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EFFECT OF HYDROCORTISONE ON AMINO ACID INCORPORATION BY MICROSOMES ISOLATED FROM MOUSE LYMPHOMA ML-388 CELLS AND RAT THYMUS*

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Abstract—The effects of hydrocortisone on ^{3}H-leucine incorporation into an acid-insoluble form by microsomes of lymphoid tissues are described. Microsomes plus cell sap isolated from cells (either ML-388 or rat thymus) pre-exposed to the steroid for a 12-hr period had a depressed ability to incorporate the label. In contrast, no effect was seen if hydrocortisone was added directly to the incubation vessel during the incorporation period. Crossover experiments in which microsomes from control cultures of ML-388 cells or from the thymus glands of control rats were incubated with cell sap from steroid-treated animals showed that the cell sap from steroid-treated cells or animals did not significantly inhibit the uptake of ^{3}H-leucine by microsomes from control cells. Likewise it was shown that the inhibitory effects of hydrocortisone on microsomes from steroid-treated cells could not be reversed by cell sap from control cells. These results indicate that hydrocortisone does not act directly on the microsomal system to inhibit amino acid incorporation. Further, the biochemical lesion would seem to be associated with the particulate (messenger RNA–ribosomal RNA complex) and not with the soluble enzyme systems, transfer RNA, or the availability of amino acids.

IN A previous study[1] hydrocortisone was reported to exert a selective toxic effect on mouse lymphoma ML-388 as compared to other cell lines. Structure acitivty studies demonstrated that the growth-inhibitory activity of steroids in ML-388 cells cultured *in vitro* is very similar to the ability of these compounds to cause thymic involution. The same report also presented data showing that the levels of protein, acid-soluble amino acids, DNA, and possibly RNA were all decreased on a per cell basis in response to hydrocortisone. Protein synthesis was shown to be inhibited very early after steroid treatment but could not be shown to occur before other alterations in cell metabolism.

More recent experiments§ in which the rate of uptake of ^{14}C-leucine, ^{3}H-thymidine, and ^{14}C-uridine was compared (cells exposed to radioactive compounds for 1 hr at

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various times after steroid addition) showed metabolic effects of hydrocortisone as early as 4 to 6 hr after steroid addition. However, the data from these experiments failed to show any significant differential effects of this steroid on protein, RNA, or DNA synthesis. Rather it was found that the uptake of all the above radioactive metabolites was affected at about the same time.

This report describes further studies of effects of hydrocortisone on protein synthesis in lymphoid tissue. ^{3}H-leucine incorporation into acid-insoluble material by microsomes isolated from ML-388 cells or rat thymus was measured in the presence or absence of hydrocortisone. Similar studies were also carried out with microsomes isolated from cells or animals which were pretreated with the steroid.

MATERIALS AND METHODS

The mouse lymphoma ML-388 cell line used was that described in a previous report.[1] Male rats of the Wistar strain, weighing 160 to 180 g, were used as a source of thymic tissue. Hydrocortisone* for cell culture experiments was prepared as previously described;[1] Cortef, an aqueous suspension of hydrocortisone, was used for the studies of rat thymus.

Isolation of microsomes

Mouse lymphoma ML-388. Replicate cultures of ML-388 cells were grown in Roux bottles inoculated with 70 ml nutrient medium containing about 1×10^6 cells. Three days after inoculation the cultures were treated with hydrocortisone (5×10^{-6} M) or the appropriate vehicle; 12 hr later the cells from each group of bottles (30 bottles per group) were combined by scraping into a 0·35 M sucrose solution containing 0·004 M $MgCl_2$, 0·025 M KCl, 0·001 M mercaptoethanol, and 0·05 M Tris buffer, pH 7·6 (medium A[2]). This and all subsequent operations were carried out at 1°–2°. The two samples so obtained were centrifuged at 550 *g* for 10 min and the supernatant discarded. The cells were then washed with 10 ml of medium A and the final pellet resuspended in 4 volumes of distilled water containing 1×10^{-3} M mercaptoethanol. Cell lysis was allowed to proceed for 20 min in an ice bath, after which time the suspension was homogenized with 10 strokes of a loose-fitting pestle in a Dounce homogenizer. Concentrated (10×) buffer–sucrose solution was then added so that the final solution was 0·25 M sucrose, 0·01 M Tris-HCl buffer (pH 7·8), 0·01 M magnesium acetate, 0·06 M KCl, and 0·001 M mercaptoethanol. The resulting suspension was then centrifuged for 20 min at 15,000 *g*. In some experiments the 15,000-*g* supernatant was further fractionated in a Spinco model L ultracentrifuge into a microsomal fraction (pellet obtained from 1-hr centrifugation at 144,000-*g*) and cell sap (144,000-*g* supernatant). Microsomes were re-suspended in a volume of 0·25 M sucrose–0·001 M $MgCl_2$–0·006 M mercaptoethanol equivalent to the volume of 15,000-*g* supernatant from which they were isolated.

