The Action of Corticosteroids on Proteolysis

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1. The corticosteroids cortisol, cortisone and corticosterone were tested for their ability to affect the hydrolysis of serum albumin, insulin and oxyhaemoglobin incubated with trypsin, chymotrypsin, papain and pepsin. 2. Corticosteroids stimulated the hydrolysis of albumin and oxyhaemoglobin with trypsin between 10% and 200% and inhibited the hydrolysis of insulin by 15% (steroid/substrate molar ratio, 5:1). 3. The degree of stimulation of proteolysis for a given substrate depended on both the nature of the steroid and the protease. Corticosterone did not increase the activity of papain and pepsin with any of the substrates tested. 4. Corticosterone stimulated (fivefold) the denaturation of oxyhaemoglobin measured spectroscopically in 2·4% (w/v) sodium hydroxide. Small changes in the absorption spectrum of haemoglobin solutions were also noted at pH 7·8 without a marked change in the basic properties of haemoglobin. 5. With regard to the action of corticosterone on the activity of trypsin, the lack of stimulation when benzoylarginine amide was used as a substrate, the lowering of the stimulation on prior heat denaturation of haemoglobin and the high temperature coefficient for stimulation suggest that the steroid resulted in improved access of the protease to susceptible bonds of the substrate.

Previous experiments indicated that corticosteroids enter cells by diffusion and are then concentrated by adsorption (Bellamy, 1963*a*). Some of the cellular steroids that are strongly attached to insoluble cell proteins are not in diffusion equilibrium with the extracellular phase. Further, a large fraction of bound steroids is non-exchangeable (Bellamy, Phillips, Jones & Leonard, 1962; Bellamy, 1963*b*). Questions arising from these results relate to the biochemical properties of non-enzymic steroid–protein complexes and the mechanism whereby strongly adsorbed steroids are removed from the cell. In this connexion, the following work was undertaken to determine whether corticosteroids could influence protein turnover by affecting the enzymic hydrolysis of various receptor proteins. From the results a model system was defined in which steroids stimulated proteolysis in a highly specific way by interacting with the substrate.

MATERIALS AND METHODS

Proteins and enzymes. Horse oxyhaemoglobin (referred to below as 'haemoglobin') was prepared by Dr M. H. Smith, Department of Biochemistry, University of Sheffield, by the method of Keilin & Hartree (1935), precipitated with ethanol and stored at below 0°. The precipitate was dissolved as required in deionized water and dialysed overnight against deionized water at 10°. Solutions contained between 1 and 2% (dry wt.) of haemoglobin and were stored at 5° for not more than 3 weeks. Bovine serum albumin (fraction V) was obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.), and crystalline ox insulin from British Drug Houses Ltd. (Poole, Dorset).

Trypsin (type I), chymotrypsin (type II) and pepsin (crystallized) were obtained from Sigma Chemical Co. Papain was obtained from British Drug Houses Ltd.

Absorption spectra and haemoglobin denaturation. The absorption spectrum of haemoglobin solutions was recorded between 400 and 650 mμ with a Beckman model DB spectrophotometer coupled with a Varicord model 43 recorder. Denaturation was determined by the methods of Brinkman, Wildschut & Wittermans (1934) and Rossi-Fanelli, Azzone & Mondovi (1955) from the increase in extinction at 615 mμ in a 1 cm. light-path (Manwell, 1959). The extent of denaturation was obtained by comparison with the maximum change (2·9-fold increase) in the extinction at 615 mμ (Manwell, 1959), obtained 15 min. after the addition of 0·1 vol. of 60% (w/v) NaOH to a 0·1% solution of haemoglobin in deionized water at 25°.

Measurement of proteolytic activity. Proteases were dissolved immediately before use in an appropriate buffer solution, so that when diluted 1:10 in the protein substrate solution the weight ratio of substrate to enzyme was not less than 100:1. Samples of the medium were removed and mixed with 0·1 vol. of 50% (w/v) trichloroacetic acid. After centrifuging, 0·1 ml. of the supernatant solution was added to 3·0 ml. of water, and 1·0 ml. was used for the estimation of amino nitrogen by the method of Rosen (1957). Corticosteroids (L. Light and Co. Ltd., Colnbrook, Bucks.) were

added dissolved in ethylene glycol. The steroid/protein ratios were calculated on a molar basis assuming mol.wt. 66000 for horse oxyhaemoglobin and 50000 for bovine serum albumin.

