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EFFECT OF EXOGENOUS PHOSPHORYLATED MONOSACCHARIDES ON HORMONE-SENSITIVE LIPOLYSIS IN RAT ADIPOSE TISSUE

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Abstract—The activity of glucose-1-phosphate, glucose-6-phosphate, fructose, fructose-1-phosphate, fructose-6-phosphate, and fructose-1,6-diphosphate was tested on rat adipose tissue. The only active one was fructose-1,6-diphosphate (FDP). It slightly increased basal lipolysis and strongly potentiated the lipolytic effect of noradrenaline and theophylline, but not that of dibutyryl cAMP. Furthermore FDP increased basal ATP levels and potentiated cAMP accumulation induced by noradrenaline. Phosphate potentiated the lipolytic effect of noradrenaline but did not increase ATP level. The activity of FDP on lipolysis seems not due to its hydrolytic products and may be related to an increase of ATP availability.

Phosphorylated monosaccharides were used in the study of cellular regulation of lipolysis.

Previous work with metabolic inhibitors [1–11], aimed at biochemical and energetic interrelations between hormone-induced lipolysis and the metabolic sources of ATP, indicated energy requirement for lipolysis. Furthermore it suggested that glycolysis could supply ATP for the synthesis of cyclic AMP, whereas oxidative phosphorylation was important in successive stages of the process [1–3, 10, 11].

In this context, a more conventional experimental approach involving the use of glucose and insulin was questionable. For instance glucose does not enter by diffusion into the fat cells. Insulin cannot provide reliable results having other effects on lipolysis unrelated to glucose transport [12–14]. Finally, data obtained with glucose at very high concentration raised questions as glycerol and FFA release were affected in a different, even opposite way [4, 15–18].

Fructose-1,6-diphosphate, by contrast, offered a new experimental means to investigate this problem. Its central regulatory role in glucose and lipid metabolism has been recently brought to the fore (see review [19]). Apart from organ- and species-dependent variations in affecting glycolytic enzymes, the net effect of FDP always has been to stimulate glycolysis. Of particular interest have been reports indicating the stimulation by extracellular FDP of cellular lipolysis in adipose tissue [20, 21] although the transmembrane penetration of this compound has not been established.

These reported concomitant effects on both pathways have prompted us to use FDP in order to reinvestigate the relationship between glycolysis and lipolysis in rat adipose tissue and adipocytes. Both under basal conditions and in the presence of noradrenaline and other stimulating drugs (theophylline and dibutyryl cAMP), the release of glycerol and free fatty acids was determined, together with the levels of ATP and the cAMP accumulation. The activity of various phosphorylated monosaccharides was tested for comparison with FDP, however, it was the only active one in stimulating lipolysis. In relation to its action mechanism, FDP was found to increase ATP level in basal conditions and to potentiate cyclic AMP accumulation induced by noradrenaline. The mechanism of action of FDP showed partial similarity to that of inorganic phosphate, which also stimulated lipolysis but did not modify cAMP and ATP levels.

MATERIALS AND METHODS

Two months old male Wistar rats fed *ad libitum* have been used for these experiments.

Materials. Extract from firefly tails, ATP and bovine serum albumin were from Sigma, St. Louis, Missouri. ^{125}I ScAMP-TME was from New England Nuclear, Dreieichenhain, W. Germany. Cyclic AMP antiserum was a generous gift of Dr. G. Krishna, NIH, Bethesda. Noradrenaline bitartrate monohydrate was from Recordati, Milano; theophylline from Carlo Erba, Milano. FDP was kindly given from Biomedica Foscama, Roma; other phosphorylated sugars were from Boehringer, Mannheim; and dibutyryl cAMP was kindly given from Maggioni, Milano. Other chemicals were from standard suppliers.

