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The Effect of Starvation on Phosphodiesterase Activity and the Content of Adenosine 3′:5′-Cyclic Monophosphate in Isolated Mouse Pancreatic Islets

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1. The concentration of cyclic AMP and the activity of phosphodiesterase were measured in isolated pancreatic islets from fed or 48h-starved mice. 2. Two different phosphodiesterases were detected. Neither the maximum activity nor the K_m values of these enzymes were changed by starvation. 3. The concentration of cyclic AMP in non-incubated islets was the same in islets from fed and starved mice. 4. Incubation with 3.3mM-glucose for 5–30min had no effect on the concentration of cyclic AMP, irrespective of the nutritional state of the mice. Incubation with 16.7mM-glucose for 5–30min raised the concentration of cyclic AMP by about 30% in islets from fed mice. This rise was prevented by addition of mannoheptulose (3mg/ml). Incubation with 16.7mM-glucose had no effect on the cyclic AMP content in islets from starved mice. 5. In islets from fed mice 10min incubation with 5mM-caffeine had no effect on the concentration of cyclic AMP in the presence of 3.3 or 16.7mM-glucose, whereas the cyclic AMP content was increased approx. 150% in islets from starved mice. 6. After 10min incubation with 1mM-3-isobutyl-1-methylxanthine in the presence of 3.3 or 16.7mM-glucose the concentration of cyclic AMP was raised by 250% in islets from fed mice and by 400% in islets from starved mice. 7. A threefold function of glucose in the insulin-secretory process is suggested, according to which the decreased islet glucose metabolism is the primary defect in the insulin-secretory mechanism during starvation.

Starvation results in an impairment of glucose-stimulated insulin secretion both *in vivo* (Grey *et al.*, 1970) and *in vitro* (Malaisse *et al.*, 1967*a*). We have shown (Hedeskov & Capito, 1974) that the decreased rate of insulin secretion during starvation is correlated with a decrease in glucose utilization in the islets and can be restored to normal values either by a very high extracellular glucose concentration or by addition of the phosphodiesterase inhibitor caffeine. These observations and the results reported by Voyles *et al.* (1973), that dibutyryl cyclic AMP as well as theophylline and tolbutamide restored the decreased secretory response to glucose seen in isolated rat islets during starvation, suggest that apart from a decreased glucose utilization, starvation may also be associated with a decreased concentration of cyclic AMP in the islets. Selawry *et al.* (1973) measured the concentration of cyclic AMP in rat pancreatic islets immediately after isolation by a collagenase method and found a decreased content of cyclic AMP after 48–72 h starvation. However, they have not investigated if glucose stimulation had any effect on the concentration of cyclic AMP. The role of cyclic AMP as a potentiator of glucose- or leucine-stimulated insulin secretion is well established (Malaisse *et al.*, 1967*b*; Ashcroft *et al.*, 1972*a*) and it has also been suggested that glucose may initiate insulin release by increasing the concentration of islet cyclic AMP (Cerasi & Luft, 1970).

The investigation of the effect of glucose on the adenylate cyclase–phosphodiesterase system has led to contradictory results as several authors, using isolated islets from mice, rats and guinea pigs, have been unable to find any effect of glucose neither on the concentration of cyclic AMP (Cooper *et al.*, 1973; Montague & Cook, 1971), nor on the activity of adenylate cyclase (Kuo *et al.*, 1973; Howell & Montague, 1973; Davis & Lazarus, 1972) nor phosphodiesterase (Ashcroft *et al.*, 1972*b*; Sams & Montague, 1972), whereas Charles *et al.* (1973) found a 2.5-fold increase in cyclic AMP concentration in rat islets after perifusion with 16.7mM-glucose and Grill & Cerasi (1973) found a stimulatory action of glucose on the adenylate cyclase activity in short-time incubations with isolated rat islets.

Howell *et al.* (1973) have shown a 40% decrease in basal adenylate cyclase activity in isolated islets from 48h starved rats and have suggested that glucose, or a glucose metabolite, may exert long-term effects on the adenylate cyclase activity.

