Enzymes Concerned in the Synthesis of Glycogen from Glucose in the Brown Adipose Tissue

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Under normal conditions glycogen cannot be detected in adipose tissue. When animals are first starved and then fed on a carbohydrate diet, glycogen appears temporarily in this tissue (see Hausberger & Neuenschwander-Lemmer, 1939). Tuerkischer & Wertheimer (1942) investigated the phenomenon in detail and found that, in the rat, this deposition of glycogen was particularly marked in the brown adipose tissue, which in this species is found between the scapulae as a light-brown butterfly-shaped mass, having the appearance of a gland. Glycogen concentrations as high as 6 g./100 g. wet tissue were sometimes observed. Fawcett (1948) has recently confirmed these observations. Mirski (1942) showed that brown adipose tissue of the rat contained phosphorylase and phosphoglucomutase and suggested that glycogen might be synthesized from glucose by a similar mechanism to that involving hexokinase and these enzymes in muscle and liver.

Cori & Green (1943) and Cori & Cori (1945) showed that suitable preparations of muscle and spleen could convert crystalline muscle phosphorylase *a* into phosphorylase *b*, which was active only in the presence of adenylic acid. This effect of muscle and spleen was attributed to the presence of a 'PR' (prosthetic group-removing) enzyme, which was considered to remove a prosthetic group from phosphorylase *a*. Cori & Cori (1943) observed that the presence of extracts of liver or heart resulted in the synthesis by phosphorylase *a* from glucose-1-phosphate of a polysaccharide similar to glycogen, while in the absence of such extracts there resulted a polysaccharide similar to amylose. They believed such extracts contained a 'Supplementary Enzyme' which, while phosphorylase *a* was forming, α-1:4-glucoside linkages catalysed the introduction of branch points formed by α-1:6-glucoside linkages.

The work described in this paper has demonstrated the presence of hexokinase in the brown adipose tissue of the rat, and has confirmed the presence of phosphorylase and phosphoglucomutase; the properties of the latter two enzymes have been investigated in more detail. Brown adipose-tissue extracts have also been found to possess PR enzyme and Supplementary Enzyme activities.

EXPERIMENTAL

Reagents

Veronal acetate buffers (0·034 M) were prepared according to Gortner (1949).

Adenosinetriphosphate (ATP). The ATP used (Boots Pure Drug Co. Ltd.) was 80% pure according to its content of acid-labile P (7 min. in N-HCl at 100°). A 0·05 M-ATP solution of the Na salt was prepared from the Ba salt.

Adenylic acid was prepared from dibarium ATP by the method of Kerr (1941).

Glucose-1-phosphate was prepared enzymically from potato starch by a modification of the method of Hanes (1940).

Cysteine HCl was obtained from Lights Ltd. Sodium β-glycerophosphate, sodium fluoride, α-methyl-D-glucoside and phlorrhizin were British Drug Houses (B.D.H.) Laboratory grade. All other chemicals used were B.D.H. Analar grade.

Aluminium hydroxide C_γ suspensions were prepared by the method of Bertho & Grassmann (1938).

Crystalline phosphorylase a. Suspensions were prepared according to Cori, Cori & Green (1943).

Glycogen was prepared from rabbit liver by the method of Somogyi (1934).

Analytical methods

Glucose was determined by the photometric method of Nelson (1944) using the Somogyi (1945) copper reagent.

Inorganic phosphorus was determined according to Fiske & Subbarow (1925) modified by Umbreit, Burris & Stauffer (1945).

Glycogen was determined by the method of Colowick & Sutherland (1942).

Preparations of adipose tissue extracts

Wistar rats were used throughout. All extracts investigated for enzyme activity were prepared as quickly as possible after killing the animal by a blow on the head. The brown adipose tissue is supported between the scapulae by strands of muscle which would contain the enzymes being sought in the tissue; particular care was therefore taken to remove all of this muscle before preparing the extracts. The brown adipose tissue was freed from white adipose tissue and stored on ice until sufficient material had been dissected out. After the fat had solidified it became much lighter in colour, and any remaining muscle could be distinguished as darker, translucent material which was easily removed. All subsequent operations were carried out in the cold room at 5° with ice-cold reagents.

Brown adipose-tissue extracts for hexokinase investigations were prepared from approximately 6·5 g. of tissue by mincing with scissors and grinding at 0° in a mortar with 10 ml. glass-distilled water. The material was centrifuged for 10 min. at 2000 rev./min., the opalescent supernatant separated from the superimposed layer of fat, and employed without further treatment. The other enzyme activities were investigated in a crude extract prepared by grinding the brown adipose tissue with sand in a mortar and extracting for 10 min. with twice its weight of water. After centrifuging for 10 min. at 2000 rev./min. the supernatant was separated off. This crude tissue extract was used without further purification for the experiments on phosphoglucomutase.

