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THE EFFECTS OF ALDOSE REDUCTASE INHIBITORS ON THE METABOLISM OF CULTURED MONKEY KIDNEY EPITHELIAL CELLS

RAYMOND BOOT-HANDFORD and HAROLD HEATH

Department of Biochemical Pathology, School of Medicine, University College London, University Street, London WC1E 6JJ, U.K.

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Abstract—Cultured monkey kidney epithelial cells were used to investigate the activities of the aldose reductase (EC 1.1.1.21) inhibitors I.C.I. 105552 (1-(3,4-dichlorobenzyl)-3-methyl-1,2-dihydro-2-oxoquinol-4-ylacetate), M-7-HEQ (7-*O*-(β-hydroxyethyl)-quercetin and HR (5,7,3′,4′-tetra-*O*-(β-hydroxyethyl)-rutin). I.C.I. 105552 5×10^{-5} M completely prevented cellular sorbitol accumulation provoked by a 4 hr incubation in 55 mM glucose, whereas under similar conditions, M-7-HEQ and HR had less of an effect, even at the higher concentration of 10^{-4} M. Intracellular glucose levels were elevated on incubation in 55 mM glucose both in the presence and absence of aldose reductase inhibitors although this effect was reduced by 10^{-4} M flavonoids (M-7-HEQ and HR). Lactate production was not affected by either the medium glucose concentration or the presence of aldose reductase inhibitors with the exception of M-7-HEQ which at 10^{-4} M reduced lactate ouput in 55 mM glucose and had a similar effect at both 10^{-4} M and 5×10^{-5} M in 5.5 mM glucose. The results indicate that the high activity of I.C.I. 105552 might make this aldose reductase inhibitor effective in preventing some of the secondary complications of diabetes.

The sorbitol pathway consists of two enzymes, aldose reductase (EC 1.1.1.21) and sorbitol dehydrogenase (EC 1.1.1.14), which catalyse the conversion of glucose to fructose by way of sorbitol:

$$\text{D-glucose} + \text{NADPH} + \text{H}^+ \xrightarrow[\text{REDUCTASE}]{\text{ALDOSE}} \text{sorbitol} + \text{NADP}^+$$

$$\text{sorbitol} + \text{NAD}^+ \xrightarrow[\text{DEHYDROGENASE}]{\text{SORBITOL}} \text{D-fructose} + \text{NADH} + \text{H}^+$$

Aldose reductase has a low affinity for glucose and so at normal tissue glucose concentrations, little sorbitol is formed. However, in diabetes, the increased availability of glucose in insulin insensitive tissues can lead to an increased synthesis of intracellular sorbitol and fructose, the osmotic consequences of which have been proposed to be of aetiological significance in the development of diabetic cataracts and diabetic neuropathy [1–3].

Tissues such as retina and kidney contain not only the sorbitol pathway but also the enzyme fructokinase (EC 2.7.1.3) [4, 5] which rapidly phosphorylates fructose to form F1P [6]. This rapid metabolism of fructose is important for two reasons; firstly it may prevent osmotically significant intracellular accumulations of sorbitol pathway intermediates and secondly, as shown in Fig. 1 the F1P produced can be channelled into glycolysis at the triose level by routes which avoid the normal glycolytic control mechanisms exerted at the hexokinase (EC 2.7.1.1) and phosphofructokinase (EC 2.7.1.11) levels. Therefore, in the diabetic state it is possible that the raised rat retinal lactate concentrations previously reported [7], which are thought to be of significance in the development of diabetic retinopathy [8], originate from a rapid and uncontrolled channelling of increased concentrations of fructose into glycolysis. Sorbitol [9, 10], fructose [10] and F1P [4] concentrations have all been shown to be significantly elevated in the diabetic rat retina which would appear to support the above hypothesis. Further, the ingestion of diets containing or yielding high levels of fructose has been shown to result in retinal lactate accumulation in short-term experiments [7] and over longer periods in the development of microvascular lesions in the normal rat similar to those found in the diabetic animal [11–13].