Intact tissue. Epididymal fat pads obtained under ether anesthesia were pooled and randomized. Samples of 100 ± 5 mg were weighted, placed in 1.9 ml of Krebs–Ringer bicarbonate buffer containing 2.5% bovine albumin (fraction V) and preincubated

Abbreviations used FFA = free fatty acids; ATP = adenosine-5′-triphosphate; cAMP = adenosine-3′5′-monophosphate; F-1-P = fructose-1-phosphate; F-6-P = fructose-6-phosphate; FDP = fructose-1,6-diphosphate; G-1-P = glucose-1-phosphate; G-6-P = glucose-6-phosphate; ^{125}I ScAMP-TME = ^{125}I Succinyl cyclic AMP-Tyrosine Methyl Ester; P_i = inorganic phosphate.

Table 1. Effect of phosphorylated monosaccharides on glycerol release in rat adipose tissue

Additions	None	G-1-P	G-6-P	F-1-P	F-6-P	FDP
None	2.32 ± 0.27 (6)	2.53 ± 0.19 (4)	2.60 ± 0.25 (4)	2.35 ± 0.26 (6)	2.61 ± 0.30 (4)	3.15 ± 0.22 (6)*
Noradrenaline 2 μM	5.19 ± 0.35 (6)	5.12 ± 0.71 (4)	5.05 ± 0.82 (4)	5.18 ± 0.51 (6)	5.03 ± 0.28 (4)	8.54 ± 0.91 (6)**

Rat epidydimal fat pads (approximately 100 mg) were incubated at 37° in a metabolic shaker in 1.9 ml of Krebs–Ringer bicarbonate containing 2.5% bovine albumin and, where indicated, 1 mM phosphorylated sugar dissolved in 0.05 ml of saline. After 30 min the stimulating drug (noradrenaline) was added dissolved in 0.05 ml of saline and samples further incubated for other 150 min. At the end of incubation glycerol was estimated as indicated under "Materials and Methods". Each value, expressed as μmol/g fresh tissue, is the mean ± S.E. of the number of assays indicated in brackets.

* $P < 0.0025$ vs control.

** $P < 0.005$ vs noradrenaline.

for 30 min at 37° in a metabolic shaker both with or without sugars dissolved in 0.05 ml of NaCl 9‰ solution.

When phosphate was tested, it was added as a mixture of Na_2HPO_4 and NaH_2PO_4, to reach the same Na^+ concentration as given by 1 mM FDP. All the phosphorylated sugars were sodium salts.

At the end of the preincubation period noradrenaline, theophylline or dibutyryl cAMP (dissolved in 0.05 ml of saline) were added and the samples further incubated at 37°. Lipolysis was determined, unless otherwise indicated, after 180 min of total incubation. The reaction was stopped with 0.1 ml of 2.5 N H_2SO_4. FFA were determined in the medium according to Dole [22] and glycerol by enzymatic test (Boehringer, Mannheim). Parallel results were always obtained on these parameters.

Samples for ATP determination were incubated (after preincubation) for 30 or 60 min and, at the end of the incubation, tissue and medium were immediately separated by filtration under vacuum. ATP extraction was performed according to Bihler and Jeanrenaud [23] with minor modifications as previously described [24]. ATP determination was performed with luciferin–luciferase method [23, 25] as previously reported [24].

Isolated adipocytes. Epididymal fat pads obtained under ether anesthesia were washed in Krebs–Ringer bicarbonate buffer containing 3% albumin, pH 7.4, and finely cut. The tissue was suspended in the same buffer in the presence of crude collagenase (1.5 mg/ml) and fat cells were isolated according to Rodbell and Krishna [26]. The number of cells was estimated by counting under microscope and suspension adjusted to a final concentration of 40,000–80,000 cells/ml. Two ml aliquots were preincubated for 15 min both with or without FDP (dissolved in saline) at 37° in a metabolic shaker.

