The Influence of Insulin on the Metabolism of Glucosamine in the Isolated Rat Diaphragm Muscle

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1. The influence of insulin on the metabolism of [$1\text{-}^{14}\text{C}$]glucosamine by diaphragm muscle from normal rats and rats rendered diabetic with streptozotocin has been studied. 2. The glucosamine was converted into glucosamine 1-phosphate, glucosamine 6-phosphate, glycogen, lactate and small amounts of other unidentified intermediates. 3. Insulin increased the incorporation of ^{14}C into glycogen in both the normal and diabetic muscle, but did not increase the formation of the glucosamine phosphate esters. 4. The ^{14}C content in the glycogen was present partly as glucose and partly as glucosamine; there was significantly more [^{14}C]glucose in the glycogen of the diabetic muscle than in that of the normal muscle.

It was first shown by Nakada, Morita & Wick (1955) that insulin stimulated glucosamine uptake by the isolated rat diaphragm muscle. From some preliminary experiments discussed previously by the present authors (Beloff-Chain & Rookledge, 1968), it appeared that insulin stimulated the phosphorylation of glucosamine to glucosamine 6-phosphate and glucosamine 1-phosphate. This conclusion was based on measurements of the ^{14}C -labelled intermediates separated by paper chromatography present in aqueous ethanolic extracts of muscle after incubation with [^{14}C]glucosamine. It was decided to make a more detailed study of the fate of [$1\text{-}^{14}\text{C}$]glucosamine in muscle by studying the intermediates formed both in aqueous ethanolic extracts of the tissue and in the incubation medium, by using the technique of column chromatography separation. Further, it was found that when the total muscle glycogen was isolated, significant amounts of ^{14}C had been incorporated from the [^{14}C]glucosamine substrate. The results of these experiments are reported in the present paper.

METHODS

Animals. Male Sprague-Dawley Rats weighing 100-130 g were used. Streptozotocin-diabetes was produced as previously described (Beloff-Chain & Rookledge, 1968). All animals were starved for 16-20 h before an experiment.

Materials. [$1\text{-}^{14}\text{C}$]Glucosamine (sp. radioactivity 3.1 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Hexokinase and phosphoglucomutase were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. The Dowex resins prepared by Bio-Rad Laboratories were obtained from Micro Bio Laboratories, London W.11, U.K.

[$1\text{-}^{14}\text{C}$]Glucosamine 6-phosphate was prepared (P. Betto & L. Longinotti, personal communication) by incubating 2.5 mmol of [$1\text{-}^{14}\text{C}$]glucosamine (1 mCi/

0.84 mmol) for 1 h at 37°C in the presence of hexokinase (approx. 200 μg) and 5 mmol of ATP (sodium salt) in 30 ml of 0.025 M- NaHCO_3 buffer, pH 8, containing 0.017 mol of MgCl_2 .

At the end of the incubation period the reaction mixture was heated for 5 min in boiling water and acidified to pH 3 with HCl. The glucosamine 6-phosphate was then separated from the unchanged glucosamine by column chromatography, as described below. Further purification of the eluted glucosamine 6-phosphate was carried out on a column (12 cm $\times$ 1 cm) of anionic resin Dowex 1 (X4; 200-400 mesh; formate form) and eluted with approx. 100 ml of 0.1 M-formic acid. The latter was then removed by vacuum distillation.

[$1\text{-}^{14}\text{C}$]Glucosamine 1-phosphate was prepared from glucosamine 6-phosphate by the action of phosphoglucomutase, as described by Brown (1953).

Incubation of diaphragm muscle. The muscle was removed, weighed and incubated as previously described (Beloff-Chain & Rookledge, 1968) with glucosamine instead of glucose as substrate. One hemidiaphragm was incubated in the absence of insulin and one in the presence of insulin (0.01 U/ml). The glucosamine concentration was 10 mM and approximately 5 μCi of [$1\text{-}^{14}\text{C}$]glucosamine was added.

Column chromatography. Separation of glucosamine, glucosamine 1-phosphate and glucosamine 6-phosphate was carried out as described by P. Betto & L. Longinotti (personal communication). A 0.2 ml volume of the tissue extract or incubation medium was acidified to pH 3 with M-HCl and passed through a column (1 cm $\times$ 12 cm) of Dowex 50 (X8, 200-400 mesh; H^+ form). The column was eluted with 1 mM-HCl at a velocity of 0.3 ml/min. The eluate contained two radioactive peaks: in the first 60 ml of eluate glucosamine 1-phosphate was present and in the second 40 ml of eluate glucosamine 6-phosphate. Glucosamine was eluted from the column with 100 ml of 0.5 M-HCl. The first radioactive peak was shown by paper chromatography to contain in addition to glucosamine 1-phosphate, glycogen, lactate and small amounts of other unidentified intermediates. This is referred to as fraction

A. To determine the ^{14}C content of glucosamine 1-phosphate the concentrated fraction A eluate was hydrolysed for 30 min in M-HCl at 100°C , so that the glucosamine 1-phosphate was quantitatively hydrolysed to glucosamine which was then separated by column chromatography as described above.