In the present study we measured the activity of phosphodiesterase and the concentration of cyclic

AMP in islets from fed and 48h starved mice. We have found that the maximum extractable activity of phosphodiesterase as well as the basal concentration of cyclic AMP is unchanged during starvation. In the starved state glucose has no influence on the cyclic AMP concentration, whereas a 36% rise in cyclic AMP content is seen in the islets from fed mice, when they are incubated with 16.7mM-glucose for 10–30min. These results indicate that the contradictory results reported on the influence of glucose on the concentration of cyclic AMP in the β-cells may be explained by the different nutritional state of the animals used in the experiments.

Materials and Methods

Materials

Firefly-luciferase was from Sigma Chemical Co., St. Louis, Mo., U.S.A. Other enzymes and unlabelled nucleotides were from Boehringer G.m.b.H., Mannheim, Germany. 3-Isobutyl-1-methylxanthine was from Aldrich Chemical Co., Milwaukee, Wis., U.S.A. [8-^{3}H]Adenosine 3′:5′-cyclic monophosphate (27.5Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. Acropore filters (pore size 0.45μm) were from Gelman Instrument Co., Ann Arbor, Mich., U.S.A. Instagel was from Packard Instrument Co., La Grange, Ill., U.S.A. Cyclic AMP-binding protein from rabbit skeletal muscle was prepared as described by Cooper *et al.* (1972). Calf thymus DNA (sodium salt) was from BDH Chemicals Ltd., Poole, Dorset, U.K. All other reagents were of purest grade obtainable.

Methods

Preparation of islets. Male white mice (12–14) with a mean weight of 21g were selected and either fed on a standard laboratory diet *ad libitum* for 24h or starved for 48h with free access to drinking water. The mice were killed by decapitation and the islets prepared by the collagenase method described by Coll-Garcia & Gill (1969). The buffer used during collagenase treatment was supplemented with glucose (3.3mM), sodium pyruvate (5mM), L-glutamic acid (5mM) and sodium fumarate (5mM).

Phosphodiesterase assay. An islet homogenate was prepared by sonication (20s at position 1, Branson Sonifier B-12) of 100–125 islets in 50μl of 0.1M-triethanolamine buffer (pH7.7) containing 10mM-$MgSO_4$ and 0.5mM-EDTA. The sonicated material was dialysed for 1h at 4°C against the same buffer and then assayed immediately for phosphodiesterase activity (Ashcroft *et al.*, 1972*b*). The rate of conversion of cyclic AMP into 5′-AMP was constant for at least 60min, and was proportional to the amount of homogenate used. Tissue and medium blanks were carried through the whole procedure. The concentration of 5′-AMP in standards as well as that of cyclic AMP in the reaction medium was spectrophotometrically determined in each experiment (E_{260}).

Assay of cyclic AMP. Islets (10–25) were incubated at 37°C in 15μl of bicarbonate-buffered medium (Krebs & Henseleit, 1932) containing the additions given in the text, Tables or Figures. After incubation the tubes were transferred to a boiling-water bath and 35μl of boiling phosphate buffer was added (0.25M-potassium phosphate, 1.5mM-3-isobutyl-1-methylxanthine, 7.5mM-2-mercaptoethanol, pH5.5). This procedure took about 3s. The tubes were boiled for 10min, then the islets were homogenized by sonication (15s at position 1 on a Branson Sonifier B-12) and the denatured protein was removed by centrifugation at 4°C for 5min. The supernatants were stored at −20°C until the concentration of cyclic AMP was measured by the protein-binding radioassay method described by Gilman (1970) and modified by Cooper *et al.* (1972). An incubation volume of 85μl of 0.25M-potassium phosphate buffer, pH5.5, containing 1mM-3-isobutyl-1-methylxanthine, 6mM-mercaptoethanol, 0.02mg of binding protein and 1pmol of cyclic[^{3}H]AMP (27.5Ci/mmol) was used. The radioactivity retained on the filters was counted in 5ml of Instagel in a Packard Tri-Carb liquid-scintillation spectrometer, model 2405. The added protein was capable of binding 35% of the added radioactive cyclic AMP. Addition of 0.1pmol of cyclic AMP resulted in displacement of 13.4±1.2% (mean±S.E.M., $n = 17$) of the added radioactive cyclic AMP. Recovery of cyclic AMP added before boiling was 96.8±6.0% (mean±S.E.M., $n = 7$). The measured cyclic AMP content was found to be proportional to the amount of islet tissue used. No interference from ATP in amounts up to 400pmol was observed. About 10pmol of cyclic GMP corresponded to the binding of 0.3pmol of cyclic AMP. Treatment of the boiled extracts with phosphodiesterase decreased the cyclic AMP content to a value which was not different from zero.