The incubation was started by adding noradrenaline dissolved in 0.01 ml of saline and stopped after 5 min by adding 0.2 ml of 50% TCA. After mixing and centrifugation (10,000 rev./min for 20min), TCA was removed from the supernatant fluid by repeated extractions with 6 ml of water saturated ether until a pH about 4.5 was reached. Cyclic AMP was then determined without further purification. Duplicate aliquots of 0.05 ml were assayed by the radioimmunoassay described by Frandsen and Krishna [27].

RESULTS

G-1-P, G-6-P, F-1-P, F-6-P and FDP were tested for their activity both on basal and noradrenaline-induced lipolysis in rat adipose tissue. Of these compounds only FDP was found to be effective. It slightly stimulated basal lipolysis but consistently potentiated the effect of noradrenaline (Table 1).

Neither fructose nor P_i, tested for comparison, had any effect on basal lipolysis, whereas noradrenaline stimulation was potentiated by either P_i or by P_i plus fructose, but not by fructose alone (Table 2).

Since noradrenaline-stimulated lipolysis was potentiated by both FDP and P_i, the problem arose whether FDP was active *per se* or after having been hydrolyzed into fructose plus P_i during the incubation period. The following experiments carried out to discriminate between the two possibilities, clearly show that FDP is active as such and not as a potential source of P_i: (i) The time course of lipolysis induced by noradrenaline in the presence and the absence of added FDP shows that lipolysis stimula-

Table 2. Effect of fructose and phosphate on glycerol release in rat adipose tissue

Additions	None	Fructose 1 mM	Phosphate 2 mM	Fructose + Phosphate
None	3.58 ± 0.22	3.87 ± 0.31	3.74 ± 0.14	3.79 ± 0.20
Noradrenaline 2 μM	14:56 ± 0.60	14.28 ± 1.56	21.32 ± 0.80*	22.01 ± 1.21**

Phosphate was added as a mixture of $Na\,H_2PO_4$ and Na_2HPO_4 to reach the same concentration of sodium given by 1 mM FDP. Other experimental conditions as in Table 1.
Each value, expressed as μmol/g fresh tissue, is the mean ± S.E. of four assays from 2 different experiments in duplicate.

* $P < 0.001$ vs noradrenaline.

** $P < 0.0025$ vs noradrenaline.

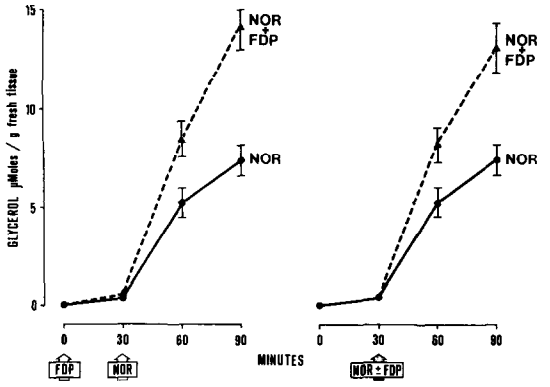

Fig. 1. Effect of FDP on noradrenaline-induced lipolysis in rat adipose tissue. Rat epididymal fat pads (100 ± 5 mg) were incubated at 37° in a metabolic shaker in 1.9 ml of modified, phosphate free, Krebs–Ringer bicarbonate solution containing 2.5% bovine albumin. In the left panel FDP (1.8 mM) dissolved in 0.05 ml of saline was added before incubation was started, while in the right panel FDP was added after 30 min of incubation. Noradrenaline (2 μM) dissolved in 0.05 ml of saline was always added after 30 min of incubation. At fixed time incubation was stopped and FFA and glycerol were determined as reported under "Materials and Methods".

Each value is the mean ± S.E. of 4 assays from 2 different experiments in duplicate.

