- Felber, J. P. (1963). *Experientia*, **19**, 227.
- Forsman, O. & Gemzell, C. A. (1959). *Acta endocr., Kbh.*, **32**, 480.
- Gemzell, C. A. (1959). *J. clin. Endocrin.* **19**, 1049.
- Gibson, R. E., Ekstedt, R. D. & Stollerman, G. H. (1962). *Fed. Proc.* **21**, 23.
- Glick, S. M., Roth, J., Yalow, R. S. & Berson, S. A. (1963). *Nature, Lond.*, **199**, 784.
- Greenspan, F. S., Cofell, J. A., Lew, W. & Peng, C. T. (1962). *J. Lab. clin. Med.* **59**, 520.
- Greenwood, F. C., Hunter, W. M. & Glover, J. S. (1963). *Biochem. J.* **89**, 114.
- Grodsky, G. M., Hayashida, T., Peng, C. T. & Geschwind, I. I. (1961). *Proc. Soc. exp. Biol., N.Y.*, **107**, 491.
- Grumbach, M. M. & Kaplan, S. L. (1962). *Ciba Found. Colloq.: Endocrinology*, **14**, 63.
- Hales, C. N. & Randle, P. J. (1963). *Biochem. J.* **88**, 137.
- Hayashida, T. & Li, C. H. (1958). *Science*, **128**, 1276.
- Hunter, W. M. (1963). *Analyst*, **88**, 251.
- Hunter, W. M. & Greenwood, F. C. (1962a). *Biochem. J.* **85**, 39p.
- Hunter, W. M. & Greenwood, F. C. (1962b). *Nature, Lond.*, **194**, 495.
- Josimovich, J. B. & MacLaren, J. A. (1962). *Endocrinology*, **71**, 209.
- Kohn, J. (1958). In *The Protides of Biological Fluids*, vol. 5, p. 120. Ed. by Peeters, H. Amsterdam: Elsevier.
- Li, C. H., Moudgal, N. R. & Papkoff, H. (1960). *J. biol. Chem.* **235**, 1038.
- Morgan, C. R. & Lazarow, A. (1962). *Proc. Soc. exp. Biol., N.Y.*, **110**, 29.
- Parker, M. L., Utiger, R. D. & Daughaday, W. H. (1962). *J. clin. Invest.* **41**, 262.
- Pastorelle, D. J. (1962). *Surg. Forum*, **13**, 375.
- Raben, M. S. (1959). *Recent Progr. Hormone Res.* **15**, 71.
- Read, C. H. & Bryan, G. T. (1960). *Ciba Found. Colloq.: Endocrinology*, **13**, 68.
- Read, C. H., Eash, S. A. & Najjar, S. (1962). *Ciba Found. Colloq.: Endocrinology*, **14**, 45.
- Read, C. H. & Stone, D. B. (1958). *Amer. J. Dis. Child.* **96**, 538.
- Richter, M., Gold, J., Rankowicz, W. & Rose, B. (1962). *Canad. J. Biochem. Physiol.* **40**, 35.
- Roth, J., Glick, S. M., Yalow, R. S. & Berson, S. A. (1963). *Science*, **140**, 987.
- Trenkle, A., Moudgal, N. R., Sadri, K. K. & Li, C. H. (1961). *Nature, Lond.*, **192**, 260.
- Unger, R. H., Eisentraut, A. M., McCall, M. S., Keller, S., Lanz, H. C. & Madison, L. L. (1959). *Proc. Soc. exp. Biol., N.Y.*, **102**, 621.
- Utiger, R. D., Parker, M. L. & Daughaday, W. H. (1962). *J. clin. Invest.* **41**, 254.
- Wolstenholme, G. E. W. & Cameron, M. P. (eds.) (1962). *Ciba Found. Colloq.: Endocrinology*, **14**, 45-136.
- Yalow, R. S. & Berson, S. A. (1960). *J. clin. Invest.* **39**, 1157.