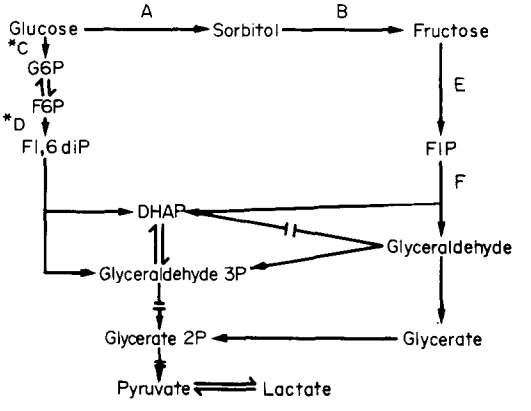

Fig. 1. The involvement of the sorbitol pathway in the metabolism of glucose. Key: (A) aldose reductase, (B) sorbitol dehydrogenase, (C) hexokinase, (D) phosphofructokinase, (E) fructokinase and (F) aldolase B (EC 4.1.2.1.3). (*) Glycolytic rate control point.

Compounds which inhibit aldose reductase, thereby preventing the conversion of glucose to sorbitol, provide a means of testing the hypothesised pathogenic effects of the increased sorbitol pathway activity found in diabetes. However, whilst exhibiting reasonable *in vitro* activities, some of these compounds have little effect *in vivo*. There are two aims to the investigation described below. Firstly, to examine under tissue-culture conditions, the efficacy of a new aldose reductase inhibitor, I.C.I. 105552 (1-(3,4-dichlorobenzyl)-3-methyl-1,2-dihydro-2-oxoquinol-4-ylacetate) and compare its activity with those of two established aldose reductase inhibitors [14], namely the flavonoids M-7-HEQ (7-*O*-(β-hydroxyethyl)-quercetin) and HR (5,7,3′,4′-tetra-*O*-(β-hydroxyethyl)-rutin). The second aim is, by examining the effects of aldose reductase inhibition on the cellular metabolism of glucose, sorbitol, fructose and lactate in high and low ambient glucose concentrations, to determine whether any link between sorbitol pathway activity and lactate production can be demonstrated. Since cultured monkey kidney epithelial cells have previously been reported to exhibit high sorbitol pathway activity [15] this cell type was chosen for the current investigation.

MATERIALS AND METHODS

Cell culture. African Green Monkey kidney epithelial (GLV3) cells were obtained from Flow Laboratories (Irvine, U.K.), medium 199 with Hanks salts (M199), and new-born calf serum (NCS) from Gibco Europe (Glasgow, U.K.). Cells were grown routinely in 50 ml plastic flasks (25 cm^2 surface area, Nunc U.K. Ltd., Stafford, U.K.) at 37° in 10 ml M199 supplemented with 5% NCS, 100 I.U./ml penicillin and 100 μg/ml streptomycin (Glaxo Laboratories Ltd., Greenford, U.K.). Confluent monolayers were detached from flasks for subculture using a 0.2% trypsin solution (Wellcome Reagents Ltd., Beckenham, U.K.).

For metabolic studies cells were grown in Falcon 3008 Multiwell tissue culture plates (Becton Dickinson, Wembley, U.K.). Each well was seeded with approximately 7.8×10^4 cells in 1 ml M199 containing 5% NCS. Medium (1 ml per well) was changed on days five and six after seeding, analyses being conducted on day seven. The multiwell plates were maintained at 37° in a container with a CO_2-enriched atmosphere (produced by a burning wick which was sealed in the container with the plates).

High glucose (55 mM) medium was prepared by adding 2.75 ml 1.8 M glucose (sterile) to 100 ml M199 containing 5% NCS. Osmolarity of the low glucose (5.5 mM) medium was adjusted to that of the high glucose medium by the addition of 2.75 ml 0.9 M NaCl (sterile) to 100 ml M199 containing 5% NCS. Glucose and NaCl solutions were sterilised using Millex disposable filter units (Millipore (U.K.) Ltd., London, U.K.).

100 mM stock solutions of I.C.I. 105552 (sodium salt) and HR were prepared in distilled water. M-7-HEQ (100 mM) was prepared in 400 mM NaOH because of this compound's low solubility in distilled water. Fresh stock solutions were sterilised by filtration as described above and then diluted with sterile distilled water so that addition of 0.1 ml of diluted solution to 10 ml of medium resulted in the medium concentrations described in the results.

