Stimulation by glucocorticoids of protein degradation in hepatocyte monolayers

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1. Protein degradation in rat hepatocytes in stationary monolayer culture was measured as release of radioactive trichloroacetic acid-soluble material from intracellular proteins labelled with [^3H]leucine. 2. Glucocorticoids, but not other steroids, stimulated protein breakdown in the hepatocyte monolayers. The effects observed were greater when the cells were preincubated with the hormones, indicating that the stimulation was not immediate. In addition, the stimulation by glucocorticoids persisted for up to 4 h after hormone removal. 3. Cycloheximide and the lysosomotropic agents leupeptin and ammonia effectively blocked glucocorticoid stimulation of protein degradation. 4. Insulin blocked dexamethasone stimulation when added at the same time as the steroid, but not when added 3 h later. 5. Stimulation of protein breakdown by dexamethasone was additive with that by glucagon or dibutyryl cyclic AMP, suggesting that its mechanism of action is different from that of the latter two agents. 6. Total activities of several lysosomal enzymes were unaffected under conditions where protein breakdown was stimulated by either glucagon or dexamethasone. 7. It is suggested that, whereas glucagon, dibutyryl cyclic AMP and insulin modulate protein breakdown in these cells via changes in autophagocytosis, the stimulation by glucocorticoids is exerted independently, perhaps by stimulating the synthesis of membrane proteins essential to the autophagic process.

Glucocorticoid administration *in vivo* leads to protein catabolic effects in muscle and other peripheral tissues. Muscle protein synthesis is decreased (Wool & Weinshelbaum, 1960; Young, 1970; Hanoune *et al.*, 1972) and muscle protein breakdown is increased (Goldberg, 1969, 1975; Tomas *et al.*, 1979). Even though the amount of liver protein increases soon after glucocorticoid injection (Goodlad & Munro, 1959; Henderson *et al.*, 1971), the amino acids produced from extra-hepatic tissues are not fully accounted for by protein accumulation in liver. Excess amino acids are utilized in gluconeogenesis leading to increased glycogen synthesis and deposition, and by oxidation, leading to an overall negative nitrogen balance (Steele, 1975). Measurements of liver protein synthesis and degradation after glucocorticoid administration have yielded conflicting results. Protein synthesis has been reported to be inhibited (Kim & Kim, 1975), unaffected (Hanoune *et al.*, 1972) or stimulated (Feigelson & Feigelson, 1963; Hanoune & Feigelson, 1970; Millward *et al.*, 1976), and protein degradation is increased (Chertow *et al.*, 1973) or unaffected (Kim & Kim, 1975). Differences of this

kind may be due to the many variables involved in studies with intact animals. Frequency of injection of the steroid and size of dose are likely to be important variables, particularly in view of the reported short plasma half-life of glucocorticoids (L'Age *et al.*, 1974). Dietary differences may also be expected to exert a significant effect on these measurements (Munro, 1964). In addition, corticosteroids may alter circulating concentrations of other hormones such as insulin (Millward *et al.*, 1976; Tomas *et al.*, 1979), which directly modulate protein synthesis or degradation (Ballard, 1980). Finally, problems of measurement of protein synthesis and degradation *in vivo* are recognized (Goldberg & Dice, 1974; Ballard, 1977; Waterlow *et al.*, 1978), but are hard to overcome. Some problems can be avoided by using the isolated perfused liver, but difficulties with tissue viability occur over the long period required for measurements of protein degradation. Experiments with hepatoma cells in culture have indicated that protein breakdown is stimulated by glucocorticoids in HTC (Gelehrter & Emanuel, 1974) and H35 (Gunn *et al.*, 1976) cells, but no studies with normal liver cells have been reported.

In the present study, parenchymal liver cells in stationary monolayer culture have been used to examine the effects of steroid hormones on protein breakdown in the absence of the complicating factors that apply to experiments *in vivo* or perfusion. The results indicate that physiological concentrations of glucocorticoids, but not other steroid hormones, significantly stimulate protein breakdown in these cells.