Biochem. J. (1964) **91**, 56

Inhibition of Glucose Phosphorylation by Mannoheptulose

BY H. G. COORE AND P. J. RANDLE

Department of Biochemistry, University of Cambridge

(Received 2 August 1963)

D-Mannoheptulose prevents glucose from stimulating the secretion of insulin by rabbit pancreas *in vitro* (Coore, Randle, Simon, Kraicer & Shelesnyak, 1963). This action of mannoheptulose may be largely responsible for the temporary diabetic state induced by injection of the sugar (Simon & Kraicer, 1957; Simon, Scow & Chernick, 1961). Identification of the enzyme in glucose metabolism inhibited by mannoheptulose might thus indicate which pathway of glucose metabolism within the β -cell provides the stimulus for insulin secretion. Because no convenient preparation is available for a study of glucose metabolism in mammalian β -cells we have investigated the effect of mannoheptulose on glucose metabolism in rat-liver slices. Inhibitory effects of mannoheptulose on glucose and mannose uptake by rat-liver slices and on crude rat-liver glucokinase and hexokinase have been demonstrated. These observations suggested that mannoheptulose may also inhibit phosphorylation of glucose in the pancreatic β -cell and therefore that

the sugar cannot be used to determine whether a particular pathway of glucose metabolism beyond glucose 6-phosphate provides the signal for insulin release.

EXPERIMENTAL

Materials. D-Mannoheptulose was given by Professor E. Simon, Rehovoth, Israel. The sources of other chemicals and enzymes were as given by Battaglia & Randle (1960) and by Newsholme & Randle (1961).

Methods. Glucose was determined with glucose oxidase (Huggett & Nixon, 1957), mannoheptulose by the method of Dische (1953) and total reducing sugar by Somogyi's (1945) modification of Nelson's method. Mannose was calculated by difference (total reducing sugar minus glucose and minus mannoheptulose when present). Solutions for sugar analysis were deproteinized with $\text{Zn}(\text{OH})_2$ (Somogyi, 1945). Glycogen was assayed by the method of Walaas & Walaas (1950) except that glucose was assayed after hydrolysis with glucose oxidase. Glucokinase and hexokinase activities were assayed at 20° or 30° by the rate of

change of extinction at 340 μ in a Beckman spectrophotometer on adding 0.05–0.2 ml. of glucokinase or hexokinase preparations to 2.95–2.80 ml. of assay medium (either 40 mM-tris–5 mM-ATP–7.5 mM-MgCl₂–20 mM-KF–0.75 mM-NADP plus 5 μ g. of glucose 6-phosphate dehydrogenase, pH 7.5, or the cysteine-containing medium of Viñuela, Salas & Sols, 1963), with glucose at concentrations of 16.7 mM, 10 mM, 0.5 mM and 0.25 mM, and, when present, mannoheptulose at a concentration of 14.3 mM.

Liver slices. Livers (from male rats of 250–300 g., starved for 16 hr. and killed by decapitation) were rinsed with incubation medium and individual lobes sliced with a Stadie & Riggs (1944) microtome. The slices were gently blotted and distributed among 25 ml. conical flasks containing 2 ml. of medium (so that each flask contained 200–300 mg. of tissue). The composition of the medium was 40 mM-KHCO₃–20 mM-MgCl₂–10 mM-CaCl₂–70 mM-KCl (Hastings, Ching-Tseng Teng, Nesbitt & Sinex, 1952) with sugars added as described in the text or Table 1. After being gassed with O₂ + CO₂ (95:5) the flasks were stoppered and incubated (with shaking) for 2 hr. at 37°. For assay of initial glycogen one slice from each lobe was dissolved immediately in boiling 30% (w/v) KOH. At the end of incubation the slices were dissolved in 30% KOH for glycogen analysis and the medium was assayed for sugars after centrifugation.

Liver glucokinase and hexokinase. A weighed amount of liver (about 10 g.) from fed rats aged 2–4 months was dispersed (after washing by perfusion with buffer) in 2 vol. (v/w) of ice-cold buffer (0.12 M-tris–5 mM-MgCl₂–5 mM-EDTA, pH 7.0, or in some experiments the extraction fluid of Viñuela *et al.* 1963) in an all-glass hand-operated Potter-Elvehjem homogenizer. The extract was freed of nuclei, debris, mitochondria and microsomes (containing the glucose 6-phosphatase activity; Appelmans & de Duve, 1955) by centrifuging at 700g for 15 min., 10 000g for 20 min. and 100 000g for 60 min. at 5°. The resulting supernatant solution containing glucokinase and hexokinase and free of glucose 6-phosphatase was tested either immediately or after dialysis at 4° against two changes of buffer in 24/32 Visking seamless cellulose tubing previously boiled to remove plasticizing substances. The crude extract was either used immediately or stored at –10°. In some experiments crude glucokinase and hexokinase were separated by fractionation with (NH₄)₂SO₄ as described by Viñuela *et al.* (1963) and used on the same day without prior dialysis.

