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Effects of Insulin on the Pattern of Glucose Metabolism in the Perfused Working and Langendorff Heart of Normal and Insulin-Deficient Rats

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1. The metabolic pattern of [U-^{14}C]glucose in the isolated rat heart has been studied, with both retrograde aortic (Langendorff) and atrially (working) perfused preparations in the presence and absence of insulin, in normal animals, animals rendered insulin-deficient (by injection of anti-insulin serum 1hr. before excision of the heart) and animals rendered diabetic by streptozotocin injection 7 days before use. 2. Radioautochromatograms of heart extracts show that the pattern of glucose metabolism in heart muscle is more complex than in diaphragm muscle. In addition to $^{14}CO_2$, glycogen, oligosaccharides, phosphorylated sugars and lactate (the main metabolites formed from [^{14}C]glucose in diaphragm muscle), ^{14}C label from [^{14}C]glucose appears in heart muscle in glutamate, glutamine, aspartate and alanine, and in tricarboxylic acid-cycle intermediates. 3. By a quantitative scanning technique of two-dimensional chromatograms it was found that a mechanical work load stimulates glucose metabolism, increasing by a factor of 2–3 incorporation of ^{14}C into all the metabolites mentioned above except lactate and phosphorylated sugars, into which ^{14}C incorporation is in fact diminished; $^{14}CO_2$ production is equally stimulated. 4. Addition of insulin to the perfusion fluid of the working heart causes increases in ^{14}C incorporation, by a factor of about 1·5 into $^{14}CO_2$, by a factor of about 3–5 into glycogen, lactate and phosphorylated sugars, by a factor of about 2–3 into glutamate and tricarboxylic acid-cycle intermediates and by a factor of about 0·5 into aspartate, whereas incorporation into alanine and glutamine is not affected. The effect of a work load on the pattern of glucose metabolism is thus different from that of insulin. 5. Increasing the concentration of glucose in the perfusion fluid from 1 to 20mM leads to changes of the pattern of glucose metabolism different from that brought about by insulin. $^{14}CO_2$ production steadily increases whereas [^{14}C]lactate and glycogen production levels off at 10mM-glucose, at values well below those reached in the presence of insulin. 6. In Langendorff hearts of animals rendered insulin-deficient by anti-insulin serum or streptozotocin, glucose uptake, formation of $^{14}CO_2$ and [^{14}C]lactate, and ^{14}C incorporation into glycogen and oligosaccharides are decreased. In insulin-deficient working hearts, however, glucose uptake and $^{14}CO_2$ production are normal, whereas incorporation of ^{14}C into glycogen and [^{14}C]lactate production are greatly decreased. 7. Insulin added to the perfusion fluid restores ^{14}C incorporation from glucose into $^{14}CO_2$, glycogen and lactate in the Langendorff heart from animals rendered insulin-deficient by anti-insulin serum; in hearts from streptozotocin-diabetic animals addition of insulin restores ^{14}C incorporation into glycogen and lactate, but $^{14}CO_2$ production remains about 50% below normal. 8. The bearing of these results on the problem of the mode of action of insulin is discussed.

Some years ago, on the basis of experimental work on the effect of insulin on glucose metabolism in the isolated rat diaphragm and adipose tissue by one of the authors (E.B.C.) and his colleagues, as well as the evaluation of experimental work reported by others, views were formulated on the mode of action of insulin (for review see Chain, 1962) of which the essential points are briefly summarized as follows.

Insulin acts by accelerating an enzymic process

catalysing the formation of the first reaction product of the intact glucose molecule in insulin-sensitive cells. It thereby raises the energy potential of the cell, enabling it to carry out more efficiently endergonic synthetic reactions, such as synthesis of glycogen and fatty acid. The process is strictly aerobic, and cannot be a simple transport mechanism, as the metabolic effects of increasing the glucose concentration do not simulate the effect of insulin.

Objections to the technique using isolated rat diaphragm have frequently been made, mainly on the basis that the tissue is damaged by cutting and therefore the experimental results with such material in which the permeability of the cells may be radically altered may give a distorted picture of the true physiological conditions. This criticism, however, was not considered valid, as the effect of insulin on glucose metabolism in the rat hemidiaphragm is quite definite and specific and therefore meaningful, and persists qualitatively unchanged even if the hemidiaphragms are cut up into very small (1mm.^{2}) pieces (Bessmann, 1966). Nevertheless, it was considered that it would be rewarding to study the effect of insulin on glucose metabolism in an intact organ, and it was decided to study the action of insulin on the fate of glucose in the perfused heart, by using a computerized version of the quantitative radiochromatographic-scanning technique previously developed for the study of metabolic patterns of radioactive metabolites in different tissues (Beloff-Chain *et al.* 1955). The investigations were started with the Langendorff (1895) retrograde aortic perfused heart, but extended to the working-heart preparation described by Morgan *et al.* (1965), in which the perfusing medium, entering via the left atrium, is pumped by the heart against an aortic hydrostatic pressure of 100cm. H_2O.