Materials and methods

Chemicals

Dexamethasone sodium phosphate ('Decadron'; 24 mg/ml) and cortisol (acetate) were supplied by Merck, Sharp and Dohme (Aust.) Pty. Ltd., Granville, N.S.W., Australia. Corticosterone, progesterone, oestradiol-17 β , β -glycerophosphate (grade II), *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide (grade III), *p*-nitrophenyl β -D-galactoside, bovine haemoglobin (type I) and dithiothreitol were from Sigma Chemical Co., St. Louis, MO, U.S.A. Cortisone, aldosterone, prednisolone, oestriol and oestrone were from Steraloids Inc., Wilton, NH, U.S.A. Testosterone was from Fluka A.G., Buchs, Switzerland. [3 H]Acetic anhydride (500 Ci/mol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The sources of other chemicals and materials were as previously described (Knowles *et al.*, 1975; Knowles & Ballard, 1976; Hopgood *et al.*, 1977, 1980).

Preparation and culture of hepatocytes

Animals, diet, lighting schedule and the preparation and culture of hepatocytes were as described previously (Hopgood *et al.*, 1980).

Measurement of protein degradation

Intracellular proteins were labelled with [3 H]-leucine for 16 h (Hopgood *et al.*, 1980) in the time period from 6 to 22 h after plating, followed by a 3 h 'chase' period and a 4 h measurement period as described by Hopgood *et al.* (1980).

In experiments where cells were preincubated with agents before the measurement period, the appropriate compound was added to the chase medium (3 h preincubation) or to the pulse and the chase media (19 h preincubation). Routine removal of the chase medium, followed by two washes with control medium to remove residual soluble radioactivity from the monolayers, also served to decrease the concentration of the preincubation agent to an insignificant value.

At the end of the degradation period, incubation media were removed and trichloroacetic acid (final concn. 1 M) was added to each sample of medium. After storage at 4°C for 16 h, radioactivity in the trichloroacetic acid-soluble and -insoluble fractions

was determined. Total radioactivity in the cells was measured after dissolving the monolayers by trituration in 0.5 M-NaOH/0.5% Triton X-100. In preliminary experiments, cell-associated trichloroacetic acid-soluble radioactivity was found to be less than 5% of that in the medium and was not modified by treatments which stimulated or inhibited protein breakdown. Thereafter this fraction was not measured independently, but was included in the total cellular radioactivity recovered in harvesting the cell monolayer. The percentage protein degradation at 4 h was calculated as 100 times the radioactivity in the trichloroacetic acid-soluble fraction of the medium divided by the total radioactivity in all fractions. Radioactivity in the trichloroacetic acid-insoluble fraction of the medium, normally less than 6% of the total trichloroacetic acid-insoluble radioactivity, was used as an index of cellular integrity.

Methods of radioactivity determination, statistical treatment of results and protein assay were as described previously (Hopgood *et al.*, 1980).

Assays of lysosomal enzymes

After removal of the incubation medium, cell monolayers were harvested in 1 ml of 0.5% Triton X-100 and frozen. The thawed samples were homogenized to produce uniform suspensions and assayed for lysosomal enzyme activities and total protein.

Acid phosphatase activity was assayed as described by Bodansky (1972). *N*-Acetyl- β -D-glucosaminidase and β -D-galactosidase were assayed by the methods of Plattner & Henning (1975). Cathepsin D was assayed by the method of Hille *et al.* (1970) and activity calculated as μ g of [3 H]acetyl-haemoglobin hydrolysed/min.