RESULTS

The results of experiments showing effects of mannoheptulose on glucose and mannose metabolism in liver slices are given in Table 1. In experiments with glucose (3 mg./ml.), mannoheptulose (3 mg./ml.) in one experiment abolished net uptake of glucose and in a further experiment increased a net output of glucose by liver slices. These changes were associated with diminished synthesis or net loss of glycogen respectively. These experiments do not distinguish between a possible inhibitory effect of mannoheptulose on glucose uptake or an accelerating effect on glucose output by liver slices. To distinguish between these possibilities mannose (5 mg./ml.) was substituted for glucose, and mannose uptake and glucose output by the slices were assayed. Mannose is rapidly phosphorylated by liver hexokinase and glucokinase (Viñuela *et al.* 1963), but mannose 6-phosphate is only slowly hydrolysed by glucose 6-phosphatase (Crane, 1955). Moreover, mannose 6-phosphate will be competing within the slice for the preferred substrate for glucose 6-phosphatase (glucose 6-phosphate formed from mannose 6-phosphate, from glycogen and by gluconeogenesis). The net uptake of mannose may therefore be expected to approximate closely to the phosphorylation of mannose by hexokinase and glucokinase. The results in Table 1 show that the uptake of mannose was markedly inhibited by mannoheptulose and, moreover, the output of glucose was slightly diminished under these conditions (presumably because of the diminished rate of formation of glucose 6-phosphate from mannose). Since there appears to be no permeability barrier to the entry of hexoses into the liver cell (Cahill, Ashmore, Earle & Zottu, 1958) we conclude that mannoheptulose inhibits the phosphorylation of glucose and mannose in the liver cell.

This conclusion is substantiated by experiments with crude liver glucokinase and hexokinase

Table 1. *Effects of mannoheptulose on uptake of mannose, uptake or output of glucose, and concentration of glycogen in rat-liver slices*

Experimental details are given in the text. The results are expressed as means $\pm$ S.E.M., with the numbers of observations given in parentheses.

Expt. no.	Sugars added	Effects (mg./g. of wet liver/2 hr.)		
		Net uptake (+) or output (–) of glucose	Change in glycogen concn.	Mannose uptake
1	Glucose (3 mg./ml.)	–0.16 $\pm$ 0.4 (6)	0.28 $\pm$ 0.17	—
	Glucose + mannoheptulose (3 mg./ml.)	–2.22 $\pm$ 0.3* (6)	–0.99 $\pm$ 0.10*	—
2	Glucose (3 mg./ml.)	+3.06 $\pm$ 0.65 (6)	2.07 $\pm$ 0.20	—
	Glucose + mannoheptulose (3 mg./ml.)	–0.50 $\pm$ 0.45* (6)	0.84 $\pm$ 0.08*	—
3	Mannose (5 mg./ml.)	–9.59 $\pm$ 0.54 (12)	—	7.20 $\pm$ 0.88 (12)
	Mannose + mannoheptulose (3 mg./ml.)	–8.24 $\pm$ 0.27† (12)	—	1.57 $\pm$ 0.48* (12)

* $P < 0.01$. † $P < 0.05$.

(assayed by the reduction of NADP in the presence of glucose 6-phosphate dehydrogenase). Crude liver glucokinase [precipitated at 60–75 % saturation with ammonium sulphate and tested at 10 mM-glucose; K_m for glucose 10 mM (Viñuela *et al.* 1963)] was inhibited 80 % by 14.3 mM-mannoheptulose. Similarly crude liver hexokinase (precipitated at 20–50 % saturation with ammonium sulphate and tested at 0.25 mM-glucose; K_m for glucose 0.01 mM) was inhibited 80 % by 14.3 mM-mannoheptulose. In unfractionated extracts 14.3 mM-mannoheptulose inhibited glucose phosphorylation 70–80 % at 16.7 mM-, 10 mM- and 0.5 mM-glucose. Because of the marked differences in K_m for glucose for the two enzymes (see above), the results with unfractionated extracts are consistent with inhibition by mannoheptulose both of glucokinase and hexokinase. Under the conditions used the rates of glucose phosphorylation in the absence of mannoheptulose (with 0.05–0.2 ml. of particular extracts) varied from 0.02 to 0.05 μ mole of NADPH₂ formed/min. Control experiments performed under the same conditions showed that mannoheptulose did not inhibit the reduction of NADP by glucose 6-phosphate (i.e. that the effect of mannoheptulose was on glucose phosphorylation and not on the reactions involved in the oxidation of glucose 6-phosphate). We conclude that mannoheptulose inhibits both glucokinase and hexokinase in rat-liver extracts.