Purification of phosphorylase. To the crude brown adipose-tissue extract 0·5 vol. of C_γ aluminium hydroxide suspension was added and the mixture allowed to stand at 0° for 30 min. The aluminium hydroxide was centrifuged off, washed twice with distilled water and the phosphorylase eluted for 1 hr. at room temperature with 1 vol. 0·25 M-sodium β-glycerophosphate. The mixture was centrifuged and the residue washed with 1 vol. 0·25 M-sodium β-glycerophosphate. The united eluate and washing solution was incubated at 30° for 20 min. after the addition of $\frac{1}{3}$th vol. 0·3 M-cysteine hydrochloride, pH 6·75 (Cori, Cori & Green, 1943).

Separation of phosphorylase from PR enzyme. All procedures were performed as rapidly as possible in the cold room at 5°. A crude extract of brown adipose tissue, prepared as previously described, was adjusted to pH 5·9 with N-HCl. After centrifuging off the precipitate which contained the PR enzyme, the supernatant was treated with C_γ aluminium hydroxide suspension. The phosphorylase preparation obtained from the aluminium hydroxide by elution with 0·25 M-sodium β-glycerophosphate as described above was investigated for phosphorylase activity with and without added adenylic acid.

PR enzyme. The precipitate referred to above and containing the PR enzyme was centrifuged, washed once with water and suspended in water (2 ml./g. tissue). After addition of N-KOH to pH 8·5, the mixture was frozen and allowed to thaw. The precipitate was separated by centrifugation, the supernatant adjusted to pH 6·7 and investigated for PR enzyme activity (cf. Cori & Cori, 1945).

Supplementary Enzyme was prepared by a modification of the method of Cori & Cori (1943). Saturated $(NH_4)_2SO_4$ (0·7 vol.) at pH 7·0 was added to a PR enzyme-free phosphorylase preparation (see above); the precipitated protein containing the Supplementary Enzyme was centrifuged, dissolved in distilled water (0·5 ml./g. tissue) and dialysed overnight against distilled water at 4–5°. This solution formed the Supplementary Enzyme preparation.

Determination of enzyme activity

Hexokinase was determined by the method of Colowick, Cori & Slein (1947) in which the glucose utilized on incubation of the enzyme solution with glucose and ATP at 28° is measured.

Phosphoglucomutase. Glucose-1-phosphate (1-ester) is completely hydrolysed by heating with N-H_2SO_4 at 100° for 10 min., whereas glucose-6-phosphate (6-ester) is unaffected. The conversion of 1-ester to 6-ester was, therefore, determined from the decrease in easily hydrolysable phosphate (Najjar, 1948).

Phosphorylase was determined from the percentage of inorganic P liberated by the enzyme solution from 1-ester, in the presence of adenylic acid to activate phosphorylase *b*, of NaF to inhibit phosphoglucomutase and phosphatase, and of glycogen to activate the phosphorylase (Cori *et al.* 1943).

PR enzyme. Crystalline muscle phosphorylase *a* was incubated at 25° with the PR enzyme preparation for 2 hr., samples being removed every 30 min. for determination of phosphorylase activity in the presence and in the absence of 0·3 mM-adenylic acid (Cori & Green, 1943).

Synthesis of polysaccharide by brown adipose-tissue extract. A crude extract of brown adipose tissue (1 ml.) containing 0·1 ml. 0·3 M-cysteine-glycerophosphate buffer (pH 6·75) was added to 0·5 ml. 0·28 M-1-ester (pH 6·75), 0·2 ml. 9 mM-adenylic acid, 0·1 ml. 2% glycogen and 0·2 ml. 0·2 M-NaF. After incubation for 1 hr. at 30° the reaction was stopped by the addition of 2·0 ml. 30% NaOH, and the polysaccharide determined as glycogen. In a separate experiment, after heating the reaction mixture with the NaOH, 1·3 vol. ethanol (95%) were added, the mixture boiled for a few seconds and the precipitated polysaccharide allowed to settle overnight. The mixture was then centrifuged and the polysaccharide dissolved in 1·0 ml. of water from which it was reprecipitated by the addition of 1·3 vol. of ethanol. This procedure was repeated twice, the polysaccharide washed with absolute ethanol, then with ether and finally dried *in vacuo* over anhydrous $CaCl_2$.