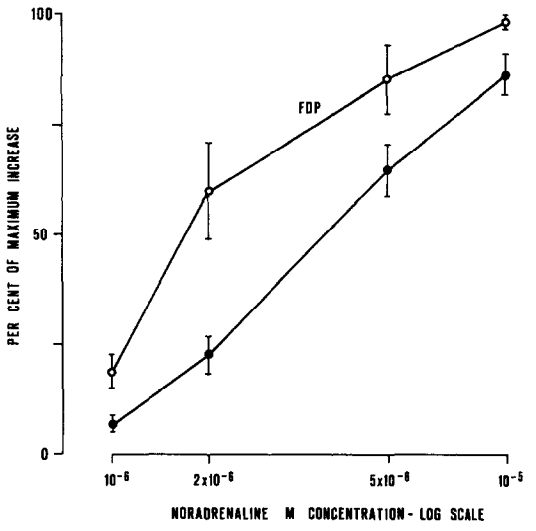

Fig. 2. Effect of FDP on lipolysis induced by various concentrations of noradrenaline. FDP concentration was 1 mM. Experimental conditions as in Table 1. Calculations were done considering FFA values, taking as 100 per cent the maximal increase induced by noradrenaline over appropriate control value.

Each value is the mean ± S.E. of 6 assays from 3 different experiments in duplicate.

tion was identical when FDP addition preceeded that of noradrenaline by 30 min or when FDP and noradrenaline were added simultaneously (Fig. 1). (ii) No significant increase in P_i was observed in the medium when 1.8 mM FDP was incubated for up to 90 min in the presence of adipose tissue (results not shown).

The relationship between FDP concentration in the medium and lipolytic rate was then studied at a fixed noradrenaline concentration (2 μM) (Table 3). 1 mM FDP yielded about 50 per cent of its maximal effect. At this FDP concentration the dose–effect curve of noradrenaline was strongly modified as shown in Fig. 2. The lipolytic response potentiation by FDP has been most marked at intermediate level (2 μM and 5 μM) of noradrenaline. Adrenaline and isoproterenol actions were similarly affected by FDP (not shown).

Table 4 illustrates that the lipolytic effect of theophylline was potentatiated by the same FDP concentration (1 mM) whereas that of dibutyryl cAMP remained unchanged.

A possible involvement of energy metabolism in the effect of FDP was investigated by evaluating ATP levels in adipose tissue in the presence and absence of added FDP. Addition of FDP resulted in an increase of ATP in basal conditions, but did not prevent the decrease in ATP induced by noradrenaline (Fig. 3). P_i failed to increase the ATP content even when simultaneously administered with fructose (Table 5).

Since FDP did not affect lipolysis induced by dibutyryl cAMP (Table 4), it seemed likely that its lipolytic action was related to changes in cAMP level. In order to test this hypothesis we measured the effect of FDP on cAMP in isolated adipocytes, which represent the most suitable tool to investigate cyclic nucleotide variations [26]. Indeed, Fig. 4 indicates that FDP did potentiate the noradrenaline effect on cAMP level at all noradrenaline concentrations tested.

Table 3. Effect of increasing concentrations of FDP on basal and noradrenaline-induced glycerol release in rat adipose tissue

Additions	FDP Molar concentration 0	5×10^{-5}	5×10^{-4}	10^{-3}	5×10^{-3}	10^{-2}
None	1.84 ± 0.21	2.06 ± 0.14	2.00 × 0.15	2.33 ± 0.19	2.45 ± 0.20	2.31 ± 0.34
		n.s.	n.s.	n.s.	$P < 0.05$	n.s.
Noradrenaline 2 μM	4.43 ± 0.41	4.20 ± 0.60	5.06 ± 0.49	6.60 ± 0.40	7.36 ± 0.48	6.65 ± 0.38
		n.s.	n.s.	$P < 0.005$	$P < 0.0025$	$P < 0.005$

Experimental conditions as in Table 1.

Each value, expressed as μmol of glycerol/g fresh tissue, is the mean ± S.E. of 4 assays from 2 different experiments in duplicate.