DNA determination. DNA content of samples of the islet homogenate was assayed by the method of Kissane & Robins (1958) with calf thymus DNA as standard. The fluorimeter used was a Photovolt multiplier fluorescence meter, model 540, with a 25μl cuvette.

Results

Phosphodiesterase activity

Lineweaver–Burk plots of rates of hydrolysis at various concentrations of cyclic AMP were curvilinear suggesting the presence of two forms of phosphodiesterase, which agrees with the results obtained by Ashcroft *et al.* (1972*b*) for mouse islets and by Sams & Montague (1972) for guinea-pig islets.

Table 1. *Effect of starvation on phosphodiesterase activity*

Dialysed islet homogenate prepared from fed or 48h-starved mice was incubated for 60min at 37°C with various amounts of cyclic AMP. The 5'-AMP formed was converted into ATP and measured with a luciferin–luciferase method. K_m and $V_{max.}$ were calculated from Lineweaver–Burk plots, which were carried out by a least-squares curve-filling computer program. Results are given as means±S.E.M. of four experiments.

	Fed	Starved
Low-K_m phosphodiesterase		
K_m (μM)	6.3 ±2.9	4.6 ±0.9
$V_{max.}$ (pmol/h per 100ng of DNA)	84.7 ±7.6	84.4 ±8.1
High-K_m phosphodiesterase		
K_m (mM)	0.26±0.02	0.28±0.01
$V_{max.}$ (pmol/h per 100ng of DNA)	387±40	449±77

Table 2. *Cyclic AMP content in isolated islets of Langerhans after incubation with glucose*

Batches of 25 islets were incubated at 37°C in 15μl of the gassed (O_2+CO_2, 95:5) bicarbonate buffer containing glucose as indicated. After incubation the cyclic AMP content was determined by a protein-binding radioassay as described in the Materials and Methods section. Results are given as means±S.E.M. of 12 batches.

Glucose concn. (mM)	Incubation time (min)	Cyclic AMP (pmol/10 islets) Fed	48h-starved
0	0	0.112±0.013	0.128±0.008
3.3	5	0.121±0.020	0.111±0.006
16.7	5	0.134±0.015	0.106±0.009
3.3	10	0.124±0.018	0.119±0.014
16.7	10	0.158±0.019	0.112±0.009*
3.3	30	0.131±0.020	0.118±0.006
16.7	30	0.144±0.015	0.106±0.009*

* $P<0.05$ versus fed control animal.

Lineweaver–Burk plots were carried out by a least-squares curve-filling computer program and K_m and $V_{max.}$ were calculated assuming the presence of two enzymes (Table 1). Both the maximum activity of the two enzymes and the K_m values are unchanged by starvation.

Content of cyclic AMP

When islets were examined immediately after isolation a basal value of 0.120pmol of cyclic AMP/10 islets was found irrespective of the nutritional state. This value is similar to the concentration found in rat islets (Charles *et al.*, 1973; Montague & Cook, 1971), but somewhat higher than that reported for mouse islets by Cooper *et al.* (1973). Incubation with 3.3mM-glucose for 5–30min had no effect on the content of cyclic AMP in islets from either fed or starved mice (Table 2). When the glucose concentration was raised to 16.7mM the cyclic AMP content was increased 28–35% in islets from fed mice during 5–30min of incubation (Fig. 1). This increase was tested statistically by means of a differential *t* test and was found to be significant after 10 and 30min incubation with a high concentration of glucose ($P<0.001$ and $P<0.05$). The increase in cyclic AMP was prevented if mannoheptulose (3mg/ml) was added to the incubation medium (Table 3).

Incubation with 16.7mM-glucose had no effect on the cyclic AMP content in islets from 48h-starved mice (Fig. 1). After 10 and 30min incubation with 16.7mM-glucose the cyclic AMP content in islets from fed mice was significantly higher ($P<0.05$) than in islets from 48h-starved mice, whereas no difference in cyclic AMP content between the fed and starved state could be observed when islets were incubated with 3.3mM-glucose.