Control experiments carried out with purified materials showed that recovery from the columns was at least 90%.

Paper chromatography. Purified samples of glucosamine 1-phosphate, glucosamine 6-phosphate and glucosamine were separated on paper by descending chromatography in two different solvents: (a) picric acid-2-methylpropan-2-ol-water (49:80:20, by vol.), (b) ethanol-ammonium acetate (5:2, v/v), pH 7.5 (Paladini & Leloir, 1952). The R_F values were determined after radioautography.

Chemical analysis. Glucosamine was determined by the method described by Elson & Morgan (1933) as modified by Neuhaus & Letzring (1957). Phosphorus analysis was carried out with the method of Berenblum & Chain (1938).

Methods used for the identification of glucosamine, glucosamine 1-phosphate and glucosamine 6-phosphate separated by column chromatography from tissue extracts and incubation media. (a) The substances isolated from the experimental material behaved identically on paper chromatography (see above) with glucosamine and purified glucosamine 1-phosphate and glucosamine 6-phosphate.

(b) The identity of glucosamine 1-phosphate was confirmed by quantitative conversion into glucosamine (determined chemically) after 30 min hydrolysis in M-HCl at 100°C .

(c) The identity of glucosamine 6-phosphate was confirmed by (i) showing that the substance contained 1 mol of acid-soluble phosphate which was not labile after 30 min hydrolysis in M-HCl at 100°C ; (ii) alkaline phosphatase transformed this substance into free glucosamine; (iii) the substance gave the same colour reaction as free glucosamine.

Preparation of samples and radioactive measurements. The method of preparation of aqueous ethanolic tissue extracts was identical with that previously described (Beloff-Chain *et al.*, 1955). The intermediates found in the tissue extracts and the incubation medium were separated by column chromatography and identified as described above. After separation on columns the eluents were concentrated and the radioactivity determined in a liquid-scintillation counter, after addition of the scintillation fluid (Scales, 1967). The efficiency of counting was determined by the channels-ratio method (Baillie, 1960).

The initial ^{14}C content of the $[1-^{14}\text{C}]\text{glucosamine}$ present in the medium was determined by counting the radioactivity in portions of the latter in a liquid-scintillation counter and the specific radioactivity of the glucosamine was then calculated: the results are expressed as nmol of glucosamine converted into the intermediates measured.

As shown in Table 1 the conversion of glucosamine into the phosphate esters was calculated from the sum of the values in the extracts and media, whereas the conversion of glucosamine into the intermediates that separated in fraction A are expressed separately for extract and media.

Glycogen. In one series of experiments the total glycogen of the muscle was isolated by the method of Walaas & Walaas (1955) and hydrolysed for 3 h at 100°C in 2M-HCl . The hydrolysate was neutralized to pH 4. The radio-

Table 1. Influence of insulin on the metabolism of glucosamine in the isolated rat diaphragm muscle

	Glucosamine 1-phosphate, lactate, glycogen and other unidentified intermediates						Glucosamine 1-phosphate			Glucosamine 6-phosphate			Glucosamine		
	Extract		Medium		No insulin	With insulin	Extract and Medium		No insulin	With insulin	Extract and Medium		No insulin	With insulin	Extract
	No insulin	With insulin	No insulin	With insulin			No insulin	With insulin			No insulin	With insulin			
Rats															
Normal	151 $\pm$ 30	257* $\pm$ 54	339 $\pm$ 13	285 $\pm$ 12	80 $\pm$ 5	85 $\pm$ 7	103 $\pm$ 14	93 $\pm$ 10	337 $\pm$ 23	414 $\pm$ 31	381 $\pm$ 15	369 $\pm$ 17	359 $\pm$ 35	341 $\pm$ 22	
Streptozotocin-treated	115 $\pm$ 10	202† $\pm$ 20	407 $\pm$ 25	430† $\pm$ 29							425 $\pm$ 30	399 $\pm$ 57			

* Influence of insulin probably significant ($P = 0.1$) but a very high biological variation.

† Influence of insulin significant ($P = 0.01$).

