Protein Degradation in Hepatocyte Monolayers

EFFECTS OF GLUCAGON, ADENOSINE 3':5'-CYCLIC MONOPHOSPHATE AND INSULIN

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1. Hepatocytes were isolated by collagenase perfusion of livers from fed rats and established in stationary monolayer culture. 2. Degradation of intracellular protein was measured in these monolayers after labelling for 16h with [³H]leucine followed by a 3h chase period in medium containing 2mM-leucine. 3. Proteolysis in this system was stimulated by physiological concentrations of glucagon and also by added dibutyryl cyclic AMP. The effects of these two agents were not additive, which is consistent with the view that they act by the same mechanism. 4. A close correlation was found between intracellular cyclic AMP concentrations generated by glucagon and the degree of stimulation of proteolysis elicited by the hormone. 5. Insulin reduced glucagon-stimulated proteolysis, but not glucagon-elevated intracellular cyclic AMP concentrations. 6. The continual presence of either insulin or glucagon was necessary for the full expression of their effects on proteolysis. 7. In the presence of cycloheximide, proteolysis was normally responsive to glucagon but not to insulin. In contrast, proteolysis was not responsive to either hormone in the presence of ammonia, an agent that blocks the final lysosomal step of protein breakdown. 8. We propose that in hepatocyte monolayers glucagon may act via cyclic AMP to increase cellular autophagy and thus increase proteolysis, whereas insulin inhibits these processes independently of cyclic AMP.

Previous studies of the hormonal regulation of intracellular protein degradation in liver have used the isolated perfused organ, in which it has been shown that proteolysis is stimulated by glucagon (Miller, 1960; Mallette *et al.*, 1969; Woodside *et al.*, 1974) and inhibited by insulin (Mortimore & Mondon, 1970). However, these measurements with the perfused liver suffer from some inherent disadvantages. When protein degradation is measured as release of radioactive amino acid from protein prelabelled *in vivo*, the amount of radioactivity incorporated *in vivo* will be small unless very large doses of radioactive isotope are administered. If, on the other hand, radioactive precursor is added during perfusion and immediately preceding the degradation measurement, proteins of short half-life will contribute markedly to measured proteolysis, because of the relatively brief time over which the perfusion can be sustained. We have shown previously that the degradation of short-lived proteins is not regulated (Knowles & Ballard, 1976). In addition, a very high concentration of unlabelled precursor must be added to minimize the effects of reincorporation of label (Mortimore & Mondon, 1970). Where amino acid release is used as an index of protein breakdown in

the absence of radioactive labelling, reutilization of amino acids by protein synthesis must be prevented by the addition of cycloheximide (Woodside *et al.*, 1974), which by itself inhibits protein degradation (Ballard, 1977). Furthermore, only one test can be carried out on each liver, and sequential measurements of intracellular metabolites such as cyclic AMP are not possible.

A detailed study of the effects of hormones on protein degradation in liver is clearly facilitated by the isolation from the organ of parenchymal cells in homogeneous suspension, with which many individual effectors can be tested and appropriate controls included with the one cell preparation (Hopgood *et al.*, 1977). In addition, such preparations will contain only the one major liver cell type, the parenchymal cell, and effects on only this single cell type will be measured. However, in preliminary experiments we have found that protein breakdown in parenchymal cell suspensions is poorly responsive to glucagon. Consequently we have established hepatocytes in stationary monolayer culture. This modification offers the advantages that relatively stable intracellular proteins can be labelled by long-term exposure to radioactive precursors, the con-

tribution of short half-life proteins to overall protein breakdown can be minimized by 'chase' incubation, and cell washing procedures and medium changes are easily accomplished. In this system the effects of glucagon and insulin on protein degradation have been examined with particular reference to the possible intermediary role of cyclic AMP.