The use of anti-insulin serum has made it possible to induce an acute transient state of insulin deficiency in animals (Armin, Grant & Wright, 1960). It has further been shown that insulin-sensitive tissues from animals treated with anti-insulin serum, such as the heart and diaphragm (Mansford, 1967; Beloff-Chain & Rookledge, 1968; Gregor, Martin, Williamson, Lacy & Kipnis, 1963), have decreased glucose metabolism owing to depletion of tissue-bound insulin. It was therefore decided to study the pattern of glucose metabolism under working and non-working conditions in the hearts of rats treated with anti-insulin serum. With the availability of the effective diabetogenic antibiotic, streptozotocin (Rakieten, Rakieten & Nadkarni, 1963), which acts by specifically destroying the β-cells of the Langerhans islets of the pancreas (Arison, Ciaccio, Glitzer, Cassaro & Pruss, 1967), and therefore produces a state of insulin deficiency uncomplicated by the toxic effects observed in acute alloxan-induced diabetes (Mansford & Opie, 1968), it was also decided to study the pattern of glucose metabolism in working and non-working perfused hearts of streptozotocin-diabetic rats.

EXPERIMENTAL

Materials

Rats. Male rats (280–320g.) from a closed breeding colony of specific pathogen-free derived Sprague–Dawley strain, originating from Charles River Inc., Wilmington, Mass., U.S.A., were used; they had unrestricted access to water and stock laboratory diet.

Chemicals. Alloxan, glucose and sorbitol were obtained from British Drug Houses Ltd., Poole, Dorset. Heparin (as Pularin 1000 units/ml.) was purchased from Evans Medical Ltd., Speke, Liverpool. Streptozotocin (batches 4621-HKJ-126D, 4858-THP-106/4) was kindly given by the Upjohn Company, Kalamazoo, Mich., U.S.A. Crystalline insulin (glucagon-free) was donated by Burroughs Wellcome and Co., Beckenham, Kent, and was dissolved in 3·3mM-HCl to yield a stock solution of 20 units/ml. which was stored at $-15°$ in 1ml. samples.

Chromatographically pure [U-^{14}C]glucose of radioactivity 2–3mc/m-mole was obtained from The Radiochemical Centre, Amersham, Bucks. [^{3}H]Sorbitol of radioactivity of 200mc/m-mole was originally obtained from New England Nuclear Corporation, Boston, Mass, U.S.A., but later was available from The Radiochemical Centre.

Preparation of diabetic rats. Streptozotocin-diabetes was induced in rats by injecting 65mg./kg. of streptozotocin into the tail vein. The solution of streptozotocin used was kept at pH4 by a few drops of citrate buffer because of the instability of streptozotocin solutions at pH7. These animals were used 7 days later without starvation. All treated animals lost weight during this period, exhibited dystrophic changes in the coat, and when used, had blood glucose concentrations greater than 300mg./100ml. The mortality in streptozotocin-treated animals was only 5% and no macroscopic evidence of liver damage was observed.

Anti-insulin serum. This was prepared and used as described by Mansford (1967).

Methods

Heart perfusion. The perfusion method and apparatus was essentially that described by Neely, Liebermeister, Battersby & Morgan (1967) modified for collection of CO_2 and incorporating an in-line Millipore filter (5μm. porosity) in the perfusion circuit. This filter was found to be essential for stable performance by the perfused working heart.

Perfusion medium. Krebs–Henseleit (1932) bicarbonate buffer, pH7·4, equilibrated with O_2+CO_2 (95:5) at 37° was used in all experiments. The precautions suggested by Umbreit, Burris & Stauffer (1964) were followed to prevent precipitation of Ca^{2+}. Additions of glucose (2mg./ml.) and sorbitol (1mg./ml.) were made as indicated below. Before use, the Krebs–Henseleit perfusion medium was carefully filtered through a Millipore filter (0·8μm porosity, type SMWP) to remove any material not in true solution.

Parameters monitored during heart perfusions. (*a*) Oxygen consumption. Oxygen consumption was measured by using a water-jacketed oxygen electrode in a holder placed immediately below the heart-perfusion chamber and a second electrode inserted into the pressure chamber over the aortic cannula. Beckman macro oxygen electrodes (type 325814) were connected to a Beckman model 160 Physiological Gas Analyser.

(*b*) Mechanical stability. The stability of the perfused heart preparations was judged by measurements of heart rate, coronary flow rate and aortic pressure as 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 this transducer was fed via an SE4912 preamplifier and SE4910 amplifier to a u.v.-light-trace six-channel recorder (SE2005).