Cell extraction. Confluent 7 day cultures of MKE cells (passage No. 167) were incubated for 4 hr in either 55 mM or 5.5 mM glucose in the presence or absence of aldose reductase inhibitors. Medium from one well was then removed and stored at −20°. The cell layer was rapidly rinsed with three 1 ml volumes of ice-cold phosphate buffered saline (pH 7.4). 1 ml ice-cold distilled water containing 4 μg α-methyl-D-mannoside (an internal standard in the subsequent g.l.c. analysis) was then added. This procedure was repeated for the remaining 17 wells used for extraction of soluble cellular contents. Processed trays were then rapidly frozen and thawed twice. The cell extracts were removed from the wells, deproteinised using 0.3 ml 0.9 M $ZnSO_4$ and 0.9 ml 0.3 M $Ba(OH)_2$, divided into two and lyophilised in flat bottom glass tubes (50 mm × 12 mm o.d., Scientific Supplies Co. Ltd., London, U.K.). Six of the 24 wells on each tray were used for determining protein. These wells were rinsed as described above and drained before the freezing and thawing process. Following the removal of the 18 cell extracts from a tray, the remaining 6 wells were dried, incubated for 24 hr at 37° with each well containing 0.5 ml 1.0 M NaOH, the resulting solutions being used to determine protein [16].

The stored medium samples were utilised to determine lactate [17] following deproteinisation as described above.

G.l.c. analysis. To prepare TMS sugar derivatives, 0.1 ml of a mixture of trimethylsilylimidazole in dry pyridine (1.5 m-equiv./ml, TRI-SIL 'Z', Pierce and Warriner, Chester, U.K.) was added to each tube containing the lyophilised cell extracts. The tubes were then sealed with polythene stoppers and incubated for 24 hr at room temperature. Distilled water (2.5 ml) was then added to the silylation mixture and the TMS sugar derivatives immediately extracted into 0.2 ml cyclohexane (Analar grade, BDH, Poole, U.K.). The cyclohexane was separated from the aqueous phase by centrifugation, removed and then concentrated approximately 10-fold under a stream of oxygen-free nitrogen.

Of this extract, 0.1 μl was used for g.l.c. analysis in a Pye Unicam GCD chromatograph fitted with a flame ionisation detector coupled to a Philips PM8251 pen recorder. The coiled glass column (2.5 m × 4 mm i.d.) was packed with acid washed diatomite C (80–100 mesh, J.J's (Chromatography) Ltd., King's Lynn, U.K.) which had been coated with 2.5% SE-52 silicone rubber gum (J.J's (Chromatography) Ltd.) using the following procedure. SE-52 silicone rubber (0.4g) gum was dissolved in 30–40 ml toluene (Analar grade, Koch-Light Laboratories, Colnbrook, U.K.). The solution was placed in a 250 ml round bottom thick walled flask and 15.6 g acid washed diatomite C slowly added. The flask was placed on a warm water bath and a moderate vacuum applied to assist solvent evaporation. The flask was shaken continuously and gently to prevent bumping, until most of the solvent had evaporated. The flask was then rotated every 5 min to ensure even distribution of the stationary

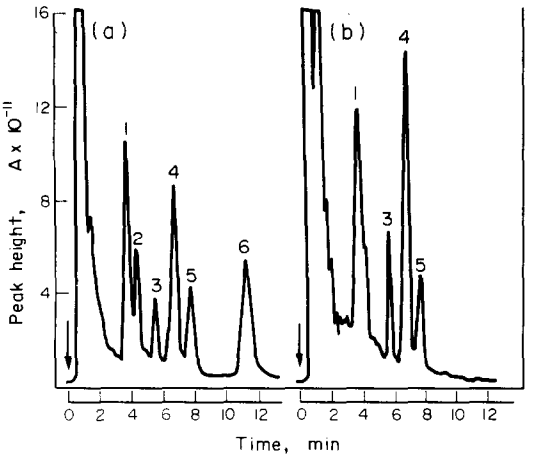

Fig. 2. Gas liquid chromatographs of (A) an equimolar mixture of trimethylsilylated (1) α-methyl-D-mannoside (internal standard), (2) D-fructose, (3 and 5) D-glucose, (4) sorbitol and (6) myo-inositol and (B) a trimethylsilylated extract of monkey kidney epithelial cells which had undergone a 4 hr incubation in medium containing 55 mM glucose and 10^{-4} M HR. Numbering of peaks as in (A). Procedures are described in Materials and Methods.

phase. After all the solvent had evaporated the remaining material was heated on the water bath at 100° for 1 hr under a moderate vacuum to remove the last traces of solvent vapour.

Injector, oven and detector temperatures were 250°, 190° and 250°, respectively. Oxygen-free nitrogen was used as the carrier gas (flow rate 40 cm^3/min). The detector gases were hydrogen and air (flow rates 40 cm^3/min and 400 cm^3/min, respectively). Readings were taken over the detector output range (at full scale deflection) of 4, 8 or 16×10^{-11} amperes. Typical chromatographs obtained during this investigation are shown in Fig. 2. TMS sugar derivatives were identified by retention time and concentrations determined by peak height analysis.