Cathepsin B activity was measured with 3 H-labelled liver-cell protein as substrate. This material was prepared by incubating monolayers with [3 H]-leucine as in the degradation experiments. Subsequently the cells were dissolved in 0.1 M-NaOH, the pH was adjusted to 5.5 with 0.5 M-HCl and the sample was passed through a Sephadex G-100 column pre-equilibrated with 50 mM-sodium citrate, pH 5.5. The high-molecular-weight protein fractions eluted first from this column contained approx. 2×10^5 d.p.m./mg of protein and were used without further treatment. Proteolysis was followed as the rate of formation of radioactive material that was soluble in 0.6 M-trichloroacetic acid. All incubations contained 1 mM-dithiothreitol and 1 mM-EDTA. Measured rates of proteolysis were corrected for that occurring in the absence of cell homogenate, which was approx. 20% of the rate observed in the presence of the homogenate. Proteinase activity inhibited by leupeptin (50 μ g/ml) is referred to as cathepsin B, even though cathepsin L and possibly

some serine proteinases would be similarly inhibited. General proteinase activity at pH 4.0 was also measured, in the absence of EDTA, dithiothreitol and leupeptin, by using the same labelled substrate as used for the cathepsin B assays.

Results and discussion

Effects of steroid hormones

Of the steroids examined, only the glucocorticoid group was found to have significant effects on protein breakdown when added to monolayers at the beginning of the measurement period (Table 1). Dexamethasone, prednisolone and the natural glucocorticoids corticosterone and cortisol all stimulated the rate of protein degradation by approx. 8% at a concentration of $1\mu\text{M}$, which is in the very high physiological range (L'Age *et al.*, 1974; Tomas *et al.*, 1979). Smaller but significant stimulation was noted with cortisone and aldosterone. Cortisone must be first converted into cortisol in order to have glucocorticoid activity (Spain, 1975), and consequently its lesser potency is not surprising. Aldosterone possesses weak physiological glucocorticoid activity (Goodman & Gilman, 1970), consistent with a direct effect on protein degradation.

Other steroids tested but without effect on protein breakdown were oestradiol- 17β , oestriol, oestrone and testosterone (results not shown). Progesterone was found to inhibit measured protein degradation,

but only at unphysiological concentrations (over $20\mu\text{M}$), which also resulted in damage to the cells.

When the sensitivity of protein breakdown to glucocorticoids was tested, significant stimulation was found with dexamethasone at $0.1\mu\text{M}$ and with cortisol and corticosterone at $1\mu\text{M}$. Maximal effects were observed at $10\mu\text{M}$ concentration of all three hormones. Protein-degradation rates were less than maximal when glucocorticoids were added at $100\mu\text{M}$. Such high steroid concentrations probably caused cell death, since some cells were observed to be lifting from the plates.

Dexamethasone effects in combination with other hormones

We have previously reported that protein breakdown in hepatocyte monolayers is stimulated by glucagon and dibutyryl cyclic AMP and inhibited by insulin (Hopgood *et al.*, 1980). Consequently we examined the stimulation by dexamethasone in the presence of each of these agents, since the observation of additive effects might indicate different mechanisms of action. Dexamethasone stimulated protein breakdown in the presence of a maximally effective concentration of glucagon (10 nM ; Hopgood *et al.*, 1980) or dibutyryl cyclic AMP (0.1 mM ; Hopgood *et al.*, 1980). The extent of stimulation was similar to that observed when dexamethasone was added alone (Fig. 1). This result is consistent with the proposition that the stimulation by the glucocorticoid occurs via a different mechanism from that of glucagon, since the latter elevates intracellular cyclic AMP concentrations in parallel with its stimulation of protein degradation (Hopgood *et al.*, 1980). In the presence of a maximally effective concentration of insulin (10 nM ; Hopgood *et al.*, 1980), the stimulation of protein degradation by dexamethasone was not observed (Fig. 1).

We have also examined the dexamethasone stimulation in the presence of other inhibitors of protein breakdown. Leupeptin, which inhibits lysosomal cathepsin B (Ikezawa *et al.*, 1971), and ammonia, which raises the intralysosomal pH (Reijngoud *et al.*, 1976), were both effective inhibitors of protein breakdown in hepatocyte monolayers (Table 2). Dexamethasone did not stimulate protein breakdown in the presence of these agents (Table 2), suggesting that the pathway of protein degradation increased by glucocorticoids is a lysosomal system. Leupeptin and ammonia also prevent glucagon stimulation of protein degradation in hepatocyte monolayers (Hopgood *et al.*, 1980; Hopgood & Ballard, 1980). The dexamethasone stimulation of protein breakdown was abolished in the presence of the protein-synthesis inhibitor cycloheximide (Table 2). This is evidence that protein synthesis is required for glucocorticoid action, and provides another indication of a fundamental dif-