In the case of working heart preparations perfused via the left atrium, the aortic output was measured as an index of stable work performance.

Collection of samples. Samples of medium were taken 2min. after the addition of [U-^{14}C]glucose or [^{3}H]sorbitol or both. This was taken as the time found necessary for equilibration with the extracellular space of the heart (Morgan, Henderson, Regen & Park, 1961). The dilution of the [^{3}H]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 [^{3}H]sorbitol. Samples of medium were also taken immediately before termination of the perfusion (normally after 30min.). In experiments on the fate of [U-^{14}C]glucose the $^{14}CO_2$ produced by oxidative metabolism was trapped by bubbling the effluent gas from the top of the oxygenating chamber through an ethanolamine–methanol (1:1, v/v) mixture. The $^{14}CO_2$ in solution as bicarbonate at the end of the perfusion was measured by rapid transfer of cooled 1ml. samples of the final medium into scintillation vials. These vials were then quickly closed with skirted rubber caps, which also held a wire support for a small glass centre well containing 0·5ml. of an ethanolamine–methanol (3:2, v/v) mixture. To liberate the $^{14}CO_2$, 0·5ml. of 5M-H_2SO_4 was then injected through the rubber caps into the sample of medium. The vials were then left for at least 3hr. 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 10ml. of Bray's (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 0·1ml. with 10ml. of the same scintillant mixture. The radioactivity of all samples was counted in a Packard Tri-Carb model 3003 liquid-scintillation spectrometer and automatically corrected for quenching by a computerized version of the channels-ratio method of Baillie (1960). The samples of medium obtained at the beginning and end of the perfusion were immediately deproteinized either with 5% (w/v) $ZnSO_4$ and 0·15M-$Ba(OH)_2$ for subsequent glucose assay by the method of Mansford & Opie (1963), or by addition to an equal volume of ice-cold 6% (w/v) $HClO_4$ for lactate assays (Hohorst, 1963).

Preparation of heart extracts. Perfusions were terminated by freezing the heart between aluminium clamping blocks cooled in liquid N_2 (Wollenberger, Ristau & Schoffa, 1960). The frozen muscle was then placed in liquid N_2 and powdered in a percussion mortar. A portion of the frozen percussed powder was transferred to a weighed container for determination of dry weight. A second portion was used for isolation of [^{14}C]glycogen by the method of Good, Kramer & Somogi (1933). A third portion (the remaining powdered frozen tissue) was placed in a weighed tube containing 20ml. of ice-cold 60% aq. (v/v) ethanol. After reweighing, the contents of the tube were thoroughly mixed by homogenization on a vortex mixer.

Chromatographic analysis of metabolites. The suspension of tissue in 60% ethanol was centrifuged and the residue resuspended in a further 20ml. of cold 60% ethanol and homogenized, and then recentrifuged. The combined ethanolic supernatants were then evaporated to 1ml. under vacuum at room temperature. Both one- and two-dimensional chromatograms (see Fig. 1) were then prepared by spotting 20μl. (one dimension) or 50μl. (two) in 2μl. portions. Samples of medium (1ml.) were evaporated to dryness and taken up in 0·2ml. of water before chromatography for measurement of [^{14}C]lactate output.

The chromatograms were developed by descending solvent, run in all-glass tanks at 20° with the solvents described by Hanes & Isherwood (1949) and Crowley, Moses & Ullrich (1963).

After the chromatograms had been allowed to dry in air, the paper was placed in contact with Kodirex X-ray film for 7 days. The radioautographs were then developed with D 19B developer (Kodak). Alternatively, the radioactive spots on one-dimensional chromatograms were detected by the spark-chamber method of Pullan, Howard & Perry (1966).

The radioactivity of all the substances separated on the chromatograms of the heart extracts and perfusion media was quantitatively measured by using a computerized form (E. B. Chain, K. R. L. Mansford & A. E. Lowe, unpublished work) of the automatic radioactive-chromatogram scanner originally developed in the Istituto Superiore di Sanità, Rome (Frank, Chain, Pocchiari & Rossi, 1959).

RESULTS

Radioautographic pattern of glucose metabolism in heart muscle. The radioautographic pattern of [U-^{14}C]glucose metabolism in heart is more complex than in diaphragm muscle. Whereas in the latter the main metabolites formed are glycogen, oligosaccharides, phosphorylated sugar intermediates, lactate and alanine, in the heart muscle, in addition to these, considerable incorporation of ^{14}C into glutamate, glutamine and aspartate occurs (Fig. 1).

