Protein Synthesis by Perfused Hearts from Normal and Insulin-Deficient Rats

EFFECT OF INSULIN IN THE PRESENCE OF GLUCOSE AND AFTER DEPLETION OF GLUCOSE, GLUCOSE 6-PHOSPHATE AND GLYCOGEN

By ERNST B. CHAIN and PETER M. SENDER*

*Department of Biochemistry, Imperial College of Science and Technology,
London SW7 2AY, U.K.*

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In the absence of glucose, insulin stimulated the incorporation of ^{14}C -labelled amino acids into protein by perfused rat hearts that had been previously substantially depleted of endogenous glucose, glucose 6-phosphate and glycogen by substrate-free perfusion. This stimulation was also demonstrated in hearts perfused with buffer containing 2-deoxy-D-glucose, an inhibitor of glucose utilization. It is concluded that insulin exerts an effect on protein synthesis independent of its action on glucose metabolism. Streptozotocin-induced diabetes was found to have no effect either on ^{14}C -labelled amino acid incorporation by the perfused heart or on the polyribosome profile and amino acid-incorporating activity of polyribosomes prepared from the non-perfused hearts of these insulin-deficient rats, which show marked abnormalities in glucose metabolism. Protein synthesis was not diminished in the perfused hearts from rats treated with anti-insulin antiserum. The significance of these findings is discussed in relation to the reported effects of insulin deficiency on protein synthesis in skeletal muscle.

It has been known for some time that the incorporation of radioactively labelled amino acids into skeletal (Krahl, 1952; Sinex *et al.*, 1952) and heart muscle protein (Wool & Manchester, 1962) is stimulated by insulin. As the major effect of insulin on insulin-sensitive tissues is on glucose, the question must be answered whether this effect on protein synthesis is a direct consequence of the stimulation of glucose metabolism and whether in fact the presence of glucose is necessary for the effect of insulin on amino acid incorporation. This was investigated in the isolated rat diaphragm, where, despite the absence of glucose from the incubation medium, insulin still increased the incorporation of amino acids into protein (Manchester & Young, 1958; Wool & Krahl, 1959); in the perfused rat heart similar findings were obtained (Wool & Manchester, 1962).

In these experiments, however, the muscle would have contained substantial glycogen stores, the metabolism of which may have been affected by the added insulin. The heart is able to contract vigorously for long periods of time when oxidizable substrates are absent from the perfusate (Fisher & Williamson, 1961), mobilizing its glycogen and triglyceride stores at a rate depending on the pressure developed (Shipp

et al., 1964; Olson & Hoeschen, 1967; Crass *et al.*, 1971) until these are exhausted.

The glucose analogue 2-deoxy-D-glucose is taken up and phosphorylated by muscle (Kipnis & Cori, 1959) and inhibits glycolysis but not [^{14}C]acetate oxidation (Wick *et al.*, 1957). De Schepper *et al.* (1965) reported an inhibition by 2-deoxyglucose of [^{14}C]leucine incorporation by rat diaphragm, with abolition of the stimulatory effect of insulin. Insulin deficiency caused by the diabetogenic antibiotic streptozotocin and by specific anti-insulin antiserum results in more physiological disturbances of glucose metabolism by the perfused rat heart (Chain *et al.*, 1969).

The experiments described in this paper were designed in an attempt to clarify the relationship between the insulin effects on glucose and on amino acid incorporation. Rates of ^{14}C -labelled amino acid incorporation, in the presence and absence of insulin, were measured under conditions of impaired glucose utilization (produced by 2-deoxyglucose and streptozotocin), and also in the absence of intracellular glucose after perfusion of the heart with a substrate-free medium.

Because protein synthesis occurs on the ribosome and is impaired in the alloxan-diabetic condition (Wool *et al.*, 1968), we decided to examine ribosomes prepared from the hearts of streptozotocin-diabetic

* Present address: Department of Human Nutrition, London School of Hygiene and Tropical Medicine, London WC1E 7HT, U.K.

rats to determine whether any similar changes could be detected.

Experimental

Materials

Animals. Male albino rats of the Sprague-Dawley strain, weighing 280–320 g, were used in all the experiments described. They were allowed unrestricted access to water and stock laboratory diet (Thompson rat cubes; Pilsbury, Birmingham, U.K.).