Supplementary Enzyme was assumed to be present in a tissue extract when the addition of that extract to a phosphorylase test system resulted in an increase in the phosphorylase activity and in the formation of a polysaccharide staining brown with iodine (Cori & Cori, 1943). The Supplementary Enzyme preparation (0·4 ml.) was added to 0·4 ml. 0·1% glycogen, 0·2 ml. 0·14 M-1-ester (pH 6·75) and 1 ml. of a solution of crystalline phosphorylase *a* in 0·03 M-cysteine-hydrochloride-glycerophosphate buffer (pH 6·75). During incubation at 30° liberation of inorganic P was determined on suitable samples as for phosphorylase activity. At the end of the reaction period the stain of the synthetic polysaccharide on addition of dilute I_2 solution was observed. Controls were also set up without added glycogen and also using boiled Supplementary Enzyme preparation. In a separate experiment using five times the above amounts the inorganic phosphate liberated indicated the conversion of 60% of the 1-ester to polysaccharide in 2·5 hr. Solid NaOH (1·5 g.) was added and the mixture heated at 100° for 30 min. The polysaccharide was then isolated as described above. The yield was 10 mg. The solution of this material in water was opalescent and stained brown with I_2. On hydrolysis with N-HCl, 4·0 mg. yielded 4·26 mg. of reducing sugar calculated as glucose.

The ability of the polysaccharide synthesized by phosphorylase *a* in the presence of the Supplementary Enzyme to prime a second phosphorylase system from which glycogen had been omitted was also investigated. The previous experiment was repeated and the incubated reaction mixture heated at 100° for 2 min. to inactivate the enzymes. After cooling, 0·4 ml. of the reaction mixture was added to 0·2 ml. 0·14 M-1-ester, 0·4 ml. water and 1 ml. of a solution of crystalline phosphorylase *a* in 0·03 M-cysteine-glycerophosphate buffer (pH 6·75) and the mixture incubated at 30°. Inorganic P liberation was determined as for phosphorylase activity. A control was set up in which the initial Supplementary Enzyme reaction mixture was heated at 100° before incubation.

RESULTS

Hexokinase

In the investigations on hexokinase activity the glucose content of the reaction mixture was not significantly changed in the absence of ATP, but in the presence of ATP two different brown adipose-tissue extracts resulted in glucose utilization of 475 and 438 μg./10 min./ml. of tissue extract. These values were of the same order as was obtained with similar extracts of muscle. The hexokinase activity of brown adipose tissue showed no definite optimum activity over the pH range 6·6–8·8. These results are in agreement with those of van Heyningen (1941) for yeast hexokinase and those of Smith (1949) for muscle hexokinase.

Phosphoglucomutase

Fig. 1 shows that in the presence of added magnesium sulphate 97·5% of the glucose-1-phosphate had been converted into glucose-6-phosphate at equilibrium. The activity was lower

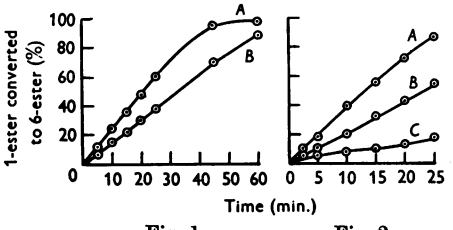

Fig. 1. Fig. 2.

Fig. 1. Phosphoglucomutase activity of 1 ml. crude extract of brown adipose tissue incubated at 30° with (*A*) 7 mM-1-ester, 2 mM-$MgSO_4$ and 25 mM-cysteine hydrochloride; pH 7·5. Total vol. = 2 ml. (*B*) $MgSO_4$ omitted.

Fig. 2. The effect of NaF on phosphoglucomutase activity of 1 ml. of a crude extract of brown adipose tissue incubated at 30° with (*A*) 7 mM-1-ester, 2 mM-$MgSO_4$ and 25 mM-cysteine hydrochloride; pH 7·5. Total vol. = 2 ml. (*B*) 1 mM-NaF added. (*C*) 3 mM-NaF added.

when the reaction mixture contained no magnesium ions other than those present in the tissue extract. Fig. 2 illustrates the inhibition of the phosphoglucomutase activity of extracts of brown adipose tissue by sodium fluoride; inhibition in the presence of 3 mM-sodium fluoride is almost complete. The initial velocity of the reaction, as measured by the amount of 6-ester phosphorus formed during the first 10 min. was plotted against the 1-ester concentration (Fig. 3). The enzyme activity was reduced at concentrations of substrate higher than 10 mM with crude aqueous extracts of adipose-tissue enzyme and above 20 mM with similar extracts of rat muscle. An apparent inhibition resulting from phosphorylase

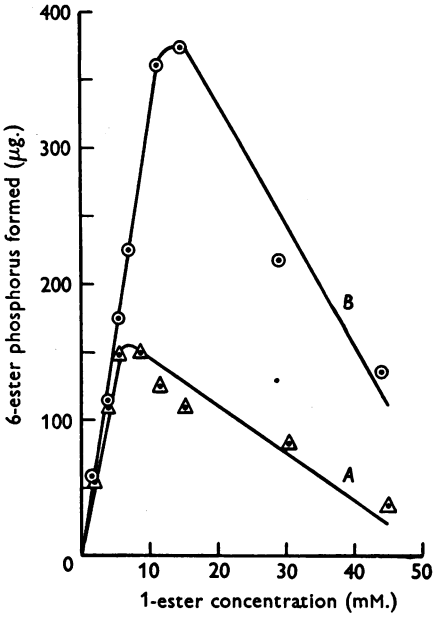

Fig. 3. Phosphoglucomutase activity expressed as μg. of 6-ester P formed in 10 min./1·0 ml. of reaction mixture when 1 ml. crude extract of brown adipose tissue (*A*) and rat muscle (*B*) was incubated at 30° with 2 mM-$MgSO_4$, 25 mM-cysteine hydrochloride and different concentrations of 1-ester; pH 7·5. Total vol. = 2 ml.

