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AN *IN VITRO* EFFECT OF PHYSIOLOGICAL LEVELS OF CORTISOL AND RELATED STEROIDS ON THE STRUCTURAL INTEGRITY OF THE NUCLEUS IN RAT THYMIC LYMPHOCYTES AS MEASURED BY RESISTANCE TO LYSIS

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SUMMARY

The ability of rat thymus cell nuclei to survive lysis of cells by hypotonic shock (dilution into 1·5 mM $MgCl_2$) is decreased by incubation of whole cells with physiological levels of glucocorticoid hormones *in vitro*.

This effect on "nuclear fragility" is first discernible after 0·5–1 h of incubation with hormone as an increase in non-sedimentible DNA in whole cell lysates. It is measureable at 2 h by counting numbers of surviving nuclei, and by changes in the distribution of DNA and protein from lysed cells on sucrose gradients.

Evidence is presented to show that the effect is initiated by a series of molecular events characteristically similar to those which initiate the metabolic effects of the hormone, including specific binding of hormone to receptors and events that are sensitive to cycloheximide.

The effect differs from effects of the hormone on many transport and biosynthetic processes which occur at the same time (1–3 h) in as much as the nuclear effect does not require the presence of a carbohydrate energy source in order to become apparent.

The specificity and time of onset of the decrease in nuclear integrity combined with the fact that it is reflective of changes in nuclear structure, make it quite likely that it is a simple, rapid assay *in vitro* of the lymphocytolic effects of cortisol.

INTRODUCTION

Although lymphocytolysis ranks as one of the best known and most widely studied responses to the administration of glucocorticoid hormones (see [1–3] for reviews) considerable gaps in our understanding of the mechanism for the generation of this cellular destruction still exist. In the case of thymic lymphocytes studies of the effects of physiological levels of glucocorticoids *in vitro* seem to favor the view, that has been sometimes challenged [4], that the lytic action is a direct one on sensitive lymphocytes [3, 5]. The demonstration that morphologic effects are not seen unless cells are metabolically healthy and are incubated at *in vivo* temperatures would seem to indicate that cell destruction requires active cellular metabolism [5].

Metabolic effects of glucocorticoids are abundant. Inhibitions in the metabolism of glucose can be measured as early as 20 min after exposure of the cells to physiological levels of the hormone *in vitro* [6, 7]. This is followed at later times (about 1–3 h) by inhibitions in many other cellular metabolic functions which include: decreases in the incorporation of precursors into proteins [8–10] and nucleic acids [9, 11–13], decreases in the transport of some amino acids and nucleosides [7, 14], in the activity of RNA polymerase [16, 17] and changes in the levels [7, 10, 11] and turnover [15] of adenine nucleotides. As interrelationships between several of these metabolic effects have been clarified [7, 11, 14] and insights into the molecular mechanisms for the generation of the metabolic inhibitions have been gained [18–22] the anticipated relationship between the metabolic effects and the morphologic changes have nevertheless not been clarified. Still unresolved is the basic question, does cell destruction lead to observed decreases in biosynthetic and

transport processes, or visa versa? Efforts to resolve this question have been especially hampered by a lack of a convenient assay for accurately assessing early structural changes. An increase in the release of DNA from surviving slices of rat thymus at 2 h after cortisol administration *in vivo* has been reported [23]. However, neither we, using chemical measurement, nor others using radiolabel [9], have been able to detect such a release as part of cortisol action on thymus cells *in vitro*.

We report here a new effect of cortisol, an increase in "nuclear fragility" that is quite possibly a direct, simple measure *in vitro* of the lymphocytolytic actions of glucocorticoids. The effect may be measured either as a decrease in the number of nuclei which survive intact when whole cells are lysed in hypotonic medium or, more precisely, by the amount of DNA released into the medium from those nuclei that are lysed along with the cells. The effect is by all available criteria a specific one. It is not secondary to decreases in the synthesis of protein. It appears to be separate from a number of the "metabolic effects" of cortisol, specifically, those that are measured by a decrease in glucose metabolism or in the incorporation or radiolabelled precursors into RNA and protein. These results raise the possibility that the mechanism for the initiation of cell destruction may be independent of these general metabolic inhibitory effects.