Rat thymus. Rats were injected with Cortef of the appropriate vehicle; 12 hr later they were sacrificed by decapitation and the thymus glands rapidly excised. This and all subsequent operations were carried out in the cold. Thymus glands from 3 to 5 rats were combined for each sample. The thymic tissue was then homogenized in 3 volumes of 0·25 M sucrose containing 0·01 M Tris–HCl (pH 7·8), 0·01 M magnesium

* Hydrocortisone was obtained from California Corp. for Biochemical Research; Cortef was obtained from Upjohn Co.

acetate, 0·06 M NH_4Cl, and 0·006 M mercaptoethanol and stored at −20° for 2 hr. The homogenate was then thawed and centrifuged at 15,000 *g* for 20 min. The 15,000-*g* supernatant was further fractionated into a microsomal component and cell sap as described for ML-388. In the case of rat thymus the microsome pellet was washed by resuspending in sucrose and centrifuged again for 1 hr at 144,000 *g*. The pellet was then resuspended in a volume of 0·25 M sucrose–0·001 M $MgCl_2$–0·006 M mercaptoethanol equivalent to the volume of the 15,000-*g* supernatant from which it was isolated.

Assay procedure for ^{3}H-leucine incorporation. The complete reaction mixture contained 46 μmoles KCl; 5 μmoles magnesium acetate; 50 μmoles Tris buffer (pH 7·5); 0·25 μmole GTP;*† 1·0 μmole ATP; 10 μmoles phosphoenolpyruvate (PEP); 0·14 mg (17·5 units) pyruvate kinase (PK); 4·3 μmoles mercaptoethanol; 0·30 ml microsomes (equivalent to 0·3 ml 15,000-*g* supernatant;‡) 0·30 ml cell sap; and 0·05 ml ^{3}H-leucine (1 mc/ml) in a final volume of 1·0 ml. The reactants were pipetted into tubes kept in an ice bath; microsomes, cell sap, and ^{3}H-leucine were the last three constituents added (in that order) just before incubation. The mixture was incubated at 37°; 0·1-ml aliquots were removed at various times and pipetted into 2 ml of ice-cold 6% trichloroacetic acid. These samples were then heated at 90° for 30 min, cooled, and filtered through a Millipore filter (type HA). The purified protein remaining on the Millipore filter was thoroughly washed with cold 6% TCA (zero-time samples contained only a very small amount of radioactivity; see Fig. 1). The filter was then dried under a heat lamp and placed in a scintillation vial to which was added 10 ml of toluene scintillation solution containing 0·1 g 2,2-*p*-phenylenebis(5-phenyloxazole) per liter and 4·0 g 2,5-diphenyloxazole per liter. ^{3}H activity was determined in a Packard Tri-Carb liquid scintillation spectrometer. Microsomal ribose was determined by the orcinol reaction of Volkin and Cohn;[4] microsomal protein was assayed by the method of Oyama and Eagle.[5]

RESULTS

Table 1 presents data characterizing the microsomal amino acid incorporating systems isolated from ML-388 cells and rat thymus. These systems were shown to be dependent upon the presence of an ATP-generating system, to require the presence of RNA, and to be inhibited by puromycin, a classic inhibitor of protein synthesis.