Effect of insulin on the pattern of glucose metabolism in normal non-working and working heart muscle. In the working heart muscle there is a pronounced effect of insulin not only on incorporation of ^{14}C from [U-^{14}C]glucose into glycogen and oligosaccharide, which is increased by a factor of 3–5, but also on [^{14}C]lactate formation, which is increased by a factor of 3 (Tables 1 and 2). In addition, $^{14}CO_2$ production from glucose and the incorporation of ^{14}C into tricarboxylic acid-cycle

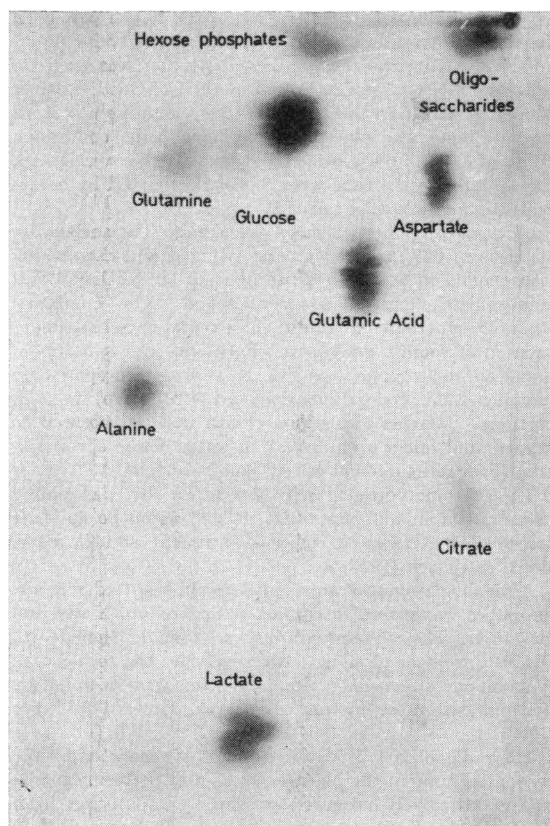


Fig. 1. Autoradiograph of two-dimensional chromatogram of metabolites of [U-^{14}C]glucose in 60% ethanol extract of rat heart muscle after atrial perfusion (20cm. H_2O pressure) for 30min. with 11mM-glucose as substrate. Solvents: first (↓), butan-1-ol–acetic acid–water (40:11:25, by vol.) for 16hr.; secondly, (←) phenol–aq. NH_3 (sp.gr. 0·88)–water (80:1:20, by vol.) for 24hr.

intermediates and one of their transamination products, glutamate, are stimulated, whereas incorporation of ^{14}C into aspartate, glutamine and alanine is not significantly affected, or only slightly increased (Table 1). The effect of insulin on [U-^{14}C]glucose metabolism in the Langendorff heart is similar to that in the working heart (Table 2).

Effect of work on pattern of glucose metabolism in heart muscle. Work itself leads to increased glucose metabolism in heart muscle, but the effect of work on the pattern of glucose metabolism is quite different from the insulin effect (Table 1). Incorporation of ^{14}C into glycogen and oligosaccharides, tricarboxylic acid-cycle intermediates and the amino acids is increased (though the maximal values are well below those obtained after insulin stimulation), but [^{14}C]lactate formation is not affected and incorporation into hexose phosphates is actually decreased.

Effect of glucose concentration on pattern of glucose metabolism in Langendorff heart muscle. Increasing the concentration of glucose in the perfusion fluid leads to changes in the pattern of glucose metabolism in the heart muscle (Table 3), but these changes are different from those brought about by insulin, inasmuch as the $^{14}CO_2$ production steadily increases but the [^{14}C]lactate production levels off at 10mM-glucose. Incorporation of ^{14}C into glycogen also increases to a maximum value at 10mM-glucose that is well below that reached in the presence of insulin.

Pattern of glucose metabolism in the perfused Langendorff and working heart from animals treated with anti-insulin serum in the absence and presence of insulin. The contribution of glucose oxidation to the oxygen consumption of the perfused working heart varies with time (see Table 4). In the following experiments a time of 30min. was chosen in which glucose oxidation represented approx. 40% of the total respiration, the rest being due to the oxidation of endogenous lipid substrates (Olson & Hoeschen, 1967).

In the Langendorff heart of animals treated by injection of anti-insulin serum the proportion of oxygen consumption due to glucose oxidation is still further decreased to about 19% (Table 4). Further, incorporation of ^{14}C into glycogen falls to almost a quarter of its normal value (Table 5), whereas [^{14}C]lactate production is almost halved. Addition of insulin *in vitro* to the perfusion medium stimulates the incorporation of ^{14}C into glycogen to values above the normal ones obtained on addition of insulin to control hearts; $^{14}CO_2$ production is restored to normal values and [^{14}C]lactate production and incorporation of ^{14}C into tissue intermediates are strongly stimulated.