Chemicals and enzymes. ATP, GTP, glucose 6-phosphate (sodium salt), fructose 1,6-diphosphate (tricyclohexylammonium salt), phosphoenolpyruvate (sodium salt), NADP, hexokinase, glucose 6-phosphate dehydrogenase and pyruvate kinase were all obtained from the Boehringer Corp. (London) Ltd. (London W.5, U.K.). Sodium pyruvate, 2-deoxy-D-glucose, Tris and Triton X-100 were obtained from Sigma (London) Chemical Co. Ltd. (London S.W.6, U.K.). Bovine pancreatic ribonuclease A (3100 units/mg) was from the Worthington Biochemical Corp. (Freehold, N.J., U.S.A.), puromycin dihydrochloride from the Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.) and casein hydrolysate from Hopkin and Williams Ltd. (Epsom, Surrey, U.K.). The scintillant, 5-(4-biphenyl)-2-(4'-t-butylphenyl)-1-oxa-3,4-diazole, was from CIBA Ltd. (Cambridge, U.K.). Heparin (as Pularin, 1000 units/ml) was purchased from Evans Medical Ltd. (Liverpool, U.K.). Streptozotocin [2-deoxy-2-(3-methyl-3-nitrosoureido)-D-glucopyranose] was donated by Upjohn Ltd. (Kalamazoo, Mich., U.S.A.). Crystalline insulin (glucagon-free), donated by Wellcome Ltd. (Dagenham, U.K.), was dissolved in 0.15M-NaCl with a few drops of 3.3 mM-HCl, to yield a stock solution of 20 units/ml, measured portions of which were stored at -15°C .

Radioactive chemicals. Sodium $[3\text{-}^{14}\text{C}]\text{pyruvate}$ (13.2 mCi/mmol), $[\text{U-}^{14}\text{C}]\text{glycine}$ (11–15 mCi/mmol) and ^{14}C -labelled reconstituted protein hydrolysate (code CFB 104, 52 mCi/mg-atom of carbon) were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.).

Perfusion medium. Krebs & Henseleit (1932) bicarbonate buffer, pH 7.4, equilibrated with $\text{O}_2 + \text{CO}_2$ (95:5), was used with the addition of 20 natural amino acids, each at $50\text{ }\mu\text{M}$. Other additions were made as indicated in the legends to the tables.

Methods

Preparation and perfusion of hearts. The technique and apparatus used for the perfusion of isolated rat hearts has been described (Chain *et al.*, 1969). For the present study two types of heart-perfusion experiment were carried out.

(i) Perfusion without recirculation (drip-through), in which hearts were perfused retrogradely through

the aorta at a pressure of $100\text{ cmH}_2\text{O}$ from a large jacketed reservoir (Langendorff, 1895). The coronary output was allowed to run to waste. A drip-through pre-perfusion period of 15 min was given to all hearts by this method before recirculation was begun. This washing (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, unless otherwise indicated) served to remove all blood and tissue-bound insulin and to allow the heart to recover from the period of anoxia associated with excision and cannulation.

(ii) Recirculation perfusions, in which hearts were switched after the pre-perfusion period to retrograde aortic perfusion with 10 ml of medium recirculated in the apparatus described by Morgan *et al.* (1961), at an aortic pressure of 60 mmHg.

Measurement of performance of heart. A number of parameters were monitored to ensure the physiological viability and stability of the hearts perfused.

(a) Cardiac output. The coronary flow rate was measured by diverting the effluent from the heart chamber into a graduated cylinder for 30 s.

(b) Heart rate, rhythm and aortic pressure. These were registered by means of a pressure transducer (SE4 Medical type, SE Laboratories Ltd., Feltham, Middx., U.K.) attached to a side arm on the aortic cannula. The output of the transducer was fed via a SE4912 preamplifier and SE 4910 amplifier to a u.v.-light trace recorder (SE 2005).

(c) Oxygen consumption. Perfusate $p\text{O}_2$ values were monitored by using Beckman oxygen macro electrodes (type 325814) connected to a Beckman model 160 Physiological Gas Analyzer (Beckman Instruments Ltd., Glenrothes, Fife, U.K.).

The isolated heart resumed spontaneous contraction during the first 15 s in the perfusion circuit. During the next minute contractions were irregular, but after that time all hearts had established a regular rhythm, with a coronary flow rate of 10–15 ml/min. The cardiac rate was 200–300 beats/min. The $p\text{O}_2$ in the coronary effluent was never lower than 150 mmHg, and the perfusate was maintained at $\text{pH } 7.45 \pm 0.05$.

Preparation of streptozotocin-diabetic rats. Streptozotocin diabetes was induced as described by Chain *et al.* (1969). Animals were used after 7 days without starvation. They showed dystrophic changes of the fur, lost weight and had blood glucose concentrations of greater than 300 mg/100 ml. The mortality of the animals after streptozotocin treatment was negligible. The animals were tested for glycosuria before removal of the heart by using Clinistix indicator tabs (Ames Co., Stoke Poges, Bucks., U.K.).