Amidase activity of trypsin. A 0·1 ml. portion of trypsin solution (1mg./ml. in 0·2M-sodium phosphate buffer, pH7·8) was added to 0·8 ml. of a 3% solution of sodium benzoyl-L-arginine amide hydrochloride (British Drug Houses Ltd.) at 37°, with and without steroid dissolved in 0·1 ml. of ethylene glycol. After the incubation 0·1 ml. of 50% trichloroacetic acid was added and the mixture centrifuged. The ammonia content of 0·5 ml. of clear supernatant solution was determined colorimetrically, after microdiffusion (Conway, 1947), with Nessler's reagent (for details see Bellamy & Jones, 1961).

RESULTS

Hydrolysis of albumin by trypsin. Trypsin had little action on native bovine serum albumin, and to obtain a convenient rate of hydrolysis it was necessary to increase the albumin concentration to about 4% (Fig. 1). Even at this concentration less than 1% of the albumin was hydrolysed in 4hr. at 37°. The addition of corticosterone increased the initial rate of proteolysis (Fig. 2). A 10% stimulation was obtained with as little as 0·36mM-steroid, equivalent to a steroid/protein molar ratio about 1:2; at a 4:1 molar ratio a 60% stimulation was obtained. The greatest effect of the steroid was obtained in the initial stages of incubation (Fig. 3), and albumin treated with trypsin in the absence of corticosterone was eventually degraded to the same extent (about 4% of the protein hydrolysed in 24hr.).

This effect of corticosterone was markedly dependent on temperature. A temperature decrease from 37° to 27°, which diminished proteolysis by 50%, completely abolished the action of the steroid.

A range of compounds of biological origin with widely different properties was tested to examine the specificity of the action of corticosterone on trypsin hydrolysis. Unfortunately, most of the compounds were not soluble in glycol to the same extent as corticosterone and only Strophanthin G

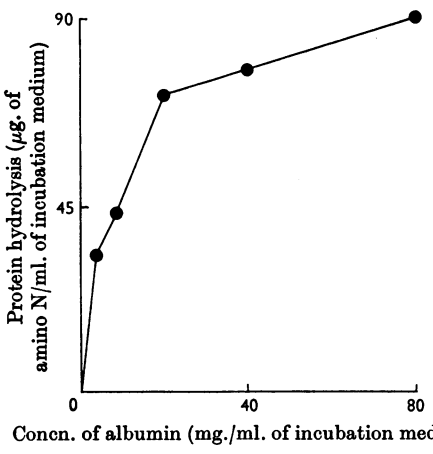

Fig. 1. Effect of albumin concentration on the activity of trypsin. Solutions of serum albumin in water (0·8 ml.) were incubated at 37° for 4hr. with 0·1 ml. of trypsin (5mg./ml. in 0·2M-sodium phosphate buffer, pH7·8) and 0·1 ml. of ethylene glycol. The reaction was stopped by the addition of 0·1 ml. of 50% trichloroacetic acid, the precipitate was centrifuged down and the amino nitrogen content of the supernatant solution was estimated as described in the text.

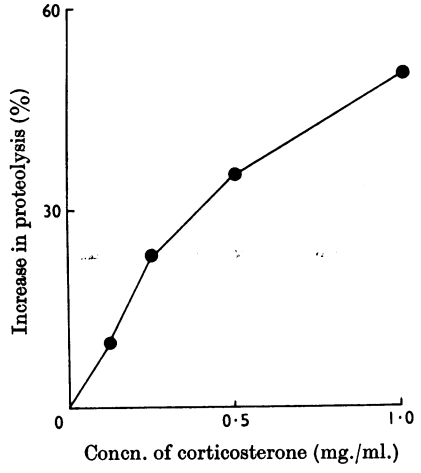

Fig. 2. Effect of corticosterone concentration on the hydrolysis of albumin by trypsin. Incubations were carried out as described in Fig. 1 with 40mg. of albumin/ml. Various amounts of corticosterone in ethylene glycol (0·1mg./ml. of incubation medium) were added and the percentage increase in amino nitrogen was determined.

