7 days the cardiac glycogen and citrate levels of the perfused streptozotocin diabetic heart were decreased while levels of G6P, F6P and FDP were no longer significantly different to normal values (see Table 12).

A comparison was then made of the pattern of cardiac glucose metabolism using rats with these two types of induced diabetes as well as the acutely insulin deficient animal described above.

In Table 13, the metabolic effects of streptozotocin diabetes and cardiac work in the perfused rat heart are summarised. Data on the fate of glucose-U-C^{14} in perfused hearts from streptozotocin diabetic rats is given in Table 14. In Langendorff perfused hearts the glucose uptake, lactate production, $^{14}CO_2$ production and incorporation of C^{14} into glycogen were all significantly reduced by streptozotocin induced diabetes whereas the pyruvate production was markedly elevated. In the corresponding working hearts, the glucose uptake, $^{14}CO_2$ production and lactate production were no longer significantly different to normal. C^{14} incorporation into glycogen however was still depressed despite the stimulation of cardiac work. The pyruvate output remained significantly elevated in streptozotocin diabetic hearts performing mechanical work.

TABLE 13

METABOLIC EFFECTS OF WORK IN THE PERFUSED HEARTS OF RATS RENDERED DIABETIC WITH STREPTOZOTOCIN

	Glucose uptake	Lactate production	Pyruvate production	Medium L/P	Glycogen
(a) Langendorff Hearts (Aortic Pressure 100 cm)					
Diabetic (12)	57.1 ± 4.1	16.0 ± 3.6	9.1 ± 1.2	2	87 ± 17
% change from normal control	- 48%	- 28% (n.s.)	+ 320%	- 80%	0
(b) Working Hearts (Atrial Pressure 20 cm)					
Diabetic (8)	246 ± 12	22 ± 5	5.2 ± 1.7	4	74 ± 9
% change from normal control	- 8.5% (n.s.)	- 18% (n.s.)	+ 175%	- 71%	+ 19% (n.s.)

All hearts preperfused for 15 mins at 100 cm aortic pressure then by recirculation for 30 mins under conditions specified.

Substrate:- glucose 11.1 mM. Initial glycogen at T0 (i.e. after preperfusion period) = 77 ± 8 µmoles glucose equivalent/g dry wt

(n.s.) indicates difference not statistically significant ($p > 0.05$)

Units:- µmoles glucose equivalent dry wt/30 min (mean ± s.e.m.)

TABLE 14

FATE OF GLUCOSE-U-C^{14} IN THE PERFUSED HEARTS OF DIABETIC RATS
EFFECTS OF CARDIAC WORK

I STREPTOZOTOCIN DIABETIC RATS

	$^{14}CO_2$	^{14}C-intermediates in tissue	^{14}C as glycogen	qO_2
(a) Langendorff Hearts (Aortic Pressure 100 cm)				
Diabetic (8)	23.7 ± 4.1	19.5 ± 2.7	2.1 ± 0.2	720 ± 33
% change from normal control	- 64%	(n.s.)	- 34%	(n.s.)
(b) Working Hearts (Atrial Pressure 20 cm)				
Diabetic (10)	199 ± 10	33.4 ± 2.5	2.4 ± 0.2	1725 ± 90
% change from normal control	(n.s.)	- 10% (n.s.)	- 67%	- 10% (n.s.)

All hearts preperfused for 15 mins and then by recirculation for 30 mins.
Substrate glucose 11.1 mM (n.s.) indicates differences not significant ($p > 0.05$)
Units:- μmoles glucose equivalent/g dry wt/30 min (mean ± s.e.m.)

Corresponding data for alloxan diabetic hearts is given in Table 15 (glucose uptake, lactate and pyruvate output, glycogen content) and Table 16 (fate of glucose-U-C^{14}). Again glucose uptake, lactate production, $^{14}CO_2$ from glucose-U-C^{14} and incorporation of C^{14} into glycogen were markedly reduced in Langendorff perfused alloxan diabetic hearts. In this case however, although the performance of cardiac work again provided a metabolic stimulus (as judged by the rise in qO_2, in glucose uptake and $^{14}CO_2$ production) alloxan diabetic working hearts still showed significantly impaired glucose uptake and oxidation compared to either normal or streptozotocin diabetic working hearts.

VII Effects of insulin (in vitro) on the pattern of glucose metabolism in hearts from diabetic rats.

Considerable differences were observed in experiments on the effects of insulin added (in vitro) to the perfusion medium, on perfused hearts from diabetic rats, (see Tables 17 and 18)

Whilst, as described above (Table 9) insulin in vitro completely restored the glucose uptake and oxidation to normal values (i.e. to give a full insulin effect) in Langendorff hearts from anti-insulin serum treated rats, in both streptozotocin induced and alloxan induced diabetic rats the perfused working heart did not show normal maximal rates of glucose oxidation on the addition of insulin. However the incorporation of C^{14}

TABLE 15

METABOLIC EFFECTS OF WORK IN THE PERFUSED HEARTS OF RATS RENDERED DIABETIC WITH ALLOXAN

	Glucose uptake	Lactate production	Pyruvate production	Medium L/P	Glycogen content
(a) Langendorff Hearts (Aortic Pressure 100 cm)					
Alloxan Diabetic (4)	68.8 ± 8.2	18.3 ± 5.8	13.8 ± 4.9	1.5	88 ± 8
% change from normal control	- 38%	- 20% (n.s.)	+ 585%	- 85%	(n.s.)
(b) Working Hearts (Atrial Pressure 20 cm)					
Alloxan Diabetic (5)	80 ± 8	59.4 ± 8.2	45.9 ± 5.7	1.4	49 ± 4
% change from normal control	- 70%	+ 115%	+ 2300%	- 90%	- 21%

Initial glycogen T0 (i.e. after preperfusion period) = 128 ± 10 µmoles glucose equiv/g.
All hearts preperfused for 15 mins and then by recirculation for 30 mins.
Substrate: glucose 11.1 mM.
Units: µmoles glucose equivalent/g dry wt/30 mins (mean ± s.e.m.).

TABLE 16

FATE OF GLUCOSE-U-C^{14} IN THE PERFUSED HEARTS OF DIABETIC RATS

EFFECTS OF CARDIAC WORK

II ALLOXAN DIABETIC RATS

	$^{14}CO_2$	^{14}C-intermediates in tissue	^{14}C as glycogen	qO_2
(a) Langendorff Hearts (Aortic Pressure 100 cm)				
Alloxan Diabetic	10 ± 4	22.0 ± 1.5	1.4 ± 0.4	714 ± 72
% change from normal control	- 85%	(n.s.)	- 56%	(n.s.)
(b) Working Hearts (Atrial Pressure 20 cm)				
Alloxan Diabetic (5)	48 ± 5	23.9 ± 1.6	1.4 ± 0.2	2227 ± 139
% change from normal control	- 76%	- 35%	- 81%	+ 17% (n.s.)

All hearts preperfused for 15 mins and then by recirculation for 30 mins.
Substrate:- glucose 11.1 mM.
(n.s.) indicates differences not significant ($p > 0.05$). All other differences significant.
Units:- µmoles glucose equivalent/g dry wt/30 mins (mean ± s.e.m.).
qO_2 as µl/g dry wt/min.

glucose into glycogen was not significantly below normal values after insulin addition to Langendorff streptozotocin diabetic hearts despite a marked depression (- 58%) of glucose oxidation to $^{14}CO_2$.

A striking feature of the results in working streptozotocin diabetic hearts was the lack of effect of insulin on glucose-U-C^{14} oxidation to $^{14}CO_2$ (cf. Table 14 and Table 18) despite an increase in glucose uptake and abnormally high lactate and pyruvate production (cf Table 13 and Table 17). A large proportion of the additional glucose uptake brought about by the presence of insulin (+ 159 µmoles/g) could be accounted for by the increase in lactate (+ 113 µmoles/g) and pyruvate (+16 µmoles/g). The lactate/pyruvate concentration ratio in the medium remained significantly below the control value. Again in working streptozotocin diabetic hearts, insulin restored the incorporation of ^{14}C from glucose into glycogen to within normal limits despite the depressed oxidation of ^{14}C-glucose. Alloxan diabetic working hearts perfused with glucose and insulin continued to show depressed glucose uptake, $^{14}CO_2$ production, and incorporation of ^{14}C from glucose to glycogen when compared to normal hearts with insulin, although all of these parameters showed some increases in the presence of insulin (cf. Table 16 and Table 18).

TABLE 17

METABOLIC EFFECTS OF INSULIN IN THE PERFUSED HEARTS OF RATS RENDERED DIABETIC

I WITH STREPTOZOTOCIN II WITH ALLOXAN

	Glucose uptake	Lactate production	Pyruvate production	Medium L/P	Glycogen
(a) Langendorff Hearts (Aortic Pressure 100 cm)					
Streptozotocin Diabetic + insulin (14)	201 ± 11	90 ± 10	18.8 ± 2	5	99 ± 13
% change from normal + insulin	-10% (n.s.)	+ 61%	+ 200%	- 70%	- 26%
(b) Working Hearts (Atrial Pressure 20 cm)					
Streptozotocin Diabetic + insulin (11)	405 ± 33	135 ± 17	21 ± 3	6	73 ± 8
% change from normal + insulin	- 25%	+ 69%	+ 180%	- 165%	- 32%
Alloxan Diabetic + insulin (8)	316 ± 25	108 ± 18	-	-	120 ± 19
% change from normal + insulin	- 41%	+35% (n.s.)			+ 11% (n.s.)

All hearts preperfused for 15 min at 100cm aortic pressure. Substrate: glucose 11.1 mM. Insulin used at 2mU/ml *in vitro*. Initial glycogen at T0 (i.e. after preperfusion period) = 77 ± 8 µmoles glucose equivalent/g dry wt for streptozotocin and 128 ± 10 µmoles glucose equivalent/g dry wt for alloxan. (n.s.) indicates differences not significant ($p > 0.05$)
Units: µmoles glucose equivalent/g dry wt/30 mins (mean ± s.e.m.)

TABLE 18

EFFECTS OF INSULIN ON FATE OF GLUCOSE-U-C^{14}
IN THE PERFUSED HEARTS OF RATS RENDERED DIABETIC
I WITH STREPTOZOTOCIN II WITH ALLOXAN

	$^{14}CO_2$	^{14}C tissue intermediates	^{14}C as glycogen	qO_2
(a) Langendorff Hearts (Aortic Pressure 100 cm)				
Streptozotocin Diabetic + insulin (8)	47.9 ± 4.8	33.7 ± 4.4	18.1 ± 1.4	721 ± 40
% change from control + insulin	- 58%	- 13% (n.s.)	- 14% (n.s.)	(n.s.)
(b) Working Hearts (Atrial Pressure 20 cm)				
Streptozotocin Diabetic + insulin (8)	187 ± 25	31.0 ± 1.0	19.0 ± 2.5	1692 ± 116
% change from control + insulin	- 44%	- 57%	- 27% (n.s.)	- 16% (n.s.)
Alloxan Diabetic + insulin (8)	194 ± 30	41.9 ± 2.2	12.1 ± 1.6	2252 ± 293
% change from control + insulin	- 42%	- 42%	- 54%	+ 11% (n.s.)

All hearts preperfused for 15 mins and then by recirculation for 30 mins.
Substrate glucose 11.1 mM. Insulin used at 2 mU/ml (in vitro).
Units:- μ moles glucose equivalent/g dry wt/30 min (mean ± s.e.m.). qO_2 as μl/g dry wt/mi
n.s. indicates differences with control (+ insulin) hearts not significant ($p > 0.05$)
Streptozotocin animals used 7 days after dosage (65 mg/kg i.v.)
Alloxan animals used 2 days after dosage (65 mg/kg i.v.)

VIII Contribution of glucose to respiration of the perfused rat heart.

As mentioned above in both Langendorff and working hearts from normal rats the percentage of total respiration which could be accounted for by exogenous glucose oxidation during the first 30 minutes was 30 - 40%. Further atrial perfusion raised the proportion to 50% beyond 30 minutes and to 80% after 45 minutes. The data from insulin deficient and diabetic hearts can be considered in the same way and a summary of the contributions of glucose to respiration is given in Table 19. Depending on the length of time of perfusion, the performance of work, the type of insulin deficiency or diabetes, and the presence or absence of added insulin (in vitro) it was possible to have as little as 6% of the respiration accounted for by glucose oxidation or as much as 80%.

