The Effects of Glucagon and Insulin on Adenosine 3':5'-Cyclic Monophosphate Concentrations in an Organ Culture of Mature Rat Liver

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1. A modified radioimmunoassay for cyclic AMP was developed from the method of Steiner *et al.* (1969). Cyclic [^3H]AMP was used as the radioactive tracer. Free and antibody-bound nucleotides were separated by adsorption of protein to Millipore filters. The assay was used to measure amounts of cyclic AMP down to 0.1 pmol in 50 μl . 2. The effect of glucagon on cyclic AMP content in pieces of mature rat liver maintained for 6 days in organ culture was studied. 3. Cyclic AMP content in the tissue reached a maximum in 5-15 min and then decreased. This may have been partly due to an inhibitor of glucagon action formed in the tissue. Small amounts of cyclic AMP were released into the incubation medium. 4. The maximal increase in cyclic AMP content produced by glucagon decreased over 6 days in culture. However, liver pieces cultured for 2 and 6 days were more sensitive to low concentrations of glucagon than were fresh liver pieces. Glucagon concentrations for half-maximal effects were approx. 1 μM and 0.05 μM for fresh liver and 2-day cultured liver respectively. 5. Insulin (3.5 μM) lowered the cyclic AMP content by 30% in the presence of a submaximal glucagon concentration in liver cultured for 2 days. No effect of insulin was demonstrated on fresh liver pieces. 6. Insulin and glucagon were rapidly destroyed by fresh liver pieces.

Cyclic AMP has been proposed as the 'second messenger' mediating many effects of glucagon on mammalian liver, after activation by the hormone of membrane-bound adenylate cyclase (Robison *et al.*, 1971). Glucagon increases hepatic cyclic AMP content *in vivo* (Pauk & Reddy, 1971) and during perfusion *in vitro* (Exton *et al.*, 1971), whereas insulin lowers cyclic AMP content under suitable conditions (Jefferson *et al.*, 1968). It is thought that the intracellular cyclic AMP concentration regulated by glucagon, adrenalin and possibly insulin is an important factor controlling 'minute-to-minute' glucose output from perfused rat liver (Park *et al.*, 1972).

Little is at present known about mechanisms of long-term metabolic control in liver as reflected by changes in enzyme concentrations. The concentrations of several regulatory enzymes of hepatic lipid and carbohydrate metabolism vary in different dietary and hormonal states (Nichol & Rosen, 1963; Weber *et al.*, 1965; Johnson & Sassoon, 1967; Wimbhurst & Manchester, 1970). Cyclic AMP has been implicated as mediator of glucagon induction of phosphoenolpyruvate carboxykinase (EC 4.1.1.32) (Sahib *et al.*, 1971) and tyrosine transaminase (EC 2.6.1.5) (Wicks, 1969; Wicks *et al.*, 1969) and of repression of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) (Rudack *et al.*, 1971).

We decided to develop a system for culturing mature rat liver *in vitro* with which to study the con-

trol of cyclic AMP content and its possible importance in regulating enzyme concentrations. Other liver systems that had been used *in vitro* appeared unsatisfactory either because the cells did not survive long enough or because the cultured tissue differed in enzyme pattern or hormonal sensitivity from normal adult rat liver. Campbell & Hales (1971) described an organ culture for mature rat liver in which living cells were maintained for at least 6 days in small pieces of tissue incubated in a defined medium. The hormonal sensitivity of liver in this organ culture has now been tested by studying the effects of glucagon and insulin on cyclic AMP content in the tissue and incubation medium.

In addition to studies on control of enzyme concentrations, the culture promised to be useful for investigating factors required for maintenance of a hormonally sensitive adenylate cyclase. Other workers have shown that thyroxine and glucocorticoids may be important in regulating the amount and hormonal sensitivity of adenylate cyclase in various tissues (Krishna *et al.*, 1968; Levey *et al.*, 1969; Bitensky *et al.*, 1970).

Finally, the organ culture appeared suitable for study of the short-term control of cyclic AMP content after hormonal stimulation. Ho & Sutherland (1971) have presented evidence for the formation of an inhibitory substance participating in a feedback control of cyclic AMP content in adrenalin-stimulated fat-cells. The possibility that a similar phenomenon may occur

during hormonal stimulation of liver has been investigated.