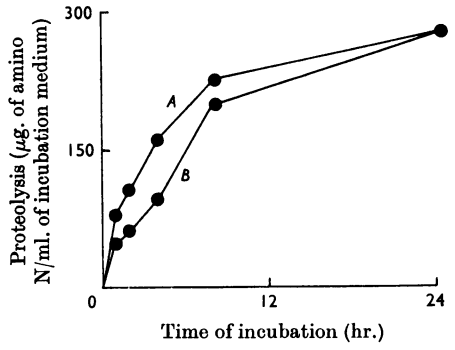

Fig. 3. Effect of corticosterone on the hydrolysis of albumin by trypsin. Incubations were carried out as described in Fig. 2 with (*A*) and without (*B*) corticosterone (1·0mg./ml. of incubation medium). Samples were removed at intervals and the amino nitrogen content was determined.

remained in solution throughout the incubation period (Table 1). Under these conditions testosterone, β-carotene and thyroxine gave a marked stimulation of proteolysis, although they were not as effective as a similar quantity of corticosterone (Table 1).

Effect of corticosteroids on the enzymic hydrolysis of other proteins. Trypsin had hardly any action on haemoglobin, particularly after the first hour of incubation (Table 2). In contrast, the addition of corticosterone, for example (steroid/protein ratio 30:1), resulted in an increased rate of proteolysis that was maintained for 4hr. (Table 2). This gave a 4–5-fold increase in protein breakdown during the last 3hr. of incubation. A similar but smaller effect was observed with bovine serum albumin but not with insulin (Table 2). When haemoglobin solution was heated at 100° for 2min. and then cooled, before adding trypsin, the rate of protein hydrolysis was increased almost 100-fold. After this treatment, however, corticosterone (steroid/protein ratio 30:1) produced only a 14% increase in trypsin activity. Corticosterone did not stimulate the hydrolysis of benzoylarginine amide by trypsin (Table 2).

Corticosterone was the most effective corticosteroid in stimulating the hydrolysis of albumin by trypsin. Cortisone was almost as effective, whereas cortisol had less than half the action of corticosterone (Table 3). When haemoglobin was used as a substrate corticosterone again gave the greatest stimulation. With this substrate, however, cortisone was the least effective compound. It was also found that besides a marked steroid- and substrate-dependence the type of response was also influenced by the nature of the protease. For example, although papain and trypsin gave similar rates of proteolysis under the same incubation conditions,

Table 1. *Effect of various compounds on the hydrolysis of albumin by trypsin*

A solution or suspension of a range of compounds in glycol (1·25mg./0·1ml.) was incubated with 0·8ml. of 5% albumin and 0·1ml. of trypsin (5·0mg./ml. of 0·2M-sodium phosphate buffer, pH7·8) for 1hr. Each experiment was carried out in duplicate. The rate of albumin hydrolysis was compared with that obtained with glycol alone by using the method described in the text.

Compound	Proteolysis (μg. of amino N/ml. of incubation medium)		Mean change in proteolysis (%)
—	50·0	59·6	—
Corticosterone	98·7	97·3	+89
Testosterone*	89·8	77·4	+52
Thyroxine*	76·4	72·0	+40
β-Carotene*	71·6	76·6	+40
Oestradiol*	64·2	63·7	+17
Bilirubin*	64·3	59·6	+13
Cholesterol*	63·5	61·9	+14
Strophanthin G	56·5	54·2	−1

* Ppt. present during the incubation.

Table 3. *Relative activation of haemoglobin and albumin by corticosteroids*

Incubations were carried out as described in Table 2 for 4hr. Relative proteolysis is expressed as the activity of trypsin in the presence of steroid divided by the activity with glycol alone. Each value is the mean ±S.E.M. from five measurements.

Steroid added	Relative proteolysis: Albumin	Relative proteolysis: Haemoglobin
Cortisol	1·25±0·04	1·35±0·02
Cortisone	1·54±0·03	1·23±0·03
Corticosterone	1·63±0·04	2·19±0·04

Table 2. *Effect of corticosterone on the proteolytic and amidase activities of trypsin*

Proteolysis. Incubations were carried out at 37° with 0·8ml. of 1% protein solution, 0·1ml. of ethylene glycol with (C) and without (N) corticosterone (1·25mg.) and 0·1ml. of trypsin solution (1mg./ml. in 0·2M-sodium phosphate buffer, pH7·8. The reaction was stopped by the addition of 0·1ml. of 50% trichloroacetic acid and the amino nitrogen was determined by the method of Rosen (1957). Amidase activity. Incubations were carried out as above except that 0·8ml. of 3% sodium benzoylarginine amide hydrochloride in deionized water was used in place of the protein solutions.