In contradistinction to these results in the Langendorff heart, no depression of glucose uptake or of $^{14}CO_2$ production from [U-^{14}C]glucose is observed in the working heart of animals treated with anti-insulin serum, the results being essentially the same as in the working hearts of normal animals. However, incorporation of ^{14}C into glycogen remains abnormally low and [^{14}C]lactate formation and ^{14}C in tissue intermediates are also decreased.

Pattern of glucose metabolism in the perfused Langendorff and working heart from streptozotocin-diabetic rats in the absence and presence of insulin.

There is a marked decrease in glucose uptake in the Langendorff perfused hearts but not in the working perfused hearts from streptozotocin-diabetic rats, showing that work can, to some degree, overcome the limitation of glucose utiliza-

Table 1. *Effects of insulin and of heart work on incorporation of ^{14}C from [U-^{14}C]glucose into metabolic intermediates in perfused rat heart muscle*

All hearts were pre-perfused without recirculation at 100cm. H_2O aortic pressure for 15min. with 11·1mM-glucose before switching to recirculation perfusion under the conditions stated. Langendorff hearts were at 100cm. H_2O aortic pressure; working hearts at 20cm. H_2O atrial pressure. The freeze-clamped heart tissue was extracted with 60% (v/v) aq. ethanol and the extract was chromatographed as described in the Methods section. The substrate was 11·1mM-glucose+insulin (2m-units/ml.). Results are given as μmoles of glucose equivalent incorporated/g. dry wt./30min. (mean±S.E.M., for the numbers of hearts given in parentheses).

	Langendorff (4)	Work (4)	Work+insulin (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
Tricarboxylic acid-cycle intermediates	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†

* Significant difference ($P \leq 0.05$) between results for working and Langendorff hearts.
† Significant difference ($P \leq 0.05$) between (work+insulin) and (work−insulin) results.

Table 2. *Fate of [U-^{14}C]glucose in the perfused rat heart; effects of insulin in Langendorff and working hearts*

All hearts were pre-perfused by the Langendorff aortic route at 100cm. H_2O pressure for 15min. before recirculation perfusion for 30min. under the conditions specified (Langendorff heart aortic pressure 100cm. H_2O; working heart atrial pressure 20cm. H_2O). Glucose uptake was measured from the change in concentration of glucose in the perfusion medium during the recirculation period. [^{14}C]Lactate output was measured by quantitative scanning of radiochromatograms of perfusion-medium samples, as described in the Methods section. [^{14}C]Glycogen was isolated from a sample of freeze-clamped tissue by the method of Good, Kramer & Somogyi (1933). $^{14}CO_2$ was measured by absorption into ethanolamine–methanol, as described in the Methods section. The substrate was 11·1mM-glucose in Krebs–Henseleit (1932) bicarbonate buffer, pH 7·4. Results are given as μmoles of glucose equivalent/g. dry wt./30min. (mean ±S.E.M., for the numbers of hearts given in parentheses).

	Glucose uptake	[^{14}C]Lactate output	^{14}C as glycogen	$^{14}CO_2$
Langendorff	111±4 (19)	25·2±2·8 (9)	3·2±0·5 (7)	65·4±6·6 (7)
Langendorff+insulin (2m-units/ml.)	228±9 (6)	57·6±6·1 (16)	21·2±4·4 (6)	112·7±6·8 (6)
Work	270±9 (18)	19·3±2·6 (4)	7·4±1·4 (7)	196·5±8·6 (24)
Work+insulin (2m-units/ml.)	538±17 (15)	83·8±17·3 (7)	26·0±3·6 (16)	333·6±18 (12)

tion imposed by diabetes (Table 6). Similarly, $^{14}CO_2$ production from [U-^{14}C]glucose is greatly decreased in the Langendorff heart, but not affected significantly in the working heart of streptozotocin-diabetic rats. Incorporation of ^{14}C into glycogen is, however, markedly decreased in both the Langendorff and the working heart in diabetic rats. Addition of insulin (Table 7) restores the incorporation of ^{14}C into glycogen to almost normal values. Lactate production from streptozotocin-perfused hearts is, in fact, increased by insulin to well above normal values whereas $^{14}CO_2$ production remains about 50% below normal values in the presence of insulin although it is doubled in the Langendorff heart in diabetic animals.

DISCUSSION

The results with working hearts rendered insulin-deficient by specific anti-insulin serum provide a striking demonstration of the specificity of the action of insulin, being confined to the

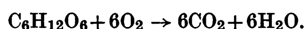
Table 3. *Effect of glucose concentration on $^{14}CO_2$ production, [^{14}C]lactate formation and glucose uptake in Langendorff perfused heart*

All hearts were perfused for 30min. by recirculation at 100cm. H_2O hydrostatic pressure. Glucose uptake, [^{14}C]lactate production, [^{14}C]glycogen and $^{14}CO_2$ were measured as described in Table 2. Results are given as μmoles of glucose equivalent/g. dry wt./30min. (mean ±S.E.M., for the numbers of hearts given).