METHODS AND MATERIALS

Male Sprague–Dawley rats (Charles River Breeding Laboratories) were adrenalectomized 1–2 weeks prior to each experiment. After adrenalectomy, they were maintained on 1% NaCl and fed Labena. Suspensions of washed thymus cells were prepared as in previous experiments [10]. Thymuses from 2 to 12 decapitated rats were minced in Krebs–Ringer bicarbonate buffer (KRB, pH 7·4 at 37° C in equilibrium with 95% O_2–5% CO_2), homogenized gently by hand, filtered through nylon mesh, and washed twice in 40 ml of buffer. Cells were resuspended in buffer and 0·5 ml aliquots were added to incubation flasks. Cells were prepared at room temperature; buffer containing cells was maintained in constant equilibrium with gas. Incubations were started within 30 min after killing the first rat.

Incubations were carried out in Neoprene-stoppered, 10 ml Erlenmeyer flasks shaken at 37° C in a Dubnoff metabolic incubator at about 100 cycles per min. Flasks were gassed at 30 min intervals during the incubation. Packed-cell volume was determined shortly after the start of the incubation by a standard microhematocrit procedure.

Glucose was added as a 5% aqueous solution to give a final concentration of 1 mg/ml; sodium pyruvate as a 6·1% aqueous solution to give a final concentration of 1·22 mg/ml. Cortisol, unless otherwise indicated, was added as a 10^{-4} M aqueous solution to give a final concentration in the incubation flasks of slightly less than 10^{-6} M. Cortexolone*, cortisone, deoxycorticosterone and dexamethasone were also added as aqueous solutions. Initial concentrations of steroids were determined by measuring optical absorbance at 250 nm, assuming a molar extinction coefficient of $1{\cdot}5 \times 10^4$.

To determine the number of whole nuclei remaining after hypotonic shock (Table 1) cells were lysed by a 50-fold dilution into 1·5 mM $MgCl_2$ at 3° C, shaken for 15 s at high speed on a vortex mixer and allowed to sit at 3° C for at least 5 min. Aliquots of the lysate were then placed in a Neubauer counting chamber and remaining nuclei were counted. At the beginning and at the end of each experiment cells were also diluted 50-fold into KRB and counted in a similar manner.

In experiments where cell fractions were collected on sucrose gradients whole cells were incubated as described above, broken by diluting the cells 50-fold into 1·5 mM $MgCl_2$ at 3° C, shaken and then forced under pressure through a 28 g Teflon needle. The shearing forces developed in the needle strip most of the cytoplasmic and membrane tabs from the nuclei. For the experiment in Fig. 1, half a millilitre of this solution was then layered on a 4 ml, 0·2–2·0 M sucrose gradient containing 1·5 mM $MgCl_2$ and spun in a Sorvall GLC-1 centrifuge for 2·5 min at 1500 ***g***. With this procedure, the nuclei migrate most of the length of the gradient and appear as a single, thin, white band [24].

All other visible material remains near the top of the gradient. Tubes were then punctured and 20 drop fractions were collected in test tubes already containing 0·5 ml of 20% trichloroacetic acid (TCA). Collected samples were then centrifuged, supernatant was discarded and precipitates were washed 4 times with 5% TCA. The washed precipitates were re-suspended in 0·5 ml aliquots of 10% TCA and heated to 90° C for 30 min to hydrolyze DNA. After samples were cooled and centrifuged, 400 μl of the supernatant was removed, added to 100 μl of 2·5 N perchloric acid, mixed with diphenylamine reagent (100 ml glacial acetic acid; 1·5 ml concentrated H_2SO_4; 1·5 g diphenylamine; 0·5 ml 0·16 mg/ml acetaldehyde in H_2O). Samples were then incubated 16–24 h at 25–30° C, and the difference between absorbance at 600 and 650 nm determined to give a measure of DNA [25]. Precipitates from the hot TCA were dissolved in 0·5 ml of 0·1 N NaOH, and pro-

* The trivial names for steroids used are: cortexolone, 17, 21-dihydroxy 4-pregnen, 3, 20-dione and dexamethasone, 9α-fluoro-16α-methyl-11β,17,21-trihydroxy 1,4-pregnadiene-3,20-dione.