Substrate.....	Proteolysis (μg. of amino N/ml. of incubation medium): Haemoglobin		Serum albumin		Insulin		Amidase activity (μg. of NH_3/ml. of incubation medium) Benzoylarginine amide	
Time (hr.)	N	C	N	C	N	C	N	C
0	1·05	1·05	7·72	7·72	7·01	7·01	8·78	8·78
1	6·32	9·60	31·5	40·5	—	—	66·2	55·6
4	11·9	33·0	59·7	89·8	23·9	20·5	—	—

corticosterone decreased the rate of albumin hydrolysis with papain by almost 25%. The effect on insulin hydrolysis was the same as with trypsin (Table 4; cf. Table 2). Pepsin gave very high rates of proteolysis but corticosterone had no action on the hydrolysis of either substrate (Table 4). Corticosterone produced effects with chymotrypsin similar to those obtained with trypsin.

Corticosterone and haemoglobin denaturation. In view of the strong possibility that corticosterone stimulated proteolysis by a process of denaturation of the substrate, this steroid was tested for its ability to denature haemoglobin in alkaline solution. It was found that the addition of corticosterone to haemoglobin solution increased the rate of denaturation in 2·4% (w/v) sodium hydroxide (Table 5). With a steroid/protein ratio 300:1 almost all of the haemoglobin was denatured in about 2min. compared with about 17% in the absence of corticosterone. The effect was dependent on the quantity of added steroid and with a corticosterone/haemoglobin ratio about 3:1 there was only a 10% change in the rate of denaturation.

Preincubation of haemoglobin with corticosterone (steroid/protein ratio 300:1) at pH7·8 brought about a 10% decrease in the extinction at 580mμ and an increase of 45% at 615mμ. These changes followed first-order kinetics and took about 2hr. to reach completion at 37°. The spectrum between 650 and 450mμ still had the same general form of oxyhaemoglobin. It was also found that the basic properties of the haemoglobin, as judged by its ability to be reversibly deoxygenated and to combine with carbon monoxide and cyanide, had not changed. The rate of alkaline denaturation of

Table 4. *Action of corticosterone on the hydrolysis of albumin and insulin by various proteases*

Papain. Incubations were carried out at 37° with 0·8ml. of 5% protein solution, 0·1ml. of ethylene glycol with (C) and without (N) corticosterone (1·25mg.) and 0·1ml. of papain (5mg./ml. of 0·2M-sodium phosphate buffer, pH7·8). Pepsin. Incubations were carried out as for papain except that the substrate concentration was 0·5% and the enzyme solution was 0·5mg./ml. of 0·2M-glycine–HCl buffer, pH2·2. Each reaction was stopped after 4hr. at 37° by the addition of 0·1ml. of 50% trichloroacetic acid and the amino nitrogen was determined as described in the text. Each estimation was carried out in duplicate.

	Proteolysis (μg. of amino N/ml. of incubation medium)			
	Albumin		Insulin	
	N	C	N	C
Papain	18·5	14·6	24·0	22·6
	17·9	13·3	24·4	23·9
Pepsin	81·2	81·8	131	122
	83·2	83·5	126	129
Chymotrypsin	61·5	77·7	13·3	12·0
	63·1	73·8	11·5	10·8

Table 5. *Effect of corticosterone on the rate of alkaline denaturation of haemoglobin*

Solutions were prepared containing 2·4ml. of 0·12% haemoglobin and 0·3ml. of sodium phosphate buffer, pH7·8. Ethylene glycol with and without dissolved corticosterone was added as indicated (0·3ml.). Incubations were carried out for 130min. at 37°. The rate of alkaline denaturation was measured as described in the text after the addition of 0·1ml. of 60% NaOH to 2·5ml. of haemoglobin solution at 24°. In the unincubated samples 60% NaOH was added 3min. after adding ethylene glycol. The extent of denaturation was calculated assuming that the extinction observed 3min. after the addition of 0·1ml. of 60% NaOH to unincubated haemoglobin (with 7·5mg. of corticosterone) was the maximum value for denatured haemoglobin.