Concn. of glucose in perfusate (mM)	No. of hearts	Glucose uptake	$^{14}CO_2$ production	[^{14}C]Lactate production	^{14}C into glycogen
1	3	35±3·7	22·4±2·2	13·3±2·2	2·1±0·3
3	3	56±4·2	35·7±4·4	23·1±4·2	2·1±0·6
5	6	119±7·6	60·2±3·6	22·9±2·1	2·8±0·4
10	6	140±12	74·9±3·6	28·7±3·5	4·2±1·2
20	3	154±17	79·8±4·8	28·0±3·2	4·2±0·8
10+ insulin (2m-units/ml.)	6	245±9	112·7±10·2	74·2±8·5	14·7±1·2

Table 4. *Contribution of glucose to respiration of the perfused rat heart*

The contribution of glucose oxidation to respiration is calculated from oxygen uptake (q_{O_2}) and yield of $^{14}CO_2$:



$$C_6H_{12}O_6+6O_2 \rightarrow 6CO_2+6H_2O.$$

For Langendorff hearts the perfusion pressure was 100cm. H_2O, and for working hearts, 20cm. H_2O. The '+ insulin' samples contain 2m-units/ml.

Animal	Insulin	Type of perfusion	Time of perfusion (min.)	% of respiration accounted for by $^{14}CO_2$ from 11·1mM-[U-^{14}C]glucose
Normal	−	Langendorff	0–30	37
	−	Work	0–15	30
			15–30	50
			30–45	80
	+	Langendorff	0–30	69
	+	Work	0–30	74
Anti-insulin-serum treated	−	Langendorff	0–30	19
	−	Work	0–30	50
	+	Langendorff	0–30	58
Streptozotocin-diabetic	−	Langendorff	0–30	14
	−	Work	0–30	48
	+	Langendorff	0–30	30
	+	Work	0–30	50

reactions of glucose metabolism concerned with glycogen synthesis. The absence of insulin has not affected permeability to glucose in general, as the values for glucose uptake and $^{14}CO_2$ production in working hearts of animals treated with anti-insulin serum are the same as in the normal working hearts.

The results support previously expressed views (Chain, 1962) that the stimulation of glucose metabolism in muscle by insulin cannot be explained by simple general acceleration of glucose transport, but is the consequence of a specific stimulation of reactions concerned with glycogen synthesis. The increase in the formation of [^{14}C]lactate in working and non-working heart muscle observed in the presence of insulin (in addition to the increased synthesis of glycogen and oligosaccharide) could arise partly as a consequence of increased turnover of glycogen. In the non-contracting rat diaphragm, as used in previous studies, the effect of insulin is confined to synthesis of glycogen and oligosaccharide and lactate production is not affected, possibly because of the slower turnover rate of glycogen in this tissue under the experimental conditions. It is noteworthy, as has recently been shown by Beloff-Chain & Rookledge (1968) that in rat diaphragm from anti-insulin-serum-treated and

Table 5. *Fate of* [U-^{14}C]*glucose in the perfused hearts of insulin-deficient rats*

All perfusions were carried out by recirculation for 30min. (Langendorff heart, aortic pressure 100cm. H_2O; working heart, atrial pressure 20cm. H_2O). [^{14}C]Glucose uptake and [^{14}C]lactate output were measured by quantitative scanning of radiochromatograms of perfusion-medium samples, as described in the Methods section. [^{14}C]Glycogen and $^{14}CO_2$ were measured as indicated in Table 2. The substrate was 11·1mM-glucose ± 2m-units of insulin/ml. *in vitro*. Results are given as μmoles of glucose equivalent/g. dry wt./30min. (mean ±S.E.M., for the numbers of hearts given).

Hearts	No. of hearts	[^{14}C]Glucose uptake	^{14}C into glycogen	[^{14}C]Lactate production	^{14}C in tissue intermediates	$^{14}CO_2$
Langendorff						
Control	9	142±20	4·2±0·8	28·7±3·3	21·2±4·1	74·9±9·1
Control+insulin	6	228±9	21·2±4·4	57·6±6·1	38·8±3·2	112·7±6·8
Anti-insulin-serum treated	6	63±8	1·1±0·6	18·2±3·3	11·7±1·3	33·6±4·6
Anti-insulin-serum treated +insulin	6	247±24	35·2±5·6	38·2±7·4	46·6±8·2	100·8±9·6
Working						
Control	6	249±21	5·9±1·5	23·5±2·9	41·6±5·2	189±21
Anti-insulin-serum treated	6	289+19	2·2±1·2	11·4±1·2	24·2±6·6	233±28

Table 6. *Metabolic effects of work in the perfused hearts of rats rendered diabetic with streptozotocin*

All hearts were pre-perfused for 15min. at 100cm. H_2O aortic pressure, followed by recirculation perfusion for 30min. under the conditions specified (Langendorff heart, aortic pressure 100cm. H_2O; working heart, atrial pressure, 20cm. H_2O). Glucose uptake was calculated from the change in concentration of glucose in the perfusion medium during the recirculation period. Lactate production was measured similarly. The substrate was 11·1mM-glucose. Results are given as μmoles of glucose equivalent/g. dry wt./30min. (mean ±S.E.M., for the numbers of hearts given.