Table 4. Effect of FDP on lipolysis induced by theophylline and dibutyryl cAMP

Drugs		Glycerol μmol/g fresh tissue		FFA μEq/g fresh tissue	
		None	1 mM FDP	None	1 mM FDP
Theophylline	0.5 mM	7.41 ± 0.70	9.96 ± 0.59 P < 0.025	23.43 ± 2.90	30.89 ± 1.29 P < 0.01
cAMP-DB	0.2 mM	5.25 ± 0.60	5.26 ± 0.81 n.s.	15.56 ± 0.42	16.82 ± 0.91 n.s.
cAMP-DB	0.5 mM	10.23 ± 0.44	10.26 ± 0.50 n.s.	30.58 ± 0.83	28.04 ± 1.89 n.s.

Experimental conditions as in Table 1. cAMP-DB = dibutyryl cAMP.
Each value is the mean ± S.E. of 4 assays from 2 different experiments in duplicate.

DISCUSSION

FDP markedly potentiated lipolysis induced by noradrenaline in rat adipose tissue and cells in accordance with previous data [20, 21]. Such a potentiation by FDP was found also when theophylline was the lipolytic drug, but not when dibutyryl cyclic AMP was used for inducing lipolysis. Consequently, within the events of the lipase cascade, the site of action for FDP could be located after the interaction of catecholamines with beta-receptors, but before protein kinase activation by cAMP. Thus, attention was focused on processes regulating the cyclic AMP synthesis and turnover. FDP was found to potentiate cAMP accumulation induced by noradrenaline. It also increased ATP levels in basal conditions, that is, in the absence of drugs stimulating lipolysis.

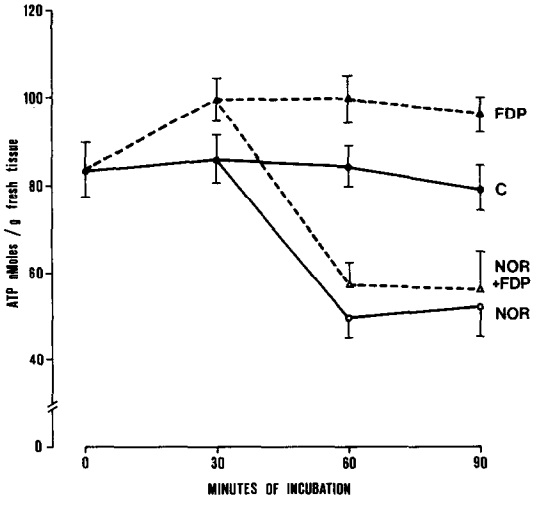

Fig. 3. Effect of FDP on ATP levels in rat adipose tissue. Rat epididymal fat pads (100 ± 5 mg) were incubated at 37° in a metabolic shaker in 1.9 ml of Krebs–Ringer bicarbonate containing 2.5% bovine albumin and, where indicated, 1 mM FDP dissolved in 0.05 ml of saline. After 30 min 2 μM noradrenaline was added and samples further incubated for 30 and 60 min. At the end of incubation ATP was extracted and determined as indicated under "Materials and Methods".

Each value is the mean ± S.E. of 8 assays from 4 experiments in duplicate.

A primary effect of FDP on enzymes controlling cAMP synthesis and accumulation (adenylate cyclase and phosphodiesterase) is improbable since FDP modified cAMP levels only in the presence of noradrenaline but not in basal conditions (Fig. 4). Further, adenylate cyclase from kidney plasma membrane [28] was unresponsive to FDP. Most probably FDP potentiated lipolysis by a primary action on carbohydrate metabolism. This is suggested by the fact that FDP activates several glycolytic enzymes and the overall rate of glycolysis [19] and inhibitors of glycolysis reduced cAMP synthesis in adipose tissue [10]. The increase in ATP by FDP may also favour cAMP synthesis. All this fits well with the observation that FDP added *in vitro* to human blood induced an increase in ATP level and a corresponding decrease in ADP, AMP and glucose [29].