Preparation and use of anti-insulin antiserum. Anti-insulin antiserum was raised in guinea pigs by the method of Mansford (1967), which is a modification of the original procedure of Moloney & Coval (1955).

Under light ether anaesthesia 0.75 ml of antiserum was injected into the femoral vein of a rat 1 h before perfusion. The vessel was sealed and the wound sutured. Before removal of the heart, the approximate blood sugar concentration was tested with Dextrostix indicator (Ames) and later measured accurately by the glucose oxidase method on a deproteinized blood sample. Control animals were injected with 0.75 ml of non-immune guinea-pig serum. Anti-insulin antiserum produced a rise of 150–200 mg/100 ml in the blood glucose of treated rats.

Tissue glucose, glucose 6-phosphate and glycogen. Heart extracts were prepared and analysed for glucose, glucose 6-phosphate and glycogen as described by Opie *et al.* (1971). In experiments to determine the specific radioactivity of [^{14}C]glycogen, precipitated glycogen was twice redissolved in water and reprecipitated with ethanol before hydrolysis.

Blood glucose. Concentrations of glucose in blood from normal and diabetic rats were determined on a neutral deproteinized filtrate [prepared by the addition of 1 ml of ZnSO_4 (5%) and 1 ml of Ba(OH)_2 to 0.1 ml of blood] by an AutoAnalyzer (Technicon Instrument Co., Chertsey, Surrey, U.K.) glucose oxidase method (Marks & Lloyd, 1963).

Preparation of heart protein fractions and myofibrils. At the end of the perfusion period the hearts were cut off the cannula, the aorta and atria were removed, and then the hearts were dropped into ice-cold perfusion fluid or medium used for the homogenization of myofibrils (39 mM-sodium borate–25 mM-KCl, pH 7.1). The muscle was minced with scissors and washed well with fresh buffer, blotted dry and homogenized in 10% (w/v) trichloroacetic acid (3 ml/g wet wt.) in a Vortex model 404 homogenizer, for measurement of the specific radioactivity of the total heart proteins.

Specific radioactivity of heart protein. After centrifugation, the trichloroacetic acid-precipitated proteins were processed to remove any radioactivity deriving from free amino acids by the method of Wool & Krah (1959). The efficiency of this procedure in removing non-peptide bound amino acid was 98–100%, as judged by the specific radioactivity of proteins from hearts perfused with radioactive amino acids for 30 s.

The dried protein was dissolved in 0.5 M-NaOH for protein determination and for liquid-scintillation counting of radioactivity. All samples for radioactivity determination were added to 15 ml of the scintillant solution of Scales (1967). They were counted with a Packard Tri-Carb liquid-scintillation spectrometer model 3003 and corrected for quenching by the channels-ratio method of Baillie (1960). Counting efficiency was 65–70% and counting time was sufficient to give an error of 0.5%.

Protein was assayed by the method of Lowry *et al.* (1951). A standard curve was always prepared for the linear portion of the assay range from a stock solu-

tion of bovine serum albumin, measured portions of which were stored frozen. Care was taken to standardize the amount of NaOH in the assay sample when proteins were dissolved in NaOH.

Preparation of ribosomes from cardiac muscle. Preparations of cardiac muscle ribosomes were always made simultaneously from the non-perfused hearts of normal and diabetic rats by the method of Earl & Morgan (1968). The total 'ribosome' population (i.e. monomers and polyribosomes) obtained by centrifugation through the 1 M-sucrose-salt solution was used in all the experiments described.

The size distributions of polyribosome preparations from normal and diabetic rats were compared by centrifugation through linear 15–30% or 15–45% (w/v) sucrose gradients. Sucrose solutions contained 0.02 M-Tris, 0.1 M-KCl, 0.04 M-NaCl, 6 mM-magnesium acetate and 1 mM-EDTA (pH 7.6 at 0°C). After centrifugation at 50000 rev./min for 35 min at 0°C in the SW50.1 rotor of the Spinco model L2-65B ultracentrifuge, the gradients were fractionated through an ISCO flow cell (Instrumentation Specialities Co. Inc., Lincoln, Nebraska, U.S.A.).

Amino acid incorporation by isolated cell-free ribosomes. The incorporation of ^{14}C -labelled amino acids into protein directed by polyribosomes from cardiac muscle prepared from normal and diabetic rats was assayed in a cell-free system. The method used was similar to those described by Chen & Young (1968), Earl & Morgan (1968) and Arnstein *et al.* (1965). The standard incubation mixture contained, in a total volume of 1.0 ml, 20 mM-Tris (pH 7.6 at 37°C), 0.1 M-KCl, 40 mM-NaCl, 10 mM-magnesium acetate, 1 mM-EDTA, 0.2 mM-GTP, 5 mM-ATP, 10 mM-phosphoenolpyruvate, 100 μg of pyruvate kinase, 2 mg of the pH 5 fraction, a complete unlabelled amino acid mixture (each 10 μM) containing 0.6 μCi of $\text{U-}^{14}\text{C}$ -labelled protein hydrolysate and 20–200 μg of polyribosomes. The pH 5 fraction was prepared from a post-microsomal supernatant, obtained by centrifuging a non-detergent-treated muscle homogenate at 100000 g for 2 h, by the method of von der Decken & Campbell (1962).