A preliminary report of these findings has been presented (Campbell & Siddle, 1972).

Experimental

Materials

Chemicals. ATP (disodium salt) and cyclic AMP (free acid) were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K.; firefly lantern extract (FLE 50) was from Sigma (London) Chemical Co., Kingston, Surrey, U.K.; basal Eagle's medium (powder form) was from Wellcome Reagents Ltd., Beckenham, Kent, U.K.; bovine serum albumin (fraction V) was from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.; human serum albumin (fraction V) was from Fluka A.G., Buchs, Switzerland; [8-³H]adenosine 3':5'-cyclic phosphate, ammonium salt (25 Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K.; Triton X-100, 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene were from BDH Chemicals Ltd., Poole, Dorset, U.K.; crystalline ox insulin (24 units/mg) was from Burroughs Wellcome and Co., Dartford, Kent, U.K. Porcine glucagon was a gift from Eli Lilly and Co., Indianapolis, Ind., U.S.A., and 2'-O-succinyl-cyclic AMP [i.e. 2'-O-(3-carboxypropionyl)-cyclic AMP] was a gift from Boehringer Corp. (London) Ltd.

Animals. Laboratory-bred male Wistar rats (60–80 g, 4–5 weeks old) were used 1 week after weaning in all experiments. They were given free access to food and water.

Methods

Culture technique. The organ culture technique was that described by Campbell & Hales (1971). Pieces of liver (0.5–1.0 mg wet wt.) for each experiment were taken from a single liver and incubated in basal Eagle's medium (Eagle, 1955) supplemented with glucose to give a final concentration of 16.7 mM (3 mg/ml). Incubation was carried out without shaking at 37°C in 5 cm (2 in) diam. covered plastic dishes [grade AA from Esco (Rubber) Ltd., London W1N 5AB, U.K.]. Each dish contained 1.5 ml of medium and 8–12 pieces of tissue. The gas phase was O₂+CO₂ (95:5), and the incubation medium was changed every 2 days. Liver that had been cultured for 2 or 6 days was preincubated for 30 min in fresh medium, containing bovine serum albumin (1 mg/ml) as protein carrier, before addition of hormones. Fresh liver was incubated for 1 h in medium containing albumin before addition of hormones. The preincubation periods before hormone treatment allowed stabilization of tissue ATP and ion content. Hormones were added from stock solutions at 50 or 100 times the required final concentration. After rapid

mixing of hormones, dishes were returned to culture jars at 37°C and not shaken further.

Tissue extraction. Liver pieces were frozen in liquid N₂ and freeze-dried in an Edwards–Pearce tissue drier. Groups of 2–3 pieces (total dry weight 0.5–1 mg) were pooled and homogenized in 25 µl of 0.6 M-HClO₄ followed by addition of 100 µl of water. The homogenate was centrifuged for 2 min in EET 23 plastic tubes (Beckman Ltd., Glenrothes, Fife, U.K.) on a Beckman Microfuge, and 100 µl of supernatant was removed and neutralized by addition of 5 M-K₂CO₃. The samples were freeze-dried and redissolved in 120 µl of 50 mM-potassium phosphate buffer, pH 7.0. After centrifugation, duplicate samples were taken for assay of cyclic AMP (30 µl) and ATP (10 µl).

Extraction of incubation medium. Samples (1 ml) of incubation medium were deproteinized with an equal volume of 10% (w/v) trichloroacetic acid, and extracted four times with 5 ml of diethyl ether. The resulting solution was freeze-dried and the powder redissolved in 50 mM-potassium phosphate buffer, pH 7.0, to give a 5-fold concentration relative to the original incubation medium.

HClO₄ was convenient for extraction of tissue, for which the volumes were small and no concentration of sample was required. Trichloroacetic acid was preferred for extraction of incubation medium because a larger volume of liquid was subsequently to be concentrated before cyclic AMP assay, and it was desirable to avoid introducing further salts into the solution.