TABLE 19

CONTRIBUTION OF GLUCOSE TO RESPIRATION OF THE PERFUSED RAT HEART

ANIMAL	Insulin	Type of perfusion	Time of perfusion	% respiration accounted for by $^{14}CO_2$ from glucose-U-C^{14} (11.1 mM)
Normal	-	Langendorff (65 cms)	0-30 mins	41
Normal	-	Langendorff (100 cms)	0-30 mins	37
Normal	-	Work	0-15 mins	30
			15-30 mins	50
			30-45 mins	80
Normal	+	Langendorff (100 cms)	0-30 mins	69
Normal	+	Work	0-30 mins	74
AIS treated	-	Langendorff (100 cms)	0-30 mins	19
AIS treated	-	Work	0-30 mins	50
AIS treated	+	Langendorff (100 cms)	0-30 mins	58
Streptozotocin diabetic	-	Langendorff (100 cms)	0-30 mins	14
"	-	Work	0-30 mins	48
Alloxan	-	Langendorff (100 cms)	0-30 mins	6
Alloxan	-	Work	0-30 mins	10
Alloxan	+	Work	0-30 mins	38
Streptozotocin	+	Langendorff (100 cms)	0-30 mins	30
	+	Work	0-30 mins	50

IX Effects of heart work and increased contractility on heart content of glycolytic intermediates, adenine nucleotides, phosphocreatine and citrate.

Because of the obvious complexity of the metabolic situation in the perfused rat working heart, with competition between endogenous and exogenous substrates and the different behaviour in different types of diabetes and insulin deficiency, analyses were carried out on the content of cardiac glycolytic intermediates, adenine nucleotides, phosphocreatine, and citrate in both working and non-working perfused hearts.

In the first series of experiments a simple non-recirculated system was used with aortic perfusion at 65 cm, or 100 cm H_2O pressure or atrial perfusion at 20 cm H_2O pressure, with a perfusion time of 15 minutes in each case. This series was perfused with glucose (5.5 mM) as substrate without addition of insulin. Analysis of the frozen extracted hearts was solely for G6P, F6P, FDP. The results are shown in Table 20. A significant and progressive decrease in the levels of the hexosemonophosphates was found with increasing contractility (65 to 100 cm H_2O aortic pressure) and the performance of cardiac work. At the same time the content of fructose 1,6-diphosphate rose so that the ratio of hexosemonophosphate/FDP fell from 2.5 to 1.8 with increased contractility and to 1.2 with performance of work.

TABLE 20

EFFECTS OF INCREASING PERFUSION PRESSURE AND CARDIAC WORK ON GLYCOLYTIC INTERMEDIATES

Perfusion conditions:-	Langendorff 65 cms (15) aortic pressure	Langendorff 100 cms (8) aortic pressure	Working 20 cm (10) Atrial pressure
Glucose-6-Phosphate	740 ± 59	638 ± 47	538 ± 30
Fructose-6-Phosphate	180 ± 13	148 ± 7	127 ± 8
Fructose-1, 6 Diphosphate	367 ± 40	444 ± 40	545 ± 45
Ratio:- $\frac{\text{Hexose monophosphate}}{\text{FDP}}$	2.5	1.8	1.2

All hearts perfused without recirculation for 15 min. Glucose 5.5 mM. No insulin added. Units: μmole/g dry wt. Number of hearts in each condition given in brackets.

A series of perfusions was then carried out using working hearts, with recirculation, for periods of 0, 15, 30 and 45 mins (subsequent to 15 mins preperfusion by aortic perfusion). In this series, the medium contained 11.1 mM glucose as substrate and the frozen extracted heart muscle was analysed for glycolytic intermediates, adenine nucleotides, citrate, and phosphocreatine. A similar series of hearts was perfused for 30 mins by the Langendorff method at 65 cm H_2O pressure for comparison of adenine nucleotide and phosphocreatine content. These results are given in Table 21 for Langendorff (65 cm) hearts and Table 22 (hexose phosphates in working hearts) Table 23 (adenine nucleotides in working hearts) and Table 24 (total adenine nucleotides and phosphocreatine in working hearts).

The changes which were apparent during the performance of external work were a fall in G6P and a rise in FDP, a fall in ATP and phosphocreatine and a rise in ADP and AMP. There was also a fall in the total content of adenine nucleotides. Similar changes were evident in the Langendorff experiments (Table 21) so that these metabolic events could not be considered as a necessary accompaniment of the performance of external work by the isolated perfused heart. All of these changes together with the heart content of glycogen and the lactate/pyruvate ratio in the perfusion medium (Table 25) became stabilised after 15 minutes of working perfusion.

TABLE 21

EFFECTS OF LANGENDORFF RECIRCULATION PERFUSION ON HIGH ENERGY PHOSPHATES IN RAT HEART

	Perfusion time: 0 min No. of hearts : (8)	30 min (4)
Adenosine triphosphate	20.9 ± 1.0	16.2 ± 1.2
Adenosine diphosphate	5.1 ± 0.3	4.6 ± 0.2
Adenosine monophosphate	0.8 ± 0.1	1.1 ± 0.1
TOTAL adenine nucleotides	26.4	21.9
ATP/AMP ratio	26	15
Phosphocreatine	28.6 ± 2.3	16.6 ± 1.4

Perfusion at 65 cm H_2O aortic pressure.

Values μmole/g dry wt (mean ± s.e.m.).

Substrate:- glucose 11.1 mM.

Zero time indicates 15 min preperfusion only at 65 cm H_2O aortic pressure.

TABLE 22

EFFECT OF WORKING TIME ON HEXOSE PHOSPHATE LEVELS IN RAT HEART

Perfusion time:- / No. of hearts :-	0 min (12)	15 min (4)	30 min (7)	45 min (8)
Glucose-6-phosphate	1148 ± 72	713 ± 128	712 ± 74	762 ± 47
Fructose-6-phosphate	248 ± 17	222 ± 20	192 ± 28	187 ± 13
Fructose 1,6-diphosphate	264 ± 24	296 ± 43	346 ± 66	345 ± 38
Hexose monophosphate/FDP	5.3	3.2	2.6	2.7

Values as n mole/g dry wt (mean ± s.e.m.)

Substrate:- glucose 11.1 mM.

Perfusion at 20 cm H_2O atrial pressure by recirculation for time indicated.

Zero time indicates 15 mins preperfusion at 100 cms H_2O aortic pressure.

TABLE 23

EFFECTS OF WORKING TIME ON ADENINE NUCLEOTIDE CONTENT OF PERFUSED RAT HEART

Time: / No. of hearts:	0 min (9)	15 min (8)	30 min (6)	45 min (4)
Adenosine triphosphate	21.4 ± 1.1	22.9 ± 1.5	17.1 ± 0.7	16.9 ± 0.9
Adenosine diphosphate	4.7 ± 0.2	5.7 ± 0.6	6.1 ± 0.8	6.6 ± 0.6
Adenosine monophosphate	1.0 ± 0.1	1.5 ± 0.2	2.1 ± 0.3	1.9 ± 0.3

Values in μmole/g dry wt (mean ± s.e.m.).
Glucose 11.1 mM. as substrate.
Perfusion at 20 cm H_2O atrial pressure by recirculation for time indicated.
All hearts preperfused for 15 mins at 100 cm aortic pressure.
Zero time indicates preperfusion only.

TABLE 24

EFFECTS OF WORKING TIME ON CONTENT OF HIGH-ENERGY PHOSPHATES IN PERFUSED RAT HEART

Perfusion time: No. of hearts:	0 min (9)	15 min (8)	30 min (6)	45 min (4)
ATP + ADP + AMP	27.1	30.1	25.3	25.4
ATP / AMP	21	15	8	9
Phosphocreatine	31.2 ± 2.3	23.8 ± 3.8	17.2 ± 0.8	18.3 ± 1.0

Values in μmole/g dry wt (mean ± s.e.m.)

Substrate:- glucose 11.1 mM.

Perfusion at 20 cm atrial pressure by recirculation for time indicated.

All hearts preperfused for 15 mins at 100 cm H_2O aortic pressure.

Zero time indicates preperfusion only.

TABLE 25

EFFECT OF WORKING TIME ON HEART GLYCOGEN CONTENT

AND MEDIUM LACTATE/PYRUVATE RATIOS

Perfusion time	0 min	15 min	30 min	45 min
Glycogen	96 ± 4 (9)	60 ± 5 (8)	63 ± 3 (6)	68 ± 5 (4)
Lactate/pyruvate (Concentration ratio in perfusion medium)	12 (4)	14 (3)	14 (4)	13 (4)

Glycogen as μmole glucose equivalent per g dry wt(mean±s.e.m.)

Substrate:- glucose 11.1 mM. No. of hearts in brackets.

Zero time indicates 15 mins preperfusion only at 100 cm H_2O aortic pressure.

TABLE 26

EFFECT OF PERFORMANCE OF WORK ON HIGH ENERGY PHOSPHATES IN PERFUSED RAT HEART

(with modified extraction procedure of Opie, Newsholme (1967))

	ATP	ADP	AMP	Phospho-creatine	Total Adenine Nucleo-tides	ATP/AMP
Time: 0 min. (4)	25.4 ± 0.1	4.5 ± 0.3	0.54 ± 0.01	34.3 ± 1.9	30.44	47
Time: 45 min. (4)	22.6 ± 0.9	5.5 ± 0.2	0.81 ± 0.06	23.7 ± 0.9	28.91	28

All hearts preperfused for 15 mins Langendorff 100 cms H_2O. (T O)

Work performed at 20 cm atrial pressure versus 100 cm aortic pressure.

Glucose 11.1 mM (without insulin) as substrate.

At end of perfusion period (T O or T45 mins) hearts clamped and percussed as described under "Experimental".

Frozen percussed powder then thrown into acetone-perchloric acid-5mM EDTA mixture (50:12:38) at -20°C. After pulverising in a mortar and centrifuging off the residue, the cold supernatant was neutralised with 30% KOH saturated with KCl, as previously described, and frozen to precipitate perchlorate. Assays then carried out immediately on neutral supernatant.

Units:- μ moles/g dry wt (mean ± s.e.m.)

It is possible that these results overemphasise the metabolic effect of work on heart content of adenine nucleotides since in a further series of experiments in which the extraction procedure for the heart tissue was modified (see Table 26) and the analysis of adenine nucleotides completed on the same day, less marked changes were observed following 45 mins of work. However the general pattern of metabolic change was very similar in the two series. The most striking difference in the two series was in the apparent AMP content and consequently on the ATP/AMP ratios.

The effect of insulin on the heart content of glycolytic intermediates

Similar studies on the levels of glycolytic intermediates were then carried out on perfused hearts from normal and diabetic rats in which the effects of insulin (2 mU/ml) added to the recirculation perfusion medium were determined. The results with normal hearts are given in Table 27 (with and without insulin) and those with hearts from streptozotocin and alloxan diabetic rats are given in Table 28 (without added insulin) and Table 29 (with insulin).

Insulin produced similar changes in both Langendorff (100 cm) and working hearts with marked elevation of the heart content of G6P and F6P, lactate and α-glycerophosphate.

TABLE 27

EFFECT OF INSULIN ON HEART CONTENT OF GLYCOLYTIC INTERMEDIATES

(a) In Langendorff Perfused Heart (Aortic Pressure 100 H_2O)

Insulin	G6P	F6P	FDP	PYR	LACT	L/P	αGP	DHAP	αGP/DHAP
+ (7)	2.19*	0.38*	0.55*	0.23	5.88*	25	1.39	0.15*	9
- (12)	0.62	0.11	0.32	0.21	4.30	21	0.78	0.06	13

(b) In Working Perfused Heart (Atrial Pressure 20 cm H_2O)

+ (9)	1.59*	0.34*	0.56	0.29	9.96*	35	2.64*	0.13	20
- (12)	0.70	0.18	0.42	0.21	6.02	29	1.28	0.13	10

All perfusions carried out for 30 mins with recirculation.
Glucose 11.1 mM as substrate.
Insulin, when present, used at 2 mU/ml.
Results as μ moles/g dry wt.
L/P denotes ratio of contents of lactate to pyruvate.
αGP/DHAP denotes ratio of contents of αglycerophosphate to dihydroxyacetone phosphate.
Number of hearts for each condition in brackets.
* denotes difference with control (-insulin) significant ($p \leqslant 0.05$)

TABLE 28

HEART CONTENT OF GLYCOLYTIC INTERMEDIATES IN DIABETIC RATS

(a) In Langendorff Perfused Heart (Aortic Pressure 100 cm H_2O)

		G6P	F6P	FDP	LACT	PYR	L/P	αGP	DHAP	αGP/DHAP
Control	(12)	0.62	0.11	0.32	4.30	0.21	21	0.78	0.06	13
Streptozotocin	(8)	0.34*	0.10	0.20*	3.16	0.33*	9.5	0.32	0.05	6
Alloxan	(4)	0.66	0.24*	0.38	6.01	0.73*	8	0.65	-	-

(b) In Working Perfused Heart (Atrial Pressure 20 cms)

Control	(12)	0.70	0.18	0.42	6.02	0.21	29	1.28	0.13	10
Streptozotocin	(8)	0.42*	0.14	0.23*	4.02	0.41*	10	0.49	0.09	5
Alloxan	(4)	0.56	0.20	0.35	5.78	1.09*	5	0.72	0.09	8

All perfusions carried out for 30 mins with recirculation.
Glucose 11.1 mM as substrate.
Results as μ moles/g dry wt.
Streptozotocin animals used 7 days after dosage.
Alloxan animals used 2 days after dosage.
Number of hearts for each condition in brackets.
* denotes difference with control significant ($p \leqslant 0.05$).