The question remains open on the site and action mechanism of FDP. Chlouverakis [20] found FDP effective in homogenates as well as in intact tissue. The present data have shown activity by FDP both on adipose tissue and cells. However it is not yet clear if phosphorylated sugars do enter into intact cells. FDP could cross the adipocyte membrane in a catalytic amount, or, alternatively, it could act extracellularly, by modifying in some way the cell membrane properties, or by releasing P_i under the effect of tissue phosphatases. Inorganic phosphates

Table 5. Effect of phosphate and phosphate plus fructose on ATP level

Additions	Incubation time, min 30	60
None	72.12 ± 4.73 (7)	76.36 ± 6.25 (7)
Phosphate 2 mM	73.76 ± 2.04 (7)	67.73 ± 5.65 (7)
Phosphate + Fructose	69.80 ± 11.60 (4)	70.76 ± 9.93 (4)
FDP 1 mM	87.71 ± 5.35 (7)*	89.92 ± 4.71 (7)**

Phosphate was added as a mixture of Na H_2PO_4 and Na_2H PO_4 to reach the same concentration of sodium given by 1 mmolar FDP. Other experimental conditions as in Fig. 3.

Each value expressed as nmoles/g fresh tissue, is the mean ± S.E. of the number of assays as indicated in brackets.

* $P < 0.025$ vs control.

** $P < 0.05$ vs control.

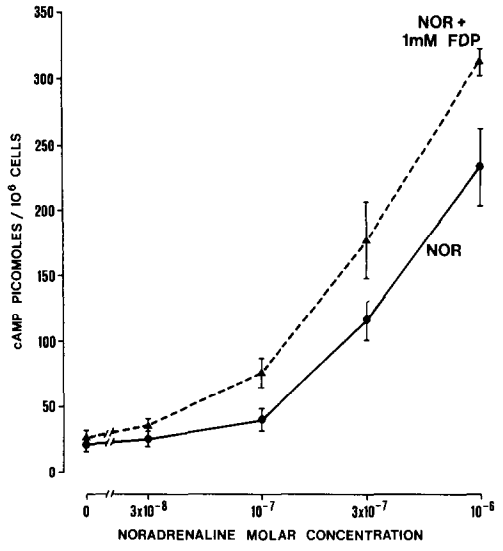

Fig. 4. Effect of FDP on cAMP levels in isolated adipocytes. Rat cells were isolated and prepared as indicated under "Materials and Methods". 2 ml aliquots of cell suspension were incubated in a metabolic shaker at 37° with or without 1 mM FDP. After 15 min noradrenaline was added and samples were further incubated for 5 min. At the end of incubation cAMP was determined as indicated under "Materials and Methods". Each value is the mean ±S.E. of 6 assays from 3 different experiments in duplicate.

potentiated noradrenaline-stimulated lipolysis (Table 2). Nevertheless a release of P_i by FDP during the incubation of adipose tissue is unlikely, as no significant increase of P_i was found in the medium after prolonged incubation; moreover, the action of FDP on noradrenaline-induced lipolysis was not dependent on incubation time which, instead, should have occurred if enzymatic hydrolysis of FDP had been involved (Fig. 1). Finally P_i ± fructose did not increase the basal levels of ATP (Table 5).

Further investigation is needed to clarify the site and action mechanism of FDP. The present data are meaningful from a physiological and pharmacological standpoint, as they support the role of energy producing metabolic pathways in modulating hormone-sensitive lipolysis [1–4]. In particular, glycolysis or one of its rate-limiting steps affected by FDP could be an important site of control and modulation for lipolysis. The foregoing is in accord also with the effects of metabolic inhibitors [1–10] and anoxia [1–3, 11]. Finally the present results provide additional evidence for the metabolic regulatory function of FDP [19].