The mixture was incubated at 37°C for 1 h and then for a further 30 min after the addition of 0.25 ml of 1 M-NaOH containing 5 g of casein hydrolysate/100 ml. The proteins precipitated with 0.5 ml of 50% (w/v) trichloroacetic acid were collected on glass-fibre filters and counted at infinite thinness with a low-background (1–2 c.p.m.) Nuclear-Chicago model 4312 automatic system with gas-flow detector.

Ribosomal RNA. The concentration of ribosomal RNA was determined from the extinction at 260 and 280 nm, by using a nomogram derived from the measurements of Warburg & Christian (1942). This method was found to give similar results to the orcinol procedure after extraction with hot 5% (w/v) trichloroacetic acid (Ogur & Rosen, 1950).

Results and Discussion

Glycogen depletion of the perfused rat heart

To establish conditions under which the incorporation of ^{14}C -labelled amino acids could be investigated in the absence of glucose or glucose precursors, hearts were perfused for various times with perfusion fluid lacking any oxidizable substrate. Perfusions were terminated by freeze-clamping the hearts with Wollenberger tongs, and measurements were made of heart glucose, glucose 6-phosphate and glycogen content. The heart glucose and glucose 6-phosphate content fell rapidly (from 459 ± 182 to $26 \pm 6 \text{ nmol/g dry wt.}$ for glucose, and 618 ± 31 to $17 \pm 2 \text{ nmol/g dry wt.}$ for glucose 6-phosphate, both results expressed as means $\pm$ S.E.M. of five hearts, after 15 min of substrate-free perfusion, and thereafter becoming undetectable with the assay used); the heart glycogen was, however, relatively slowly depleted (Fig. 1). After 45 min of substrate-free perfusion, some

Fig. 1. Effects of substrate-free perfusion on the glucose (○), glucose 6-phosphate (Δ) and glycogen (●) content of the rat heart

Hearts were perfused without recirculation by using Krebs–Henseleit buffer lacking any oxidizable substrate (Langendorff preparation; $100 \text{ cmH}_2\text{O}$ aortic pressure). Each point represents the mean of at least four perfusions. Zero-time hearts were freeze-clamped within 30 s of the commencement of perfusion.

hearts began to show signs of failure, with increase in coronary flow rates and cardiac arrhythmias. Therefore a period of 35 min was chosen as a compromise, at which time endogenous glycogen had been substantially depleted, but the heart had not suffered any detectable ill-effects. To study protein synthesis in the glycogen-depleted heart, hearts were therefore first perfused with substrate-free buffer for 35 min, and then provided with pyruvate as a substrate for the incorporation studies.

Although glycogen concentrations were very low after 35 min of substrate-free perfusion, it was thought advisable to investigate the possibility of very rapid glycogen turnover occurring under these conditions. It is known that a fall in heart glycogen content causes a marked activation in glycogen synthetase I activity in the perfused rat heart (Larner *et al.*, 1968). Although it is generally accepted that striated muscle is unable to resynthesize major quantities of carbohydrate from pyruvate, and the activity of key enzymes of gluconeogenesis, particularly fructose 1,6-diphosphatase (Krebs & Woodford, 1965; Opie & Newsholme, 1967), is very weak in muscle, these measurements of enzyme activity were made with homogenates from normal rat hearts. It was considered worthwhile to ascertain whether any incorporation of radioactivity into glycogen from ^{14}C -pyruvate took place in the glycogen-depleted heart (see Table 1).

There was no net change in glycogen content during pyruvate perfusion of the glycogen-depleted heart; also the flux of radioactivity from pyruvate through glycogen was insignificant, when compared with the total pyruvate uptake (here measured by disappearance of radioactivity from the perfusate, which would underestimate the true uptake because of the perfusate ^{14}C lactate content).

^{14}C Amino acid incorporation by the glycogen-depleted perfused rat heart

Table 2 presents the results of experiments in which glucose-, glucose 6-phosphate- and glycogen-depleted rat hearts were perfused with either glucose or pyruvate as substrate, and the incorporation of ^{14}C -labelled amino acids into protein was measured. The experiments were initially performed with ^{14}C -glycine as the tracer, and then repeated with a mixture of ^{14}C -labelled amino acids.