Statistics. Data are represented as the mean of six independent observations (±S.E.). All the results for each criterion determined were compared by 2-way analysis of variance, differences between the means being evaluated by computing *t*-values from the residual variation. Probabilities were estimated using the Studentised Range [18].

RESULTS

Controls. The intracellular sorbitol concentration in controls was increased to 181.9 ± 13.0 nmole/mg protein from 91.0 ± 8.2 nmole/mg protein by a 4 hr incubation in 55 mM compared with 5.5 mM glucose ($P < 0.01$). The high glucose incubation also caused the intracellular glucose levels to increase to 168.4 ± 21.0 nmole/mg protein from 13.1 ± 1.0 nmole/mg protein found in the low glucose-treated cells ($P < 0.01$). Lactate production, however, was not affected by the medium glucose concentration, production over 4 hr being 7.75 ± 0.80 μmole/mg protein in 55 mM glucose and 7.82 ± 0.96 μmole/mg protein in 5.5 mM glucose.

I.C.I. 105552. The results are presented in Table 1. Both concentrations of I.C.I. 105552 caused a reduction in the intracellular sorbitol concentrations detected, after the high glucose incubations in comparison with controls ($P < 0.01$ in each case) and the inhibition was greater when the higher (5×10^{-5} M) concentration of drug was used. I.C.I. 105552, at both concentrations tested, slightly reduced intracellular sorbitol levels in the low glucose incubations. This drug, however, did not have any effect on either intracellular glucose concentration or lactate production in comparison with controls when tested under the described conditions (Table 1).

M-7-HEQ. The results are presented in Table 2. Incubation in medium containing 10^{-4} M M-7-HEQ and 55 mM glucose resulted in a decrease in intracellular sorbitol concentration ($P < 0.01$), a decrease in intracellular glucose level and a decrease in lactate production ($P < 0.01$) in comparison with controls.

Table 1. The effects of I.C.I. 105552 on monkey kidney epithelial cell sorbitol and glucose concentrations and lactate production in 55 mM and 5.5 mM glucose*

	Medium glucose (mM)	Control (no drug)	I.C.I. 105552 5×10^{-5} M	I.C.I. 105552 10^{-5} M
Intracellular sorbitol concn (nmole/mg protein)	55	181.9 ± 13.0	68.3 ± 8.8†	101.7 ± 6.5†
	5.5	91.0 ± 8.2†	69.0 ± 8.1	71.4 ± 8.6
Intracellular glucose concn (nmole/mg protein)	55	168.4 ± 21.0	140.1 ± 18.3	127.6 ± 13.6
	5.5	13.1 ± 1.0†	9.7 ± 0.9	12.7 ± 0.9
Lactate production (μmole/mg protein)	55	7.75 ± 0.80	7.93 ± 0.31	5.91 ± 0.18
	5.5	7.82 ± 0.96	8.29 ± 0.58	6.67 ± 0.30

* Cells had been incubated for 4 hr in the medium glucose and drug concn indicated.
† vs 55 mM glucose control $P < 0.01$.
Values are means ± S.E. of 6 determinations.

Table 2. The effects of the flavonoids M-7-HEQ and HR on monkey kidney epithelial cell sorbitol and glucose concentrations and lactate production in 55 mM and 5.5 mM glucose*

	Medium glucose (mM)	Control (no drug)	M-7-HEQ		HR	
			10^{-4} M	5×10^{-5} M	10^{-4} M	5×10^{-5} M
Intracellular sorbitol concn (nmole/mg protein)	55	181.9 ± 13.0	71.1 ± 2.3†	89.4 ± 11.1†	135.0 ± 5.8‡	139.5 ± 14.3‡
	5.5	91.0 ± 8.2†	58.8 ± 2.4	64.7 ± 1.9	67.0 ± 4.6	79.8 ± 3.2
Intracellular glucose concn (nmole/mg protein)	55	168.4 ± 21.0	85.0 ± 2.6§	132.0 ± 15.5	99.0 ± 6.8§	138.9 ± 17.9
	5.5	13.1 ± 1.0†	7.9 ± 0.9	10.4 ± 1.2	11.6 ± 1.7	12.7 ± 1.1
Lactate production (μmole/mg protein)	55	7.75 ± 0.80	3.77 ± 0.37†	4.93 ± 0.19	6.48 ± 0.31	6.33 ± 0.24
	5.5	7.82 ± 0.96	2.65 ± 0.24‖	4.17 ± 0.29‖	6.76 ± 0.49	6.03 ± 0.32