Incubation for 10min with 1mM-3-isobutyl-1-methylxanthine and 3.3 or 16.7mM-glucose resulted in approx. 250% rise in cyclic AMP in islets from fed mice (Table 3). Addition of 1mM-3-isobutyl-1-methylxanthine increased the cyclic AMP content about 400% in islets from starved mice and established a cyclic AMP content that was similar to or even higher than that seen in the fed state. Addition of 5mM-caffeine had no effect on the cyclic AMP content in islets from fed mice whereas the cyclic AMP content in islets from starved mice was increased approx. 150%.

Discussion

It is assumed in this work that the number of cells in islets from fed and starved animals is the same, as we

have shown that these islets contain the same amount of DNA (Hedeskov & Capito, 1974). Whether the proportion of β-cells change with the nutritional state is not known. Studies with isolated islets from rats (Voyles *et al.*, 1973) and mice (Hedeskov & Capito, 1974) suggested that the impaired insulin-secretory response to glucose during starvation may at least in part be correlated with a diminished concentration of cyclic AMP in the starved state.

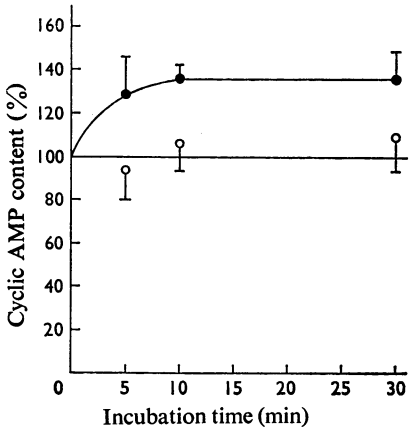

Fig. 1. *Effect of incubation with glucose on cyclic AMP content in pancreatic islets from fed and 48 h-starved mice*

Batches of 25 islets were incubated at 37°C in 15μl of gassed (O_2+CO_2; 95:5) bicarbonate buffer containing 3.3 or 16.7mM-glucose. After incubation the cyclic AMP content was determined by a protein-binding radioassay as described in the Materials and Methods section. In each assay high-glucose incubations were compared with low-glucose incubations and tested statistically against each other by means of a differential *t* test. Cyclic AMP content in the high-glucose incubations is expressed as %±S.E.M. ($n = 7$) of the corresponding cyclic AMP content in low-glucose incubations. A statistically significant rise in cyclic AMP content was obtained in islets from fed mice after 10 ($P<0.001$) and 30 ($P<0.05$)min. ○, Islets from starved mice; ●, islets from fed mice.

This hypothesis was confirmed by the present study, as we found a significantly higher concentration of cyclic AMP in islets from fed mice than in islets from starved mice after 10–30min of exposure to 16.7mM-glucose. However, we could not show any effect of starvation on cyclic AMP content if the islets were assayed immediately after isolation or after 5–30min incubation with 3.3mM-glucose.

We have thus been unable to confirm the results obtained by Selawry *et al.* (1973) who found a decreased concentration of cyclic AMP in non-incubated islets isolated from 48–72h starved rats. The observation that incubation with 16.7mM-glucose increased cyclic AMP only in islets from fed animals offers an explanation of the contradictory results published on this subject. An examination of the reports in question reveals that in those cases where a glucose regulation of cyclic AMP concentration has been reported (Charles *et al.*, 1973; Grill & Cerasi, 1973; Howell *et al.*, 1973) the investigators used fed animals. Unfortunately, the nutritional state of the animals is only stated in one (Howell & Montague, 1973) of the reports that postulates that glucose has no effect on cyclic AMP concentration or adenylate cyclase activity, and it has thus not been possible to confirm our hypothesis by literature studies.

A glucose-induced increase in cyclic AMP content can be created in two ways, either by inhibition of the phosphodiesterase or by stimulation of the adenylate cyclase.

Our finding that mannoheptulose, which is a known inhibitor of glucose phosphorylation in islets (Ashcroft *et al.*, 1970), can prevent the glucose-induced rise in cyclic AMP content in the fed state, makes it seem likely that the effect is not due to glucose itself

Table 3. *Effect of mannoheptulose and phosphodiesterase inhibitors on cyclic AMP content*

Batches of 10–25 islets were incubated for 10min at 37°C in 15μl of gassed (O_2+CO_2; 95:5) bicarbonate buffer with the additions given in the table. After incubation the cyclic AMP content was determined by a protein-binding radioassay as described in the Materials and Methods section. Results are given as means±S.E.M. of ten batches.