Table 1. *Effect of steroid hormones on protein breakdown in hepatocyte monolayers*

Hepatocytes were established in monolayer culture, their intracellular proteins labelled and proteolysis was measured over a 4 h incubation period as described in the Materials and methods section. Steroids added at the beginning of the degradation period were dissolved in dimethyl sulphoxide and the final concentration of this agent in all incubations was 1% (v/v). Values are means $\pm$ S.E.M. for the numbers of dishes given in parentheses. Two separate livers were used for preparation of monolayers.

Additions	Degradation (% in 4 h)
None	9.44 ± 0.05 (15)
Dexamethasone ($1\mu\text{M}$)	10.29 ± 0.06 (6)
Dexamethasone ($10\mu\text{M}$)	10.16 ± 0.06 (6)
Cortisol ($1\mu\text{M}$)	10.10 ± 0.10 (6)
Cortisol ($10\mu\text{M}$)	10.14 ± 0.07 (6)
Corticosterone ($1\mu\text{M}$)	10.17 ± 0.07 (9)
Corticosterone ($10\mu\text{M}$)	10.23 ± 0.09 (6)
Cortisone ($1\mu\text{M}$)	9.82 ± 0.09 (6)
Cortisone ($10\mu\text{M}$)	9.89 ± 0.08 (6)
Aldosterone ($1\mu\text{M}$)	9.95 ± 0.11 (6)
Aldosterone ($10\mu\text{M}$)	10.18 ± 0.11 (6)
Prednisolone ($1\mu\text{M}$)	10.26 ± 0.12 (6)
Prednisolone ($10\mu\text{M}$)	10.47 ± 0.13 (6)

Table 2. *Lack of stimulation in proteolysis by dexamethasone in the presence of inhibitors of protein breakdown*

Experimental details were as given in Table 1, except that dexamethasone was added in aqueous solution at a concentration of $1\mu\text{M}$. Values are means $\pm$ S.E.M. for the numbers of dishes given in parentheses. The number of cell preparations was three.

Additions	Degradation (% in 4h)
None	10.21 ± 0.08 (24)
Dexamethasone	11.12 ± 0.14 (18)
Ammonia (10 mM)	5.92 ± 0.14 (12)
Ammonia (10 mM) + dexamethasone	5.88 ± 0.19 (12)
Leupeptin (50 $\mu\text{g}/\text{ml}$)	7.08 ± 0.12 (12)
Leupeptin (50 $\mu\text{g}/\text{ml}$) + dexamethasone	7.10 ± 0.27 (6)
Cycloheximide (3 μM)	7.26 ± 0.08 (12)
Cycloheximide (3 μM) + dexamethasone	7.30 ± 0.07 (12)

Fig. 1. *Stimulation of protein breakdown by dexamethasone alone and in the presence of dibutyl cyclic AMP, glucagon or insulin*

Hepatocytes were established in monolayer culture, their intracellular proteins were labelled, and protein degradation was measured over a 4 h incubation period as described in the Materials and methods section. ●, Control plus or minus dexamethasone; □, dibutyl cyclic AMP (0.1 mM) plus or minus dexamethasone; ○, glucagon (10 nM) plus or minus dexamethasone; ■, insulin (10 nM) plus or minus dexamethasone. Values are means $\pm$ S.E.M. for 12 dishes in the absence or six dishes in the presence of dexamethasone. Two cell preparations were used.

ference between the mechanism of stimulation of protein degradation by glucocorticoids and by glucagon. We have previously shown that glucagon stimulation of protein degradation persisted in the presence of cycloheximide (Hopgood *et al.*, 1980).