When ML-388 cells in log-phase growth were incubated with hydrocortisone (5×10^{-6} M) for 12 hr, the microsomes isolated from these cells had a decreased ability to incorporate ^{3}H-leucine as compared to control (vehicle-treated) cells (Table 2). The decreased activity manifested itself on every basis of comparison used (i.e. cell number, microsomal protein, and microsomal ribose). Microsomes from rats pretreated with hydrocortisone 12 hr prior to sacrifice also showed a depressed

* In some cases GTP was replaced by a mixture of triphosphates (CTP, UTP, and GTP), 0·03 μmole each as described by Weinstein and Schechter.[3] Both systems incorporated equally well.

† GTP, ATP, PEP, PK (125 units/mg) and *p*-CMB were all obtained from California Corp. for Biochemical Research; DL-leucine-4,5-^{3}H-hydrochloride (5,000 mc/μmole) was obtained from New England Nuclear Corp.; puromycin was obtained from Nutritional Biochemicals Corp.; RNase and DNase were obtained from Worthington Biochemicals Corp.

‡ In some cases 0·3 ml of a 15,000-*g* supernatant + 0·3 ml distilled water was used in place of washed microsomes and cell sap.

ability to incorporate ^{3}H-leucine (Fig. 1). The effect was dose dependent and appeared to reach a maximal effect at about 15 mg/kg; doses of 50 mg hydrocortisone/kg (not shown) produced no greater inhibition than the 15 mg/kg dose. Incorporation of ^{3}H-leucine in this system appeared to be almost linear for 10 min, falling off sharply thereafter; ML-388 cells incorporate ^{3}H-leucine at a linear rate for 15 to 20 min.

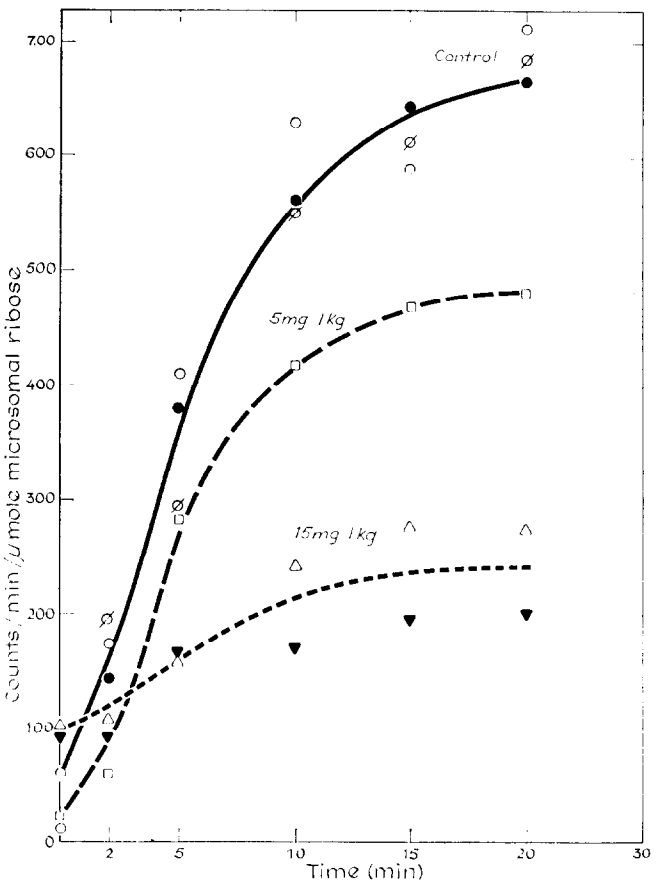

FIG. 1. Effect of hydrocortisone on ^{3}H-leucine incorporation into rat thymus microsomes. Groups of 5 rats each were given i.m. injections of hydrocortisone (□= 5 mg/kg; △, ▲ = 15 mg/kg) or vehicle (○, ●, Ø); 12 hrs later the animals were sacrificed and the thymus glands pooled for each group. Washed microsomes and cell sap were prepared as described under Methods and tested for their ability to incorporate ^{3}H-leucine into an acid-insoluble form.