DISCUSSION

The results reported in the present paper appear to show that mannoheptulose can inhibit the phosphorylation of glucose and mannose by rat-liver slices and that mannoheptulose can inhibit the phosphorylation of glucose by glucokinase and hexokinase in extracts of rat liver. Chernick, Scow, Simon & Stricker (1962), in experiments carried out before the demonstration of glucokinase in rat liver by Viñuela *et al.* (1963), were unable to obtain clear-cut evidence for an inhibitory effect of mannoheptulose on glucose metabolism in liver slices. The experimental conditions that we have used differ from those of Chernick *et al.* (1962) in a number of respects. The concentration of glucose was higher (16.7 mM as opposed to 6.2 and 1.3 mM); the concentration of mannoheptulose was greater (14.3 mM as opposed to 4.5 or 5 mM); the high-K⁺ medium of Hastings *et al.* (1952) (which favours glycogen formation from glucose) was used as opposed to Krebs bicarbonate medium (which favours breakdown of glycogen to glucose); changes in glucose phosphorylation with mannoheptulose were demonstrated by alterations in net uptake or output of glucose or uptake of mannose by liver slices as opposed to measure-

ments based on the disappearance of [U-¹⁴C]glucose from the medium or incorporation of ¹⁴C into carbon dioxide or lipid. We do not know which of these differences in experimental technique has enabled us to obtain definite inhibitory effects of mannoheptulose, but we suggest that the concentration of glucose used and the method of determining glucose and mannose phosphorylation in the slice may be important. Viñuela *et al.* (1963) have suggested that glucokinase may be responsible for the major part of glucose phosphorylation in liver. The K_m for glucose for this enzyme is high (10 mM), and the concentration of glucose which we have used (14.7 mM) is above the K_m , whereas those used by Chernick *et al.* (1962) (6.2 and 1.3 mM) were below the K_m . Moreover, it seems possible that much of the [¹⁴C]glucose taken up by liver under the conditions of Chernick *et al.* (1962) may not have been phosphorylated by glucokinase or hexokinase. It may, for example, have been lost from the medium by equilibration with the pool of non-radioactive glucose in the slice or entered metabolic pathways through an exchange reaction catalysed by glucose 6-phosphatase (Haas & Byrne, 1960).

Our observations provide further support for the view that mannoheptulose can inhibit hexokinases, first shown with extracted hexokinases of brain, yeast and adipose tissue by Sols & Crane (1954), Sols, de la Fuente, Villar-Palasi & Asensio (1958) and Hernandez & Sols (1963). Whether or not mannoheptulose inhibits glucose phosphorylation in tissues may depend in particular on the presence or absence of a permeability barrier for sugars in different tissues. In liver, as mentioned above, the cells appear to be freely permeable to hexoses, and they are presumably permeable to mannoheptulose. In adipose tissue and brain (and muscle) permeability to hexoses is restricted, and it may be that mannoheptulose does not readily enter cells in these tissues (cf. Chernick *et al.* 1962; Hernandez & Sols, 1963). There is evidence, based on studies with phlorrhizin, that pancreatic β -cells may also be freely permeable to hexose, and it seems reasonable to suggest that they may also be permeable to mannoheptulose (Coore & Randle, 1962).

If what we have observed in liver is applicable to the β -cell, then phosphorylation of glucose in the β -cell may also be inhibited by mannoheptulose. Since mannoheptulose has now been shown to be an inhibitor of five enzymes phosphorylating glucose (hexokinases of brain, adipose tissue liver and yeast, and glucokinase of liver) it seems reasonable to suggest that it may inhibit glucose phosphorylation in β -cells. Such an effect of mannoheptulose could contribute to or be responsible for its inhibitory effect on glucose-induced insulin secretion by rabbit pancreas *in vitro* (Coore *et al.* 1963).

SUMMARY

1. Mannoheptulose has been shown to decrease the net uptake of glucose and of mannose by rat-liver slices.

2. This effect has been attributed to an inhibition of hexose phosphorylation in the liver because inhibition of glucokinase and hexokinase by mannoheptulose in extracts of rat liver has been demonstrated.

3. The possibility that a similar inhibition of glucose phosphorylation in β -cells of isolated rabbit pancreas could contribute to inhibitory effects of mannoheptulose on glucose-induced insulin secretion, observed previously, is discussed.