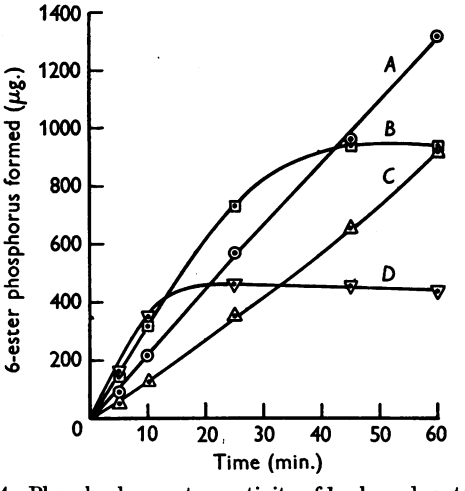

Fig. 4. Phosphoglucomutase activity of 1 ml. crude extract of brown adipose tissue incubated at 30° with 2 mM-$MgSO_4$, 25 mM-cysteine hydrochloride and different concentrations of 1-ester; pH 7·5. Total vol. = 2 ml. (*A*) With 31·6 mM-1-ester; (*B*) with 15·9 mM-1-ester; (*C*) with 47·7 mM-1-ester; (*D*) with 7·5 mM-1-ester.

or phosphatase activity becoming manifest at the higher concentrations of substrate was excluded because no inorganic phosphate was liberated. Fig. 4 shows the formation of 6-ester at different

1-ester concentrations. After 200 μg. of 6-ester phosphorus had been formed the reaction rate (as given by the slope of the curve) at a concentration of 7·55 mM-1-ester was much greater than that when the substrate concentration was 47·75 mM. The inhibition, therefore, appears due to the 1-ester itself rather than to the formation of 6-ester. The mathematical criteria of substrate inhibition suggested by Lineweaver & Burke (1934), case III, were applied to the curves in Fig. 3, but with inconclusive results.

Phosphorylase

In agreement with the observations of Cori *et al.* (1943) on muscle phosphorylase, the phosphorylase activity of purified brown adipose-tissue extracts show little or no phosphorylase activity in the absence of added cysteine. Fig. 5 shows that the

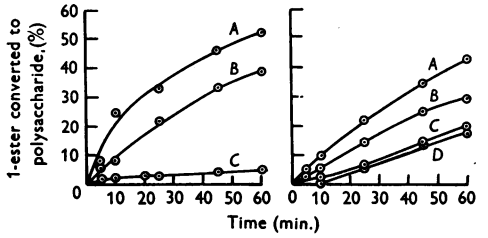

Fig. 5.

Fig. 6.

Fig. 5. Effect of glycogen concentration on the phosphorylase activity of 1 ml. brown adipose-tissue eluate containing 0·25 M-sodium β-glycerophosphate, incubated at 30° with 14 mM-1-ester, 0·3 mM-adenylic acid and 15 mM-cysteine hydrochloride; pH 6·75. Total vol. = 2 ml. (*A*) In the presence of 0·5 % glycogen; (*B*) in the presence of 0·05 % glycogen; (*C*) in the presence of 0·005 % glycogen.

Fig. 6. Phosphorylase activity of 1 ml. brown adipose-tissue eluate in the presence of inhibitors. Extract incubated at 30° with 14 mM-1-ester, 15 mM-cysteine hydrochloride, 0·3 mM-adenylic acid, 1 % glycogen; pH 6·75. Total vol. = 2 ml. (*A*) With no inhibitor; (*B*) with 0·05 M-α-methyl-D-glucoside; (*C*) with 0·05 M-glucose; (*D*) with 2·6 mM-phlorrhizin.

phosphorylase activity of brown adipose-tissue eluate is dependent on the concentration of added glycogen. Significant amounts of inorganic phosphate were not liberated in the absence of glycogen, showing that the inorganic phosphate had not been liberated by the successive action of phosphoglucomutase and phosphatase. The phosphorylase from brown adipose tissue thus appeared to have been freed from phosphoglucomutase and phosphatase by the absorption on C_γ aluminium hydroxide. Fig. 6 shows that the phosphorylase in brown adipose tissue is inhibited by glucose, phlorrhizin and α-methyl-D-glucoside.