Effect of preincubation with dexamethasone

Current concepts of glucocorticoid action are based on evidence of entrance of the steroids into the cell, interaction with specific cytoplasmic receptors and the initiation by the receptor-steroid complexes of intra- and extra-nuclear synthetic events such as DNA synthesis, transcription and translation (Litwack & Singer, 1972; Thompson & Lippman, 1974; Cake & Litwack, 1975). Action of a steroid by this sequence of reactions might take a significant period of time in relation to the 4 h period over which we measure protein breakdown, and consequently the stimulation observed during an 'acute' 4 h exposure to the hormones may have been less than maximal. Accordingly, monolayers were exposed to $10\mu\text{M}$ -dexamethasone for 19 h (during pulse and chase periods) or for 3 h (during chase period only) before the 4 h measurement period. Preliminary results indicated that stimulation of protein breakdown after either of these preincubation periods was double that observed with an acute 4 h exposure to the glucocorticoid. Since the stimulation was the same for both periods of preincubation (results not shown), a regime of 3 h preincubation was adopted for subsequent experiments.

In monolayers exposed to dexamethasone for 3 h before and also during the 4 h measurement period, protein degradation was stimulated by up to 20% (Fig. 2). Significant stimulation was evident with 10 nM-dexamethasone and a maximal stimulation was observed at 100 nM, much lower concentrations than were effective with acute 4 h exposure to the steroid. Similar results were obtained by preincubation of the cells with corticosterone. Protein degradation was significantly stimulated by 0.1 μM -corticosterone, and a maximal stimulation of 18% was observed at a concentration of 1 μM (Fig. 2). In contrast, acute exposure to corticosterone indicated that protein degradation was stimulated no more than 10% by 10-fold higher concentrations of the hormone (Fig. 2). Since the plasma corticosterone concentration in normal rats is approx. 0.1 μM (L'Age *et al.*, 1974; Tomas *et al.*, 1979), it is evident that changes in plasma corticosteroid concentrations in the normal physiological range might be expected to exert a considerable influence on protein breakdown in hepatic parenchymal cells. Protein degradation in some cell types may, of course, be insensitive to glucocorticoid stimulation, as was reported for rat embryo fibroblasts in culture (Warburton & Poole, 1977). However, these fibroblasts were exposed to dexamethasone only for the

2h period over which measurements were conducted, and the lack of response may have been due to insufficient time being allowed for the hormone to act.

The stimulation of protein breakdown by dexamethasone was not observed when cycloheximide was added with the steroid during the preincubation period (Fig. 3). This confirms that the glucocorticoid effect is dependent on protein syn-

thesis, as previously indicated for the acute-exposure experiments (see Table 2).

When monolayers were preincubated for 3h with dexamethasone and then incubated in its absence for the 4h measurement period (Table 3, line 3), the degree of stimulation of protein breakdown was greater than in the 4h acute effect and similar to the response observed when dexamethasone was present for the total 7h period (Table 3, line 4). Hence the glucocorticoid effect, once initiated, persists for some hours even after removal of the steroid. Here again, points of difference between glucocorticoid and glucagon stimulation of protein degradation emerge. We have shown previously that protein breakdown is fully stimulated within 1h after

Fig. 2. Stimulation of protein breakdown by 7h exposure to corticosterone or dexamethasone

Experimental details were as given in Fig. 1., except that monolayers were incubated with added agents for 3h before and during the 4h measurement period as described in the Materials and methods section. ■, Corticosterone; ●, dexamethasone. Values for 4h exposure to corticosterone (□) or dexamethasone (○) with the same preparations of cells are included for comparison. Values are means ± s.e.m. for measurements on 12 dishes in the absence or six dishes in the presence of glucocorticoids. Two cell preparations were used.

Fig. 3. Stimulation of protein breakdown by 7h exposure to dexamethasone in the presence and absence of cycloheximide

Experimental details were as given in Fig. 2. ●, Dexamethasone; ○, dexamethasone plus cycloheximide. Values are means ± s.e.m. for measurements on twelve dishes in the absence or six dishes in the presence of dexamethasone. Two cell preparations were used.