Hearts	No. of hearts	Glucose uptake	Lactate production	$^{14}CO_2$	[^{14}C] as glycogen
Langendorff					
Diabetic	12	57·1±4·1	16·0±3·6	23·7±4·1	2·1±0·2
Normal control	19	110·5±4·3	22·3±3·4	65·4±6·6	3·2±0·5
Working					
Diabetic	8	246 ±12	22 ±5	199 ±10	2·4±0·2
Normal control	18	270 ±9	28 ±9	196 ±9	7·4±1·4

Table 7. *Metabolic effects of insulin in the perfused hearts of rats rendered diabetic with streptozotocin*

All hearts were pre-perfused for 15min. at 100cm. H_2O aortic pressure, followed by recirculation perfusion for 30min. under the conditions specified (Langendorff heart, aortic pressure 100cm. H_2O; working heart, atrial pressure 20cm. H_2O). Streptozotocin-diabetic rats were used 7 days after a single intravenous dose of 65mg./kg. Glucose uptake and lactate production were measured as described in Table 6. The substrate was 11·1mM-glucose; insulin was used *in vitro* at 2m-units/ml. Results are given as μmoles of glucose equivalent/g. dry wt./30min. (mean ±S.E.M., for the numbers of hearts given).

Hearts	No. of hearts	Glucose uptake	Lactate production	$^{14}CO_2$	^{14}C as glycogen
Langendorff					
Streptozotocin-diabetic+insulin	14	201±11	90±10	47·9±4·8	18·1±1·4
Working					
Streptozotocin-diabetic+insulin	11	405±33	135±17	187 ±24	19·0±2·5
Normal control+insulin	12	538±17	80±11	334 ±18	26·0±3·6

streptozotocin-diabetic animals only synthesis of glycogen and oligosaccharide is decreased whereas production of $^{14}CO_2$ and [^{14}C]lactate are not significantly affected. Therefore, under these conditions of insulin insufficiency there is a specific decrease in those metabolic reactions that are stimulated by insulin in the normal rat diaphragm.

The fact that in insulin-deficient hearts and those of streptozotocin-diabetic rats, a specific biochemical defect, i.e. decreased glycogen synthesis, can be

restored to normality by addition of insulin to the perfusion medium, is also worthy of note. This appears to be the first case in which a metabolic defect in diabetes can be corrected immediately by addition of insulin *in vitro*.

The exact point of action of insulin in the pathway of glycogen synthesis remains to be established. There are involved an anaerobic series of reactions leading to glycogen from phosphorylated glucose, by the well established scheme of Leloir & Cardini (1957), and an aerobic energy-dependent series of reactions concerned with bringing about the phosphorylation step. The effect of insulin is observed only under aerobic conditions in diaphragm (Beloff-Chain *et al.* 1959) and in the perfused rat heart (Williamson, 1962) with the optimum insulin concentration of 2m-units/ml. (Bleehen & Fisher, 1954). Further, 2-deoxyglucose (Kipnis & Cori, 1957), which cannot be polymerized to glycogen, can be phosphorylated in the rat diaphragm and there is a strong effect of insulin on the phosphorylation. It is considered probable therefore that insulin is concerned with the aerobic, energy-dependent reactions involved in the phosphorylation of glucose. No conclusive evidence is available on the nature of the first product of phosphorylation of glucose on the way to glycogen, although it has been shown that radioactive glucose 6-phosphate, at least when added to diaphragm, is not incorporated into glycogen, but is oxidized, whereas the addition of labelled glucose 1-phosphate does lead to the formation of labelled glycogen (Beloff-Chain *et al.* 1964).