It is apparent that insulin significantly stimulates amino acid incorporation into the protein of hearts perfused with pyruvate. In fact the insulin stimulation of ^{14}C glycine incorporation is greater with pyruvate than in the control glucose-perfused hearts. The significance of this latter finding is not clear, as with the amino acid mixture insulin stimulation is the same for glucose- and pyruvate-perfused hearts.

Table 1. *Glycogen concentrations and ^{14}C incorporation into glycogen in glycogen-depleted rat hearts perfused with $[3\text{-}^{14}\text{C}]\text{pyruvate}$*

All hearts were perfused for 35 min with substrate-free buffer (Langendorff preparation; 100 cmH₂O aortic pressure, flow-through). The experimental group were then perfused by recirculation with 10 ml of buffer containing $[3\text{-}^{14}\text{C}]\text{pyruvate}$ (10 mM, sp. radioactivity 91 $\mu\text{Ci/nmol}$) and insulin (5 munits/ml). Results are expressed as means $\pm$ S.E.M. (number of perfusions).

Glycogen content (μmol of glucose equiv./g dry wt.)				
Control (35 min of substrate-free perfusion only)	Pyruvate-perfused	^{14}C Pyruvate uptake ($\mu\text{mol}/30\text{ min per g dry wt.}$)	^{14}C Pyruvate incorporation into glycogen (nmol/30 min per g dry wt.)	^{14}C Pyruvate uptake (%)
31 $\pm$ 8 (3)	28 $\pm$ 7 (7)	156 $\pm$ 9	388 $\pm$ 46	0.25

Table 2. *Effect of insulin on incorporation of radioactive amino acids into protein of glucose-, glucose 6-phosphate- and glycogen-depleted perfused rat hearts*

Results are presented from two series of experiments, in which the incorporation of either ^{14}C glycine or of the ^{14}C -labelled amino acid mixture was measured. All hearts were pre-perfused for 35 min without recirculation at 100 cmH₂O aortic pressure with substrate-free Krebs–Henseleit buffer, before being switched to recirculation perfusion for 1 h. The recirculation buffer contained, in addition to the substrate indicated, 20 natural amino acids (each 50 μM) with either 4.5 μCi of $[\text{U-}^{14}\text{C}]\text{glycine}$ or 1.5 μCi of the ^{14}C -labelled amino acid mixture in 10 ml. Results are expressed as means $\pm$ S.E.M. (number of observations). The differences between insulin and basal incorporation are significant ($P < 0.05$).

Substrate	Insulin (munits/ml)	Amino acid incorporation		Insulin stimulation (% of basal incorporation)	
		Glycine (pmol/h per mg of protein)	Amino acid mixture (pCi/h per mg of protein)	Glycine	Amino acid mixture
5 mM-Glucose	—	61 $\pm$ 9 (6)	591 $\pm$ 160 (3)	51	61
5 mM-Glucose	5	92 $\pm$ 11 (6)	960 $\pm$ 99 (3)		
10 mM-Pyruvate	—	76 $\pm$ 5 (8)	636 $\pm$ 47 (9)	109	49
10 mM-Pyruvate	5	159 $\pm$ 12 (7)	951 $\pm$ 28 (9)		

Table 3. *Effect of 2-deoxy-D-glucose on the insulin-stimulated incorporation of radioactive amino acids into protein by the perfused rat heart*

All hearts were pre-perfused for 15 min without recirculation at 100 cmH₂O aortic pressure with substrate-free Krebs–Henseleit buffer before being switched to recirculation perfusion for 1 h. The recirculation buffer contained, in addition to the substrate indicated, 5 mM-2-deoxy-D-glucose, 20 amino acids (each 50 μM) and either 4.5 μCi of $[\text{U-}^{14}\text{C}]\text{glycine}$ or 1.5 μCi of ^{14}C -labelled amino acid mixture in 10 ml. (Results are presented of two series of experiments in which the incorporation of either ^{14}C glycine or of ^{14}C -labelled amino acid mixture was measured.) The results are expressed as means $\pm$ S.E.M. (number of perfusions). Differences between insulin-stimulated and basal incorporation are significant ($P < 0.05$).

Substrate	Insulin (munits/ml)	Amino acid incorporation		Insulin stimulation (% of basal incorporation)	
		Glycine (pmol/h per mg of protein)	Amino acid mixture (pCi/h per mg of protein)	Glycine	Amino acid mixture
5 mM-Glucose	—	73 $\pm$ 3 (4)	—	171	—
5 mM-Glucose	5	198 $\pm$ 10 (5)			
10 mM-Pyruvate	—	81 $\pm$ 3 (5)	673 $\pm$ 24 (5)	56	53
10 mM-Pyruvate	5	126 $\pm$ 7 (4)	1027 $\pm$ 38 (5)		

¹⁴C-labelled amino acid incorporation in the presence of 2-deoxyglucose

In another method to establish whether the insulin stimulation of ¹⁴C-labelled amino acid incorporation into protein depended on intracellular glucose metabolism, use was made of 2-deoxy-D-glucose, an inhibitor of glucose utilization.