Materials and Methods

Chemicals

Gentamycin (Schering Corp.) was supplied by Essex Laboratories Pty. Ltd., Baulkham Hills, N.S.W., Australia. Cyclic AMP assay kits were from The Radiochemical Centre, Amersham, Bucks., U.K. Dibutyl cyclic AMP, cycloheximide and 3-isobutyl-1-methylxanthine were from Sigma Chemical Co., St. Louis, MO, U.S.A. Porcine glucagon was a generous gift from Eli Lilly and Co., Indianapolis, IN, U.S.A. The sources of all other chemicals and materials were as previously described (Knowles *et al.*, 1975; Knowles & Ballard, 1976; Hopgood *et al.*, 1977).

Animals

Male Wistar rats (160–200 g) were fed *ad libitum* on a high-carbohydrate diet containing 20% protein and 4% fat. Darkness was maintained in the colony from 19:00 to 07:00h and the rats were routinely used at 14:00h.

Preparation and culture of hepatocytes

Suspensions of isolated hepatocytes were prepared by the technique of Berry & Friend (1969) as modified by Hopgood *et al.* (1977). The cells were washed and plated in 50mm plastic Petri dishes as described previously (Gunn *et al.*, 1977). Non-viable cells, which did not attach immediately to the dishes, were removed after 1h by aspirating the medium, and fresh medium was added (Laishes & Williams, 1976). After incubation for a further 23h, the adherent cells had flattened out on the surface of the dishes to form confluent areas of touching polygonal cells (Laishes & Williams, 1976). Each Petri dish contained about 1 mg of cell protein.

Measurement of protein degradation

The method has been described by Gunn *et al.* (1977) and only the main points are reported here. At 24h after plating, cell proteins were labelled for 16h in leucine-free medium plus [3 H]leucine (1 μ Ci; 0.02nmol/ml). The labelling medium was then replaced by a medium consisting of Eagle's (1959) minimal essential medium with 2mM-leucine and antibiotics added ('Chase MEM'; Gunn *et al.*, 1977) and incubation continued for a further 3h. Cell monolayers were then washed twice with Earle's salts and incubated in 'chase MEM' medium for

the 4h degradation period. At this time staining of the monolayers with Trypan Blue indicated that non-viable cells, rounded up but still attached to the cell monolayer, accounted for about 5% of the total cell population.

At the completion of the degradation period Triton X-100 was added to the monolayers to give a final concentration of 0.5%, after which cells plus medium were removed by aspiration, and vortex-mixed vigorously. Protein was precipitated from this mixture by the addition of trichloroacetic acid (final concentration 1M), and the radioactivity in trichloroacetic acid-soluble and -insoluble fractions determined as described previously (Hopgood *et al.*, 1977; Gunn *et al.*, 1977). The percentage protein degradation at 4h is calculated as 100 times the radioactivity in the trichloroacetic acid-soluble fraction divided by the total radioactivity. A zero-time value is subtracted (Hopgood *et al.*, 1977). Variability between dishes in the amount of cell protein and initial protein labelling is minimized in the above calculation (Knowles *et al.*, 1975; Table 1 below).

In experiments where hormones were added for short periods and then removed, the initial incubation medium containing the hormone was aspirated at $\frac{1}{2}$, 1 or 2h and sampled, the cell monolayer washed twice with 'chase MEM' medium and incubation continued in this medium for the remainder of the incubation period. All control plates in which hormones were never present or present for the entire 4h period were subjected to the same washing procedure. The percentage protein degradation at 4h was in this case calculated as 100 times the sum of the trichloroacetic acid-soluble radioactivity in the medium at the intermediate sampling time and the trichloroacetic acid-soluble radioactivity in cells plus medium at 4h divided by the total radioactivity in all fractions. At all sampling times, cell-associated trichloroacetic acid-soluble radioactivity was less than 5% of that in the medium, and trichloroacetic acid-insoluble radioactivity in the medium was less than 6% of that in the cells.

Statistical treatment of results

From each preparation of cell monolayers, duplicate or triplicate incubations were established for each experimental treatment. Percentage-degradation values shown are means $\pm$ S.E.M. for the total number of dishes used for each treatment and the number of cell preparations given.