* Cells had been incubated for 4 hr in the medium glucose and drug concn indicated.
† vs 55 mM glucose control $P < 0.01$.
‡ vs 55 mM glucose control $P < 0.05$.
§ 10^{-4} M flavonoid vs control $P < 0.05$.
‖ vs 5.5 mM glucose control $P < 0.01$.
Values are means ± S.E. of 6 determinations.

Cells treated with 10^{-4} M M-7-HEQ in medium containing 5.5 mM glucose exhibited lower intracellular concentrations of both sorbitol and glucose and a decrease in lactate production ($P < 0.01$) when compared with controls.

Cells incubated in medium containing 5×10^{-5} M M-7-HEQ and 55 mM glucose showed a decreased intracellular sorbitol concentration ($P < 0.01$), no change in intracellular glucose level and a reduced lactate production compared with controls. Incubations in medium containing 5×10^{-5} M M-7-HEQ and 5.5 mM glucose caused a lowering of intracellular sorbitol concentration, no change in intracellular glucose level but a decrease in lactate production ($P < 0.01$) in comparison with controls.

HR. The results are presented in Table 2. HR (10^{-4} M) in medium containing 55 mM glucose caused decreases in the concentrations of intracellular sorbitol ($P < 0.01$) and intracellular glucose but did not alter lactate production in comparison with controls. Cells incubated in medium containing 10^{-4} M HR and 5.5 mM glucose exhibited a slight decrease in the concentration of intracellular sorbitol but no change in either intracellular glucose level or lactate production.

In medium containing 5×10^{-5} M HR and 55 mM glucose, cells showed a decreased intracellular sorbitol concentration ($P < 0.01$) but no change in either intracellular glucose concentration or lactate production when compared with controls. HR (5×10^{-5} M) in medium containing 5.5 mM glucose did not cause changes in any of the metabolite concentrations measured.

Preliminary investigations revealed that MKE cells incubated for 24 hr in 55 mM glucose contained intracellular fructose concentrations of 4 nmole/mg protein in comparison with <1 nmole/mg protein found in their 5.5 mM glucose-treated counterparts. The large difference between the cellular sorbitol and glucose levels, and that of fructose precluded assessment of the latter by the methods described.

DISCUSSION

The first aim of this investigation was to determine the efficacy of I.C.I. 105552 and compare it with the action of the two flavonoid aldose reductase inhibitors. The results showed that I.C.I. 105552 was the most potent drug tested since, at a concentration of 5×10^{-5} M, this compound completely prevented intracellular sorbitol accumulation provoked by high glucose incubations, an effect which could not be achieved using the flavonoid aldose reductase inhibitors even at concentrations of 10^{-4} M.

Of the two flavonoids tested, M-7-HEQ appeared to be the most active against aldose reductase in that intracellular sorbitol concentrations following treatment with M-7-HEQ were consistently lower than those found under similar conditions using HR (Table 2). However, the aldose reductase inhibitory capacity of M-7-HEQ cannot be properly assessed under the conditions described due to other metabolic effects produced by this flavonoid which are discussed below.

The second aim of this investigation was, by examining certain aspects of MKE cell metabolism, to

determine whether the level of sorbitol pathway activity had any influence on lactate production. Control cultures incubated in medium containing 55 mM glucose showed an increase in intracellular sorbitol and glucose concentrations, but no increase in lactate production. Further, treatment of cultures with 5×10^{-5} M I.C.I. 105552 resulted in a complete inhibition of sorbitol accumulation provoked by high glucose medium but no change in lactate production. These two sets of data indicate that there is no apparent link between the level of sorbitol pathway activity and lactate production in the MKE cell.

Cultures incubated in the presence of M-7-HEQ exhibited several metabolic alterations (Table 2), including a decrease in lactate production which cannot be attributed to aldose reductase inhibition since similar changes were not observed in the presence of I.C.I. 105552. These effects of M-7-HEQ may have resulted from an inhibition of lactate efflux with a concomitant fall in intracellular pH which has been previously shown to occur in Ehrlich ascites tumour cells treated with various bioflavonoids [19]. Since it is not possible to dissociate the effects that this flavonoid may have had on the intracellular environment from its specific action as an aldose reductase inhibitor, quantitative assessment of the latter is difficult.