When hearts were perfused with Krebs-Henseleit buffer containing 5 mM-2-deoxyglucose, they soon showed signs of failure and developed arrhythmias, diminished force of contraction and cardiac output in working hearts. Cardiac arrest occurred in 5–10 min. The addition of either pyruvate (10 mM) or of glucose (5 mM) to the 2-deoxyglucose-poisoned heart before final cardiac arrest rapidly reversed the failure, and the aortic output of working hearts and the general condition of the heart, as assessed by the physiological criteria used, returned to normal after 2–3 min.

The following experimental design was therefore adopted to measure ¹⁴C-labelled amino acid incorporation in the presence of 2-deoxyglucose. Hearts were pre-perfused for 15 min with substrate-free buffer to decrease intracellular glucose concentrations and then were switched to recirculation perfusion with 5 mM-2-deoxyglucose, amino acids and either glucose or pyruvate. These experiments were again performed with both [¹⁴C]glycine and the ¹⁴C-labelled amino acid mixture.

A comparison of the basal values for amino acid incorporation (i.e. in the absence of insulin) recorded in Tables 2 and 3 shows that 2-deoxyglucose did not significantly inhibit protein synthesis in the presence of either glucose or pyruvate. Although the stimulation of [¹⁴C]glycine incorporation was greater in

glucose-perfused hearts, insulin caused a significant increase in ¹⁴C-labelled amino acid incorporation in the pyruvate-perfused hearts.

Effect of insulin deficiency on ¹⁴C-labelled amino acid incorporation by the isolated perfused rat heart

The initial phase of the investigation established that, although quantitatively small in comparison with the insulin effect on glucose metabolism, the effect on amino acid incorporation could not be shown to be dependent on the presence of glucose. There have been reports of the impairment of amino acid incorporation in diaphragm muscle from alloxan-diabetic rats (Manchester & Young, 1960). *In vivo*, however, there are not any cardiac abnormalities in diabetics which are thought to be attributable to an impairment of protein synthesis as a direct consequence of insulin deficiency. To study the possible effect of insulin deficiency on protein synthesis in cardiac muscle, ¹⁴C-labelled amino acid incorporation was measured in hearts removed from animals rendered insulin-deficient by streptozotocin-induced diabetes in the absence and presence of insulin (Table 4). No difference was observed in the incorporation of [¹⁴C]glycine or of ¹⁴C-labelled amino acid mixture into heart protein between these insulin-deficient animals (with blood glucose concentrations of 450 ± 30 mg/100 ml) and normal controls. The insulin stimulation was unimpaired.

A study of protein synthesis in the rat heart previously treated with anti-insulin antiserum showed that no difference could be detected between this and the normal heart in amino acid incorporation (Table 4).

Table 4. Incorporation of ¹⁴C-labelled amino acid into protein by perfused hearts from insulin-deficient rats

Rat hearts (Langendorff preparations; 100 cmH₂O aortic pressure) were pre-perfused for 15 min without recirculation and then switched to recirculation perfusion for 1 h. The recirculation buffer (volume 10 ml) contained 20 amino acids (each 50 μM) and either 4.5 μCi of [U-¹⁴C]glycine or 1.5 μCi of ¹⁴C-labelled amino acid mixture. Glucose (5 mM) was present in pre-perfusion and recirculation fluids. Streptozotocin-treated animals were used 7 days after intravenous administration of 65 mg of streptozotocin/kg. Animals injected with serum were killed 1 h after intravenous administration of 0.75 ml. Results are expressed as means ± s.e.m. (number of perfusions). Differences between means similarly marked by asterisks (*, **) are not significant ($P > 0.05$).

	Insulin (munits/ml)	¹⁴ C-labelled amino acid incorporation	
		Glycine (pmol/h per mg of protein)	Amino acid mixture (pCi/h per mg of protein)
Normal	—	72 ± 9 (4)*	936 ± 29 (5)**
	5	138 ± 11 (4)	Not done
Streptozotocin-diabetic	—	61 ± 5 (5)*	898 ± 38 (6)**
	5	174 ± 14 (4)	Not done
Non-immune control serum	—		1095 ± 43 (3)**
Anti-insulin antiserum	—		987 ± 41 (5)**

Sucrose-gradient analysis of polyribosomes prepared from the hearts of normal and streptozotocin-diabetic rats

In addition to the report of diminished ^{14}C -labelled amino acid incorporation by incubated diaphragms from alloxan-diabetic rats, cited earlier, evidence has also been presented which suggests that ribosomes from the skeletal muscle of alloxan-diabetic rats show abnormal profiles on sucrose gradients and catalyse protein synthesis less efficiently in an assay system *in vitro* than do ribosomes from normal skeletal muscle (Wool *et al.*, 1968).