Protein measurements

The protein content of monolayers was measured in the Triton extract after the addition of an equal volume of 1M-NaOH. Subsamples of the NaOH digest were assayed in the presence of sodium dodecyl sulphate as described by Dulley & Grieve

(1975). Crystalline bovine serum albumin in Triton X-100 was used as the reference standard.

Cyclic AMP measurements

Cyclic AMP in the cell monolayers was measured after removal of the incubation medium and addition of 2ml of ice-cold 5% (w/v) trichloroacetic acid. Cells were harvested by scraping, and the suspension was vortex-mixed and centrifuged (1000g, 5min). A sample (1ml) of the supernatant was applied to a Bio-Rad AG50W (X8; 200-400 mesh) column of 2ml bed volume, and cyclic AMP eluted with water (Steiner, 1974). The portion of the column eluate containing cyclic AMP was evaporated to dryness at 40°C, and dissolved in 20mM-Tris/HCl buffer, pH7.5. These reconstituted samples were assayed as described by Gilman (1970) by using The Radiochemical Centre assay kit.

Results

Effects of glucagon and dibutyryl cyclic AMP

Preliminary experiments showed that protein degradation in hepatocyte suspensions was not responsive to glucagon. However, glucagon inhibited glycolysis, stimulated gluconeogenesis (Clark, 1976) and stimulated glycogenolysis (Clark *et al.*, 1977) in identical preparations. Since protein breakdown in control cells in suspension occurs at a rate of 3.5% of intracellular protein degraded/h (Hopgood *et al.*, 1977), a rate nearly double that observed *in vivo* (Garlick *et al.*, 1976; Scornik & Botbol, 1976), we considered that glucagon may have had no effect because proteolysis was already maximally stimulated in these cells. Control rates of protein breakdown in monolayer hepatocytes were approx. 2.5% of labelled protein degraded/h (Table 1), about two-thirds of the rate for cells in suspension and approx. 75% of that observed with the perfused liver (3.1%/h; Mortimore & Mondon, 1970). Glucagon was found to stimulate proteolysis strongly in the monolayers (Table 1), and the effect was of the same magnitude as the inhibitory effect of insulin previously observed in these cultures

(Gunn *et al.*, 1977). Dibutyryl cyclic AMP also stimulated protein breakdown in hepatocyte monolayers (Table 1).

A half-maximal effect was observed at a glucagon

Fig. 1. Stimulation of protein degradation by glucagon (a) and dibutyryl cyclic AMP (b)

Hepatocytes were established in monolayer culture, their proteins labelled, and proteolysis was measured over a 4h incubation period as described in the Materials and Methods section. Values are means $\pm$ S.E.M. for measurements on a minimum of four dishes from two cell preparations.

Table 1. Stimulation of intracellular protein degradation by glucagon and dibutyryl cyclic AMP in hepatocyte monolayers

Hepatocytes were established in monolayer culture, their intracellular proteins labelled and proteolysis was measured over a 4h incubation period as described in the Materials and Methods section. Values, except for the zero-time control, are means $\pm$ S.E.M. for six dishes. The data are taken from a single cell preparation.

Additions	Incubation time (h)	10 ⁻⁶ $\times$ Radioactivity per dish (d.p.m.) in:		Degradation (%)	Increment
		Amino acids	Protein		
—	0	0.012 (2)	2.216 (2)	0.52 (2)	—
—	4	0.230 $\pm$ 0.002	2.028 $\pm$ 0.029	10.20 $\pm$ 0.09	9.68 $\pm$ 0.09
Glucagon (5nM)	4	0.272 $\pm$ 0.005	1.891 $\pm$ 0.025	12.57 $\pm$ 0.22	12.05 $\pm$ 0.22
Dibutyryl cyclic AMP (50 μ M)	4	0.275 $\pm$ 0.007	1.948 $\pm$ 0.027	12.36 $\pm$ 0.15	11.84 $\pm$ 0.15

Table 2. *Correlation between glucagon-mediated intracellular cyclic AMP concentration and proteolysis*

Experimental details were as given in Table 1, except that intracellular cyclic AMP was measured at 1, 2, 5, 10 and 15 min of the incubation period as described in the Materials and Methods section. The concentration of 3-isobutyl-1-methylxanthine was 50 μ M. Values are means $\pm$ S.E.M. for the numbers of dishes given in parentheses. The number of cell preparations was three.