PR enzyme

Table 1 shows the phosphorylase activity, in the presence and in the absence of adenylic acid, of crystalline muscle phosphorylase before and after incubation at pH 6·75 with the PR enzyme preparation from brown adipose tissue. The activities are

Table 1. *The activity of phosphorylase* a *after incubation with PR enzyme from brown adipose tissue*

(0·1 ml. suspension crystalline phosphorylase *a*, 0·5 ml. 0·3 M-cysteine hydrochloride (pH 6·75), 4·0 ml. PR enzyme preparation, 0·5 ml. water. Control contained water instead of PR enzyme. Incubated at 25° for 2 hr. Samples tested for phosphorylase activity with and without 0·3 mM-adenylic acid.)

	Initial activity* in the absence of PR enzyme		Activity after 30 min. incubation† with PR enzyme	
	With adenylic acid	Without adenylic acid	With adenylic acid	Without adenylic acid
1-ester converted in 5 min. (%)	57	31	50	2·1
‡$K \times 10^3$	108	43	85	2·3
Activity (%)	100	40	79	2·1

* The phosphorylase activity in the absence of PR enzyme remained unchanged during the 2 hr. incubation.
† During the next 90 min. no further change in the phosphorylase activity occurred.
‡ K is the 1st order velocity constant (Cori *et al.* 1943).

expressed as percentages of the activity, in the presence of adenylic acid, of the phosphorylase not incubated with the PR enzyme. The activity of the crystalline phosphorylase incubated in the absence of the PR enzyme remained constant during the 2 hr. period. After 30 min. incubation with the PR enzyme the activity in the absence of adenylic acid was practically abolished, but in the presence of adenylic acid the activity was still 79 % of that of the control. The phosphorylase *a* had, therefore, been converted completely to phosphorylase *b*, showing that brown adipose tissue in the rat must contain PR enzyme.

Attempts were made to remove the PR enzyme rapidly from the crude adipose-tissue extract before any phosphorylase *a*, present in the intact tissue, was converted into phosphorylase *b*. Five different extracts containing phosphorylase were prepared by the method intended to remove PR enzyme as quickly as possible. Of these extracts, four apparently contained phosphorylase *a*, i.e. they were active in the absence of added adenylic acid. Their activity was, however, increased by the addition of this substance. Fig. 7 shows the results obtained with one of these extracts, as well as those given by the fifth extract which contained phosphorylase *b*, i.e. it showed activity only in the presence of added

adenylic acid. No inorganic phosphate was liberated when glycogen was omitted from the reaction mixture so that phosphate liberation, when it occurred, must have been due to phosphorylase. It is possible that the four extracts active without added adenylic acid contained only phosphorylase *b* together with suboptimal amounts of adenylic acid derived from the original tissue. This view finds support in the fact that similar results were obtained with phosphorylase preparations in which no attempt had been made to separate PR enzyme, and in which conditions had been highly favourable for the conversion of phosphorylase *a* into phosphorylase *b*. In an attempt to free the extracts of adenylic

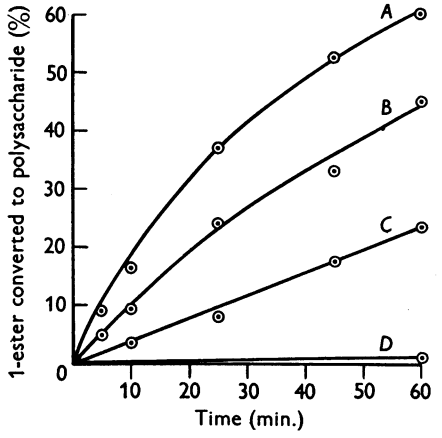

Fig. 7. Phosphorylase activity of PR-enzyme-free brown adipose-tissue extract. (*A*) and (*B*), apparent phosphorylase *a* activity of an extract; (*A*) with 0·3 mM adenylic acid; (*B*) without adenylic acid. (*C*) and (*D*), phosphorylase *b* activity of a different extract; (*C*) with 0·3 mM adenylic acid; (*D*) without adenylic acid. In each case 1 ml. of extract was incubated at 30° with 14 mM-1-ester, 15 mM-cysteine hydrochloride and 1 % glycogen; pH 6·75. Total vol. = 2 ml.

acid, several were dialysed in cellophan tubes against cysteine-glycerophosphate buffer (0·03 M, pH 6·75) at 4–5°, yet even after 8 hr. dialysis against repeatedly changed buffer solution, phosphorylase activity was still present in the absence of added adenylic acid.

Nature of the synthetic polysaccharide

Mirski (1942) showed that eluates prepared from the white adipose tissue of rats were able to catalyse the synthesis of glycogen from glucose-1-phosphate. Owing to the presence of amylase some difficulty was experienced in detecting the increase in polysaccharide concentration. Ostern, Herbert & Holmes (1939) encountered a similar difficulty in attempting to demonstrate the synthesis of polysaccharide by liver brei. By using high concentrations of glucose-1-phosphate and by adding sodium fluoride, which partially inhibited the phosphoglucomutase, Ostern *et al.* succeeded in demonstrating the synthesis of polysaccharide. Table 2 shows that under similar conditions approximately 30 % of the 1-ester was converted to polysaccharide by a crude extract of brown adipose tissue. When the polysaccharide was isolated, it behaved like glycogen in giving an opalescent solution in water and staining brown with iodine.