Addition	Cyclic AMP (pmol/10 islets)	
	Fed	48h-starved
Glucose (3.3mM)	0.124±0.018	0.119±0.014
Glucose (16.7mM)	0.158±0.019	0.112±0.009
Glucose (16.7mM)+mannoheptulose (3mg/ml)	0.070±0.014*	0.077±0.007
Glucose (3,3mM)+3-isobutyl-1-methylxanthine (1mM)	0.322±0.039	0.499±0.050†
Glucose (16.7mM)+3-isobutyl-1-methylxanthine (1mM)	0.360±0.037	0.452±0.027
Glucose (3.3mM)+caffeine (5mM)	0.121±0.011	0.192±0.027†
Glucose (16.7mM)+caffeine (5mM)	0.150±0.019	0.178±0.028

* $P<0.005$ versus glucose control; tested by differential *t* test.
† $P<0.05$ versus fed control; Student's *t* test.

but rather to a metabolite of glucose. This was also suggested by Howell *et al.* (1973), who found that the observed long-term regulation by glucose of adenylate cyclase activity in rat islets could at least partly be reproduced by pyruvate and mannose, whereas galactose and 2-deoxyglucose, which are not metabolized in islets, had no effect.

As both the maximum activity as well as the K_m (cyclic AMP) value of phosphodiesterase is unchanged during starvation it is not likely that glucose exerts its cyclic AMP increasing effect through this enzyme. This conclusion would also agree with studies with islets from both mice (Ashcroft *et al.*, 1972*b*; Bowen & Lazarus, 1972) and guinea pigs (Sams & Montague, 1972) where no effect of glucose on phosphodiesterase was observed. It is unlikely that the inability of glucose to increase cyclic AMP content in the starved state is due to a decreased concentration of adenylate cyclase during starvation as addition of the phosphodiesterase inhibitors caffeine or 3-isobutyl-1-methylxanthine produces a significantly higher concentration of cyclic AMP in islets from starved animals even at 3.3mM-glucose.

The observed glucose-induced increase in cyclic AMP content in the fed state is probably not due to an increased concentration of the substrate for the adenylate cyclase either, as we have shown that 30min incubation of mouse pancreatic islets with 16.7mM-glucose produces a significantly lower ATP content in the islets from fed animals than in starved controls (Hedeskov & Capito, 1974). Besides, a small change in ATP concentration in the islets would not cause any change in the adenylate cyclase activity, as the islet ATP concentration is much above the K_m (ATP) value for mouse islet adenylate cyclase reported by Davis & Lazarus (1972).

It is more likely that the diminished islet glucose utilization seen during starvation (Hedeskov & Capito, 1974) caused a decreased concentration of a glucose metabolite or cofactor that activates the islet adenylate cyclase and thereby prevented an activation of the enzyme in the starved state. The higher content of cyclic AMP in islets from starved animals in the presence of low glucose and 3-isobutyl-1-methylxanthine or caffeine may mean that phosphodiesterase displays an increased susceptibility for inhibition by methylxanthines under these conditions.

The precise role of cyclic AMP in insulin secretion is unknown, but it has been suggested that the insulinotropic action of cyclic AMP could be due to translocation of calcium within the β-cell (Brisson *et al.*, 1972) or to an increase in the activity of a cyclic AMP-dependent protein phosphokinase (Montague & Howell, 1972). Evidence has accumulated showing that no direct correlation exists between the concentration of cyclic AMP in the islets and the insulin secretion rate (Kuo *et al.*, 1973; Cooper *et al.*, 1973). This is also the case in the present study, e.g. 3.3mM-glucose+5mM-caffeine, which do not stimulate insulin secretion in our system, produces a concentration of cyclic AMP in islets from starved mice which is higher than that seen after incubation with 16.7mM-glucose (Table 3), which clearly stimulates insulin secretion in islets from starved mice (Hedeskov & Capito, 1974). On the other hand we have shown that 5mM-caffeine potentiates glucose-stimulated insulin release in islets from fed mice (Hedeskov & Capito, 1974), but this is not correlated with an increase in cyclic AMP content. This indicates the existence of another function of caffeine in the β-cell than that as phosphodiesterase inhibitor. This function may be a direct effect on Ca^{2+} allocation in the cells (C. J. Hedeskov, K. Capito & B. Formby, unpublished work).