Table 1. Effect of a 2·5 h incubation with cortisol on the recovery of nuclei from cells lysed at end of incubation

	Cells after 50× dilution into KRB at 0 min		Cells after 50× dilution into KRB at 150 min		Intact nuclei after 50× dilution into 1·5 mM $MgCl_2$ at 150 min	
	Cortisol	Control	Cortisol	Control	Cortisol	Control
Experiment A	1155	1184	1123	1055	983	1033
	1112	1120	1022	1133	841	967
	1308	1371	1242	1246	940	1291
	1313	1233	877	1007	1029	1224
Mean	1222	1227	1066	1110	948	1129
						$P < 0·1$
Experiment B	1245	1286	1180	1137	822	859
	1210	1223	1103	1186	825	946
	1269	1155	1146	1114	826	831
	1194	1283	1111	1159	774	948
Mean	1230	1237	1135	1148	812	896
						$P < 0·05$
Experiment C	1523	1410	1462	1473	1029	1441
	1575	1610	1507	1528	960	1174
	1559	1521	1559	1440	1019	1212
	1540	1594	1509	1515	965	1168
Mean	1549	1534	1509	1489	993	1249
						$P < 0·01$

Cell suspensions (0·5 ml) were incubated with or without cortisol (10^{-6} M) in Erlenmeyer flasks for 150 min. Packed cell volume of cell suspensions was $\simeq 10\%$ in each experiment. Cells from each flask were diluted 50-fold into KRB at 3°C at the start of the experiment, and again at 150 min. An aliquot from each diluted sample was then placed in a Neubauer counting chamber and a 0·1 mm^2 area counted under "high dry" (× 400) magnification. At 150 min cells were also diluted into 1·5 mM $MgCl_2$ at 3°C and whole nuclei from these samples were counted in the same manner as were the intact cells. Individual values from flasks, means for each group of values from three experiments are shown. Where differences are significant (by two-tailed *t*-test) the level of significance is shown.

tein in each fraction was determined by a modification of the Lowry method [25].

In all other experiments, whole cells were incubated and broken as described above, but instead of being layered over sucrose, the lysate was spun at high speed in an Eppendorf centrifuge for 30 s and aliquots of the supernatant collected and assayed for DNA or DNA and protein, as described above.

Cortisol, cortisone and desoxycorticosterone were purchased from Calbiochem; 11-desoxycortisol (cortexolone) and dexamethasone from Sigma.

RESULTS

In these experiments a brief exposure of thymus cells to 1·5 mM $MgCl_2$ (nearly pure H_2O) is used to produce rapid lysis of 100% of the cells. This procedure has been used previously to achieve a rapid lysis of thymus cells and subsequent separation of nuclear and cytoplasmic components [24, 26]. The small amount of Mg^{2+} present maintains the integrity of most of the nuclei which are recovered intact. When viewed with the electron microscope, the nuclei, apart from the fact that they are swollen, are indistinguishable from those in cells [27].

Data from cell counts in Table 1 show that incubation with cortisol for 2·5 h has no effect on the number of whole cells but decreases the number of nuclei surviving cell lysis. This decreased ability of nuclei to withstand hypotonic shock provides evidence for early hormone-induced structural changes in some of the nuclei, perhaps at the level of the membrane.

These morphological results are confirmed by the biochemical data in Fig. 1 compiled from several similar experiments. Here, nuclei are separated on a sucrose gradient from the remainder of the cell lysate. Cortisol leads to an increased release of both protein and DNA into the non-nuclear, "cytoplasmic", fraction at the expense of the nuclear fraction.

In intact cells more than 99% of the DNA is within the nucleus. With this in mind in the remainder of the experiments we have used the amount of DNA which becomes non-sedimentible by high speed centrifugation as a measure of nuclear breakage. This provides a measure similar to DNA in the "cytoplasmic fraction" in Fig. 1. This is a close correspondence (in the experiments in Figs. 2–8) between the DNA present in the post-nuclear supernatant and the loss of nuclear numbers. However, measurement of DNA yields a