Additions	Degradation (%)	Peak cyclic AMP concentration (pmol/mg of protein)
None	11.0 $\pm$ 0.3 (31)	7.0 $\pm$ 0.6 (11)
3-Isobutyl-1-methylxanthine	11.6 $\pm$ 0.2 (21)	8.2 $\pm$ 1.5 (8)
Glucagon (0.01 nM)	11.4 $\pm$ 0.2 (10)	6.7 $\pm$ 0.8 (3)
Glucagon (0.01 nM) + 3-isobutyl-1-methylxanthine	12.9 $\pm$ 0.3 (13)	10.3 $\pm$ 1.4 (4)
Glucagon (0.1 nM)	12.7 $\pm$ 0.2 (16)	10.6 $\pm$ 1.1 (5)
Glucagon (0.1 nM) + 3-isobutyl-1-methylxanthine	13.4 $\pm$ 0.3 (18)	25.3 $\pm$ 4.4 (6)
Glucagon (1 nM)	13.3 $\pm$ 0.1 (10)	32.0 $\pm$ 8.6 (3)
Glucagon (1 nM) + 3-isobutyl-1-methylxanthine	13.7 $\pm$ 0.3 (7)	110*

* Mean of two observations, 108 and 112.

Table 3. *Stimulatory effects on proteolysis of glucagon and dibutyryl cyclic AMP alone and when added together*

Experimental details were as given in Table 1, except that the concentration of glucagon was 10 nM and that of dibutyryl cyclic AMP was 0.1 mM. Values are means $\pm$ S.E.M. for the numbers of dishes given in parentheses. The number of cell preparations was three.

Additions	Degradation (%)
None	10.4 $\pm$ 0.1 (16)
Glucagon	12.7 $\pm$ 0.3 (8)
Dibutyryl cyclic AMP	13.0 $\pm$ 0.3 (8)
Glucagon plus dibutyryl cyclic AMP	13.1 $\pm$ 0.5 (8)

concentration of about 0.5 nM, and a maximal stimulation of about 25% occurred at 10 nM (Fig. 1a). Dibutyryl cyclic AMP increased protein degradation to a similar degree to that which occurred with glucagon. In this case half-maximal stimulation was observed at about 5 μ M, with a maximal response at about 0.1 mM-dibutyryl cyclic AMP (Fig. 1b).

Because glucagon and dibutyryl cyclic AMP each stimulate proteolysis in hepatocyte monolayers, and since glucagon is known to raise intracellular cyclic AMP concentrations in liver (Shikama & Ui, 1976; Exton *et al.*, 1971a,b; Christoffersen & Berg, 1974), the possible association between intracellular cyclic AMP concentration and protein degradation was investigated. Table 2 shows that the addition of glucagon in the range 0.01–1 nM resulted in a parallel increase in both proteolysis and the maximum concentration of intracellular cyclic AMP generated by the hormone. This association was also noted in experiments in which the cyclic AMP phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine was added. Over the above range of glucagon concentrations, the addition of the inhibitor produced increases in both intracellular cyclic AMP and

protein degradation to the same extent as were observed with glucagon at a 10-fold higher concentration (Table 2).

Since the 25% stimulation of proteolysis observed with 1 nM-glucagon plus 3-isobutyl-1-methylxanthine was the maximum that could be achieved even with pharmacologically high concentrations of glucagon (1 μ M; Fig. 1), only a 4–6-fold stimulation of intracellular cyclic AMP concentrations above basal values appeared necessary for full stimulation of protein degradation by glucagon. If the action of glucagon and dibutyryl cyclic AMP in stimulating proteolysis is indeed effected via an increase in intracellular cyclic AMP, it would be expected that optimal concentrations of each hormone added together would not increase proteolysis further than that increase observed with the most effective of the two agents. This prediction is confirmed in Table 3, where the addition of 10 nM-glucagon with 0.1 mM-dibutyryl cyclic AMP does not stimulate proteolysis above that observed with dibutyryl cyclic AMP alone.