Table 3. Effects of dexamethasone preincubation on the stimulation of proteolysis by dexamethasone, glucagon or dibutyryl cyclic AMP

Experimental details were as given in Table 2, except that some plates were incubated with dexamethasone (10μM) for 3h before the 4h measurement period as described in the Materials and methods section. In the 4h measurement period, dexamethasone was added at 1μM, glucagon at 10nM and dibutyryl cyclic AMP at 0.1mM.

Dexamethasone preincubation	Incubation additions	Degradation (% in 4h)
—	None	9.22 ± 0.11 (15)
—	Dexamethasone	10.10 ± 0.12 (12)
+	None	10.79 ± 0.14 (11)
+	Dexamethasone	10.74 ± 0.17 (11)
—	Glucagon	11.33 ± 0.14 (12)
+	Glucagon + dexamethasone	11.98 ± 0.18 (6)
—	Dibutyryl cyclic AMP	11.29 ± 0.15 (6)
+	Dibutyryl cyclic AMP + dexamethasone	13.09 ± 0.15 (6)

glucagon addition, but continual presence of the hormone is necessary for persistence of its action (Hopgood *et al.*, 1980). Protein degradation, although increased by preincubation of cells with a maximally effective concentration of dexamethasone, was further stimulated by the addition of glucagon (Table 3, compare line 6 with lines 4 and 5). A similar result was observed with dibutyryl cyclic AMP (Table 3). These results confirm the evidence provided from Fig. 1, but in an experimental situation in which the dexamethasone effect is much larger.

Mechanism of glucocorticoid stimulation

Glucocorticoids have a stabilizing effect on lysosomes, although very high concentrations of steroid are required both *in vivo* (Weissmann & Thomas, 1964) and *in vitro* (Weissmann, 1964; Symons *et al.*, 1969) for the effect to be observed. Subsequent investigations have shown that glucocorticoid administration *in vivo* leads to an increase in the numbers of lysosomes in liver (Wiener *et al.*, 1968). In addition, glucocorticoids have been found to increase liver lysosomal-enzyme activities *in vivo* (Bowness *et al.*, 1971; Kyaw & Mellors, 1972) and in various cells in culture (Suzuki *et al.*, 1969; Carubelli & Griffin, 1970; Kyaw & Mellors, 1972). These results have led to the suggestion that glucocorticoids may stimulate protein degradation via an increase in cellular content of lysosomes (Ballard, 1980; Hopgood & Ballard, 1980). However, when hepatocyte monolayers were incubated with dexamethasone under the same conditions as those in which protein breakdown was stimulated (Table 3), there was no change in the total activities of several lysosomal enzymes (Table 4). These negative results differ from those cited above, but agree with other measurements made

after glucocorticoid administration *in vivo* (Beaufay *et al.*, 1959) or to cells in culture (Lundgren, 1972; Cristofalo & Kabakjian, 1975). Where increases in lysosomal enzymes have been observed, exposure times have generally been much longer (12h to 30 days) than those where increases in protein breakdown are observed in hepatocyte monolayers.

The stimulation of protein degradation by dexamethasone is slow in onset, persists after removal of the steroid and is completely prevented by cycloheximide. These effects differ from the glucagon response (Hopgood *et al.*, 1980), and point to an involvement of protein synthesis in glucocorticoid action. Nevertheless, glucocorticoid administration is not accompanied by increases in lysosomal proteolytic enzymes, so perhaps proteins other than lysosomal enzymes are vital to the glucocorticoid effect. One possibility is that dexamethasone may increase the cellular content of neutral proteinases, present in the extra-lysosomal fraction of the cell (Goldberg & St. John, 1976; Etlinger & Goldberg, 1977; DeMartino & Goldberg, 1979; Rose *et al.*, 1979; Ciechanover *et al.*, 1980; Hershko *et al.*, 1980). However, this explanation is unlikely, because the lysosomotropic agents leupeptin and ammonia prevented the glucocorticoid effect.