TABLE 29

EFFECT OF INSULIN ON HEART CONTENT OF GLYCOLYTIC INTERMEDIATES IN DIABETIC RATS

		G6P	F6P	FDP	LACT	PYR	L/P	αGP	DHAP	αGP/DHAP
(a) In Langendorff Perfused Heart (Aortic Pressure 100 cm H_2O)										
Control (+ insulin)	(7)	2.19	0.38	0.55	5.88	0.23	25	1.39	0.15	9
Streptozotocin (+ insulin)	(14)	1.29*	0.20*	0.29	10.47*	0.95*	11	0.76	0.08	10
(b) In Working Perfused Heart (Atrial Pressure 20 cm H_2O)										
Control (+ insulin)	(9)	1.59	0.34	0.56	9.96	0.29	35	2.64	0.13	20
Streptozotocin (+ insulin)	(9)	1.03*	0.24*	0.33*	9.73	0.67*	14	1.48	0.12	12
Alloxan (+ insulin)	(5)	1.91	0.55*	0.49	14.14	4.37*	3	1.00	0.20	5

All perfusions carried out for 30 mins with recirculation.

Glucose 11.1 mM as substrate. Results as μ moles/g dry wt.

L/P denotes ratio of lactate content to pyruvate and α6P/DHAP denotes ratio of α-glycerophosphate content to dihydroxyacetone phosphate.

Insulin used at 2mU/ml. Streptozotocin animals used 7 days after dosage.

Alloxan hearts used 2 days after dosage.

Number of hearts for each condition in brackets.

* denotes difference with control significant ($p \leqslant 0.05$).

Since the content of pyruvate and dihydroxyacetone phosphate was not greatly influenced by the presence of insulin, both cytoplasmic redox couples (lactate-pyruvate and α-glycerophosphate-dihydroxyacetone phosphate) gave increased ratios with insulin (Table 27) in the working heart.

In the hearts from streptozotocin-diabetic rats the content of G6P was markedly reduced whilst there was a significant increase in pyruvate content and a decrease in the tissue lactate/pyruvate ratios. These changes were similar in both Langendorff and working heart preparations (Table 28).

In alloxan diabetic hearts perfused without added insulin, the most marked difference from the controls was in the increased pyruvate content which gave rise to very low lactate/pyruvate ratios in both Langendorff and working hearts.

When insulin was added to the medium for perfusion of streptozotocin diabetic hearts it brought about the expected increase in G6P, F6P and FDP, but the increases were not sufficient to raise the heart content of these intermediates to those observed in control perfusions with added insulin (cf.Tables 27 and 29). Low lactate/pyruvate ratios were again observed in hearts both from alloxan and streptozotocin diabetic rats with markedly elevated tissue pyruvate levels in working hearts (Table 29). The tissue content of α-glycerophosphate was decreased in diabetic working hearts and remained low even in the presence of insulin so that the α-glycerophosphate/dihydroxyacetone ratio was also low with both alloxan and streptozotocin diabetic hearts.

TABLE 30

CITRATE CONTENT OF HEARTS FROM DIABETIC RATS PERFUSED WITH GLUCOSE AND INSULIN

		Langendorff	Working
	T0	T30	T30
Control (8)	1250 ± 140	1960 ± 121	2007 ± 164
Streptozotocin (10)	531 ± 75*	1582 ± 118*	1035 ± 148*
Alloxan (4)	1708 ± 149*	-	2244 ± 106

All hearts preperfused for 15 mins (T0)

Langendorff hearts preperfused at 100 cm H_2O. aortic pressure.

Working hearts preperfused at 20 cm H_2O atrial pressure.

Glucose 11.1 mM + insulin 2 mU/ml.

Units:- n moles/g dry wt (mean ± s.e.m.)

* indicates significant difference from control ($p \leq 0.05$)

Because of the suggested importance of citrate (Randle et al. 1968) as a metabolic regulator of glycolysis, the heart content of citrate was determined in normal and diabetic rats perfused in the presence of insulin. Whereas alloxan hearts showed an abnormally high citrate content at the end of the pre-perfusion period, streptozotocin hearts showed a marked decrease in heart citrate which did not reach control levels after 30 mins subsequent perfusion with glucose (11.1 mM) and insulin (2 mM/ml) in either Langendorff or working hearts (Table 30).

Effects of lack of substrate on cardiac metabolic intermediates.

In the experiments already described, where perfused hearts performed external work in the absence of exogenous substrate, there was a marked decline in mechanical performance so that after 15 - 20 minutes no external work was being performed. At this point, when the contractions of the heart were no longer sufficient to maintain the aortic pressure of 100 cm of H_2O, but before the coronary flow decreased (with consequent risk of anoxia) the hearts were clamped with the tongs cooled in liquid N_2. Analysis of the extracted tissue for glycolytic intermediates, citrate, adenine nucleotides and phosphocreatine was then carried out. The results are shown in Tables 31 (hexose phosphate and citrate) 32 (redox couples) and 33 (adenine nucleotides). There was a general fall in the level

TABLE 31

SUBSTRATE-LACK IN ISOLATED

WORKING RAT HEART

EFFECTS ON HEXOSE PHOSPHATES AND CITRATE

	CONTROL (4)	SUBSTRATE-LACK (8)
Glucose-6-phosphate	718	317
Fructose-6-phosphate	222	188
Fructose-1,6-diphosphate	307	264
Citrate	1240	384

Units = n mole per gram dry weight.

Control = hearts working for 15 min.

Number of hearts in brackets.

Substrate free hearts perfused with atrial perfusion (20 cm H_2O) until unable to perform further mechanical work (15-20 min).

TABLE 32

SUBSTRATE-LACK IN ISOLATED

WORKING RAT HEART

EFFECTS ON REDOX-COUPLES

	CONTROL (4)	NO SUBSTRATE (8)
LACTATE/PYRUVATE	31	12
αGP/DHAP	7	4

Ratios in heart tissue

Conditions as in Table 31

TABLE 33

SUBSTRATE-LACK IN ISOLATED WORKING RAT HEART

EFFECTS ON HIGH-ENERGY-P

	CONTROL (4)	SUBSTRATE-LACK (8)
Adenosine triphosphate	22.9	12.0
Adenosine diphosphate	5.7	6.7
Adenosine monophosphate	1.5	5.4
Phosphocreatine	23.8	5.5

Units = µmole per gram dry weight.

Control = hearts working for 15 min.

Number of hearts in brackets.

Conditions as given in Table 31.

of all the metabolic intermediates measured, except for ADP and AMP, the changes being particularly marked in the case of citrate and phosphocreatine where only 25% to 30% of the control values were detected. Both lactate/pyruvate and α-glycerophosphate/dihydroxyacetone phosphate ratios fell (Table 32).

Effect of anoxia on cardiac metabolic intermediates

Similar measurements of adenine nucleotides and phosphocreatine were made in tissue extracts prepared from hearts which had been preperfused for 15 mins aerobically by the Langendorff method (100 cm H_2O) and then switched to atrial perfusion under anaerobic conditions (95% N_2:5%CO_2). Perfusion was terminated by freeze-clamping with the Wollenberger tongs after periods of 5 secs, 60 secs or 240 secs. The data shown in Table 34 indicates that after only 5 secs the phosphocreatine content of the heart fell to less than half whilst the AMP content doubled. The fall in ATP level was less marked at 5 secs, but only half remained after 60 secs of anoxia.

These hearts never exhibited even in the first five secs the full mechanical activity of the aerobic controls. Whereas the aortic flow in control hearts perfused aerobically was 25 ml/min (5 secs after switching to atrial perfusion) the anoxic hearts gave a mean aortic flow of only 9.7 mls/min. This rapidly declined so that after 15 secs of anoxia the heart

TABLE 34

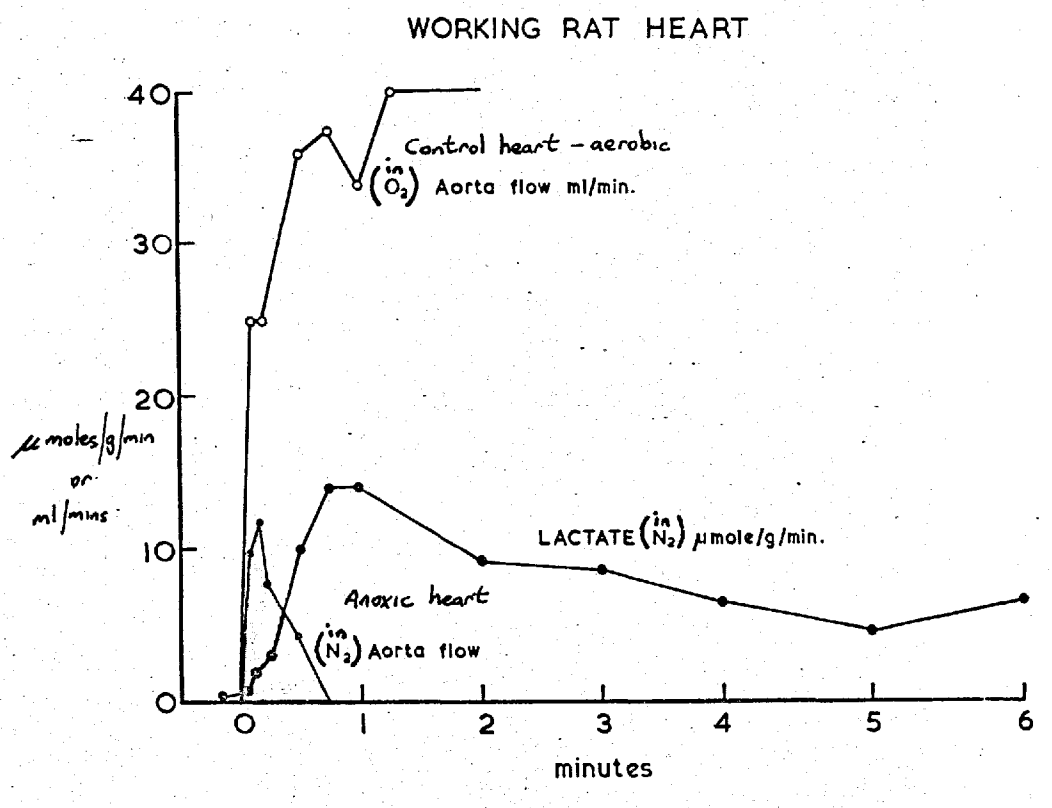
ANOXIC PERFUSION OF WORKING RAT HEART

TIME secs	0	5	60	240
	Aerobic	Anaerobic		
Adenosine triphosphate	3.76	2.95	1.61	1.75
Adenosine diphosphate	0.66	0.90	1.37	1.05
Adenosine monophosphate	0.08	0.17	0.61	0.39
Phosphocreatine	5.08	2.15	0.19	0.54
Aorta flow ml/min	0	9.7	0	0

All hearts preperfused aerobically (O_2/CO_2) for 15 mins at 100 cm aortic pressure, then switched to atrial perfusion (20 cm pressure) with N_2:CO_2 (95% 5%) for time indicated. Glucose 11.1 mM as substrate.

Values as µmole/g frozen wt. Mean of 4 hearts at each time.

Fig. 22 Effect of anoxia on performance of mechanical work and lactate production by perfused rat heart (20 cm atrial pressure).



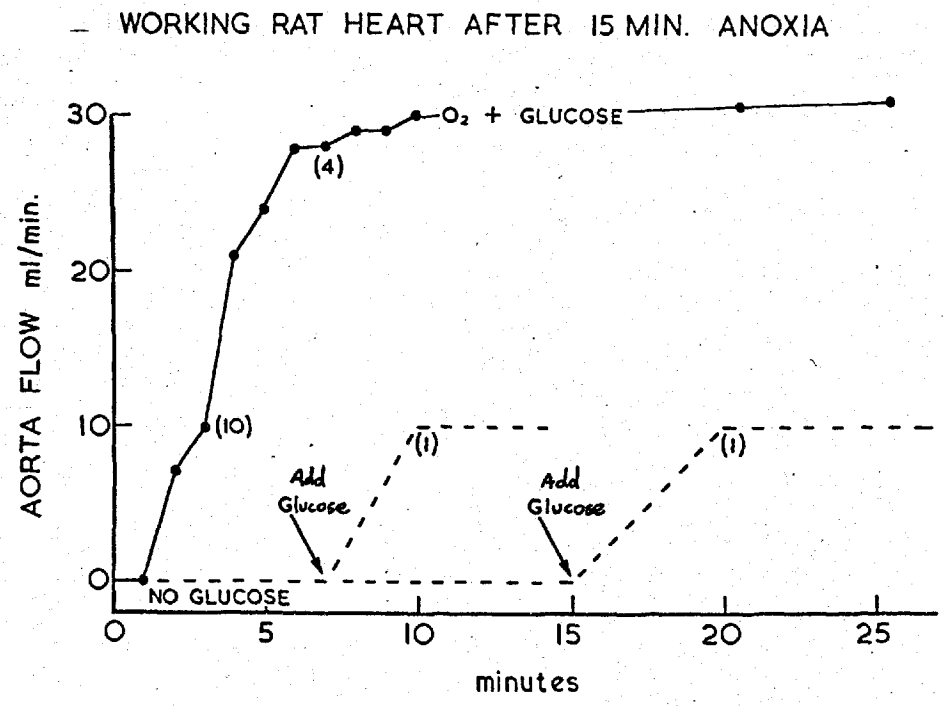
Heart preperfused 15 mins (Langendorff 100 cm.H_2O) with O_2/CO_2 then switched to N_2/CO_2 and atrial (20cm. H_2O) perfusion at zero time.
Glucose 11.1 mM as substrate.

was unable to perform any external work. Lactate production during anoxic perfusion rose steeply and then after 2-3 mins declined to a steady rate which was still over five times that of aerobic controls (Fig. 22).