Table 2. *Polysaccharide synthesis by crude extracts of brown adipose tissue*

(The enzyme solution was incubated at pH 6·75 and 30° with 0·07 M-1-ester, 0·9 mM-adenylic acid, 0·1 % glycogen, 0·02 M-NaF and 0·015 M-cysteine-glycerophosphate buffer.)

	Exp. 1	Exp. 2	Exp. 3
Polysaccharide in reaction mixture at zero time (mg.)	2·9	2·5	3·4
Polysaccharide in reaction mixture after 60 min. incubation (mg.)	11·0	10·1	12·9
Polysaccharide synthesized (mg.)	8·1	7·6	9·5
Substrate converted to polysaccharide (%)	31·0	29·1	34·8

Supplementary Enzyme

Six different Supplementary Enzyme preparations from brown adipose tissue were examined.

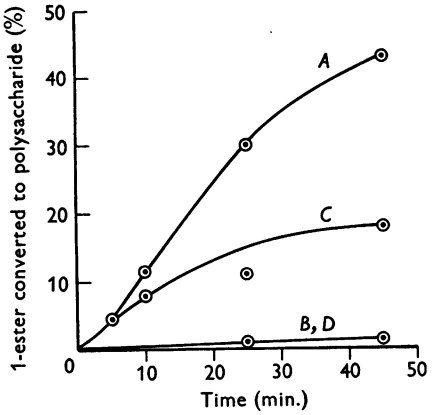

Fig. 8. Effect of brown adipose-tissue Supplementary Enzyme preparation on polysaccharide synthesis by crystalline phosphorylase *a*. Phosphorylase *a* incubated at 30° with 14 mM-1-ester, 15 mM-cysteine-glycerophosphate buffer; pH 6·75 and (*A*) Supplementary Enzyme and 0·02 % glycogen; (*B*) Supplementary Enzyme; (*C*) boiled Supplementary Enzyme and 0·02 % glycogen; (*D*) Supplementary Enzyme and 0·02 % glycogen; phosphorylase *a* omitted.

Provided a small quantity of glycogen was present to prime the reaction, all increased the activity of phosphorylase *a* and resulted in the synthesis of a

polysaccharide staining brown with iodine. Fig. 8, which is typical of the results, shows that in the absence of added glycogen there was no polysaccharide synthesis (curve *B*); after 45 min. incubation in the presence of 0·02 % glycogen, 43 % of the

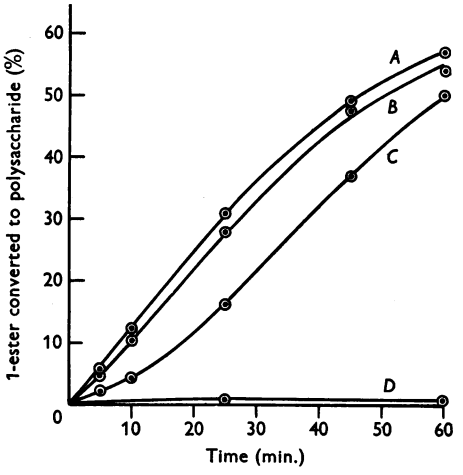

Fig. 9. Effect of brown adipose-tissue Supplementary Enzyme preparation on polysaccharide synthesis by crystalline phosphorylase *a*. Phosphorylase *a* incubated at 30° with 14 mM-1-ester, 15 mM-cysteine-glycerophosphate buffer; pH 6·75, Supplementary Enzyme and (*A*) 0·015 % glycogen; (*B*) 0·01 % glycogen; (*C*) 0·005 % glycogen; (*D*) no glycogen.

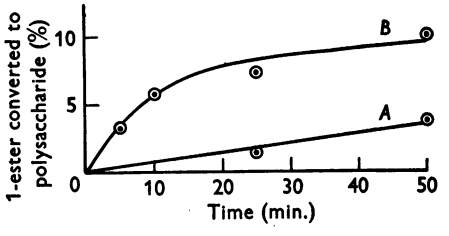

Fig. 10. Crystalline phosphorylase *a* primed with the product of simultaneous action of Supplementary Enzyme and crystalline phosphorylase *a* on 1-ester. Phosphorylase *a* incubated at 30° with 14 mM-1-ester, 15 mM-cysteine-glycerophosphate buffer (pH 6·75) and (*A*) boiled reaction mixture from experiment of Fig. 9, curve *C*, before incubation; (*B*) boiled reaction mixture from experiment of Fig. 9, curve *C*, after 1 hr. incubation.