Effects of insulin

We have previously shown (Gunn *et al.*, 1977) that insulin is an effective inhibitor of proteolysis in hepatocyte monolayers, with a half-maximal effect occurring at about 1 nM. To investigate possible opposing interactions between insulin and glucagon, we have examined effects of the two hormones on proteolysis over a wide range of concentration (Fig. 2). When insulin was present at low concentrations (0.01 and 0.1 nM), the inhibitory effect on protein degradation was altered very little by added glucagon at one-tenth of the insulin concentration, but was nullified by an equal concentration of glucagon. With a 10-fold molar excess of glucagon, a significant stimulation of degradation was observed. This pattern of equimolar competition between the two hormones was not observed at higher insulin

concentrations. With 1 and 10nM-insulin a net inhibition of proteolysis remained when equimolar amounts of the two hormones were added, and a 10-fold excess of glucagon was required to prevent the inhibition by insulin.

Although insulin inhibits proteolysis in hepatocyte monolayers by up to 25 % (Gunn *et al.*, 1977), we have not been able to show any effect of insulin addition on intracellular cyclic AMP concentrations. A decrease in basal cyclic AMP is difficult to measure with any certainty, however, because the cyclic AMP

concentration in unstimulated cells is very close to the lower limit of the assay. Consequently, we examined the effect of insulin on cyclic AMP concentrations and proteolysis in monolayers where both parameters were increased by glucagon. It is clear from the data in Table 4 that the addition of insulin at one-tenth to ten times the concentration of glucagon caused a progressive inhibition of protein degradation

Fig. 2. Effect of insulin and glucagon mixtures on proteolysis

Experimental details are as given in Fig. 1. Glucagon/insulin molar ratios were 10:1 (□), 1:1 (●) or 1:10 (○). Values are means ± s.e.m. for measurements on a minimum of four dishes from two cell preparations.

Fig. 3. Effect on proteolysis of short-term exposure to glucagon or insulin

Experimental details are as given in Fig. 1, except that monolayers were exposed to hormone for ½, 1 or 2h, washed with hormone-free medium and then reincubated in hormone-free medium for the remainder of the 4h measurement period. ○, Glucagon (10nM); ●, insulin (10nM). Values are means ± s.e.m. for measurements on a minimum of eight dishes from three cell preparations.

Table 4. Effects of insulin on glucagon-elevated intracellular cyclic AMP concentrations and on glucagon-elevated proteolysis

Experimental details are given in Table 1. Values are means ± s.e.m. for the numbers of dishes in parentheses. The number of cell preparations was three.

Additions	Degradation (%)	Intracellular cyclic AMP concentration (pmol/mg of protein)		
		5 min	10min	15 min
None	9.1 ± 0.1 (11)	4.8 ± 1.2 (4)	4.9 ± 1.2 (4)	2.4 (2)
Glucagon (1 nM)	11.8 ± 0.3 (10)	37.9 ± 4.2 (6)	34.0 ± 4.9 (6)	20.4 ± 6.2 (3)
Glucagon (1 nM)+insulin (0.1 nM)	10.8 ± 0.7 (6)	24.8 ± 4.1 (3)	35.7 ± 8.5 (3)	20.2 (2)
Glucagon (1 nM)+insulin (0.5 nM)	9.3 ± 0.2 (6)	31.3 ± 3.1 (3)	37.9 ± 5.5 (3)	23.0 (2)
Glucagon (1 nM)+insulin (1 nM)	8.7 ± 0.2 (6)	32.7 ± 5.6 (3)	46.6 ± 6.1 (3)	22.7 (2)
Glucagon (1 nM)+insulin (10nM)	8.4 ± 0.4 (6)	37.0 ± 3.8 (5)	35.1 ± 5.5 (5)	18.3 ± 5.2 (3)

without a parallel decrease in the glucagon-generated 'spike' of cyclic AMP. Measurement of intracellular cyclic AMP concentrations through the 4h incubation period also showed no effect of insulin on the decay of glucagon-induced cyclic AMP concentration.