	Denatured haemoglobin (%)					
	Not preincubated		Preincubated			
Corticosterone added (mg.).....	0	3·2	0	0·032	0·32	3·2
Time after addition of NaOH (min.)						
0	0	0	0	0·90	2·24	11·9
1	10·5	77·4	18·9	22·0	38·6	84·1
2	16·6	95·6	25·0	28·0	43·5	92·0
3	21·4	100	29·2	32·6	48·5	92·6
4	25·6	—	33·0	36·0	53·3	92·4
6	32·4	—	39·0	41·5	58·2	92·6
8	35·8	—	42·0	44·7	59·7	—
10	38·2	—	44·9	46·6	62·0	—
12	39·9	—	46·5	49·5	64·0	—
15	42·6	—	48·3	50·7	65·0	—

haemoglobin preincubated with 0·032mg. of corticosterone did not differ greatly from that of the control, taking into account that preincubation with ethylene glycol alone apparently made the haemoglobin more labile under alkaline conditions (Table 5). With 3·2mg., preincubation decreased the degree of stimulation by about 40%. However, the spectrum of the alkaline-denatured preincubated haemoglobin differed slightly from that of haemoglobin denatured in the presence of corticosterone without a preincubation period. In particular, the maximal extinction change at 615mμ was almost 10% greater.

DISCUSSION

In general, steroid hormones show a greater tendency to interact with proteins than with lipids (Bischoff, Stauffer & Gray, 1954; Bellamy, 1963*b*). The adsorption of steroids to plasma proteins has been investigated in great detail (Antoniades, 1960) and it has been suggested that steroids are attached to a wide range of proteins by hydrogen bonds (Westphal, 1961). This probably leads, in turn, to the breakage of intramolecular bonds linking peptide chains, and the process may be interpreted as a form of denaturation. In keeping with this the effect of steroids on individual enzymes is generally one of inhibition (Leiberman & Teich, 1953; Tomkins & Maxwell, 1963; Table 4 for trypsin with benzoylarginine amide as substrate), although a particular steroid may both stimulate and inhibit a given enzyme depending on the steroid/protein ratio (Dirscherl, 1962; Jones & Bellamy, 1964). However, steroids might be expected to produce alterations in the structure of proteins without changing their basic biological properties. With respect to corticosterone–haemoglobin mixtures, the formation of a new type of protein structure may be inferred from the different spectrum, the increased tendency to denature in alkaline solution and the ease with which the steroid–protein mixture was hydrolysed with trypsin. The basic properties of the haemoglobin, however, were not changed appreciably at a concentration of steroid that stimulated proteolysis.

When considering the action of corticosteroids on the proteolytic activity of trypsin, the substrate-specificity, taken together with the absence of stimulation with benzoylarginine amide as substrate, argues against a direct activation of the enzyme. Further, the small effect on denatured haemoglobin, the spectral changes on treatment of this protein with corticosterone and the marked temperature-dependence of the stimulation are strongly in favour of an interaction with the substrate that results in an improved access of the protease to susceptible bonds.

Changes in protein tertiary structure seem to occur with a wide range of molecules (allosteric modifiers; Bitensky, Yielding & Tomkins, 1965) and it would not be surprising for non-polar compounds in general to change proteins sufficiently to affect the rate of enzymic hydrolysis. The question is whether the present observations throw light on the specific action of corticosteroid hormones. In this connexion, there can be little doubt that the system steroid–receptor protein–protease, with two highly variable protein components, offers, theoretically, an ideal situation for bringing about an increase in the turnover of specific protein receptors. Therefore, investigations into intracellular mechanisms related to the effect of cortisol on protein degradation (Hoberman, 1950; Russell, 1951) should take into account the effects of a substrate level interaction of the steroid as well as the likelihood of a direct activation of cathepsins.

Turning to the question of the removal of protein-bound steroids from the cell, there are two possibilities to be considered. The steroid may be loosely bound and in equilibrium with extracellular steroid. This appears to be true for some of the biologically active cortisol in liver (Bellamy & Leonard, 1964). If, on the other hand, the steroid is strongly bound in a tissue such as muscle (Bellamy, 1963*b*), with no possibility of metabolism, the hormone would have a chance of leaving the cell only on turnover of the receptor protein. The present work opens up the possibility that, in the latter situation, attachment of the steroid may shorten the life span of the receptor.

It is appreciated that the concentration of steroid required to produce the effects was at least 100 times normal blood concentrations. This was a consequence of the high concentration of substrate necessary for an accurate measurement of protein hydrolysis. Changes in proteolysis were obtained with a steroid/substrate protein molar ratio between 1:1 and 5:1, and at the moment there is no reason to suppose that the intracellular relationship between steroid and receptor renders this relationship invalid.

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