Effects of re-oxygenation of anaerobic hearts on cardiac metabolic intermediates

In a further series of hearts, preperfusion was carried out for 15 mins by the Langendorff method under anaerobic conditions with the perfusion medium and heart chamber gassed with N_2:CO_2 (95%:5%) before switching to atrial perfusion (20 cm H_2O pressure) under the normal fully aerobic conditions with O_2:CO_2 (95%:5%). There was a lag period of approximately 1-2 min after switching to the aerobic condition before the heart was capable of sustaining an aortic pressure of 100 cm H_2O and performing external mechanical work. Full restoration of mechanical function was not achieved after 15 mins of re-oxygenation; the aortic flow and oxygen consumption both reached only 70% of the levels normally found at this time in working hearts not subjected to anoxia. The provision of glucose as exogenous substrate was found to be essential for even this partial recovery of mechanical function (see Fig. 23). Hearts switched to perfusion aerobically with substrate-free medium failed to recover the ability to perform any external work until glucose was added to the medium (Fig. 24).

Fig 23. Recovery of perfused rat heart from anoxia ; performance of mechanical work and dependence on glucose as substrate.



Hearts preperfused 15 mins (Langendorff 100 cm. H_2O) with glucose 11.1 mM under anoxic conditions (N_2:CO_2). Then switched to atrial perfusion (20 cm. H_2O) under aerobic conditions (O_2:CO_2) ± glucose (11.1mM)

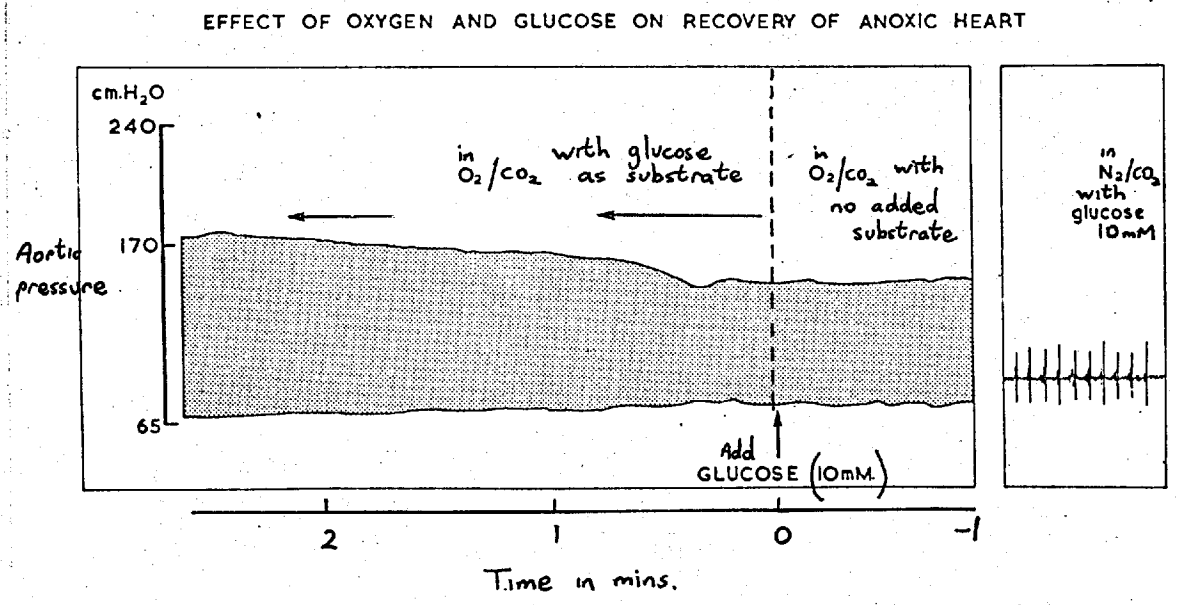


Fig. 24 Aortic pressure trace during recovery from anoxia and the effects of added glucose (10 mM).

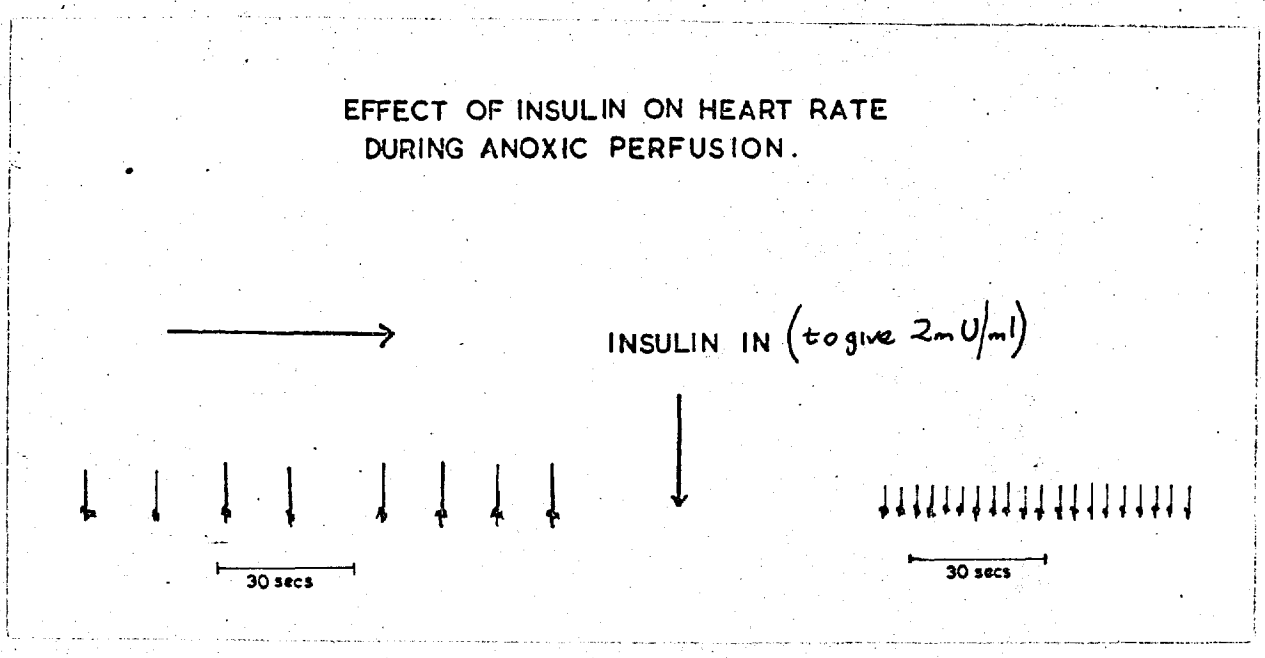
Conditions as given in Fig. 23.

Heart perfused anaerobically for 15 mins (Langendorff, 100 cm H_2O) to give aortic pressure trace shown at right. Then switched to atrial perfusion (20 cms. H_2O pressure) under aerobic conditions (O_2/CO_2) but without added substrate. Glucose (10mM) added at time 0. Aortic pressure (reading from right to left) then increases as shown.

Hearts preperfused anaerobically in the absence of glucose failed to work when switched to aerobic conditions even in the presence of glucose in the medium for the aerobic phase. It was noticeable that the rate of beating of anaerobically perfused hearts was significantly less in the absence of glucose and significantly increased by the presence of insulin in the anaerobic medium (see Fig. 25).

Analysis of extracts prepared from anoxic hearts reoxygenated for periods of 0, 1, 3, 5 minutes revealed a rapid rise at 1 min in the G6P, F6P and a fall in the FDP content. There was then a subsequent fall in G6P and F6P as the heart performed external work (see Table 35).

There was a progressive rise in the cardiac content of ATP with a corresponding fall in AMP and ADP but the total adenine nucleotide content of heart remained low, (Table 36). There was a rapid and marked rise in the phosphocreatine content (Table 37) and a fall in the lactate/pyruvate and α-glycerophosphate/dihydroxyacetone phosphate ratios (Table 38). The glycogen content of these hearts was very low and did not rise as the mechanical performance improved (Table 37).



Aortic pressure trace from heart perfused anaerobically (Langendorff 100 cm. H_2O) in presence of glucose 11.1 mM

Fig. 25

TABLE 35

REOXYGENATION
OF ANOXIC PERFUSED RAT HEART
HEART HEXOSE PHOSPHATE LEVELS

TIME (mins)	0	1	3	5
Number of hearts	5	4	4	4
Glucose-6-phosphate	104	371	174	200
Fructose-6-phosphate	30	80	39	29
Fructose-1,6-diphosphate	132	82	103	68
Aorta flow ml/min	0	0	16	24

Units = μmole per gram frozen wt.
15 minutes anoxic preperfusion (Langendorff 100 cm), then reoxygenation via atrial perfusion 20 cm.

Substrate:- glucose 11.1 mM.

TABLE 36

REOXYGENATION

OF ANOXIC PERFUSED RAT HEART

ADENINE NUCLEOTIDES IN HEART

TIME (mins)	0	1	3	5	Normal work 15 min
Adenosine triphosphate	0.99	1.46	1.59	1.70	3.07
Adenosine diphosphate	0.78	0.48	0.52	0.49	0.77
Adenosine monophosphate	0.37	0.23	0.32	0.12	0.20
TOTAL	2.14	2.17	2.43	2.31	4.04
Aorta flow ml/min	0	0	16	24	40

Units = μmole per gram frozen wt.

Conditions as given in Table 35.

TABLE 37

REOXYGENATION

OF ANOXIC PERFUSED RAT HEART

HEART CONTENT OF PHOSPHOCREATINE AND GLYCOGEN

TIME (mins)	0	1	3	5	Normal work 15 min
Phosphocreatine	0.36	2.05	1.80	2.86	2.73
Glycogen	3.1	0.8	1.0	1.0	8.0
Aorta flow ml/min	0	0	16	24	40

Phosphocreatine as µmole per gram frozen wt.

Glycogen as µmole glucose equivalent per gram frozen wt.

Conditions as given in Table 35.

TABLE 38

REOXYGENATION

OF ANOXIC PERFUSED RAT HEART

HEART REDOX RATIOS

Time (mins)	0	1	3	5
L/P	69	12	23	7
αGP/DHAP	31	33	24	15
Aorta flow ml/min	0	0	16	24

15 min anoxic preperfusion (Langendorff, 100 cm), then reoxygenation with atrial perfusion (20 cm H_2O)
Substrate:- glucose 11.1 mM.

DISCUSSION

1. Stability of the perfused heart preparations

Despite the advantages of the isolated perfused rat heart as listed earlier, various workers have experienced difficulty in obtaining a stable preparation. In the removal of the heart from the animal there is release of adrenaline, and during the period of transfer from the animal to the beginning of the perfusion there is inevitably, anoxia. The initial oxygen consumption of the Langendorff preparation is high, but then declines to a steady level. Fisher and Williamson (1961a) reported that this steady state was not reached until 15 minutes but that subsequently the oxygen consumption and coronary flow remained constant for 40 - 90 minutes. Zachariah (1961) was able to prolong the period of normal contraction and oxygen consumption to about 2 hours provided Ca^{++} and Mg^{++} concentrations in the Krebs-Henseleit medium were halved and bovine serum protein (or certain extracts thereof containing lipid material) was present with glucose or pyruvate. Willebrands and Van der Veen (1967) reported a progressive decline in the coronary flow of hearts perfused for 60 minutes with glucose, lactate, pyruvate and acetoacetate, although fatty acid (1 mM) gave a small increase in coronary flow.