‡ Difference between normal and diabetic significant ($P = 0.001$).

activity in a portion of the latter was counted in a liquid-scintillation counter as described above to determine the total ^{14}C present in the glycogen. Another portion of the hydrolysate was put on to a column (1 cm $\times$ 12 cm) of Dowex 50 (X8, 200–400 mesh; H^+ form). The ^{14}C content of the first eluate, obtained with 100 ml of 1 M-HCl, was shown by paper chromatography (Beloff-Chain & Rookledge, 1968) to be present as glucose. The second fraction eluted, with 100 ml of 0.5 M-HCl, was shown to contain ^{14}C as glucosamine, identified as described above by paper chromatography.

RESULTS AND DISCUSSION

The results given in Table 1 show that insulin increased the ^{14}C content of fraction A (containing glucosamine 1-phosphate, lactate, glycogen and other unidentified intermediates of the tissue extract). Although the statistical significance of the insulin effect with normal animals was not very high this was due to the wide variation in the ^{14}C incorporation into this fraction. However, the effect of insulin was observed in each of the ten experiments. The results show that this effect was not due to an increase in ^{14}C of glucosamine 1-phosphate. Further, no insulin effect was observed on the ^{14}C content of glucosamine 6-phosphate nor on free glucosamine in the tissue. Similar results were found with diaphragm muscle from streptozotocin-diabetic rats (Table 1). In this group the values obtained for the ^{14}C content of fraction A isolated from the media were significantly higher in the streptozotocin-treated animals than in the normal animals. The results in Table 1 show that glucosamine metabolism in muscle is slower than that of glucose. Thus under similar conditions approx. 1500 nmol of glucose was metabolized by 100 mg of muscle in 90 min (Beloff-Chain & Rookledge, 1968) whereas about 800 nmol of glucosamine was metabolized under similar conditions (see Table 1). As was to be expected there was a much higher accumulation of the phosphate esters of glucosamine than is obtained with glucose as substrate, the values being approximately 400 nmol

and 50 nmol respectively. The ratio of glucosamine 1-phosphate to glucosamine 6-phosphate was about 1:4, as shown in Table 1. This is in good agreement with the findings of Brown (1953), which showed that when glucosamine 6-phosphate was incubated with phosphoglucomutase at pH 7.5, an equilibrium of glucosamine 1-phosphate to glucosamine 6-phosphate of 1:4 was attained.

The results given in Table 2 show that the incorporation of ^{14}C from [$1\text{-}^{14}\text{C}$]glucosamine into glycogen was significantly increased by insulin. This is in agreement with the findings of Chambaut, Eboué-Bonis, Hanoune & Clauser (1969). The effect was shown both in normal muscle and in muscle from diabetic rats. The ^{14}C in the glycogen was shown to be partly present as glucose and partly as unchanged glucosamine. This suggests that part of the glucosamine is deaminated to glucose in muscle. It was noteworthy that both the incorporation of the glucose and the glucosamine were significantly stimulated by insulin, although no significant effect of insulin on the phosphorylation of glucosamine to glucosamine 6-phosphate and glucosamine 1-phosphate was observed. The incorporation of glucose, formed from glucosamine, into glycogen is significantly increased in the muscle from diabetic rats, although the glucosamine incorporation into glycogen did not differ significantly in the two groups of animals. The insulin effect on fraction A, shown in Table 1, is probably due to this effect on the incorporation into glycogen, as any glycogen present in the tissue extract would separate out in this fraction. In this connection it is of interest that Maley, McGarrahan & Del Giacco (1966) have shown that glucosamine can be incorporated into liver glycogen. Further, they have found that glycogen synthetase partially purified from rat muscle was active with uridine diphosphate glucosamine, transferring glucosamine into glycogen *in vitro*.

The observation that the incorporation of glucosamine into glycogen is increased by insulin is further

Table 2. Influence of insulin on the incorporation of glucosamine into glycogen on rat diaphragm muscle

Results are expressed as nmol of glucosamine converted into total glycogen and into glucose and glucosamine of hydrolysed glycogen/100 mg of tissue. Incubation conditions were as for Table 1. Mean values $\pm$ S.E.M. of ten experiments with normal rats and five experiments with streptozotocin-diabetic rats are given.

Rats	Total glycogen		Glucose from hydrolysed glycogen		Glucosamine from hydrolysed glycogen	
	No insulin	With insulin	No insulin	With insulin	No insulin	With insulin
Normal	82 $\pm$ 7	147* $\pm$ 11	53 $\pm$ 4	76* $\pm$ 4	32 $\pm$ 2	44* $\pm$ 3
Streptozotocin-treated	103† $\pm$ 5	157* $\pm$ 5	88† $\pm$ 3	133†* $\pm$ 13	39 $\pm$ 3	49* $\pm$ 2

* Influence of insulin significant ($P = 0.01$).

† Difference between normal and diabetic significant ($P = 0.01$).

evidence that in muscle the effect of insulin is specifically on the pathway of glycogen synthesis.

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