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REFERENCES

1. G. Fassina, P. Dorigo and R. M. Gaion, *Pharmac. Res. Commun.* **6**, 1 (1974).
2. G. Fassina, P. Dorigo and R. M. Gaion, in *Lipids, Lipoproteins and Drugs* (Eds D. Kritchewsky, R. Paoletti and W. L. Holmes) *Adv. Exp. Med. Biol.* vol. 63, p. 105, Plenum Press, New York (1975).
3. G. Fassina, in *Advances in General and Cellular Pharmacology* vol. 2 (Eds T. Narahashi and C. P. Bianchi) p. 155, Plenum Press, New York (1977).
4. Y. Giudicelli, R. Pecquery, D. Provin, B. Agli and R. Nordmann, *Biochim. biophys. Acta* **486**, 385 (1977).
5. B. Mosinger, in *Handbook of Physiology—Adipose Tissue* (Eds A. E. Renold and G. F. Cahill), p. 601. Am. Physiol. Soc. Washington. (1965).
6. P. Björntorp, *Am. J. Physiol.* **210**, 733 (1966).
7. G. Fassina, I. Maragno and P. Dorigo, *Biochem. Pharmac.*, **16**, 1439 (1967).
8. C. Hollenberg and R. L. Patten, *Metabolism* **19**, 856 (1970).
9. J. N. Fain, *Molec. Pharmac.* **7**, 465 (1971).
10. G. Fassina, P. Dorigo, G. Perini and E. Tóth, *Biochem. Pharmac.* **21**, 2295 (1972).
11. G. Fassina, P. Dorigo and R. M. Gaion, in *Atherosclerosis III, Proceeding 3rd International Symposium* (Eds G. Schettler and A. Weizel) p. 851 (Abstract) Springer Verlag, Berlin (1974).
12. K. L. Zierler and D. Rabinowitz, *Trans. Assoc. Am. Physns* **76**, 245 (1963).
13. J. N. Fain, V. P. Kovačev and R. O. Scow, *Endocrinology* **28**, 773 (1966).
14. A. H. Kissebah, P. Clarke, N. Vydelingum, H. Hope-Gill, B. Tulloch and T. R. Fraser, *Eur. J. clin. Invest.* **5**, 339 (1975).
15. P. R. Bally, H. Kappeler, E. R. Froesch and A. Labbahrt, *Ann. N.Y. Acad. Sci.* **131**, 143 (1965).
16. F. Camu, *Archs int. Pharmacodyn. Thér.* **178**, 370 (1969).
17. S. Efendic and J. Ostman, *Acta med. scand.* **187**, 485 (1970)
18. J. J. Heindel, L. Orci and B. Jeanrenaud, in *Pharmacology of Lipid Transport and Atherosclerotic Processes* (Ed. E. J. Masoro). IEPT Sect. 24, vol. 1, p. 175. Pergamon Press, Oxford (1975).
19. M. E. Kirtley and M. McKay, *Molec. cell. Biochem.* **18**, 141 (1977).
20. C. Chlouverakis, *Metabolism* **17**, 708 (1968).
21. J. Bornstein, *Israel J. Med. Sci.* **8**, 407 (1972).
22. V. P. Dole, *J. clin. Invest.* **35**, 150 (1956).
23. J. Bihler and B. Jeanrenaud, *Biochim. biophys. Acta*, **202**, 496 (1970).
24. M. Prosdocimi, L. Caparrotta and K. Szyska, *Life Sci.* **20**, 961 (1977).
25. B. L. Strehler and J. R. Totter, *Archs Biochem. Biophys.* **40**, 28 (1952).
26. M. Rodbell and G. Krishna, in *Methods in Enzymology, Biomembranes* vol. XXXI (Eds S. Fleisher and L. Packer), Academic Press, New York (1974).
27. E. K. Frandsen and G. Krishna, *Life Sci.* **18**, 529 (1976).
28. Y. Fuyino and I. Yasumasu, *Biochem. biophys. Res. Commun.* **65**, 1067 (1975).
29. S. I. Magalini, A. Bondoli and S. Scrascia, *Resuscitation* **5**, 103 (1977).