In the experiments reported here, there was an initial fall in oxygen consumption and coronary flow during the Langendorff preperfusion period but this took place over the first 10 minutes. Thereafter both the oxygen consumption and coronary flow were reasonably stable in working and in Langendorff perfused hearts provided substrate and oxygen were supplied. Switching to atrial perfusion after 15 minutes of preperfusion at 100 cm aortic pressure, gave total cardiac outputs of over 60 ml/min. which could be maintained for at least 2 hours without change of medium. This level of cardiac output is similar to that reported for the rat in vivo (Bezanak, 1964). The concentrations of Ca^{++} and Mg^{++} used in the Krebs-Henseleit solution were not lowered nor was it found necessary to incorporate heparin and Ca-EDTA into the medium used during the preperfusion period as suggested by Morgan et al., (1965).

The most important factors in achieving a stable preparation were found to be filtration and the removal of air bubbles. The filtration of all solutions before use through 0.8 μ Millipore filters and the in-line Millipore filter in the perfusion fluid circuit prevented the introduction of any suspended solids into the coronary circulation. Bleehen and Fisher (1954) stressed the need for adequate filtration during perfusion and Zachariah (1961) suggested the use of fine porosity sintered glass filters.

2. Oxygen consumption under working and non-working conditions

A considerable range of cardiac respiration rates was obtained by using asystolic hearts, produced by a high K^+ medium, Langendorff hearts perfused at 65 cm. H_2O pressure and 100 cm. H_2O pressure, and the working heart preparation with atrial perfusion at 20 cm. H_2O pressure. This graded increase in left ventricular systolic pressure development (see Neely et al. 1967a) brought about a five-fold increase in oxygen uptake. The calculated oxygen consumption values for Langendorff hearts (100 cm. H_2O) pressure agree with the results of Neeley et. al, (1967a) but similar q_{O_2} values have been reported for hearts perfused at 54 cm. H_2O (Fisher and Williamson, 1961a)*. The q_{O_2} values for atrially perfused hearts are in reasonable agreement with those for working hearts at 20 cm. atrial pressure reported by Neely et al., (1967a) although there were several differences between their perfusion conditions and those reported here. In particular, under their conditions, hearts were working against an aortic pressure of 70 cm. H_2O whereas 100 cm. was used in the present study.

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* These values obtained with hearts submerged in medium to minimise any temperature gradient effects (Fisher, personal communication)

3. Relationship between aortic pressure and qO_2

Although the cardiac output might be supposed to be directly proportional to the respiration rate, Neely et al., (1967a) point out that cardiac output and work are in fact poor indications of the rate of energy consumption. Evans and Matsuoka (1914) recognised that the heart is more efficient in pumping fluid against a low resistance than if pumping against a high resistance. Neely et al., (1967a) provided an example of this whereby a twofold increase in cardiac output and external work, associated with a decrease in mean aortic pressure, was performed with little or no increase in oxygen utilization. On the other hand, elevated aortic pressure in association with a constant cardiac output, resulted in a doubling of oxygen consumption.

In the present experiments the differences between the oxygen consumption of Langendorff (100 cm.) and working perfused hearts cannot be correlated directly to the amount of work performed but may be related to the increased pressure development by the heart (Opie, 1965; Arnold et al., 1968). This is particularly evident in the experiments carried out in the absence of exogenous substrate where the amount of work performed fell before any change in the qO_2 could be detected. The fact that changes in oxygen consumption are associated with changes in aortic pressure has not been realised in many previous studies and aortic pressures have not been strictly controlled.

4. Effect of substrate on oxygen consumption in the perfused heart

(a) in Langendorff hearts. Fisher and Williamson (1961b) demonstrated that the oxygen consumption of the Langendorff perfused heart was essentially independent of the substrate when glucose, succinate, acetoacetate or β-hydroxybutyrate were used. More recently Challoner and Steinberg (1966) have suggested that the cardiac oxygen consumption was increased when the perfusate contained palmitate. Willebrands and Van der Veen (1967), in a series of Langendorff perfusions at 85 cm H_2O pressure, investigated the effect of glucose, lactate, pyruvate, acetoacetate (10 mM) or fatty acid (1 mM complexed to 1.5% defatted human albumin) and found that glucose as substrate gave a 25% reduction in oxygen consumption compared to other substrates. However under their experimental conditions, in the presence of glucose the performance of the heart invariably clearly declined during the course of the 60 minute perfusion and the coronary flow decreased considerably. Randle, Denton and England (1968) have reported a small increase in qO_2 when Langendorff hearts were perfused with acetate (5 mM) as compared to glucose (5.5 mM) in the presence of insulin.

(b) in working hearts. Neely et al., (1967b) found that oxygen consumption and cardiac work were generally unaltered in perfused working hearts over the range of atrial pressures (5 - 20 cm H_2O) with 5 mM glucose, acetoacetate or acetate as substrate. In the studies reported here, the initial oxygen consumption of the perfused working rat heart was the same in the presence of glucose and in the absence of any exogenous substrate. Whereas in the presence of glucose, however, this oxygen consumption remained steady for periods of several hours, in the absence of exogenous substrate the working heart showed a reduced oxygen consumption and a loss of mechanical performance after 15-20 minutes. The rate of oxygen consumption of working hearts was not affected by the use of pyruvate (5 mM) as substrate in place of glucose (11.1 mM).

5. Effects of anoxia and reoxygenation in working hearts - dependence of recovery on glucose

The rapid decline of the capability of the atrially perfused rat heart to perform external mechanical work when perfused anaerobically, was accompanied by pronounced losses of phosphocreatine and adenosine triphosphate. During anoxia, however, it was of considerable interest that glucose and insulin enhanced the rate of contraction and that the presence of glucose during anoxic perfusion was apparently essential for subsequent recovery on restoring the oxygen supply. Whether or not lactate is also capable of supporting anaerobic cardiac metabolism requires further experiments. Although it can be assumed that the substantial endogenous stores of triglyceride were substantially intact during anaerobic perfusion, this endogenous substrate could not apparently be mobilised during subsequent reoxygenation since exogenous substrate (glucose or pyruvate) was necessary for recovery of the heart. The effects reported here in which glucose and insulin enhanced the rate of heart beat under anoxic conditions and led to substantial mechanical recovery during subsequent reoxygenation are similar to those reported by Weissler, Kruger, Baba, Scarpelli, Leighton and Gallimore (1968) and Cascarano, Chick and Seidman (1968) using Langendorff hearts.

Weissler et al. (1968) found in electron microscope sections of the hearts exposed to anoxia in the absence of glucose, morphological changes in mitochondria and dilation of the longitudinal tubules. These morphological changes during anoxia were averted by inclusion of glucose in the perfusion fluid.

Austen, Greenberg and Piccinini (1965) reported enhanced ventricular function and increased contractile force in the dog heart when a period of cardiac ischemia, induced by aortic occlusion, was preceded by infusion of glucose through the coronary circulation.

The clinical interest in the use of infusions of potassium-glucose-insulin in patients with ischemic heart disease (Sodi-Pallares, Testelli, Fishleder, Bisteni, Medrano, Friendland and De-Micheli, 1962) has centered round the potassium content of the "polarising mixture". The possible beneficial effects of this treatment on the electrocardiogram and stabilisation of cardiac arhythmias may be due at least as much to the glucose and insulin and their support to anaerobic metabolism. The variable incidence of improvement reported following such treatment might well depend on the extent of irreversible cardiac damage prior to the infusion.

The metabolic changes which accompanied reoxygenation of the working heart showed considerable reversal of the effects of anoxia. Although there was a rapid six-fold increase in the

phosphocreatine content, five minutes after reoxygenation, the ATP content remained abnormally low and the total level of adenine nucleotides was also considerably depressed. This is in contrast to the recent preliminary findings of Scheuer and Stezoski (1967) who found that in reoxygenated Langendorff hearts (aortic perfusion pressure not stated) there was a complete return to normal levels of both phosphocreatine (after 20-30 secs.) and ATP (after only 10 secs.). This difference is not surprising because of the much heavier demand for high energy phosphate in hearts performing external mechanical work. Danforth, Naegle and Bing, (1960) found steady rises in myocardial ATP during reoxygenation only when the aerobic perfusion medium contained 15 mM KCl to prevent cardiac activity.

The response of the lactate/pyruvate ratio to reoxygenation was more rapid than that of the ratio of α-glycerophosphate/dihydroxyacetone phosphate indicating that LDH equilibrates much faster than α-glycerophosphate dehydrogenase with the cytoplasmic $NADH_2$ pool during transition states. Similar differences in the times of response of these two redox couples were found by Williamson (1966) when investigating metabolic changes during the onset of anoxia.

Since either lack of substrate or lack of oxygen caused the isolated working rat heart to fail it is of interest to compare the metabolic changes in the two cases. The mechanical

failure due to lack of substrate was slow; with anoxia it was very rapid indeed. The metabolic changes were similar in that a pronounced loss of phosphocreatine and adenine nucleotides was observed in both cases. However, whereas in anoxic conditions the tissue lactate/pyruvate ratio showed the expected rise, when the hearts failed for lack of substrate both lactate/pyruvate and α-glycerophosphate/dihydroxyacetone phosphate ratios fell. This may possibly reflect the very different rates of glycolysis in the two conditions.

6. Oxygen availability in the perfused working heart preparation

The question of oxygen availability in the perfused heart revolves around whether or not oxygen consumption is limited by oxygen transfer from the extracellular fluid to the cells. Although the effluent oxygen tension in working hearts was high, the possibility still arises that, at the high cardiac outputs achieved in the working heart preparation, there was insufficient flux of oxygen across the cell membrane to satisfy the oxygen demand by the high levels of oxidative metabolism. Fisher and Williamson (1961b) found that oxygen consumption remained constant in the Langendorff preparation when coronary flow was increased by perfusing with erythritol tetranitrate. In addition, they showed that the Langendorff preparation perfused in the presence of 10^{-5} M dinitrophenol could reduce the effluent oxygen tension to very low values without a change in coronary flow. In this way they concluded that the perfused heart was not limited in oxygen availability and that coronary flow *per se* was not determining oxygen consumption. In the case of the working heart preparation, Neely et al., (1967a) showed that working hearts extract more oxygen at the same coronary flow than the Langendorff preparation.

In the experiments in which adrenaline was added to the perfusion fluid, the results (Fig. 14) show that the working heart was still capable of even higher rates of coronary flow and oxygen extraction so that oxygen availability was unlikely to be limiting to oxidative metabolic processes.

7. Participation of glucose in oxidative heart metabolism under working and non-working conditions

The present experiments have shown that when glucose is present as the sole exogenous substrate, the oxidation of glucose accounts initially for approx. 40% of the oxygen consumption under all three conditions examined (65 cm and 100 cm Langendorff, 20 cm atrial working) i.e. over a range of a three-fold increase in oxygen uptake. Consequently both exogenous and endogenous substrate utilisation were stimulated equally, initially, by cardiac work. However, as expected, prolonged perfusion under working conditions led to depletion of endogenous substrate and the contribution of glucose rose until 80% (Table 5) of the respiration could be accounted for by glucose oxidation. In the initial period (up to 30 mins.) therefore, approximately half of the fuel for respiration continued to come from endogenous sources.

Fisher and Williamson (1961a) calculated that in the absence of exogenous substrate, the glycogen of the Langendorff (65 cm.) perfused rat heart would be used in 14 - 19 minutes and Shipp, Matos, Knizley and Crevasse (1964b) demonstrated that perfused hearts from fed rats were depleted of glycogen in 10 minutes. Several investigators (Williamson and Krebs, 1961; Shipp et al., 1964b) have demonstrated that amino acids normally contribute very little to the energy

metabolism of the heart. The fact that tissue lipids play a key role in sustaining the contractility of the perfused heart has now been well documented (Cruickshank and Kosterlitz, 1941; Olson and Hoeschen, 1967; Denton and Randle, 1965, 1967). Most of the reports indicate mobilisation of neutral lipid (i.e. mainly triglyceride) although Shipp, Thomas and Crevasse (1964c) suggested on the basis of studies with perfused rat hearts pre-labelled in vivo with (1 - C^{14}) palmitate, that phospholipids and triglyceride were the major sources of cardiac fuel for oxidation.

Because of the availability of heart triglyceride as respiratory fuel, Langendorff perfused hearts can retain their mechanical performance for some 60 minutes without provision of external substrate (Fisher and Williamson, 1961a; Zachariah, 1961). Since the working heart has a qO_2 over three times that of the Langendorff (65 cm. H_2O pressure) perfused heart it is to be expected that its mechanical performance rapidly declines after approximately 15 - 20 minutes, at which time, however, a substantial proportion (30% approx.) of its triglycerides are still intact (unpublished data, Cassar and Mansford). This may be because only part of the total heart triglyceride is available as fuel - the remaining "compartment" representing perhaps structural lipid essential to cellular organisation; alternatively this may indicate that the energy supply rate has fallen below the critical level for the performance of external work.

8. Metabolic effects of increased perfusion pressure and work.

(a) Effects on the pattern of glucose metabolism.