glucose-1-phosphate was converted into a polysaccharide staining brown with iodine (curve *A*). In the presence of boiled brown adipose-tissue extract, the reaction rate curve was typical of phosphorylase activity with low concentrations of glycogen; only 18 % of the glucose-1-phosphate was converted into polysaccharide (curve *C*) and the synthesized polysaccharide stained blue with iodine. Although 'blue' and 'A–V values' (Bourne, Haworth, Macey & Peat, 1948) were not measured, the blue colour was considered to indicate an unbranched polysaccharide molecule, whereas the brown colour was considered to indicate a highly branched polysaccharide such as glycogen. When the brown adipose-tissue Supplementary Enzyme preparation was incubated with the substrate without phosphorylase *a*, polysaccharide synthesis did not occur (curve *D*). The effect of decreasing glycogen concentration on the course of polysaccharide synthesis by the mixture of Supplementary Enzyme preparation and phosphorylase *a* is shown in Fig. 9. In the absence of glycogen no polysaccharide synthesis occurred. In the presence of 0·005 % glycogen, an autocatalytic form of curve (curve *C*) was occasionally, but not regularly, observed. Fig. 10 shows that the product of the simultaneous action of Supplementary Enzyme and crystalline phosphorylase *a* on 1-ester can prime the phosphorylase reaction. This suggests that an increase in the concentration of branched polysaccharide had occurred.

DISCUSSION

The fact that the brown adipose tissue contains hexokinase, phosphoglucomutase, phosphorylase and Supplementary Enzyme suggests that in this tissue the synthesis of glycogen from glucose most probably occurs by a process similar to that which is known to occur in muscle. The behaviour of these enzymes is similar to that of the corresponding enzymes in muscle in regard to activators and inhibitors as far as they have been examined. Thus muscle phosphorylase is inhibited by glucose and phlorrhizin (Cori *et al.* 1943) and by α-methyl-D-glucoside (Campbell & Creasey, 1949). From the evidence presented it is impossible to decide whether brown adipose-tissue extracts possess phosphorylase *a* or phosphorylase *b* activity; although the failure to inactivate the extracts by dialysis suggests that the enzyme is in form *a*, the presence of PR enzyme in the extracts suggests that the enzyme would be in form *b*. The increase in activity of five of the preparations in the presence of adenylic acid is in agreement with the work of Cori *et al.*, who showed that phosphorylase *a* has only 71 % of its maximum activity in the absence of adenylic acid. In similar experiments, Cori & Green (1943) were unable to demonstrate phosphorylase *a* activity in heart and liver because of the high concentrations of PR enzyme in these tissues. Despite precautions to prevent the action of PR enzyme, all their extracts of heart and liver showed only phosphorylase *b* activity. The Supplementary Enzymes prepared from liver and heart by Cori & Cori (1943) showed phosphorylase *b* activity. Of the six preparations of Supplementary Enzyme from brown adipose tissue one showed no phosphorylase activity even in

the presence of adenylic acid, one showed typical phosphorylase *b* activity, while the remainder showed phosphorylase activity which was increased by the addition of adenylic acid. Similar results were obtained with seven Supplementary Enzyme preparations from liver, of which four possessed no phosphorylase activity while the other three showed activity in the absence of adenylic acid which was increased by the addition of this substance. The results are thus as equivocal as those of the experiments investigating whether phosphorylase occurs in the *a* or *b* form in brown adipose tissue.

Cori & Cori (1943) observed polysaccharide synthesis from glucose-1-phosphate by phosphorylase *a* and heart or liver Supplementary Enzyme preparations even in the absence of added glycogen, the rate curve being autocatalytic in form. In the present experiments, minute amounts of glycogen have been essential for polysaccharide synthesis (Fig. 9), and since this was also found to be the case with phosphorylase *a* and Supplementary Enzyme from liver, it may well be that Cori's preparations were not entirely free from glycogen, or other primer. Bailey, Whelan & Peat (1950) have shown that tri- and tetra-saccharides may function as primers in the synthesis of amylose by potato phosphorylase, but no information is available concerning the ability of such short chains to prime polysaccharide synthesis by animal phosphorylases, nor as to the relation of the Supplementary Enzymes of animal tissues to the corresponding *Q* enzyme of plant tissues (Barker, Bourne, Wilkinson & Peat, 1950).

Throughout the present work an alkali-stable polysaccharide staining brown with iodine has been assumed to be glycogen. It is unfortunate that we had no opportunity of measuring the optical rotation nor of the 'blue' and 'A–V' value as defined by Bourne *et al.* (1948), especially in view of the recent work of Bailey & Whelan (1950), who have shown that the *iso*phosphorylase of the potato described by Bernfeld & Meutémédian (1948) does not exist. Bernfeld & Meutémédian claimed to have prepared *iso*phosphorylase which, in the presence of potato phosphorylase and inorganic phosphorus, converted amylose into a polysaccharide presumed to possess a branched structure because it stained red with iodine. Bailey & Whelan showed that the red stain was due not to a polysaccharide with a branched structure, but to the formation of dextrins by α-amylase associated with a reduction in the amount of amylose staining blue with iodine by the action of phosphorylase in the presence of phosphate. It is clear that in future work concerning Supplementary Enzymes, care must be taken to confirm by rotation and 'A–V' measurements that the substances formed actually have branched structures.