These results in connexion with the observed correlation during starvation between decreased glucose utilization and impaired glucose-stimulated insulin secretion (Hedeskov & Capito, 1974), the failure of glucose to increase the content of cyclic AMP during starvation and the fact that mannoheptulose prevents glucose from increasing the cyclic AMP content in the fed state have led us to suggest a triple function of glucose in the insulin-secretory process: (1) to give rise to formation of a metabolite or cofactor which triggers the insulin-release mechanism; (2) to cause formation of a metabolite which activates adenylate cyclase and production of cyclic AMP in the islets. This would also explain that glucose potentiates its own effect on insulin release (Grodsky *et al.*, 1970); (3) to supply energy for the secretory process.

According to this suggestion the primary defect in the insulin-secretory mechanism during starvation would be the decreased islet glucose metabolism.

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References

Ashcroft, S. J. H., Hedeskov, C. J. & Randle, P. J. (1970) *Biochem. J.* **118**, 143–154

Ashcroft, S. J. H., Bassett, J. M. & Randle, P. J. (1972*a*) *Diabetes* **21**, *Suppl.* **2**, 538–545

Ashcroft, S. J. H., Randle, P. J. & Täljedal, I.-B. (1972*b*) *FEBS Lett.* **20**, 263–266

Bowen, V. & Lazarus, N. R. (1972) *Biochem. J.* **128**, 97P

Brisson, G. R., Malaisse-Lagae, F. & Malaisse, W. J. (1972) *J. Clin. Invest.* **51**, 232–241

Cerasi, E. & Luft, R. (1970) *Horm. Metab. Res.* **2**, 246–249

Charles, M. A., Fanska, R., Schmid, F. G., Forsham, P. H. & Grodsky, G. M. (1973) *Science* **179**, 569–571

Coll-Garcia, E. & Gill, J. R. (1969) *Diabetologia* **5**, 61–66

Cooper, R. H., McPherson, M. & Schofield, J. G. (1972) *Biochem. J.* **127**, 143–154

Cooper, R. H., Ashcroft, S. J. H. & Randle, P. J. (1973) *Biochem. J.* **134**, 599–605

Davis, B. & Lazarus, N. R. (1972) *Biochem. J.* **129**, 373–379
Gilman, A. G. (1970) *Proc. Nat. Acad. Sci. U.S.* **67**, 305–312
Grey, N. J., Goldring, S. & Kipnis, D. M. (1970) *J. Clin. Invest.* **49**, 881–889
Grill, V. & Cerasi, E. (1973) *FEBS Lett.* **33**, 311–314
Grodsky, G. M., Landahl, H., Curry, D. & Bennett, L. (1970) in *Structure and Metabolism of the Pancreatic Islets* (Falkmer, S., Hellman, B. & Täljedal, I.-B., eds.), pp. 409–420, Pergamon Press, Oxford
Hedeskov, C. J. & Capito, K. (1974) *Biochem. J.* **140**, 423–433
Howell, S. L. & Montague, W. (1973) *Biochim. Biophys. Acta* **320**, 44–52
Howell, S. L., Green, I. C. & Montague, W. (1973) *Biochem. J.* **136**, 343–349
Kissane, J. M. & Robins, E. (1958) *J. Biol. Chem.* **233**, 184–188
Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
Kuo, W.-N., Hodgins, D. S. & Kuo, J. F. (1973) *J. Biol. Chem.* **248**, 2705–2711
Malaisse, W. J., Malaisse-Lagae, F. & Wright, P. H. (1967*a*) *Amer. J. Physiol.* **213**, 843–848
Malaisse, W. J., Malaisse-Lagae, F. & Mayhew, D. (1967*b*) *J. Clin. Invest.* **46**, 1724–1734
Montague, W. & Cook, J. R. (1971) *Biochem. J.* **122**, 115–120
Montague, W. & Howell, S. L. (1972) *Biochem. J.* **129**, 551–560
Sams, D. J. & Montague, W. (1972) *Biochem. J.* **129**, 945–952
Selawry, H., Gutman, R., Fink, G. & Recant, L. (1973) *Biochem. Biophys. Res. Commun.* **51**, 198–204
Voyles, N., Gutman, R. A., Selawry, H., Fink, G., Penhos, J. C. & Recant, L. (1973) *Horm. Res.* **4**, 65–73