Although increased contractility and the performance of external work brought about dramatic rises in the formation of $^{14}CO_2$ from glucose-U-C^{14} the pattern of glucose metabolism obtained from the radioactive chromatographic scanning data was very similar. The increased incorporation of C^{14} into tissue intermediates from glucose in working hearts probably indicated more rapid turnover of glycolytic and citric acid cycle intermediates.

If one considers "transport" of glucose to be the process(es) by which extracellular glucose is made available for subsequent metabolism intracellularly, it is apparent that the stimulus of work produced a coordinated increase in both the transport and oxidation of glucose. Acceleration of uptake of sugars in working muscle has been studied earlier in whole animals and skeletal muscle. Ingle, Nezamis and Rice (1950) found that stimulation of the leg muscles of diabetic or normal rats caused a rapid fall in blood glucose levels. Goldstein, Mullick, Huddlestun and Levine (1953) suggested a specific effect of exercise on transport by showing a lowering of the concentrations of non-metabolised sugars, such as D-xylose and L-arabinose, in the blood of eviscerated rats undergoing exercise. More recently Holloszy and Narahara (1965) have shown that uptake of 3-O-methyl glucose by frog muscle was accelerated as the

frequency of stimulation was raised. At the highest rate of stimulation, uptake rose to a rate equal to that found with insulin treatment, (Narahara and Ozand, 1963).

(b) Effects on glycogen turnover.

When hearts performed mechanical work there was both glycogen breakdown and increased synthesis as measured by incorporation of radioactivity from C^{14} glucose into glycogen so that glycogen turnover was accelerated. The nett effect however was for glycogen breakdown to exceed synthesis. The major part of the glycogen broken down was apparently oxidised (as judged by the relative values of C^{14} and "cold" lactate production, Tables 2. and 3). Based on the proportion of the respiration accounted for by glucose oxidation, this utilisation of glycogen provided a maximum of 7% of the oxidative metabolism of perfused working hearts. Neely, Bowman and Morgan (1968) have shown that when both glucose and palmitate are present in the perfusion medium, the glycogen level can rise during heart work and in the present studies insulin prevented glycogen loss, so that glycogen breakdown does not invariably accompany the performance of heart work.

The explanations for the increased turnover of glycogen are to some extent conflicting. Glucose-6-phosphate has two effects on enzyme reactions involving glycogen; on the one hand a decreased heart content of G6P could be one factor

in increasing tissue levels of phosphorylase a (Neely et al., 1968) whilst on the other hand a drop in G6P content might be expected to decrease the activity of glycogen synthetase (Leloir et al., 1959). Neither of these effects could explain the increase in glycogen synthesis which is probably due to the reciprocal relationship between the percentage of glycogen synthetase I activity and the cardiac glycogen level (Larner et al., 1968 and Danforth, 1965). The situation is complicated by the considerable evidence in diaphragm muscle that the conversion of glucose to glycogen need not proceed via glucose-6-phosphate (Beloff-Chain et al., 1955, 1964; Chain 1962, Threlfall 1966, and Pocchiari 1968) and a possible explanation for these experimental findings has been proposed by Smith, Taylor and Whelan (1967) in which glucose 1,6-diphosphate, the coenzyme of phosphoglucomutase, is implicated.

(c) Effect on lactate production

There was no significant effect of increased perfusion pressure or work on either total lactate production or C^{14}-lactate formation from C^{14}-glucose. It is of interest, however, to consider the data on the formation of C^{14}-lactate from the point of view of possible tissue hypoxia in the working heart preparation.

The percentage of the glucose uptake appearing as lactate can be derived from the C^{14}-lactate formation using the chromatographic scanning data.

In perfusion conditions in which glycogenolysis did not occur, the output of labelled and unlabelled lactate was practically the same (see Tables 2 and 3). Total production of C^{14} lactate was 25.2 and 19.3 µmoles of glucose equivalent/g dry wt/30 minutes for Langendorff and working heart perfusion conditions respectively. These figures accounted for 23% and 7% of the glucose uptake whilst oxidation in turn accounted for 59% and 73% of the glucose uptake. Thus, the performance of external work increased glucose uptake and the percentage of the glucose taken up which was oxidised but decreased the proportion of this glucose which formed lactate. This is unlikely to be the case if substantial tissue hypoxia existed in the working heart preparation.

Similarly, one could argue from the basis of the lack of significant change in the medium lactate-pyruvate ratios (Table 25). However, the present work confirms the findings of Glaviano (1965) and Garland, Newsholme and Randle (1964) that there is a gradient of lactate from the intra- to extracellular space so that it would appear that the heart cell membrane is not freely permeable to lactate ions. Consequently, the use of medium lactate/pyruvate ratios by Williamson (1965), in calculating extramitochondrial NAD-NADH potential in the

perfused rat heart is open to criticism. By the same token a lack of change in medium L/P ratio during the performance of cardiac work cannot be considered any reliable index of absence of tissue hypoxia.

There are conflicting reports concerning the value of "normal" lactate/pyruvate (L/P) ratios in heart tissue extracts. Kraupp, Adler-Kastner, Niessner and Plank (1967) report in vivo tissue ratios of 14. Thorn, Gercken and Hürter (1968) report tissue L/P ratios in vivo of 110. In perfused Langendorff heart preparations, values of 26 (Williamson, 1966), 24.5 in the presence of insulin, (Garland, Newsholme and Randle, 1964) and 35, also in the presence of insulin, (Williamson, 1965) have been reported. The fact that in the atrially perfused working heart the values of both lactate/pyruvate (12 to 14) and α-glycerophosphate/dihydroxyacetone phosphate ratios (7 to 8) in tissue extracts remained essentially unaltered after 45 minutes of work could be interpreted as evidence against substantial tissue anoxia in the working heart preparation since both of these ratios are markedly increased under anoxic conditions (see Table 38 and Garland et al., 1964; Williamson, 1966).

The use of lactate/pyruvate ratios as the sole index of anaerobic metabolism by the myocardium is, however, open to considerable criticism. Huckabee (1961) has proposed that

production of lactate in "excess" of the amount calculated from the equilibrium constant and the steady state concentrations of lactate and pyruvate, is indicative of anaerobic metabolism. However, as Opie (1968) has pointed out, the use of lactate/pyruvate ratios in this way ignores the effect of various factors which are known to alter the ratio. These include oxidation of FFA by the heart (Garland et al., 1964), the presence of insulin (Williamson, 1965), the nutritional state (Opie et al., 1963), and alloxan-diabetes (Garland et al., 1964).

Furthermore, as stressed by Olson (1963) the lactate/pyruvate ratio reflects the cytoplasmic but not the mitochondrial redox potential. Although there is evidence of direct oxidation of NADH by heart mitochondria in both pigeon (Griffith and Blanchaer, 1967; Rasmussen, 1968), rabbit (Deshpande, Hickman and von Korff, 1961), and guinea pig (Blanchaer, 1964), this does not imply that the NAD:NADH ratio in the cytoplasm is in direct equilibrium with that in the mitochondria (Williamson, 1965). Consequently, an increase or decrease in total lactate/pyruvate ratio can only be interpreted as a change in the rate of glycolysis relative to the transfer of cytoplasmic hydrogen into the mitochondria. As shown by Krebs (1967) a comparison of redox couples in normal fed and alloxan diabetic rat livers reveals, in contradistinction to heart,

a rise in the lactate/pyruvate ratio, but a fall in both the glutamate/[α-oxoglutarate].[NH_4^+] ratio and β-hydroxybutyrate/acetoacetate ratios. The three systems studied, lactate dehydrogenase, glutamate dehydrogenase and β-hydroxybutyrate dehydrogenase, are located in the cytoplasm, mitochondrial matrix and mitochondrial cristae respectively. Consequently, the cytoplasm of diabetic tissues can be in a more reduced (liver) or less reduced (heart) state than that of normal tissues and the mitochondria can show changes simultaneously in the reverse direction (Williamson, Lund and Krebs, 1967). To interpret these changes as indicative of the degree of anaerobic metabolism is obviously a misleading simplification.

(d) Effects on glycolytic intermediates, adenine nucleotides and phosphocreatine.

It is of interest to compare the levels of glycolytic intermediates, adenine nucleotides, and phosphocreatine, found in the present experiments in the isolated perfused rat heart performing external work with those reported recently by Thorn, et al., (1968) who perfused rabbit hearts in situ with an artificial blood to which various substrates were added individually. As controls for these experiments (in which the ability of various substrates to maintain both mechanical function and metabolite content was compared), Thorn et al., provide values for glycolytic intermediates, adenine nucleotides and phosphocreatine of the rabbit heart in vivo. After 2 hours of perfusion with 5 mM glucose, they report that the heart maintained normal output of 134 ml/min. despite a fall of 25% of the initial value of ATP, 50% of the initial glycogen and 50% of the initial phosphocreatine content. In the experiments reported here, mechanical function and rate of respiration were unimpaired in isolated hearts performing external mechanical work for 45 minutes at which time the heart content of ATP had fallen by 15 - 30%, phosphocreatine by 30 - 40%, G6P by nearly 50%, and glycogen by 60%. It would appear, therefore, that these

metabolic changes cannot be explained solely by poor tissue oxygen availability resulting from the use of an artificial perfusion medium of oxygen content about ten times lower than normal arterial blood, since similar changes were obtained by Thorn et al., using a medium containing washed erythrocytes. Furthermore, the changes observed in cardiac glycogen and intermediates tended to stabilise after 15 minutes of work suggesting a new steady state resulting from the metabolic stimulus of cardiac work.

The data on heart content of glycolytic intermediates show that the increased flow through glycolysis observed in working hearts with increased contractility is accompanied by decreases in the heart content of glucose-6-phosphate and fructose-6-phosphate and a rise in the fructose -1-6-diphosphate content.

In view of the fact that the enzymes fructose-1-6, diphosphatase and glucose-6-phosphatase are virtually absent from heart (Newsholme and Randle, 1962), and that direct oxidation of glucose-6-phosphate via the pentose shunt is thought to be normally minimal in heart tissue, these changes in hexose phosphates are consistent with a stimulation of the effective phosphofructokinase activity.

The interpretation of this type of data, however, requires various assumptions if it is to be related to the

activity of regulatory enzymes such as phosphofructokinase in the intact tissue. Firstly, it is assumed that all the major metabolic pathways for the disposal of intracellular glucose are known. The demonstration of the formation of 1,2-bisphosphoenolpyruvate from glucose in the rat diaphragm by van Heijningen (1966) is an example of an intermediate without any suggested function in the published metabolic schemes. Secondly, it is assumed that estimates of total tissue levels of substrates and intermediates are at least proportional to the concentrations to which the enzymes are exposed in the various compartments of the cell. As Regen et al., (1964) have pointed out in discussing estimates of cardiac hexokinase activity (where the Km for glucose phosphorylation in the intact tissues is about two orders of magnitude higher than in muscle extracts), possible regulation by as yet unidentified factors cannot be ignored. Because glucose-6-phosphate is a potent inhibitor of hexokinase in a cell-free system (Newsholme and Randle, 1961; Crane and Sols, 1955), it has frequently been proposed that a similar type of inhibition may control the rate of glucose phosphorylation in the intact cell. Williamson (1965), however, showed that in Langendorff hearts perfused with insulin and glucose the mean intracellular concentration of glucose-6-P rose to about 1 mM, a level theoretically sufficient to inhibit heart muscle hexokinase by 90% whereas in fact, no inhibition of glucose

phosphorylation occurred. Similarly, an increased rate of hexokinase activity associated with substantial increase of glucose-6-phosphate has been observed in frog skeletal muscle during anoxia (Ozand, Narahara and Cori, 1962). Furthermore, Beloff-Chain et al. (1964), showed in diaphragm that G6P-U-C^{14} could participate directly in oxidative metabolism without affecting the pattern of glucose metabolism. In the present study, working hearts perfused in the presence of insulin showed accumulation of G6P but glucose phosphorylation was not affected. These findings and similar observations during stimulation of glycolysis by glucagon (Kreisberg and Williamson, 1964) or adrenaline (Williamson, 1964) may possibly be explained by the concomitant rise in the intracellular concentration of inorganic phosphate which has been shown to relieve G-6-P inhibition of hexokinase in a heart mitochondrial fraction (Mayer, Mayfield and Hass, 1961). The situation is further complicated by the possibility of compartmentation of e.g. glucose-6-phosphate, for which evidence has been given by Shaw and Stadie (1959), Sims and Landau (1966), Landau and Sims (1967), Figueroa, Pfeiffer and Niemeyer (1962), Threlfall (1966). Much of this evidence could equally well be explained by alternative pathways to glycogen (Beloff-Chain et al., 1955, 1964) which do not directly involve glucose-6-phosphate.