Cyclic AMP assay. Cyclic AMP was measured by a radioimmunoassay modified from the procedure of Steiner *et al.* (1969). Antibodies were obtained by injecting 2'-O-succinyl-cyclic AMP coupled to human serum albumin into rabbits. Sera were screened for ability to bind cyclic [³H]AMP, rate of dissociation of the antibody-cyclic AMP complex, and cross-reactivity of antibody with ATP, AMP, cyclic GMP and theophylline. One antiserum showing favourable characteristics in all these tests was selected for use in the assay. Serum was stored frozen at –20°C in 10 µl batches. It was thawed and diluted in 50 mM-potassium phosphate buffer, pH 7.0, immediately before use. Assay incubations contained 0.5 pmol (12.5 nCi) of cyclic [³H]AMP, antiserum at a final dilution of 1:500 (v/v) (sufficient to bind 40% of added tracer) and sample or standard, in a final volume of 50 µl of 50 mM-potassium phosphate buffer, pH 7.0, containing 5 mg of bovine serum albumin/ml. The range of standards normally used was 0.1–8 pmol, the standard solutions having been treated in exactly the same way as tissue or medium samples as described above. Incubation was carried out in EET 23 tubes overnight at 0°C. To separate free and antibody-bound cyclic AMP, 300 µl of ice-cold 50 mM-potassium phosphate buffer, pH 7.0, was then added to the assay mixture and the whole was rapidly transferred,

by using an ice-cold 500 μ l Hamilton syringe, to a filter disc under suction [2.5 cm diam. HAWP filters, of 0.45 μ m pore size, were from Millipore (U.K.) Ltd., London NW10 7SP, U.K.]. Syringe and incubation tube were immediately washed with a further 300 μ l of the cold buffer which was also transferred to the filter. The filter was finally washed with 3 ml of the cold buffer, transferred to 1 ml of methoxyethanol in a counting vial and left for 1 h to dissolve. Antibody-bound cyclic AMP adsorbed by the filter material was counted for radioactivity after addition of 10 ml of scintillation fluid [1 litre of toluene, 500 ml of Triton X-100, 4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene] in a Packard Tri-Carb liquid-scintillation spectrometer. A minimum of 10000 counts was recorded. Counting efficiency was 30%.

ATP assay. ATP was assayed by a modification of the firefly luciferase method of Addanki *et al.* (1966). The incubation volume (2 ml) contained firefly lantern extract (0.5 mg/ml), 20 mM-sodium arsenate, pH 7.4, and 2 mM-MgCl₂. The reaction was carried out at 8°C in a Packard Tri-Carb liquid-scintillation spectrometer. A plot of counts in 0.5 min against (pmol of ATP)² was linear.

Insulin assay. Insulin in incubation media was assayed by method C of Hales & Randle (1963). Standard solutions were diluted in basal Eagle's medium to make them equivalent to the experimental samples.

Results

Cyclic AMP assay

A typical standard curve for assay of cyclic AMP is shown in Fig. 1; 0.1 pmol of cyclic AMP in the assay decreased the counts bound by the antibody by approx. 10%. Recalculation of these results for a Scatchard (1949) plot gives a value of $3 \times 10^8 \text{ M}^{-1}$ for the mean association constant of cyclic AMP and antibody. Affinities for ATP, cyclic GMP and theophylline were estimated to be less than for cyclic AMP by factors of 80000, 30000 and 1000000 respectively. Thus the antibody was sufficiently specific to measure cyclic AMP in deproteinized tissue extracts without further purification. However, it appears that there will be a contribution of about 10–20% to the apparent basal cyclic AMP content of tissues owing to the other adenine nucleotides, which are present in 10000–20000-fold excess over cyclic AMP. This calculation was supported by treatment of extracts of rat adipose tissue and liver with cyclic AMP phosphodiesterase. In extracts of unstimulated tissue, only 80% of the apparent cyclic AMP content was destroyed by this enzyme, but when the cyclic AMP concentration was raised by suitable hormone treatment, the proportion of the cyclic AMP content of tissue extracts that was resistant to phosphodiesterase fell to negligible values.

Fig. 1. Standard curve for assay of cyclic AMP

Assay conditions were as described in the Experimental section, with a final incubation volume of 50 μ l. Standards were taken through the HClO₄ extraction procedure as used for tissue samples. Each point is the mean of two determinations. Total cyclic [³H]AMP per assay tube was 8500 c.p.m. (0.52 pmol).