Persistence of insulin and glucagon effects

Hepatocyte monolayers were exposed to insulin or glucagon for various times, the medium was removed and the monolayers were washed with hormone-free medium and incubated in hormone-free medium for the remainder of the 4h experimental period. The results of this experiment (Fig. 3) showed that exposure of the cells to either hormone for up to 2h followed by its removal was not sufficient for the full effect of the hormone to be expressed. Indeed the magnitude of the glucagon-induced stimulation and the insulin-induced inhibition of protein breakdown

was approximately proportional to the exposure time of the cells to each hormone.

Effects of cycloheximide and ammonia

The protein-synthesis inhibitor cycloheximide has also been found to be an inhibitor of proteolysis in several cell types (Goldberg & St. John, 1976; Ballard, 1977). The relationship between the mechanism of action of this agent and of insulin and glucagon was examined by testing the effect of each hormone on proteolysis in the presence of $3\mu\text{M}$ -cycloheximide, a concentration that gives a maximal (30%) inhibition of proteolysis (Fig. 4a). We found that in the presence of cycloheximide, protein breakdown remained normally responsive to glucagon, even though cycloheximide reduced the basal rate of

Fig. 4. Inhibition of protein degradation by cycloheximide (a) and ammonia (b)

Experimental details are as given in Fig. 1. Values are means $\pm$ S.E.M. for measurements on a minimum of three dishes (a) or six dishes (b). The number of cell preparations was one in (a) and two in (b).

Fig. 5. Glucagon and insulin effects on proteolysis in the presence of cycloheximide (a) or ammonia (b)

Experimental details are as given in Fig. 1, except that $3\mu\text{M}$ -cycloheximide (a) or 5mM - NH_4Cl (b) was present in all dishes for the 4h measurement period. $\circ$, Glucagon; $\bullet$, insulin. Values are means $\pm$ S.E.M. for measurements on a minimum of seven dishes (a) or four dishes (b). The number of cell preparations was three in (a) and two in (b).

proteolysis (Fig. 5a). However, insulin at a concentration up to 10 nM produced very little inhibition of proteolysis additional to that observed with cycloheximide alone. Control dishes in these experiments showed that the cells used were fully responsive to glucagon (27% stimulation at 10 nM) and to insulin (17% inhibition at 10 nM) in the absence of cycloheximide.

Protein breakdown in liver cells in suspension is inhibited by ammonia (Seglen, 1975; Hopgood *et al.*, 1977). We also observe this effect in liver monolayers, in which 5 mM-NH₄Cl produces a maximal inhibition of proteolysis of 37% (Fig. 4b). However, in contrast with the result with cycloheximide, glucagon did not stimulate protein degradation in the presence of 5 mM-NH₄Cl (Fig. 5b). Insulin at 10 nM produced only a small additional inhibition of protein breakdown in the presence of NH₄Cl, similar to its effect in the presence of cycloheximide. As with the cycloheximide experiments above, these cells in the absence of ammonia showed normal responsiveness to glucagon (24% stimulation at 10 nM) and to insulin (22% inhibition at 10 nM).

Discussion

We have previously reported that protein degradation in hepatocyte monolayers is inhibited by insulin with a half-maximal effect occurring at approx. 1 nM (Gunn *et al.*, 1977). The data in Fig. 1(a) show that glucagon stimulates proteolysis in the same culture system, and that half-maximal stimulation occurs at approx. 0.5 nM. Thus protein degradation in hepatocyte monolayers is responsive to both hormones in a similar way to that observed in the perfused liver (Mortimore & Mondon, 1970; Woodside *et al.*, 1974), and the sensitivity in each case is sufficiently high for concentrations within the normal physiological range to have measurable effects.