9. Rates of lipolysis as determined by glycerol output

Garland and Randle (1964b) have used the glycerol output by Langendorff perfused hearts as an index of the rate of lipolysis, as suggested by Vaughan (1961). Using a perfusion period of 15 minutes they found apparent glycerol outputs of 1.8 - 4.6 μM/g wet wt/hr, i.e. 0.45 - 1.15 μM/g during the 15 minutes.

The results of glycerol assays on perfusion medium after only two minutes of recirculation indicated glycerol outputs of 0.4 - 1.12 μM/g which were not increased by further recirculation perfusion. In view of these results and the demonstration by Robinson and Newsholme (1967) of glycerol kinase activity in heart tissue, the accuracy of estimations of rates of lipolysis based on glycerol release is in doubt. Furthermore, Kreisberg (1966) has shown that C^{14} glycerol can be directly oxidised to $^{14}CO_2$ in perfused heart. As Robinson and Newsholme (1967) point out, the increase in glycerol output observed in perfused heart from starved rats could equally well be explained by inhibition of glycerol-kinase activity as by an increase in a lipase.

10. Effects of insulin on the pattern of glucose metabolism in perfused heart

An examination of the effects of cardiac work and added insulin on the pattern of glucose metabolism in the perfused rat heart reveals several similarities. Work and insulin both increase glucose uptake, glucose oxidation and the incorporation of glucose carbon into glycogen. However, cardiac work and insulin can be distinguished by their effects on the total oxygen uptake and on lactate production. Insulin has no significant effect on the oxygen consumption whereas cardiac work brings about a doubling in qO_2; work has no significant effect on total lactate production whereas insulin brings about a 3-fold increase in lactate output with a concomitant sparing of cardiac glycogen. Insulin also increases the heart content of hexose phosphates and of lactate. These differences are consonant with a major effect of insulin on the processes of glucose uptake with glycolysis being stimulated to a greater extent than the rate of disposal of pyruvate into the citric acid cycle. A work load on the other hand appears to give rise to a primary stimulation of the oxidative processes.

The results obtained with insulin in heart muscle differ strikingly from those obtained in diaphragm (Beloff-Chain et al. 1955) where insulin was found to stimulate

specifically the synthesis of glycogen and oligosaccharides whilst the production of $^{14}CO_2$ and lactate were not affected. On the basis of observations in diaphragm and adipose tissue, Chain (1962) proposed that insulin action involved the elevation of the energetic potential of the cell. One possible manifestation of this in e.g. fat synthesis, might be an increase in the turnover of NADP/NADPH. In cardiac muscle, however, the pentose shunt, although present (Glock and McClean, 1954; Crevasse et al., 1964) is normally quiescent (Williamson and Kreisberg, 1965; Shipp et al., 1964a). Williamson and Kreisberg (1965) have shown that the content of NADP in rat heart is normally only 5% that of NAD and the proportion is unchanged in the presence of insulin. Any effects of insulin on the pentose shunt in specific tissues are probably secondary in any case to the primary action by which glucose is made available for oxidative metabolism.

Fisher (1962) has produced convincing evidence of an effect of insulin on the penetration of non-metabolised pentoses (L-arabinose, D-xylose) into heart muscle and has shown that the findings are consistent with the hypothesis that pentose enters the cells in combination with a specific carrier (Widdas, 1952; 1954) in the cell membrane. The accelerating effect of insulin on pentose penetration has been shown to be explicable in terms of a reduction in the

affinity of this carrier for the pentose (Fisher and Zachariah, 1961). Park et al., (1956, 1959) have produced evidence in support of a similar membrane carrier system for glucose transport, indicating saturation kinetics, stereospecificity and competition between pairs of sugars. In addition, the observation which favours most strongly the presence of a carrier in the membrane is the phenomenon of 'uphill' transport by counterflow in which non-metabolised 3-O methyl glucose has been used (Morgan, Regen and Park, 1964). Morgan et al., (1965) postulate the existence of the carrier in an "active" and "inactive" form. The increase in Vmax which they find following insulin or anoxic stimulation of transport could, they think, be due to conversion of inactive to active carrier.

This "activation" could result from a modification of the primary structure of the carrier molecule itself, resulting in a catalytically active form (see also Randle and Smith, 1960) or insulin could function as an allosteric activator of the carrier (Morgan et al., 1965). Such concepts overcome the difficulty of the original Levine and Goldstein (1955) hypothesis of increased "permeability" as the primary action of insulin, which failed to explain why insulin action cannot be reproduced by increasing the extracellular concentration of glucose (Table 8). The carrier system exhibits saturation kinetics and only an

alteration in the carrier-substrate affinity could raise the transport rate sufficiently to produce intracellular accumulation of glucose which does not occur even in non-working cardiac muscle in the absence of insulin, even with the external glucose concentration raised to 44 mM (Morgan et al., 1961b).

A further hypothesis has recently been proposed by Katzen (1967) on the basis of experimental evidence of the effect of insulin deficiency on the proportion of hexokinase isoenzymes (Katzen and Schimke, 1965; Katzen, Soderman and Nitowsky, 1965; McLean, Brown, Greenslade and Brew, 1966; Grossbard and Schimke, 1966). Tissues known to be insulin-sensitive have a greater proportion of hexokinase II (Km, 2×10^{-4}M) than hexokinase I (Km, 2×10^{-5}M) and diabetes has been shown to reduce the amount of hexokinases, especially hexokinase II, in heart muscle (Katzen, 1966), adipose tissue (McLean et al., 1966), and mammary gland (Walters and McLean, 1967; McLean, Brown, Walters and Greenslade, 1967). Katzen proposes the existence of a "bucket-brigade" of hexokinases of increasing affinity with the lowest affinity isoenzyme being external to the cell plasma membrane and the highest affinity hexokinase III (Km 5×10^{-6}M) being internally located. Glucose transport is thus seen as a handing on of glucose from one hexokinase to the other with

the possibility of metabolic conversion from carrier-glucose pools at each stage. In this proposed model, insulin acts as an alternative to hexokinase I in the sequence thereby accelerating the process of glucose transport in those tissues with low amounts of hexokinase I.

All of these hypotheses can be considered to have certain common ground. The essential feature of a carrier is that it should be capable of combining reversibly with its substrates. Any enzyme (or multi-enzyme system as suggested by Chain, 1968) should be able to act as a carrier of its substrate (e.g. glucose) or of its competitive inhibitors (e.g. 3-O-methyl glucose, pentoses). Such an enzyme-complex might transport its substrate without appreciable transformation (e.g. phosphorylation) especially if the location in the cell membrane was not favourable for the access of the co-factors required for enzymic action (e.g. ATP, Mg^{++}).

It is of considerable interest that the "permease" system of sugar transport in bacteria, previously considered to be based on selective permeability, has recently been shown in mutant studies to be a multi-enzyme system (Kundig, Kundig, Anderson and Roseman, 1966) involving a phosphotransferase reaction with phosphoenolpyruvate.

11. Metabolic effects of acute insulin deficiency produced by anti-insulin treatment

The isolated heart as obtained from the normal rat contains endogenous insulin. This was suggested by Zachariah (1961) who observed an initial fall in heart cell permeability to L-arabinose over the first 30 minutes of Langendorff perfusion. The basal level of permeability achieved at 30 minutes perfusion could be reproduced by pretreatment of the rat with anti-insulin serum. This observation together with the results reported here which indicate that glucose metabolism by the isolated Langendorff heart is greatly reduced by removal of this endogenous insulin by anti-insulin serum, probably account for the variable rates of glucose uptake and size of insulin effect reported in the literature (e.g. Morgan et al., 1961b; Williamson, 1962; Opie et al., 1962).

Several authors have studied the metabolic behaviour of tissues taken from animals pretreated with anti-insulin serum (Wright, 1961). Gregor, Martin, Williamson, Lacy and Kipnis (1963) have shown in rats treated with anti-insulin serum, that isolated rat diaphragm removed ninety minutes after the injection showed a significantly reduced rate of phosphorylation of 2-deoxyglucose to 2-deoxyglucose-6-phosphate. These authors found that reduction of the oxidation of glucose-1-C^{14} by isolated adipose tissue required two injections of anti-insulin serum. In liver slices from rats taken one to two hours after anti-insulin serum administration, Mahler and Ashmore (1963) showed that fatty acid synthesis from glucose was markedly

decreased whereas acetoacetate production was increased. Kalkhoff and Kipnis (1966) have shown that hepatic lipogenesis is rapidly impaired following acute insulin deprivation and have produced evidence to show that the block in lipogenesis occurs at the acetyl-CoA carboxylase step. Kalkhoff, Hornbrook, Burch and Kipnis (1966) have also shown that acute insulin deprivation of three hour duration resulted in an impairment of glycolysis at the phosphofructokinase step, activation of glycogenolysis and a decreased activity of the hexose-monophosphate shunt pathway in liver. Tissue levels of citrate and malate were not elevated under these conditions. Williamson, Wright, Malaisse and Ashmore (1966) reported an increase in plasma FFA and ketones two hours after anti-insulin treatment and an associated diminished level of CoA and elevated acetyl-CoA in liver. Stimulation of hepatic gluconeogenesis as observed by Williamson et al. (1966), and by Wagle and Ashmore (1963), following administration of anti-insulin serum, was thought to be secondary to enhanced lipolysis. Tarrant, Thompson and Wright (1962) also showed increased serum FFA one hour after a massive dose (5 ml) of anti-insulin serum given intraperitoneally. All of these metabolic changes in experimental insulin deficiency may be induced by hormones with actions normally

counterbalanced by insulin (Wright, 1965). Such hormones are glucagon, adrenaline, ACTH and corticosteroids (Mahler, Stafford, Tarrant, and Ashmore, 1964; Jungas and Ball, 1963). The fact that the effects of anti-insulin serum in producing insulin deficient hearts could be completely reversed by the in vitro addition of insulin in the perfusion fluid (see Table 9) does not necessarily argue against this view. Both endogenous glucagon and adrenalin are likely to have only a short lived effect before inactivation and any such effects might not necessarily be apparent following on the 15 minute preperfusion stabilising period. In addition, the hearts were removed only 1 hour after treatment of the donor rat with anti-insulin serum so that although the hyperglycaemia had reached its maximum (Fig. 15) the possibility of such secondary metabolic changes from opposing hormones was kept to a minimum.

There has been conflicting evidence of the ability of anti-insulin serum to neutralise insulin bound to tissue. Wright (1959) reported that the in vitro addition of anti-insulin serum to the isolated rat diaphragm did not reduce the basal glucose uptake. Wardlaw and Weidinger (1965), however, found that anti-insulin serum did cause a diminution of glycogen deposition during incubation of mouse hemi-diaphragms that had previously been exposed to insulin.

Wohltmann and Narahara (1967) found anti-insulin serum incapable of neutralising the effect of insulin on the permeability of frog sartorius muscle to 3-O-methyl glucose after the hormone had become bound to the muscle. Beloff-Chain, Catanzaro and Chain (1967), found a consistent *in vitro* effect of anti-insulin serum on the oxidation of glucose-1-C^{14} by epididymal fat pads.

In the present experiments with perfused heart, the use of anti-serum *in vitro* (i.e. in the preperfusion fluid) led to technical difficulties because of excessive foaming during the oxygenation but the results reported here on the use of anti-insulin serum *in vivo*, together with the earlier studies of Zachariah (1961), suggest that the endogenous insulin of the heart muscle had in fact been neutralised.

The results from the working perfused hearts of such rats pretreated *in vivo* with anti-insulin serum differed markedly from the corresponding Langendorff perfused hearts. The stimulation to glucose uptake and oxidation imparted by the performance of cardiac work was sufficient to overcome the restriction on these processes imposed by the lack of insulin which however was still essential for maximal rates of incorporation of C^{14} into glycogen and lactate.
In these circumstances therefore there was a specific effect of endogenous insulin on the metabolic fate of glucose

entering the cell and this effect was apparently independent of the effects of insulin on glucose uptake and oxidation already described.

In heart muscle virtually the complete range of transport rates are apparently controlled by metabolic and hormonal regulatory mechanisms. Morgan et al., (1965) have related glucose uptake as a function of tension development (measured by integrating the area under aortic or ventricular pressure curves) and by extrapolation have found that when zero tension is produced, the rate of glucose uptake is only 25 μmoles/hr/g dry wt. It may well be that any measurable rate of glucose uptake in the perfused rat heart indicates a degree of stimulation by mechanical work, incomplete oxygenation or hormonal action.