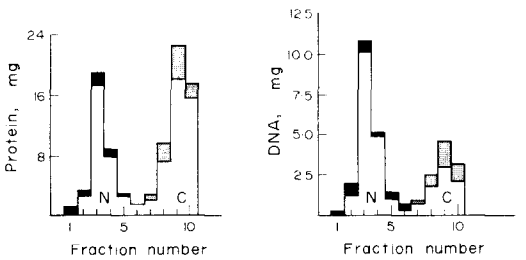

Fig. 1. Distribution of DNA and protein between nuclear and cytoplasmic fractions isolated on sucrose gradients. Cells (0·5 ml) were incubated either with or without cortisol (10^{-6} M) for 150 min in Neoprene stoppered, 10 ml. Erlenmeyer flasks. At 150 min, cells were broken by diluting 50-fold into cold (3°C) 1·5 mM $MgCl_2$, and forcing the lysate through a 1 in., 28 G. Teflon needle. Lysate (0·5 ml) was layered over a 4 ml 0·2–2·0 M sucrose gradient containing 1·5 mM $MgCl_2$ (uniform concentration) and spun for 2·5 min at 1500 *g*. The gradients were collected as 10 fractions in test tubes containing 0·5 ml of 20% TCA. Resulting precipitates were washed 4 times with 1 ml of 5% TCA, and the amount of DNA and protein collected in each fraction was determined. The first peak (in dense sucrose) on the gradient is termed the nuclear fraction (N), the second, the cytoplasmic fraction (C). Solid areas represent a decrease from control values produced by cortisol, shaded areas an increase produced by cortisol. The data are the means of results from six experiments.

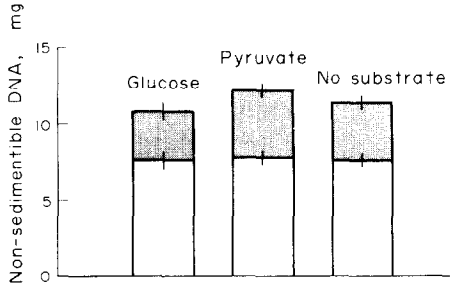

Fig. 2. Effect of cortisol on amounts of DNA recovered in supernatant fractions from cells incubated with glucose, pyruvate, or no substrate. Cell suspensions (0·5 ml) were incubated with or without cortisol (10^{-6} M) for 150 min. At 120 min, glucose (1 mg/ml), sodium pyruvate (1·22 mg/ml) or no substrate was added to flasks. Cells were broken at 150 min. Lysates of cells were spun at high speed in an Eppendorf centrifuge for 20 s, and the DNA remaining in the supernatant was measured by determination with diphenylamine [25]. The data are expressed as the amount of DNA remaining in the supernatant fraction from 1 ml of packed cells. Open bars represent the DNA from supernatants of control cells, shaded areas, the increase in DNA above control values produced by cortisol. Each value is the mean of determinations from five flasks. ±1 S.E. The difference between control and cortisol in each case is $P < 0{\cdot}01$.

more precise measurement of nuclear breakage than counting the remaining nuclei since the inaccuracy inherent in collecting data as a small difference between large numbers, is circumvented. The limitations on precise timing and sample numbers that are imposed upon the experimentor by either cell counting or centrifugation through sucrose, are also overcome.

It has been repeatedly observed that the addition of carbohydrate (or an amino acid such as glycine that is

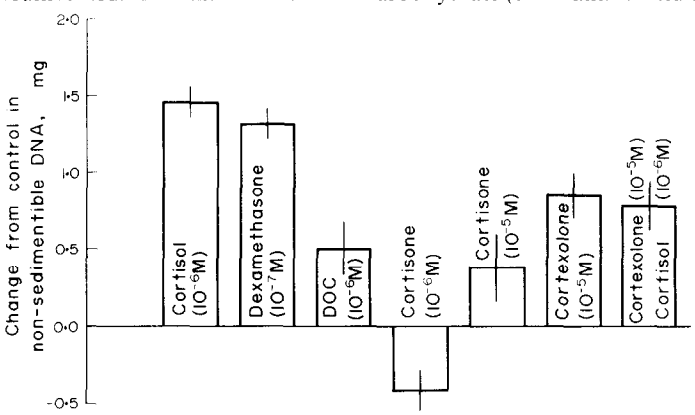

Fig. 3. Effects of dexamethasone, cortisol, desoxycorticosterone, cortisone and cortexolone on DNA recovered in the supernatant fraction: the ability of cortexolone to block the increase in non-sedimentible DNA produced by cortisol. Cell suspensions (0·5 ml) were incubated in flasks for 150 min. Before the start of incubation, dexamethasone (10^{-7} M, final concentration), cortisol (10^{-6} M), desoxycorticosterone (DOC, 10^{-6} M), cortisone (10^{-6} or 10^{-5} M), cortexolone (10^{-5} M), or cortexolone (10^{-5} M) and cortisol (10^{-6} M) together were added to flasks. All steroids were added to flasks at 10-fold higher than final concentration as solutions in KRB. Flasks not receiving steroid were given an equal volume of KRB. Cells were lysed and the supernatant fractions collected at 150 min. The data are expressed as the difference from control in the DNA recovered from the supernatant fraction of cells given different steroids (in mg of DNA per ml of packed cells). Each value represents the mean difference between five sets of experimental and control flasks. ±1 S.E. The control value for non-sedimentible DNA is 6·73 ± 0·126 mg. Difference from control with cortisol or dexamethasone is significant at $P < 0{\cdot}001$, desoxycorticosterone, $P < 0{\cdot}05$, cortexolone and cortexolone with cortisol, $P < 0{\cdot}01$. Neither 10^{-6} nor 10^{-5} M cortisone produces values significantly different from controls.