Standards taken through the HClO₄ tissue-extraction procedure gave a curve almost superimposable on a standard curve set up in phosphate buffer. This implied that yields of cyclic AMP after extraction were almost 100%, and that any residual acid or salts did not appreciably affect the binding equilibrium. A significant displacement of the standard curve was found for standards taken through the trichloroacetic acid extraction procedure as used for the incubation medium. This appeared to be because of decreased cyclic AMP binding with the high concentrations of salts in the final sample (5-fold concentrated relative to the original medium), since the effect was not observed if standards extracted in this way were assayed at a greater dilution of residual salts from the medium.

Time-course of cyclic AMP content in response to glucagon

The concentrations of cyclic AMP in tissue and in medium were measured after incubation of fresh liver and 2-day and 6-day cultures with 12 μ M- (40 μ g/ml)

and 12 nM-glucagon. In fresh liver (Fig. 2) and 2-day cultures (Fig. 3), tissue cyclic AMP content increased up to about 10–15 min and then decreased, returning to near basal values by 2 h. Basal tissue cyclic AMP content did not change significantly during this period. Only very low concentrations of cyclic AMP were detectable in the medium after glucagon stimulation, and it appeared that these did not decrease until after 1 h. The time-course with 6-day cultures (Fig. 4) followed a similar pattern, although the peak content of tissue cyclic AMP was consistently reached sooner. Insufficient cyclic AMP was released into the medium from 6-day cultures for reliable measurement.

Experiments were carried out to test whether the peak observed in the cyclic AMP time-course could be due to destruction of glucagon or to a loss of tissue responsiveness to glucagon. In the first type of experiment, pieces of fresh liver (approx. 2 mg dry wt. of tissue in 1.5 ml of medium) were incubated with 6 μ M-glucagon for 2 h. Medium from several incubations was pooled ('old' medium) and 1.5 ml portions were transferred to new dishes with various added glucagon

concentrations. A parallel series of dishes with the same added glucagon concentrations in fresh medium was also set up. Meanwhile, fresh liver pieces from a second rat were preincubated for 1 h in medium without glucagon. After this preincubation period they were transferred for 15 min to the two series of dishes containing various glucagon concentrations in 'old' and 'fresh' medium. Dose-response curves obtained with the two types of medium are shown in Fig. 5. They are almost superimposable, indicating that the 2 h incubation of 'old' medium with tissue had destroyed almost all (>99%) the glucagon it originally contained, and that there had been no significant accumulation of any inhibitor of glucagon action in the 'old' medium. In a similar experiment, evidence was obtained for a small residual amount of glucagon (about 3%) after incubation of fresh liver pieces with 12 μ M-hormone for 2 h.

A second type of experiment involved reincubating pieces of liver previously exposed to glucagon in fresh medium containing glucagon (Table 1). Fresh

Fig. 2. Time-course of glucagon action on cyclic AMP content in fresh liver

Fresh liver pieces were preincubated for 1 h before addition of 12 μ M-glucagon, and samples were taken at various times after this. Two or three liver pieces, total dry weight about 0.5–1 mg, were pooled for each determination of tissue cyclic AMP. Extraction procedures were as described in the Experimental section. ●, Tissue cyclic AMP; ○, total cyclic AMP in 1.5 ml of medium. Vertical bars indicate $\pm$ S.E.M. of three determinations.

Fig. 3. Time-course of glucagon action on cyclic AMP content in 2-day cultured liver

Liver pieces from a single rat after culture for 2 days were preincubated for 30 min in fresh medium before addition of 12 μ M-glucagon (a) or 12 nM-glucagon (b). Symbols are as given in Fig. 2.

liver was preincubated for 1 h, and 12 μ M-glucagon was then added. This caused the normal increase in cyclic AMP content after 15 min followed by a decrease to approx. 3 times the basal value at 2 h. Control pieces were incubated without glucagon for this period. Both sets of pieces were then transferred to new medium containing 12 μ M-glucagon for a further 15 min. The tissue cyclic AMP content in this second incubation was much higher in the control pieces than in pieces that had previously been exposed to glucagon.