Experiments with the perfused liver (Glinsman & Mortimore, 1968; Mackrell & Sokal, 1969; Parrilla *et al.*, 1974; Exton *et al.*, 1971b) and with hepatocytes in suspension (Pilkis *et al.*, 1975; Claus & Pilkis, 1976) have shown that several metabolic pathways in liver are modulated in opposite directions by insulin and glucagon, whereas, when present together, these two hormones act as competitive antagonists. Thus the stimulation of hepatic gluconeogenesis, ketogenesis, ureogenesis and glycogenolysis by glucagon is inhibited by insulin, although the sensitivity of these different metabolic pathways to insulin varies over a wide range.

Our results suggest that a similar competition between the two hormones exists with respect to the control of intracellular protein degradation in liver cells. At 0.1 nM-insulin, proteolysis in hepatocyte monolayers is inhibited when glucagon is absent

(Gunn *et al.*, 1977) or if present at one-tenth of the insulin concentration (Fig. 2). A molar excess of glucagon stimulates protein degradation (Fig. 2). The response to glucagon is seen throughout a 100-fold range of insulin concentrations from 0.01 to 1 nM. Thus our results suggest that in the intact animal changes in the portal glucagon/insulin ratio in the normal physiological range would be expected to have marked effects on hepatic protein breakdown. This conclusion differs somewhat from the results of Parrilla *et al.* (1974), who found that net proteolysis by the perfused liver, measured as valine production in the perfusate, was increased as the glucagon/insulin ratio was raised from 2.6 to 2600 but not at lower more physiological ratios. Also Parrilla *et al.* (1974) did not find a net inhibition of proteolysis, even when insulin was present in 40-fold molar excess over glucagon.

Current evidence on the mechanism of action of glucagon argues that the hormone acts by binding to specific receptors on the outside of the plasma membrane leading to a stimulation of membrane-bound adenylate cyclase. Cyclic AMP thus produced is responsible for the modulation of several metabolic pathways (Hems, 1977). In agreement with such concepts, we have found three lines of evidence that indicate that glucagon stimulation of intracellular protein degradation in hepatocyte monolayers may be mediated via cyclic AMP as 'second messenger'. First, proteolysis is elevated by added dibutyryl cyclic AMP to a similar degree to the stimulation observed with glucagon (Figs. 1a and 1b). Second, when optimal concentrations of these two effectors are added together, proteolysis is not stimulated significantly above that observed with either agent alone (Table 3), suggesting that they may share a common mechanism of action. Finally, we have found a close correlation between glucagon-mediated increases in proteolysis and intracellular cyclic AMP concentrations (Table 2).

Increases in intracellular cyclic AMP in liver upon addition of glucagon have been observed to be of short duration, even when the tissue remains exposed to the hormone (Exton *et al.*, 1971b; Christoffersen & Berg, 1974; Dich & Gluud, 1976; Claus & Pilkis, 1976; De Rubertis & Craven, 1976). Removal of glucagon from liver perfusates resulted in a rapid fall in tissue cyclic AMP concentrations as well as decreases in gluconeogenesis and glycogenolysis (Exton *et al.*, 1971b). With protein degradation we find a similar result, since the pathway remains increased only when glucagon is present (Fig. 3). Analogous results were found with insulin. This requirement for continuous exposure to the hormones would, of course, be observed if they were rapidly degraded by the cells during the incubation period. However, we have measured the degradation of ¹²⁵I-labelled insulin under the above conditions and

find this rate to be too low to account for such an explanation. Insulin at 10nm is degraded by hepatocyte monolayers at a rate of less than 1%/h per mg of cell protein (Ballard & Gunn, 1979).