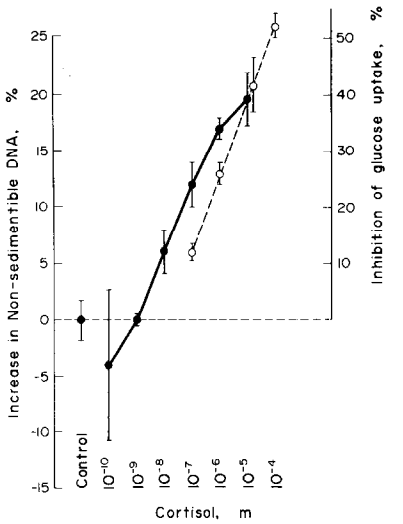

Fig. 4. Dose–response to cortisol of the effect increasing nuclear fragility. Before the start of incubation, solutions of cortisol (in 50 μl of KRB) were added to flasks at 10-fold less than final concentrations. To start incubations, 0·45 ml of cell suspension (packed cell volume = 10%) was added to each incubation flask. Flasks were incubated 150 min. At 150 min 20 μl aliquots of cells were taken from flasks and diluted 50-fold into 1·5 mM $MgCl_2$ for cell lysis (see methods and materials). Aliquots (0·5 ml) of the "post-nuclear" supernatants were assayed for DNA by diphenylamine determination. The data is plotted as the per cent increase from control values produced by cortisol (closed circles). Each point is the mean of values from six flasks, $\pm$1 S.E. For sake of comparison, data from Kattwinkel and Munck [31] showing the inhibition of glucose uptake during the first hour of exposure to cortisol (open circles) are included. Absolute value for controls in this experiment was 7·21 $\pm$ 0·187 mg DNA/ml packed-cell volume.

convertible to carbohydrate) to the incubation medium is essential to elicit *in vitro* glucocorticoid-induced inhibitions in the incorporation of labelled precursors into RNA and protein [9–11]. The data in Fig. 2 indicate that the development of the effect on nuclear fragility neither requires nor is influenced by the presence of a carbohydrate during the incubation. (The somewhat larger effect in the presence of pyruvate in this experiment is not significant and is not a constant finding.) These results in Fig. 2 may be compared directly with data from previous experiments relating effects on macromolecular metabolism to effects on carbohydrate metabolism in which the same experimental protocol was used (see Fig. 5 in Ref. [10] and Fig. 1–3 in Ref. [11]).

Several criteria were applied to determine whether the effect on nuclear fragility was a "specific" one, as opposed to one of the non-specific effects of steroids. The latter are unrelated to binding of hormones to specific glucocorticoid receptors [18]. They are effects produced by all steroids when present at concentrations of 10^{-5} M and higher, probably through interactions with lipid-aqueous interfaces [28]. First, a specific effect should be produced only by glucocorticoids and not by non-hormonal steroids with high surface activity. The results presented in Fig. 3 indicate that this is the case. Here the ability of a number of steroids to increase the amount of DNA released from the nuclei are compared. The nucleolytic effect of dexamethasone, which *in vitro* is 10 times more potent than cortisol in thymus cells [18], is equal to cortisol when 10-fold less dexamethasone is used. On the other hand steroids that are not active as glucocorticoids but are much more surface active than cortisol, here represented by deoxycorticosterone (DOC), do not duplicate the effects of either cortisol or dexamethasone even at concentrations, 10-fold higher than cortisol. Cortisone is inactive as a glucocorticoid here since it is not metabolized to cortisol by thymus cells [18].