Changes in maximal response over the 6-day culture period

The amount of stimulation of tissue cyclic AMP contents caused by 12 μ M-glucagon at given stages

Fig. 4. Time-course of glucagon action on cyclic AMP content in 6-day cultured liver

Liver pieces after culture for 6 days were preincubated for 30 min in fresh medium before addition of 12 μ M-glucagon. Symbols are as given in Fig. 2.

in the culture period varied between experiments. However, there was a clear tendency for the stimulation to decrease over 6 days in culture (Figs. 2-4 and

Fig. 5. Evidence of the destruction of glucagon after incubation with fresh liver pieces

Medium containing 6 μ M-glucagon was used for incubating liver pieces for 2 h (2 mg dry wt. of fresh liver/1.5 ml of medium). This medium was pooled and used for incubating fresh liver pieces with various additional concentrations of glucagon for 15 min ($\square$). As a control, tissue from the same rat was incubated with the same glucagon concentrations in fresh medium ($\blacksquare$). Each point is the mean $\pm$ S.E.M. of four determinations.

Table 1. Effect of a second incubation with glucagon on cyclic AMP content in tissue previously incubated with glucagon

Fresh liver pieces were incubated with and without 12 μ M-glucagon for 2 h (first incubation). Pieces were then immediately transferred to fresh medium containing 12 μ M-glucagon for 15 min (second incubation). Results are tissue cyclic AMP content (nmol/g dry wt.), each value being the mean $\pm$ S.E.M. of four determinations.

Total incubation time (min) ...	First incubation		Second incubation 135
	15	120	
Exposed to glucagon at 120min	2.8 $\pm$ 0.5	2.8 $\pm$ 0.2	33.0 $\pm$ 7.3
Exposed to glucagon throughout	69.2 $\pm$ 5.9	9.6 $\pm$ 1.4	10.8 $\pm$ 1.2

Fig. 6). The effect is shown more clearly in an experiment where pieces of tissue from a single liver were used for all culture times (Table 2). Liver cultured for 1 h, 2 days and 6 days was exposed to glucagon ($12\mu\text{M}$) and theophylline (1mM), and cyclic AMP content was measured after 15 min. The effect of glucagon alone decreased with increasing culture time. Theophylline alone had no significant effect on fresh liver or 2-day cultures, and caused a further doubling of cyclic AMP content in the presence of glucagon. In 6-day cultures, theophylline appeared to increase basal cyclic AMP content 2–3-fold, and to cause a 4–5-fold increase compared with glucagon alone if added with the hormone. Although the scatter of values in a given experiment was sometimes appreciable, the same pattern of results, including the effects of theophylline at 6 days, was consistently observed on several occasions. ATP content of the tissue was not significantly affected by glucagon or theophylline, and the mean content showed little change during the culture period (Table 2).

Dose-responses

Pieces of liver were incubated for 15 min with various glucagon concentrations after the usual culture periods. Dose-response curves are shown in Fig. 6. Glucagon concentrations for half-maximal response were $0.5\text{--}1\mu\text{M}$ with fresh liver, and $0.03\text{--}0.05\mu\text{M}$ with 2- and 6-day cultures.

Effect of insulin

Various concentrations of insulin were tested in the presence of submaximal concentrations of glucagon, the two hormones being added simultaneously and tissue incubated with them for 15 min. With fresh liver, in the presence of $0.6\mu\text{M}$ - ($2\mu\text{g/ml}$) or $0.03\mu\text{M}$ -glucagon, no inhibitory effect of insulin could be shown at concentrations between 0.14 nM and $1.4\mu\text{M}$ ($20\mu\text{units/ml}$ to $200\mu\text{units/ml}$). With 2-day cultures $3.5\mu\text{M}$ -insulin in several experiments consistently produced a 30–40% decrease in cyclic AMP content, increased by $0.03\mu\text{M}$ -glucagon (Table 3). Immuno-

Fig. 6. Dose-response curves for cyclic AMP content of liver pieces incubated with glucagon

Fresh liver pieces (a) were preincubated for 1 h and 2-day (b) and 6-day (c) cultured liver pieces for 30 min before addition of glucagon. Tissue cyclic AMP content was measured after 15 min incubation with hormone. Experiments (a), (b) and (c) used tissue from three different rats. Each point is the mean $\pm$ S.E.M. of three or four determinations.