There is good evidence that glucagon or dibutyryl cyclic AMP stimulate, and insulin inhibits, protein breakdown in liver via modulation of the rate of autophagocytosis (Deter *et al.*, 1967; Rosa, 1971; Arstila *et al.*, 1972; Shelburne *et al.*, 1973; Neely *et al.*, 1974, 1977; Grammeltvedt & Berg, 1976; Pfeifer, 1978). A possible regulatory site for glucocorticoid action may be the supply of precursors for autophagy, e.g. the synthesis of membrane proteins for use in the autophagic process. Such synthetic events would be expected to be sensitive to protein-synthesis inhibitors and to be relatively slow-acting

Table 4. *Effect of dexamethasone on the activities of lysosomal enzymes*

Experimental details were as given in Table 3, except that cell monolayers were harvested in 0.5% Triton X-100 and lysosomal enzymes assayed as described in the Materials and methods section. Values are means $\pm$ S.E.M. for the numbers of dishes given in parentheses. The number of cell preparations was three.

	Control		Dexamethasone (1 μ M)	
	3 h	7 h	3 h	7 h
Acid phosphatase (nmol/min per mg of protein)	6.3 $\pm$ 0.4 (8)	5.2 $\pm$ 0.3 (7)	6.2 $\pm$ 0.4 (7)	5.9 $\pm$ 0.3 (14)
N-Acetyl- β -D-glucosaminidase (nmol/min per mg of protein)	18.5 $\pm$ 0.7 (8)	16.6 $\pm$ 0.7 (8)	17.9 $\pm$ 0.3 (8)	17.4 $\pm$ 0.4 (16)
β -D-Galactosidase (nmol/min per mg of protein)	13.5 $\pm$ 0.1 (4)	13.4 $\pm$ 0.3 (4)	14.0 $\pm$ 0.1 (4)	14.6 $\pm$ 0.3 (8)
Cathepsin D (μ g/min per mg of protein)	5.3 $\pm$ 0.1 (4)	5.3 $\pm$ 0.1 (4)	5.2 $\pm$ 0.1 (4)	5.0 $\pm$ 0.1 (8)
Cathepsin B (μ g/min per mg of protein)	0.116 $\pm$ 0.005 (8)	0.093 $\pm$ 0.003 (8)	0.109 $\pm$ 0.003 (8)	0.095 $\pm$ 0.002 (16)
Proteinase activity at pH 4.0 (μ g/min per mg of protein)	0.230 $\pm$ 0.014 (12)	0.263 $\pm$ 0.009 (12)	0.219 $\pm$ 0.005 (12)	0.235 $\pm$ 0.007 (24)

Fig. 4. Inhibition of protein degradation by insulin in control and in dexamethasone-preincubated monolayers. Experimental details were as given in Fig. 1, except that some plates were incubated with dexamethasone ($10\mu\text{M}$) for 3h before the 4h measurement period as described in the Materials and methods section. ●, Control dishes plus or minus insulin; ○, dexamethasone-preincubated dishes plus or minus insulin. Values are means $\pm$ S.E.M. for measurements on eight dishes in the absence or four dishes in the presence of insulin. Two cell preparations were used.

and persistent. In addition, the rapid action of insulin to inhibit the formation of new autophagic vacuoles (Pfeifer, 1979) should prevent glucocorticoid stimulation of protein breakdown when both hormones are added at the same time (Fig. 1). In contrast, when hepatocyte monolayers were preincubated with dexamethasone before insulin addition, protein breakdown remained stimulated over a subsequent 4h period (Fig. 4). This result is to be expected if insulin inhibits the formation of new autophagic vacuoles, but has no effect on subsequent degradative events such as the fusion of autophagic vacuoles with primary lysosomes and proteolysis in secondary lysosomes (autophagosomes). Finally, if maximal stimulation of autophagocytosis and hence protein degradation by glucagon or dibutyryl cyclic AMP were limited by the availability of membranes for autophagy, then the suggested action of glucocorticoids to increase this supply would lead to an additional stimulation of autophagy and of protein breakdown, an effect

which is indeed observed (Table 3). Since glucocorticoid stimulation of protein degradation is effectively blocked by the lysosomotropic agents leupeptin and ammonia, it is apparent that the pathway stimulated by glucocorticoids ultimately involves lysosomal proteolysis.

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