A second criterion used to determine specificity was to see whether cortexolone (11-deoxycortisol), a competitive inhibitor of specific glucocorticoid binding to receptors in thymus cells [18, 20], blocks the development of effects of cortisol on nuclear integrity. The fact

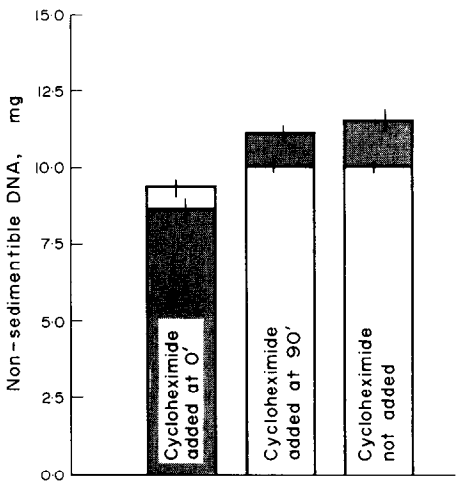

Fig. 5. Protection by cycloheximide from the effect of cortisol increasing the fragility of thymus cell nuclei. Cell suspensions (0·5 ml) were incubated with or without cortisol (10^{-6} M). To some flasks, cycloheximide was added to cells at 0 or 90 min of incubation as a 10^{-3} M solution in H_2O to give a final concentration of 10^{-5} M. Cells were lysed at 120 min, lysates centrifuged and the supernatant fractions collected and assayed for DNA. Bars representing DNA recovered from supernatants of control and cortisol-treated flasks are superimposed. Open bars are from controls; closed bars from cortisol-treated cells. Data presented are the means of values from six flasks, $\pm$1 S.E. When cycloheximide was added at 90 min, cortisol values were different from controls at the $P < 0{\cdot}05$ level. Without cycloheximide the level of significance was at the $P < 0{\cdot}01$ level.

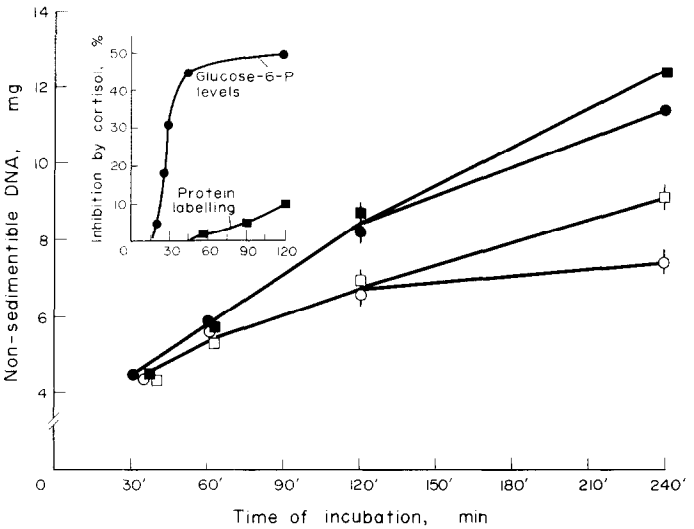

Fig. 6. Time-course of the development of the effect of cortisol increasing nuclear fragility in the presence and absence of added glucose. Cell suspensions (0·5 ml) were incubated with or without cortisol (10^{-6} M) and with or without glucose (1 mg/ml) for 240 min. Aliquots of cell suspensions were lysed at the times indicated in the Figure. Closed circles represent the amount of DNA recovered in supernatant fractions of cells incubated with glucose and cortisol; open circles, with glucose and without cortisol; closed squares, without glucose and with cortisol; open squares, without glucose or cortisol. Data presented are the means of determinations from five flasks, ± 1 S.E. The insert, included for comparison, shows the time of onset and course of development of effects of cortisol on glucose transport (levels of glucose-6-phosphate) and on rates of incorporation of radiolabelled valine into protein [11]. The difference between cortisol and controls is significant at the $P < 0{\cdot}05$ level by 60 min, at the $P < 0{\cdot}01$ level by 2 h and at the $P < 0{\cdot}001$ level by 4 h, both in the presence and in the absence of added glucose. The difference between glucose and no glucose, either in the presence or absence of cortisol, does not become significant until 4 h, when the difference is significant at $P < 0{\cdot}01$ level.

that in Fig. 3 cortexolone by itself at high concentrations (10^{-5} M) produces an appreciable effect may in part be the result of non-specific steroid effects at this high concentration. Alternatively, cortexolone by itself may be a weak agonist since it binds to specific nuclear receptors [29]. However, just as previously reported for effects of cortisol on glucose metabolism [20] and on uridine incorporation into RNA [30], the presence of cortexolone with cortisol blocks at least part of the cortisol effect on nuclear breakage. This reduction by an antagonist of specific cortisol binding is strong evidence that the effects of physiological concentrations (10^{-6} M or less) of glucocorticoid hormones on nuclear integrity are produced by specific hormone interactions with cellular receptors.