Cultures treated with HR did not exhibit the alterations in lactate production detected in the presence of M-7-HEQ. However, 10^{-4} M HR in comparison with 5×10^{-5} M HR did induce a considerable drop in the intracellular glucose concentration of cultures incubated in medium containing 55 mM glucose. Since the intracellular sorbitol levels detected in the presence of both concentrations of HR in 55 mM glucose were virtually identical (Table 2), it would appear that the glucose concentration within cells treated with 10^{-4} M HR (99 nmole/mg protein) was sufficient to saturate the sorbitol synthesising capacity of the MKE cell.

Whilst lactate production was not affected by either increased sorbitol pathway activity provoked by hyperglycaemic conditions, or aldose reductase inhibition, it should be noted that intracellular fructose concentrations remained extremely low. This is a crucial point with respect to demonstrating a metabolic link between the sorbitol pathway and lactate production since the low levels of fructose may have been due to an absence of any appreciable sorbitol dehydrogenase activity in the MKE cells. If this were so, the metabolism of glucose in hyperglycaemic conditions, by way of fructose into glycolysis (as shown in Fig. 1) resulting in increased lactate production, would not be feasible in this cell type. Alternatively, the low levels of fructose may have been due to the rapid metabolism of this sorbitol pathway product [6], in which case it would appear that the uncontrolled channelling of increased quantities of fructose into glycolysis does not necessarily result in elevated lactate production. These two possibilities are under further investigation.

The results indicate that the use of cultured monkey kidney epithelial cells provides a rapid means of assessing the efficacy of aldose reductase inhibitors in a biologically viable system. Intracellular sorbitol accumulation provoked by high glucose medium has previously been shown to be partially reduced in primary cultures of MKE cells by 5×10^{-3} M 3,3'-tetramethyleneglutarate [15]—one of the first aldose reductase inhibitors tested—but this concentration is clearly too high to be readily achieved in intact animals. The fact that a 100-times lower concentration of I.C.I. 105552 (5×10^{-5} M) completely prevents MKE cell sorbitol accumulation in high-glucose medium indicates that a potentially useful compound is now available with which to investigate, *in vivo*, the relationship between sorbitol pathway activity and the aetiology of the secondary complications of diabetes.

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REFERENCES

1. R. van Heyningen, *Nature, Lond.* **184**, 194 (1959).
2. J. H. Kinoshita, *Invest. Ophthal.* **13**, 713 (1974).
3. K. H. Gabbay, *New Engl. J. Med.* **288**, 831 (1973).
4. Y. C. Hamlett and H. Heath, *IRCS Med. Sci.* **5**, 510 (1977).
5. C. Bublitz and E. P. Kennedy, *J. biol. Chem.* **211**, 968 (1954).
6. H. F. Woods, *Acta med. scand. Suppl.* **542**, 87 (1972).
7. H. Heath, S. S. Kang and D. Philippou, *Diabetologia* **11**, 57 (1975).
8. G. Imre, *Albrecht v. Graefes Arch. Ophthal.* **201**, 263 (1977).
9. J. C. Hutton, P. J. Schofield, J. F. Williams and F. C. Hollows, *Aust. J. exp. Biol. med. Sci.* **52**, 361 (1974).
10. H. Heath and Y. C. Hamlett, *Diabetologia* **12**, 43 (1976).
11. D. Papachristodoulou, H. Heath and S. S. Kang, *Diabetologia* **12**, 367 (1976).
12. A. M. Cohen, A. Teitelbaum and E. Rosenman, *Metabolism* **26**, 17 (1977).
13. R. P. Boot-Handford and H. Heath, *Metabolism* **29**, 1247 (1980).
14. S. D. Varma and J. H. Kinoshita, *Biochem. Pharmac.* **25**, 2505 (1976).
15. J. C. Hutton, J. F. Williams, P. J. Schofield and F. C. Hollows, *Eur. J. Biochem.* **49**, 347 (1974).
16. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
17. P. Tfelt-Hansen and O. Siggaard-Andersen, *Scand. J. clin. Lab. Invest.* **27**, 15 (1971).
18. P. Armitage, *Statistical Methods in Medical Research*, pp. 205–206. Blackwell, Oxford (1971).
19. J. A. Belt, J. A. Thomas, R. N. Buchsbaum and E. Racker, *Biochemistry*, **18**, 3506 (1979).