There are always at least two possible explanations of the biosynthetic formation of different end products from a common precursor substrate: one is the assumption of separate pathways involving different intermediates, the other compartmentation with a common intermediate. With glycogen synthesis from glucose it is possible to visualize either the formation of an initial phosphorylated intermediate different from glucose 6-phosphate (possibly more closely related to glucose 1-phosphate) that on formation is immediately transferred via UDPG to glycogen, or alternatively a separate compartment for glucose 6-phosphate that makes it different from the bulk of glucose 6-phosphate, and in which thermodynamically particularly favourable conditions prevail for its isomerization to glucose 1-phosphate. A tightly coupled multienzyme system for glycogen synthesis, bound closely to the glycogen particles would represent such a compartment. In either case some kind of glucose–ATP phosphotransferase is involved; in the latter case, this would form part of the multienzyme complex. It is now well documented that glucose–ATP phosphotransferases of mammalian tissue occur in a soluble and a particulate form; further, the soluble glucose–ATP phosphotransferases occur as isoenzymes, four different isoenzymes having so far been demonstrated by electrophoretic and chromatographic separation techniques (Grossbard & Schimke, 1966). These have been termed types I–IV in order of increasing electromobility. They differ widely in their K_m values. It has been shown by Katzen (1966) that in insulin-sensitive tissues such as skeletal and heart muscle and adipose tissue, the pattern of glucose–ATP phosphotransferases differs significantly from that in insulin-insensitive tissues, in that in the former, glucose–ATP phosphotransferase type II is present, but it is absent in the latter. A similar situation has been shown by Walters & McLean (1967) to occur in the mammary gland; this tissue exhibits cyclic insulin-sensitivity, being insulin-insensitive before lactation, becoming increasingly insulin-sensitive as lactation develops, and returning to insulin-insensitivity after termination of lactation. Walters & McLean (1967) have demonstrated that concomitantly with insulin-sensitivity, glucose–ATP phosphotransferase activity and the ratio of type II enzyme to type I increase threefold in mammary gland during lactation, the enzyme returning to type I after cessation of lactation. Further, Katzen (1966) has shown that in hearts of streptozotocin-diabetic rats, glucose–ATP phosphotransferase type II activity is low or absent, and can be restored by insulin; however, reactivation can also be brought about by substances containing thiol groups, such as mercaptoethanol or GSH. Walters & McLean (1968) have shown that in the lactating mammary gland of animals treated with anti-insulin serum there is a change in the distribution of insoluble and soluble glucose–ATP phosphotransferases, the insoluble particle-bound fraction decreasing in amount to 11% of the control value and the soluble fraction increasing by about 50%.

The significance of these findings for the mode of action of insulin is not clear. It is suggested that the glucose–ATP phosphotransferases represent a set of alternative enzymes utilizing aerobically generated ATP by a linked process, catalysing the terminal reaction of this whole process; by analogy, bacterial isoenzyme systems have been shown to have the function of directing metabolic reactions along different pathways, thus causing a sort of compartmentation in the cell (Kaplan, 1963). Whether insulin acts by activating type II phosphotransferase directly or acts on a point further back in the system responsible for the phosphorylation of glucose and by doing this keeps the glucose–ATP phosphotransferase in the reactive reduced state, remains to be established.

Inactivation of one component of a tightly

coupled multi-enzyme system could, of course, lead to impaired function of other components, particularly if redox systems are involved, as they are in the processes of oxidative phosphorylation. If the synthesis of glycogen from glucose via a phosphorylated intermediate represents such a multi-enzyme system, it is conceivable that a defect at the beginning of the enzyme chain, caused by lack of insulin, may reflect itself in a defect of the terminal reaction, i.e. the transfer of glucose via UDPG to glycogen by glycogen synthetase. In this respect, Danforth (1965) demonstrated that the ratio of the I form of glycogen synthetase to the D form in the isolated rat diaphragm was markedly raised by insulin. Similarly Williams & Mayer (1966) reported that insulin raised the I/D ratio in heart without affecting the total content of glycogen synthetase.

Evidence has been produced by several groups that insulin stimulates ATP turnover in the rat diaphragm and in adipose tissue (Clauser, Volfin & Eboue-Bonis, 1962; Hepp, Challoner & Williams, 1968). In this context it should be remembered that energy-rich phosphates other than ATP could be involved in the phosphorylation of glucose. In bacterial systems, phosphoenolpyruvate has been shown to be one of the components of the glucose permeases, acting as phosphate donor for the phosphorylation of glucose (Kundig, Kundig, Anderson & Roseman, 1966). It is noteworthy that van Heijningen (1966) has isolated bis-phosphoenolpyruvate from diaphragm muscle and shown that this compound, whose role in metabolism has not yet been established, is increased in amount in the presence of insulin.

Although the problem of the mode of action of insulin has not been solved by the studies reported above, it appears to have been narrowed down to the specific initial reaction involved in the first product of glucose metabolism and it is in this direction that future research in this field should be directed.

The fact that the deficiency of glycogen synthesis occurring in hearts of anti-insulin-serum-treated or streptozotocin-diabetic animals can be reversed by addition of insulin should make it possible to investigate which of the enzymes involved in glycogen synthesis are inactive in insulin insufficiency and reactivated by addition of insulin.

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