One more test of the specificity of hormone effects is a demonstration that the dose-response curve *in vitro* occurs within the physiological range of concentrations of hormone *in vivo*, and that such a curve is similar to the curve for hormone–receptor binding. Both hold true for the effect of cortisol on nuclear integrity. This is shown in Fig. 4. It can be seen that a half-maximal effect occurs at approximately 5×10^{-8} M cortisol, a concentration which is physiological, and half-saturates specific cortisol-binding receptors [18]. The effect reaches maximum proportions at concentrations just sufficient to saturate binding (10^{-6} M), levels similar to the highest concentrations of cortisol seen *in vivo*. Data replotted from Kattwinkel and Munck [31], included in Fig. 4 for comparison, show that the effect on nuclear fragility is at least as sensitive to lower concentrations of cortisol as the effect on glucose uptake.

The demonstration of a period of sensitivity to cycloheximide that is confined to only the time of emergence of the effect on glucose transport (15–35 min) has suggested that the action of glucocorticoids that ultimately limits glucose entry into thymus cells, involves the early synthesis of a new protein [21]. Inhibitors of protein synthesis also block cortisol action on the transport of amino acids and nucleosides [14, 32]. A similarity between these metabolic actions of cortisol and the effect on nuclear fragility is revealed by the data in Fig. 5 which show that the latter action of cortisol is also abolished by the presence of cycloheximide from the start of incubation. In the absence of hormone cycloheximide presence for the full 2·5 h does not change the number of nuclei that survive lysis. Nor

does late addition of cycloheximide (90 min) interfere with the expression of the cortisol effect once developed. The amount of cycloheximide added is sufficient to block about 62% of protein synthesis within 5 min and 97% by 20 min [21]. The fact that an inhibitor blocks the expression of an inhibitory, or "toxic" action of a hormone suggests that the mode of action of the inhibitor, here cycloheximide, is through its specific block or the synthesis of a protein induced by cortisol rather than through some unknown non-specific toxic effect of the inhibitor. Our experiments therefore support the interpretation that the action of cortisol on nuclear integrity requires the synthesis of new protein.

The data in Fig. 6 demonstrate the time-course of the nuclear effect, both in the presence, and in the absence of added carbohydrate substrate. The effect is first measureable 0·5 h after cortisol addition and increases steadily in magnitude. It does not have a sudden burst of onset starting at 20 min like the effect on glucose metabolism (insert, Fig. 6) but develops more slowly becoming measureable at 1–2 h, similar to the effects on the labelling of RNA and protein and effects on the accumulation of alpha-aminoisobutyric acid (AIB) [7,9]. Although at incubation times after 2 h, the presence of glucose does itself influence nuclear fragility, neither the time of onset nor the course of development of the cortisol effect are influenced by glucose. Moreover, the effect of cortisol is greater than that produced by the absence of carbohydrate. These data, as well as those in Fig. 2, indicate that the effect of cortisol on nuclear integrity is not secondary to effects on carbohydrate metabolism.

DISCUSSION

This study demonstrates a new type of effect of glucocorticoid hormones on thymic lymphocytes, an increase in the breakage of nuclei when whole cells are subjected to hypotonic lysis. For want of a better term we have called this new effect increased "nuclear fragility". While its physiological significance is difficult to assess the effect may provide an important handle for the further study on the mechanism of lymphocytolysis. In *in vitro* studies of lymphocytolysis an altered chromatin pattern and nuclear edema are seen in some of the cells *via* electron microscopy as early as 2 h after cortisol [5]; it seems possible if not likely that these are the nuclei that are subject to rupture when the cells are lysed.

A hypothesis [33] that has guided research in several laboratories including our own is that early endocrine effects on the metabolism of carbohydrates (especially glucose) result, possibly through decreases in carbohydrate-dependent ATP, in a decreased synthesis of macromolecules. The decreased synthesis in turn is thought to be responsible for cell destruction. The finding by several laboratories [9–11] that glucocorticoid effects on RNA, protein and ATP are observed only when cells are incubated in the presence of an exogenous source of carbohydrate (glucose, lactate, pyruvate or glycine), in spite of appreciable synthetic rates and ATP production in the absence of this source, supports this hypothesis.* Effects on ATP turnover may also be interpreted within this framework [15]. The insensitivity of the cortisol effect on nuclear fragility to the presence or absence of carbohydrates (Figs. 2 and 6) provides evidence that the lytic effect of cortisol on thymus cells may not be related to either effects on the metabolism of carbohydrates or to previously-studied decreases in overall rates of anabolic processes. The finding (Fig. 5) that cycloheximide at levels sufficient to almost completely inhibit protein synthesis does not increase nuclear fragility, but if anything protects nuclei, also supports this view.