Table 2. Cyclic AMP and ATP content of liver pieces during 6 days of culture

Fresh liver pieces, and 2-day and 6-day cultured liver pieces, all from a single rat, were preincubated in the usual way and then incubated for 15 min with glucagon ($12\mu\text{M}$) or theophylline (1mM) or both. Values are the means $\pm$ S.E.M. for three determinations (cyclic AMP, nmol/g dry wt.) or for twelve determinations (ATP, $\mu\text{mol/g dry wt.}$).

	Additions	Fresh liver	2-Day culture	6-Day culture
Cyclic AMP (nmol/g)	None	1.29 ± 0.31	1.77 ± 0.11	1.56 ± 0.51
	Theophylline	1.67 ± 0.07	1.83 ± 0.34	4.50 ± 0.65
	Glucagon	33.5 ± 5.80	13.9 ± 1.33	6.65 ± 1.47
	Glucagon + theophylline	75.8 ± 23.30	26.1 ± 2.12	25.0 ± 11.33
ATP ($\mu\text{mol/g}$)	—	3.53 ± 0.38	4.34 ± 0.19	4.91 ± 0.40

Table 3. *Effect of insulin on glucagon-stimulated cyclic AMP content*

Insulin and glucagon were added simultaneously to fresh liver (a), or 2-day cultured liver (b), and cyclic AMP content (nmol/g dry wt.) was measured after 15 min incubation with hormones. Experiments (a) and (b) used liver from different rats. Values are means $\pm$ S.E.M. of six determinations.

	Concn. of insulin (μM)	Glucagon concn. (μM)	Cyclic AMP content		
			...	0	0.03
(a) Fresh liver	0		1.82 $\pm$ 0.07	3.23 $\pm$ 0.30	23.2 $\pm$ 1.3
	0.014		—	3.17 $\pm$ 0.27	27.0 $\pm$ 3.8
	1.4		—	4.78 $\pm$ 0.19	25.7 $\pm$ 2.8
(b) 2-day culture	0		1.90 $\pm$ 0.24	10.44 $\pm$ 1.56	—
	0.035		—	10.69 $\pm$ 1.51	—
	3.5		—	7.57 $\pm$ 0.41	—

assayable insulin was measured during incubation with fresh liver pieces (2 mg dry wt. of tissue in 1.5 ml of medium). A concentration of 0.035 μ M-insulin (5 munits/ml) was lowered to an undetectable value (<10% of initial value) within 15 min, 0.35 μ M-insulin was 80% destroyed after 2 h and destruction of 3.5 μ M-insulin was complete within 48 h.

Discussion

A problem in expressing results from the organ culture was necrosis of cells at the centre of the tissue pieces because of inadequate oxygenation (Campbell & Hales, 1971). The content of ATP, K⁺ or soluble protein provides a measure of living cells, but only ATP can be conveniently assayed in samples extracted for assay of cyclic AMP. Content of DNA, RNA or of total protein is not a satisfactory index of surviving tissue (Campbell & Hales, 1971). Dry weight was used as a routine basis for expression of results as the amount of viable tissue, indicated by ATP content, was roughly proportional to tissue weight. ATP content was also measured when an index of the amount of living tissue was required.

The tissue content of cyclic AMP after addition of glucagon reached a maximum within 5–15 min and then decreased, without any marked increase in the concentration of cyclic AMP in the incubation medium (Figs. 2–4). This is comparable with the response of cyclic AMP content to hormones in other systems *in vitro*, including rat fat-cells (Manganiello *et al.*, 1971; Ho & Sutherland, 1971), rat liver cells (Johnson *et al.*, 1972), rat heart (Robison *et al.*, 1965), rat skeletal tissue (Chase & Aurbach, 1970) and mouse fibroblasts (Manganiello & Vaughan, 1972). Glucagon was rapidly destroyed in cultures of fresh liver (Fig. 5) and this would be expected to lead eventually to a decrease in cyclic AMP content (Exton *et al.*, 1971). Also an 'inhibitor' of glucagon action may have been formed because of the increased cyclic AMP concentration. This might cause

the unresponsiveness of tissue to a second addition of glucagon after previous exposure to the hormone (Table 1), by inhibiting adenylate cyclase or stimulating phosphodiesterase. Ho & Sutherland (1971) demonstrated the accumulation of an 'inhibitor' in the incubation medium after treatment of isolated fat-cells with adrenalin. In liver cultures, such an inhibitor, if present, remained predominantly in the tissue (Fig. 5). Bitensky *et al.* (1971) described a macromolecular inhibitor of glucagon-stimulated adenylate cyclase found in the cytoplasm of rat liver, which differed in properties from the compound studied by Ho & Sutherland (1971). It is not certain whether the accumulation of an 'inhibitor' would be important *in vivo* as a feedback control of cyclic AMP concentration, or whether it is an artifact of tissue incubation *in vitro*.