We thank the Elmore Research Fund, University of Cambridge and the British Diabetic Association for contributing to the cost of these investigations.

REFERENCES

- Appelmans, F. & de Duve, C. (1955). *Biochem. J.* **59**, 438.
 Battaglia, F. C. & Randle, P. J. (1960). *Biochem. J.* **75**, 408.
 Cahill, G. F., Ashmore, J., Earle, A. S. & Zottu, S. (1958). *Amer. J. Physiol.* **192**, 491.
 Chernick, S. S., Scow, R. O., Simon, E. & Stricker, F. A. (1962). *Proc. Soc. exp. Biol., N.Y.*, **109**, 589.
 Coore, H. G. & Randle, P. J. (1962). *Biochem. J.* **84**, 78r.
 Coore, H. G., Randle, P. J., Simon, E., Kraicer, P. F. & Shelesnyak, M. C. (1963). *Nature, Lond.*, **197**, 1264.
 Crane, R. K. (1955). *Biochim. biophys. Acta*, **17**, 443.
 Dische, Z. (1953). *J. biol. Chem.* **204**, 983.
 Haas, L. F. & Byrne, W. L. (1960). *J. Amer. chem. Soc.* **82**, 947.
 Hastings, A. B., Ching-Tseng Teng, Nesbett, F. B. & Sinex, F. M. (1952). *J. biol. Chem.* **194**, 69.
 Hernandez, A. & Sols, A. (1963). *Biochem. J.* **86**, 166.
 Huggett, A. St G. & Nixon, D. A. (1957). *Biochem. J.* **66**, 12p.
 Newsholme, E. A. & Randle, P. J. (1961). *Biochem. J.* **80**, 65.
 Simon, E. & Kraicer, P. F. (1957). *Arch. Biochem. Biophys.* **69**, 592.
 Simon, E., Scow, R. O. & Chernick, S. S. (1961). *Amer. J. Physiol.* **201**, 1073.
 Sols, A. & Crane, R. K. (1954). *J. biol. Chem.* **210**, 581.
 Sols, A., de la Fuente, G., Villar-Palasi, C. & Asensio, C. (1958). *Biochim. biophys. Acta*, **30**, 92.
 Somogyi, M. (1945). *J. biol. Chem.* **160**, 69.
 Stadie, W. C. & Riggs, B. C. (1944). *J. biol. Chem.* **154**, 687.
 Viñuela, E., Salas, M. & Sols, A. (1963). *J. biol. Chem.* **238**, 1175.
 Walaas, O. & Walaas, E. (1950). *J. biol. Chem.* **187**, 769.

Biochem. J. (1964) **91**, 59

The Fractionation of Proteins from Ox-Heart Mitochondria Labelled *in vitro* with Radioactive Amino Acids

By D. E. S. TRUMAN*

Wenner-Gren Institute for Experimental Biology, University of Stockholm, Sweden

(Received 29 July 1963)

The incorporation of radioactive amino acids into protein by isolated ox-heart mitochondria has been described by Simpson, Skinner & Lucas (1961) and Truman & Löw (1963), incorporation by submitochondrial particles from ox heart has been demonstrated by Kalf & Simpson (1959) and Kroon (1963), and mitochondria from various other sources have been found capable of incorporating amino acids into their proteins (McLean, Cohn, Brandt & Simpson, 1958; Roodyn, Reis & Work, 1961a; Truman & Korner, 1962; Kalf, 1963), but there is still little evidence about which of the mitochondrial proteins becomes labelled and of whether the incorporation represents the synthesis of any specific protein. Roodyn, Suttie & Work (1962) have studied the proteins

labelled by the incorporation of radioactive amino acids into isolated rat-liver mitochondria, which they fractionated to yield purified catalase, malate dehydrogenase and cytochrome *c*. However, they found no significant incorporation into any of these proteins, and Roodyn (1962) concluded that the major site of the incorporation of amino acids was into insoluble lipoprotein. Simpson *et al.* (1961) were unable to demonstrate any incorporation of radioactive valine into cytochrome *c* in isolated ox-heart mitochondria.

In the experiments described below the proteins of ox-heart mitochondria were fractionated after incubation of the mitochondria with ¹⁴C-labelled amino acids. Ox-heart mitochondria were chosen because they were available in sufficient quantities and showed an appreciable incorporation of amino acid into protein under relatively simple incubation

* Present address: Institute of Animal Genetics, University of Edinburgh.