Notwithstanding these dissimilarities, the effect on nuclear fragility does have some characteristics (apart from similarities in dose-response and hormonal specificity) in common with certain of the well-known effects of glucocorticoids. The inhibition of the non-metabolizable amino acid analogue α-aminoisobutyric acid (AIB) [9] is similarly unrelated to effects on carbohydrate metabolism [7]. It also emerges with the similar kinetics and appears to be similarly sensitive to inhibitors of protein synthesis [14,34]. Apart from the earlier time of appearance and clearly different kinetics of onset [7] the effect on glucose transport also shows these characteristics [21]. Further studies are in progress (using approaches similar to those in references 20 and 21) to determine to what extent common molecular events subsequent to hormone binding are involved in the initiation of these 3 distinct effects.

It is known that not only do large doses of glucocorticoids lead to widespread lymphocytolysis, but also

* Drews and Wagner (*Eur. J. Biochem.* **16**, 545, 1970) have concluded that there are effects of glucocorticoid hormones on the labelling of RNA that are unrelated to effects on carbohydrate (glucose). In our view, their comparison between the evolution of the effects of prednisone administered to the whole animal and the events that occur when the cells are incubated *in vitro* in the complete absence of glucose is not a valid one. It is also apparent that in their study "RNA synthesis" declines rapidly after the first 40 min (compare their Fig. 1 with our Fig. 1, reference 11). In our view this makes their estimate of the magnitude of the effect of glucose on the synthesis of RNA invalid. Indeed their results (Fig. 1) indicate that the labelling of RNA is almost completely dependent on glucose after the first 40 min.

that physiological levels in the absence of stress exert chronic suppressive effects on lymphoid tissues, most of which hypertrophy if glucocorticoids are deficient [2]. What is not clear is whether or not the suppressive effects on lymphoid tissues occur by means other than the effects which lead to cell lysis. For example, the slowing down of cellular metabolic functions as measured by generalized effects on transport processes and biosynthesis of RNA and protein may lead to the growth suppression. The findings reported here that the effect on nuclear fragility appears under conditions when the generalized metabolic inhibitions are not seen (e.g. in the absence of carbohydrate) suggests that the two types of actions, metabolic and lymphocytolytic, may be separable and for that matter, may not even occur within the same functional cell population. Steroid resistant strains of lymphatic cancer cells appear to be resistant to the lymphocytolytic action of glucocorticoids [35]. The simplicity of the assay for nuclear fragility may make it a useful screening test for the susceptibility of lymphoid cancers to glucocorticoid suppression therapy. In fact, as an assay for the lytic effects, such a test may prove to be more specific than the measurement of "metabolic" effects of glucocorticoids on cancer cells, such as changes in rates of synthesis or transport.

While data in this paper indicate that the effects on nuclear fragility are not secondary to the inhibitory effects of cortisol on either glucose metabolism or protein synthesis they provide no alternative mechanism. Studies by Bowers and DeDuve [36] at late times after cortisol (8 h) and by Nakagawa, Dvorkin and White [37] at earlier times (3 h), provide inconclusive yet suggestive evidence that there is an increase in the activity of free hydrolytic enzymes normally held inert within lysosomal particles in thymus cells. More recent work by Szego *et al.* [38] showing that estrogens induce the migration of lysosomes to the nuclear envelope in uterine target cells and lead to the labilization of degradative enzymes again suggest that lysosomes may somehow be involved in the early effects of glucocorticoid hormones on nuclear fragility. It is important to note, however, that neither we nor others have been able to find increases in rates of breakdown of cellular protein in thymus cells during the first 6 h of hormone action [9]. Another possibility, suggested by Turnell *et al.* [35] is that cortisol increases the titre of free fatty acids in lymphoid cells, and these have a detergent effect on the nuclear membrane, and thus cause nucleolysis. Increased activity of hydrolytic enzymes and increased levels of free fatty acids are certainly not mutally exclusive events, and both events may either produce, or be associated with lymphocytolysis.

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