SUMMARY

1. Hexokinase, phosphoglucomutase and phosphorylase have been demonstrated in extracts of the brown adipose tissue of the rat.

2. The phosphoglucomutase of brown adipose tissue requires magnesium ions for its optimum activity, and is inhibited by sodium fluoride and by high concentrations of glucose-1-phosphate.

3. The phosphorylase of brown adipose tissue has been prepared free from phosphatase and phosphoglucomutase; it is activated by cysteine and glycogen and inhibited by glucose, α-methyl-D-glucoside and by phlorrhizin. It was impossible to demonstrate unequivocally whether the phosphorylase in the adipose tissue was in the *a* or *b* form since the preparation obtained may have been contaminated with suboptimal concentrations of adenylic acid.

4. Extracts of brown adipose tissue contain PR enzyme.

5. The polysaccharide synthesized by the phosphorylase of brown adipose tissue stained brown with iodine and was assumed to possess a branched structure.

6. Extracts of the brown adipose tissue of the rat contained Supplementary Enzyme which, in conjunction with phosphorylase *a*, catalysed the synthesis from glucose-1-phosphate of a branched polysaccharide similar to glycogen.

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Direct Oxidation of Glucose-6-phosphate, 6-Phosphogluconate and Pentose-5-phosphates by Enzymes of Animal Origin

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The existence of a direct oxidative pathway, distinct from the glycolytic route, for the oxidation of glucose-6-phosphate was demonstrated in yeast extracts by Warburg & Christian (1936, 1937), Negelein & Gerischer (1936), Lipmann (1936) and Dickens (1936, 1938*a*). Although the oxidation products were not fully identified, oxidation was considered to proceed by stepwise C-1 and C-2 oxidation and decarboxylation via 6-phosphogluconate and a pentose phosphoric ester, and Dickens (1938*a*) suggested that this was D-ribose-5-phosphate rather than D-arabinose-5-phosphate. Although the latter ester would be expected to arise from D-glucose-6-phosphate (Lipmann, 1936), of the pentose phosphates examined by Dickens (1938*a*, *b*) only D-ribose-5-phosphate was attacked by yeast enzymes at a rate justifying its assumption as an intermediate product, and the occurrence of an inversion was postulated to account for its formation.

Recent work from other laboratories, full details of which have not yet been published, tends to support this hypothesis. Cohen & McNair Scott (1950*a*), using the same yeast system as Dickens (1938*a*), identified chromatographically D-ribose-5-phosphate and possibly D-arabinose-5-phosphate among oxidation products of 6-phosphogluconate, although 50% of the pentose phosphate fraction remained unidentified. Horecker (1950) reported an enzyme preparation from yeast which converted 6-phosphogluconate quantitatively into pentose phosphate. At equilibrium (Horecker & Smyrniotis, 1950) 75% of this pentose phosphate was in the form of D-ribose-5-phosphate.

The occurrence in animal tissues of a similar oxidative pathway was indicated by preliminary experiments of Dickens (1936, 1938*a*) and the oxidation of glucose-6-phosphate by erythrocytes had already been demonstrated by Warburg & Christian (1931). Indirect physiological evidence (see Stotz, 1945) also indicated the occurrence of a non-glycolytic pathway of carbohydrate oxidation. Apart from more recent work of Barkhash & Demianovskaya (1946), Lindberg (1946) and Wainio (1947), confirming its existence, this direct oxidative pathway has not been systematically investigated in animal tissues. The present work was designed with this object in view. A preliminary account has already been published (Dickens & Glock, 1950).

MATERIALS

Hexose monophosphate (HMP). The Ba salt was prepared by the method of Ostern, Guthke & Terszakowec (1936) using starch instead of glycogen as substrate (cf. Fantl & Anderson, 1941). (Found: organic P, 7·5; inorganic P, 0·0; total hexose (Hagedorn & Jensen as modified by Robison & King, 1931), 41·6; aldose (hypoiodite method of Macleod & Robison, 1929), 29·3. Calc. for $C_6H_{11}O_5.PO_4Ba$: P, 7·8; hexose, 44·8%.)

D-*Glucose-6-phosphate* (G-6-P). The Ba salt was kindly synthesized by Dr C. T. Beer by the method of Levene & Raymond (1931). (Found: organic P, 7·5; inorganic P, 0·0; total hexose, 38·3; aldose, 39·2. Calc. for $C_6H_{11}O_5.PO_4Ba$: P, 7·8; hexose, 44·8%.)