In view of the negative findings reported in the previous section, and the fact that alloxan in addition to its diabetogenic action exerts many other toxic side effects, we decided to extend the investigation to ribosomes isolated from cardiac muscle rendered diabetic by the action of the non-toxic streptozotocin.

Polyribosomes were prepared from the non-perfused hearts of normal and streptozotocin-diabetic rats. The E_{260}/E_{280} ratios were both approx. 1.7, corresponding to an RNA/protein ratio of 1.25 (Breuer & Florini, 1965); the yields were not significantly different.

The sedimentation profiles of ribosomes prepared from cardiac muscle from normal and diabetic rats are compared in Fig. 2. The position of the monosome peak was identified by comparison with a sample of rabbit reticulocyte monosomes [a gift from Dr. H. J.

Gould, prepared by the method of Arnstein *et al.* (1965)]. Limited ribonuclease digestion of muscle polyribosomes (performed by the addition at 0°C of ribonuclease A, $5\text{ }\mu\text{g}$ of rRNA/mg, to the ribosomal suspension immediately before layering on the sucrose gradients) caused disappearance of the more rapidly sedimenting u.v.-absorbing material, leaving essentially only the monoribosome peak. This confirmed that the heavy material sedimenting towards the bottom of the gradients consisted of ribosomes held together by RNA, and not a non-specific aggregation. The two patterns were indistinguishable. Visual comparison is, however, rather unsatisfactory, and an attempt to quantitate the amount of u.v.-absorbing material was made by measurement of the areas under the peaks. There was no significant difference in the percentage of u.v.-absorbing material in the polyribosome region in the two preparations.

Comparison of protein synthesis directed in vitro by polyribosomes from heart muscle of normal and diabetic rats

Rat heart-muscle polyribosomes stimulated incorporation into protein of radioactivity from ^{14}C -labelled amino acids. Fig. 3 compares the kinetics of incorporation and the Mg^{2+} -dependence for polyribosomes prepared from the hearts of normal and diabetic rats. The rates of ^{14}C -labelled amino acid

Fig. 2. Sucrose-gradient analysis of aggregation patterns of heart polyribosomes from normal (—) and streptozotocin-diabetic (---) rats

Approx. $75\text{ }\mu\text{g}$ of rRNA was layered on linear 15–30% (w/v) sucrose gradients. Details of centrifugation are given in the text. E_{254} values of the gradient were recorded in a 5 mm flow-cell with the ISCO u.v. analyser. Four representative preparations are shown in (a) and (b); the monomer peak is marked M .

Fig. 3. Comparison of (a) kinetics and (b) Mg^{2+} -dependence of amino acid incorporation directed by heart muscle ribosomes from normal (●, 78 μ g of rRNA) and streptozotocin-diabetic rats (○, 67 μ g of rRNA)

(a) Reaction mixtures were prepared and incubated at 37°C. At the times indicated samples were withdrawn and the radioactivity incorporated into protein was determined. (b) Standard reaction mixtures containing different amounts of magnesium acetate were prepared, polyribosomes were added and the mixtures incubated and analysed.

incorporation were not significantly different (6480 ± 315 and 6011 ± 393 c.p.m./mg of rRNA $\pm$ S.E.M. for five preparations from normal and diabetic rats respectively).

There was thus no difference, with regard to the parameters investigated, between cardiac polyribosomes from normal and streptozotocin-diabetic rats.

Our initial interpretation of the absence of any

defect in protein synthesis in the hearts of streptozotocin-diabetic rats was that the abnormalities reported by Wool *et al.* (1968) in the skeletal muscle of alloxan-diabetic rats were a consequence of the well-known toxic side effects associated with the use of alloxan (Lukens, 1948). Rannels *et al.* (1970) showed that although there are abnormalities in the synthesis of skeletal muscle protein by alloxan-diabetic rats, the hearts of these animals in fact show normal protein synthetic activity. These findings emphasize the individual metabolic responses of different muscle types. Rannels *et al.* (1970) suggest that there is some factor protecting protein synthesis in the diabetic heart of the animal, and their preliminary findings are that elevated concentrations of free fatty acids found in the diabetic may have such an effect.