Various workers have observed reduced liver cyclic AMP concentrations in the presence of insulin. Thus Exton *et al.* (1971a) have reported that insulin reduces basal cyclic AMP concentrations by about 15% in perfused liver. In addition, insulin has been observed to decrease cyclic AMP concentrations elevated by glucagon (or adrenaline) in perfused liver (Exton *et al.*, 1971a) and in isolated liver cells (Pilkis *et al.*, 1975; Claus & Pilkis, 1976). Since cyclic AMP is synthesized only by adenylate cyclase and degraded only by cyclic AMP phosphodiesterase, both enzyme activities have been examined to see if they are modulated by insulin. Loten *et al.* (1978) reported that insulin treatment of isolated hepatocytes resulted in an increase in the activity of the low- K_m cyclic AMP phosphodiesterase. This effect was similar to that noted with isolated adipocytes (Loten & Sneyd, 1970) and thus provided a possible explanation for the lowering of liver cyclic AMP concentrations by insulin.

In contrast with these reports, our experiments indicate that insulin can inhibit glucagon-stimulated proteolysis to a considerable degree in hepatocyte monolayers, even though intracellular cyclic AMP concentrations remain elevated (Table 4), suggesting that the inhibitory effect of insulin on proteolysis is not exerted via cyclic AMP. However, if cyclic AMP is compartmentalized within the liver cell, total intracellular cyclic AMP concentrations, as measured in these and other studies, would not necessarily correlate with effective intracellular concentrations or with the metabolic effects of cyclic AMP (Exton *et al.*, 1971a; Claus & Pilkis, 1976). Evidence has also been presented that insulin may enter cells and provoke direct actions on the cell nucleus (Goldfine *et al.*, 1977), and it has been suggested that the long-term effects of the hormone may be exerted by such a mechanism (Goldfine *et al.*, 1977). Because the entry of insulin into cells might reasonably be expected to occur via endocytosis, this would also provide an entry point for insulin action on the lysosomal system, leading to an inhibition of lysosomal proteolysis. Indeed, it has been found that the number of autophagic vacuoles in liver is reduced by insulin (Grammeltvedt & Berg, 1976; Neely *et al.*, 1977; Pfeifer, 1978), and it has been proposed that a major pathway for turnover of general cell proteins is via the autophagic lysosome system (Knowles & Ballard, 1976; Amenta *et al.*, 1977). Since autophagocytosis is also inhibited by cycloheximide (Neely *et al.*, 1974), it has been suggested that the inhibition of proteolysis by cycloheximide may occur by restricting autophagic vacuole formation (Knowles & Ballard, 1976). That this may indeed be the case is

indicated by the data in Fig. 5(a) which show that proteolysis, already maximally inhibited by cycloheximide, is not further inhibited by normally effective concentrations of insulin. In addition, the two agents that stimulate proteolysis in hepatocyte monolayers, namely glucagon and dibutyryl cyclic AMP, have been shown to stimulate the lysosomal autophagic system of liver (Deter *et al.*, 1967; Rosa, 1971; Arstila *et al.*, 1972; Shelburne *et al.*, 1973). Although the mechanism by which the stimulation of autophagocytosis occurs after addition of glucagon is not understood, it is clearly unrelated to protein synthesis, since cycloheximide does not reduce the response (Fig. 5a).

Ammonia and other weak bases can enter the lysosome and accumulate therein (Reijngoud *et al.*, 1976), and it has been suggested that the resultant increase in intralysosomal pH may interfere with lysosomal proteinase action (Seglen & Reith, 1976). This idea is supported by the observations that ammonia is an effective inhibitor of protein breakdown in liver cells in suspension (Seglen, 1975; Hopgood *et al.*, 1977) or culture (Fig. 4b) and of autophagy-associated proteolysis in cultured fibroblasts (Amenta *et al.*, 1978). We also find that, in the presence of ammonia, no additional inhibitory effect of insulin on proteolysis is observed, and the stimulatory effect of glucagon is completely abolished (Fig. 5b). These results are in accord with the concept that inhibition by ammonia of the final lysosomal hydrolysis steps of protein breakdown would abolish the effects of agents that stimulate autophagy or other earlier steps in the protein breakdown sequence.

We conclude that glucagon stimulates proteolysis in hepatocyte monolayers via an increase in intracellular cyclic AMP, leading in some way to increased cellular autophagy. On the other hand, inhibition of proteolysis by insulin and by cycloheximide leads to a reduction in autophagy by a mechanism independent of cyclic AMP.

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