The peak cyclic AMP content in fresh liver cultures after addition of glucagon was variable, but generally similar to that found *in vivo* (Pauk & Reddy, 1971) and in perfused liver (Exton *et al.*, 1971). The magnitude of the cyclic AMP response decreased over 6 days of culture (Fig. 6 and Table 2). This was not a result of increased cell death as the ATP content of the tissue was stable throughout the culture period (Table 2). The increased effect of theophylline after 6 days of culture suggests that an increase in phosphodiesterase activity may have been partly responsible for the lower peak of cyclic AMP. It is also possible that there was a decrease in adenylate cyclase activity. There was no histological evidence for a decrease in the ratio of parenchymal to non-parenchymal cells.

Tissue cultured for 2 days was more sensitive than fresh liver to low doses of glucagon, the concentrations for a half-maximal effect being about 0.05 μ M and 1 μ M respectively (Fig. 6). By using perfused rat livers, Exton *et al.* (1971) found that a glucagon concentration of 0.01 μ M gave a half-maximal increase in cyclic AMP content. Glucagon concentrations of 0.004 μ M (Pohl *et al.*, 1971) and 0.06 μ M (Hepp *et al.*, 1970) have been reported for half-maximal activation

of adenylate cyclase in liver membranes. The relative insensitivity of liver cultures to glucagon, particularly the fresh liver, may have been caused by hormone destruction together with restricted diffusion into the tissue pieces. The cyclic AMP content of fresh and 2-day cultured liver after addition of glucagon did not reach a maximum for 10–15 min (Figs. 2 and 3) compared with 3 min in perfused livers (Exton *et al.*, 1971), confirming that the hormone did not have rapid access to all the tissue in the organ cultures. The degradation of polypeptide hormones by liver has been well documented (Kenny, 1960; Pohl *et al.*, 1972; Freychet *et al.*, 1972) and glucagon and insulin were rapidly destroyed by fresh liver cultures. The tissue may become sensitive to lower concentrations of glucagon (Fig. 6) and insulin (Table 3) during culture because of decreased hormone destruction or more rapid diffusion of hormone into the pieces.

Even with 2-day cultures a high concentration of insulin was required to produce a small decrease in cyclic AMP (Table 3). Insulin inhibits glucagon-stimulated adenylate cyclase in liver and adipose tissue membranes (Hepp & Renner, 1972; Illiano & Cuatrecasas, 1972) and stimulates phosphodiesterase in adipose tissue (Loten & Sneyd, 1970), but it is not certain which action of the hormone is primarily responsible for its effect on tissue cyclic AMP content.

It is hoped that the organ culture will be useful for further studies of the control and regulatory importance of cyclic AMP content in liver. Perfused liver has advantages over the organ culture for study of acute hormone effects because of its greater hormone sensitivity and because it may be possible to measure cyclic AMP released into the perfusate as an index of the free, metabolically active nucleotide in the tissue (Park *et al.*, 1972). The concentration of cyclic AMP released in the organ culture was very low. One advantage of the organ culture is that many tissue pieces may be obtained from a liver and a large number of variables can be investigated in a single experiment.

The fact that liver remained sensitive to glucagon during 6 days of culture suggests that the system may be valuable for studies requiring observations over relatively long periods, such as investigation of the role of cyclic AMP in enzyme induction. It will also be interesting to study the control of the concentrations of adenylate cyclase and phosphodiesterase, as the balance between these enzymes apparently changes during the culture period. Perfused livers cannot be maintained for more than a few hours, and the usefulness of cultures of foetal liver and hepatomas is limited by the biochemical differences between these preparations and adult rat liver (Greengard, 1970; Gelehrter & Tomkins, 1970).

Preliminary work has shown that adult human liver can be cultured by the procedure described for rat. This may provide a unique system with which to

study the control of metabolism in normal and diseased human liver *in vitro*.

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