Conclusion

Under the conditions of disturbed glucose metabolism investigated, and even in the virtual absence of intra- or extra-cellular glucose, the stimulating effect of insulin on amino acid incorporation still occurred to its full extent. This demonstrates the important fact that insulin can exert an effect on reactions not connected with glucose metabolism, and has obvious implications for any theory on the mechanism of insulin action.

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References

- Arnstein, H. R. V., Cox, R. A., Gould, H. J. & Potter, H. (1965) *Biochem. J.* **96**, 500–506
- Baillie, L. A. (1960) *Int. J. Appl. Radiat. Isotop.* **8**, 1–6
- Breuer, C. B. & Florini, J. R. (1965) *Biochemistry* **4**, 1544–1550
- Chain, E. B., Mansford, K. R. L. & Opie, L. H. (1969) *Biochem. J.* **115**, 537–546
- Chen, S. C. & Young, V. R. (1968) *Biochem. J.* **106**, 61–67
- Crass, M. F., McCaskill, E. S., Shipp, J. C. & Murthy, V. K. (1971) *Amer. J. Physiol.* **220**, 428–435
- De Schepper, P. J., Toyoda, M. & Bessman, S. P. (1965) *J. Biol. Chem.* **240**, 1670–1674
- Earl, D. C. N. & Morgan, H. E. (1968) *Arch. Biochem. Biophys.* **128**, 460–469
- Fisher, R. B. & Williamson, J. R. (1961) *J. Physiol. (London)* **158**, 86–101
- Kipnis, D. M. & Cori, C. F. (1959) *J. Biol. Chem.* **234**, 171–177
- Krahl, M. E. (1952) *Science* **116**, 524
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–36
- Krebs, H. A. & Woodford, M. (1965) *Biochem. J.* **94**, 436–445
- Langendorff, O. (1895) *Pflügers Arch. Gesamte Physiol.* **61**, 291–332

- Larner, J., Villar-Palasi, C., Goldberg, N. D., Bishop, J. S., Huijing, F., Weager, J. I., Sasko, H. & Brown, N. B. (1968) *Contr. Glycogen Metab., Proc. Meet. FEBS 4th* 1-18
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Lukens, F. D. W. (1948) *Physiol. Rev.* **28**, 304-329
- Manchester, K. L. & Young, F. G. (1958) *Biochem. J.* **70**, 353-358
- Manchester, K. L. & Young, F. G. (1960) *Biochem. J.* **77**, 386-394
- Mansford, K. R. L. (1967) *Diabetes* **16**, 475-477
- Marks, V. & Lloyd, K. (1963) *Proc. Ass. Clin. Biochem.* **2**, 176-179
- Moloney, P. J. & Coval, M. (1955) *Biochem. J.* **59**, 179-185
- Morgan, H. E., Henderson, M. J., Regen, D. M. & Park, C. R. (1961) *J. Biol. Chem.* **236**, 253-261
- Ogur, M. & Rosen, G. (1950) *Arch. Biochem.* **25**, 262-276
- Olson, R. E. & Hoeschen, R. J. (1967) *Biochem. J.* **103**, 796-801
- Opie, L. H. & Newsholme, E. A. (1967) *Biochem. J.* **103**, 391-399
- Opie, L. H., Mansford, K. R. L. & Owen, P. (1971) *Biochem. J.* **124**, 475-490
- Rannels, D. E., Jefferson, L. S., Hjalmarson, A. C., Wolpert, E. B. & Morgan, H. E. (1970) *Biochem. Biophys. Res. Commun.* **40**, 1110-1116
- Scales, B. (1967) *Int. J. Appl. Radiat. Isotop.* **18**, 1-6
- Shipp, J. C., Matos, O. E., Knizley, H. & Crevasse, L. E. (1964) *Amer. J. Physiol.* **207**, 1231-1236
- Sinex, F. M., MacMullen, J. & Hastings, A. B. (1952) *J. Biol. Chem.* **198**, 615-619
- von der Decken, A. & Campbell, P. N. (1962) *Biochem. J.* **82**, 448-454
- Warburg, O. & Christian, W. (1942) *Biochem. Z.* **310**, 384-421
- Wick, A. N., Drury, D. R., Nakada, H. I. & Wolfe, J. B. (1957) *J. Biol. Chem.* **224**, 963-969
- Wool, I. G. & Krah, M. E. (1959) *Amer. J. Physiol.* **196**, 961-964
- Wool, I. G. & Manchester, K. L. (1962) *Nature (London)* **193**, 345-346
- Wool, I. G., Stirewalt, W. S., Kurihara, K., Low, R. B., Bailey, P. & Oyer, O. (1968) *Recent Progr. Horm. Res.* **24**, 139-208