12. Comparison of metabolic abnormalities in streptozotocin and alloxan-induced diabetes in the rat.

Streptozotocin is a broad spectrum antibiotic extracted from Streptomyces achromogenes (Herr, Eble, Bergy and Jahnke, 1959). Its structure has recently been shown to contain a N-nitrosomethylamide group present as a urea derivative involving the nitrogen of glucosamine (Herr, Jahnke, Argoudelis, 1967) (see Fig. 26). The substance has also been shown to exert antitumour activity in leukamia L 51784, Ehrlich carcinoma and Walker 256 carcinosarcoma (Evans, Gerritsen, Mann, Owen, 1965). In 1963, Rakieten et al. reported that intravenous administration of streptozotocin to rats and dogs led to diabetes and on the basis of histological studies, they attributed this diabetogenic action to a specific effect on the pancreatic β cells. However, Evans et al. (1965) while confirming the diabetogenic action, suggested that it might not result from permanent damage or necrosis of the β cells but rather from an inhibition of production and/or secretion of insulin. Similarly, Arison, Ciaccio, Glitzer, Cassaro, Pruss (1967) concluded from electron microscopy studies that streptozotocin produced degranulation of cells without necrosis. These authors also found a high incidence of bilateral cataracts

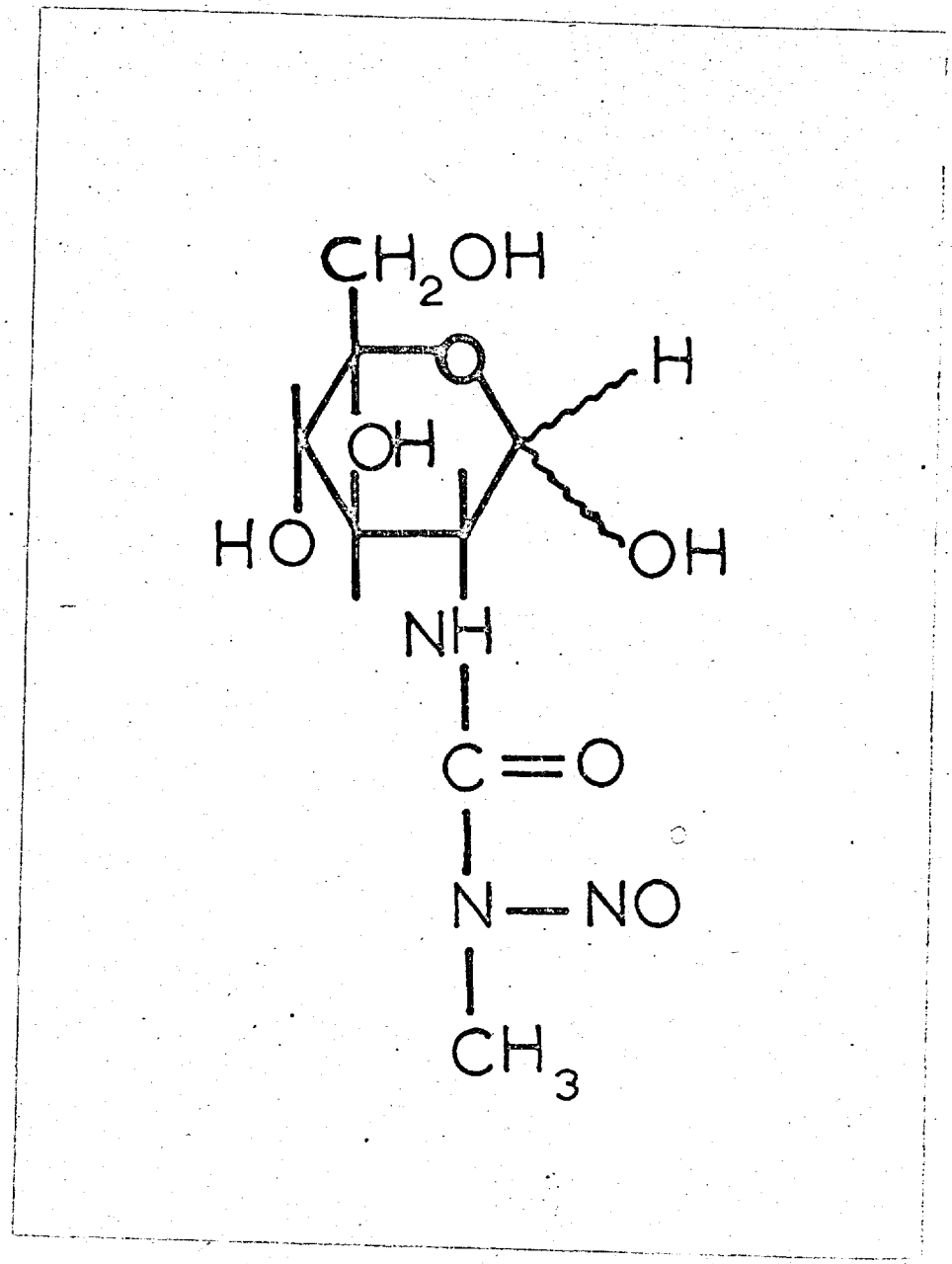

Fig. 26 Structure of Streptozotocin

4 months after 65 mg/kg of streptozotocin (an observation confirmed in the present study) together with accumulation of glycogen in the proximal convoluted tubules of the kidney. Histological examination of the pancreas indicated some evidence of β cell regeneration only when the dosage was lowered to 50 mg/kg. In all of these studies, however, some doubt existed as to the purity of the streptozotocin preparations since Evans et al. (1965) stated that many preparations made available up to 1965 were contaminated with as much as 15% of another compound, Zedalan (3- oximinoacetamido - acrylamide). Indeed these authors postulated synergistic action between streptozotocin and Zedalan with regard to the diabetogenic and antitumoral activity. Junod, Lambert, Orci, Pictet, Gonet and Renold (1967) have shown, however, that pure streptozotocin has a rapid specific and irreversible cytotoxic action upon pancreatic β cells. Only 7 hours after a single 65 mg/kg injection of pure streptozotocin into rats, Junod et al. (1967) were able to demonstrate massive necrosis of the β-cells whilst the α-cells and the exocrine pancreatic tissue remained intact. Pancreatic insulin levels decreased to levels below 5% of normal controls within 24 hours and were still only 2% of normal values after one month. Because of this specific action, streptozotocin

has considerable advantages over alloxan. As Lukens (1948) has pointed out, the LD_{50} (medial lethal dose) of alloxan in the rat corresponds with the ED_{50} (medial effective or diabetogenic dose). Consequently, the mortality associated with diabetogenic doses of alloxan is very high. Alloxan diabetes in the rat (Gomori and Goldner, 1943) is almost invariably accompanied by anaemia and elevation of blood non-protein nitrogen. Damage to the renal tubules and focal central lobular necrosis in the liver are also frequently encountered in the alloxan diabetic rat (Herbut, Watson, Perkins, 1946). In contrast to the extremely narrow margin (or overlap) between diabetogenic and generally toxic or lethal doses seen with alloxan, streptozotocin produced a clear cut diabetes, although of lesser severity, with doses as low as 25 mg/kg whilst the LD_{50} in the rat has been reported to be about 130 mg/kg (Rakieten et al. 1963).

Despite the toxic side effects known to be inevitable in alloxan induced diabetes, the use of hearts from animals 48 hours after alloxan treatment has been very widespread. (Morgan et al. 1961a; Regen et al. 1964; Greenman and Shipp, 1965; Randle et al. 1964; Garland and Randle, 1964b; Kraupp et al. 1967).

The changes reported in carbohydrate metabolism of heart muscle in alloxan diabetes are similar to but more marked than those observed in hearts from fasting animals (Morgan et al. 1961a, Randle, Garland, Hales and Newsholme, 1963; Kreisberg, 1966). The major effects noted have been depression of glucose uptake and increased cardiac glycogen (Cruickshank, 1913; McLeod and Prendergast, 1921; Evans, 1934). Newsholme and Randle (1962) suggested that both the increased cardiac glycogen and the inhibition of glycolysis observed in hearts of both diabetic and fasted rats resulted from inhibition of the phosphofructokinase reaction. A similar phosphofructokinase inhibition was shown in hearts from normal rats perfused with fatty acids or ketone bodies (Bowman, 1962; Newsholme, Randle, Manchester, 1962). Furthermore, citrate, an inhibitor of phosphofructokinase, was increased in hearts of alloxan-diabetic and fasted rats, and in hearts of normal rats perfused with palmitate (Parmeggiani and Bowman, 1963; Garland, Randle and Newsholme, 1963). In the studies reported here with streptozotocin induced diabetic rats the increase in blood free fatty acids and ketones was observed for only the first 3 days after treatment. During this period, hearts perfused with glucose and insulin (Fig.21)

showed evidence of phosphofructokinase inhibition, together with elevated cardiac glycogen and citrate levels. In these respects, therefore, streptozotocin-diabetic hearts resembled alloxan-diabetic hearts although the effects were less marked. After 5-7 days, however, the streptozotocin-diabetic rats although markedly hyperglycaemic and glucosuric no longer exhibited elevated free fatty acid levels in the blood nor were the blood ketone levels significantly elevated. Hearts from these animals no longer had elevated glycogen or citrate levels and the hexose monophosphate content remained normal after perfusion with glucose and insulin.

These results, therefore, show an important difference between the metabolic abnormalities accompanying streptozotocin and alloxan induced diabetes. After the initial phase of rapid lipolysis during which free fatty acid and ketone bodies are elevated, streptozotocin diabetic rats show an apparent specific insulin deficiency hyperglycaemia. In contrast to this, any alloxan animals which survive the first toxic acute phase continue to show elevated blood ketone levels together with increased cardiac contents of G-6-P, F-6-P and citrate, two to seven weeks after alloxan treatment (Kraupp et al. 1967).

13. Effect of work on glucose metabolism in the perfused heart of diabetic rats.

Hearts from streptozotocin diabetic animals (7 days after treatment) when made to perform external mechanical work utilised normal amounts of glucose as measured by glucose uptake and oxidation whereas similar hearts perfused by the Langendorff method showed a very considerable reduction in both of these parameters. In this respect, therefore, the streptozotocin diabetic heart closely resembled the behaviour of the insulin deficient heart obtained by pre-treatment with anti-insulin serum. The stimulation to rates of glucose uptake and oxidation brought about by the performance of cardiac work was much less marked in the alloxan diabetic hearts despite a normal increased oxygen consumption. This could be explained by the increased cardiac citrate and triglyceride (Denton and Randle, 1967) and circulating FFA and ketone levels in alloxan diabetes since it is known that the inhibitory effects of palmitate on glucose uptake and oxidation (Newsholme and Randle, 1961) are observed even in the 'working' heart (Neely et al., 1968).

14. Metabolic effects of insulin in the diabetic perfused heart:

(a) on glycolysis and glucose oxidation.

In Langendorff perfused hearts rendered diabetic with streptozotocin, insulin stimulated the glucose uptake to within normal limits and restored the incorporation of C^{14} from glucose to glycogen. The oxidation of glucose-U-C^{14} however remained abnormally low and there was a striking increase in lactate and pyruvate production. Despite the stimulus to glucose oxidation brought about by the performance of cardiac work, similar results were obtained in streptozotocin diabetic working hearts perfused with glucose and insulin. This lack of effect of insulin on glucose oxidation together with the very marked increases in lactate and pyruvate production and accumulation in heart tissues suggests a defect in the entry of pyruvate into the citrate cycle. This would be consistent with the low heart content of citrate observed in streptozotocin hearts. Normally, however, high concentrations of pyruvate would be expected to give rise to large quantities of citrate as is seen in alloxan diabetes (Garland and Randle, 1964b), since pyruvate promotes the formation of both oxaloacetate and acetyl CoA (Williamson, 1965; 1966). It would appear, therefore, that the mechanism by which pyruvate accumulates in both streptozotocin and alloxan diabetic

hearts may be different in the two cases. Oxidation of pyruvate by perfused rat heart is inhibited by fatty acids and ketone bodies (Garland, Newsholme and Randle, 1964) and in alloxan diabetes but in all of these cases there is a concomitant rise in heart citrate and of acetyl-CoA relative to that of CoA (Garland and Randle, 1964b). Since heart citrate does not accumulate in streptozotocin diabetic hearts, further investigations on the direct oxidation of pyruvate by streptozotocin diabetic hearts and on heart levels of acetyl CoA, which can act as a competitive inhibitor of heart pyruvate dehydrogenase in vitro (Garland and Randle, 1964a) would be of interest.

(b) on glycogen

Alloxan diabetes and streptozotocin diabetes showed important differences in relation to the effects of insulin on incorporation of C^{14} glucose into cardiac glycogen. Streptozotocin diabetic hearts showed near to normal incorporation with added insulin whilst alloxan diabetic hearts continued to show depressed incorporation, even in the presence of insulin. These results should be considered with those on the total cardiac glycogen content in the two cases. In alloxan diabetic rats, cardiac glycogen remained high even with work whilst in streptozotocin diabetes, cardiac glycogen was depressed. Consequently, the restriction on glycolysis imposed by the

phosphofructokinase block in alloxan diabetes (with the associated elevated citrate) presumably reduced glycogen turnover whereas in streptozotocin diabetes there was near to normal turnover with a smaller glycogen "pool". This is consistent with the results of Larner et al., (1968) on the inverse relationship between glycogen content and transferase I activity in perfused rat heart.

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