Crossover experiments, in which microsomes from control (vehicle-treated) cells were incubated with cell sap (144,000-*g* supernatant) from steroid-treated cells and microsomes from steroid-treated cells were incubated with cell sap from control cells, were performed. Cell sap from steroid-treated cells did not decrease the ability of microsomes from control cells to incorporate ^{3}H-leucine ($M_V + S_F$ in Table 3). Likewise, cell sap from control cells failed to reverse significantly the depressed ability to incorporate ^{3}H-leucine of steroid-treated microsomes ($M_F + S_V$ in Table 3).

The rather large variations seen for rat thymus in Table 3 are most likely attributable to variations in the condition of the thymus glands for control animals at the time they were exercised. ^{3}H-leucine incorporation into control microsomes varied as much as twofold for the four experiments shown. This variation might well reflect different levels of endogenous adrenal steroids present at the time of sacrifice.*

TABLE 1. CHARACTERIZATION OF MICROSOMAL AMINO ACID-INCORPORATING SYSTEM

		^{3}H-leucine incorporation by microsomes	
		ML-388	Rat thymus
		(cpm/0·3 ml)	
Complete system		13,803	3,229
Complete system	+ 5×10^{-6} M puromycin	2,555	
	1×10^{-5} M puromycin		561
	+ 5×10^{-5} M *p*-CMB	2,726	814
	+ 1 μg DNase	12,780	4,183
	+ 1 μg RNase	3,670	761
	− ATP and PEP	2,516	802

The microsomes for this experiment were obtained as a 15,000-*g* supernatant of a 25% homogenate of either ML-388 cells or rat thymus as described under Methods. Additions to or deletions from the complete incubation system (see Methods) were made as indicated above. Puromycin, *p*-chloromercuribenzoate (*p*-CMB), deoxyribonuclease (DNase), and ribonuclease (RNase) were added to the incubation mixture just prior to the addition of microsomes. The figures above represent ^{3}H incorporated in 15 min at 37°.

TABLE 2. INCORPORATION OF ^{3}H-LEUCINE BY ML-388 MICROSOMES

Exp.	^{3}H incorporated by 15,000-*g* supernatant from steroid-treated cells as % of control		
	per 10^7 cells	per mg prot. N	per μmole ribose
1		69	
2		61	49
3	68	64	68
4	34	30	36
5	40	51	43

Microsomes in the form of a 15,000-*g* supernatant of a 25% homogenate of ML-388 cells (steroid-treated and control) were prepared as described under Methods. Incorporation of ^{3}H-leucine was essentially linear for 20 min. The vehicle was without measurable effects as compared to the uptake by microsomes from untreated cells. ^{3}H-leucine in acid-insoluble material was assayed at zero time and after 15 min. Control incorporation during the 15-min period averaged 6,902 ± 1,393 cpm per incubation tube for the five experiments shown above.

Table 4 shows that hydrocortisone, when added directly to the *in-vitro* microsomal amino acid incorporation systems (from ML-388 cells and from rat thymus) in concentrations up to 5×10^{-4} M, failed to inhibit the incorporation of ^{3}H-leucine.

* Rats were kept in our animal quarters under normal conditions for at least 7 days before use to allow recovery from shipping stress. No other precautions were taken to stabilize these animals.

TABLE 3. EFFECT OF CORTISOL ON UPTAKE OF ^{3}H-LEUCINE BY MICROSOMES FROM STEROID-TREATED CELLS

Incubation mixture	^{3}H-leucine incorporated expressed as % of control ($M_V + S_V$)			
	Rat thymus			
$M_V + S_V$	100	100	100	100
$M_F + S_F$	22	12	24	67
$M_F + S_V$	12	27	55	35
$M_V + S_F$	87	172	79	76
	ML-388 cells			
$M_V + S_V$	100	100		
$M_F + S_F$	55	53		
$M_F + S_V$	67	78		
$M_V + S_F$	102	103		

Microsomes and cell sap from rat thymus and ML-388 cells were obtained as described under Methods; 0·3 ml of microsome suspension and 0·3 ml cell sap were added to the complete incubation mixture, followed by the addition of 50 μc ^{3}H-leucine (1 mc/ml). The incorporation of ^{3}H-leucine into acid-insoluble material was followed for 15 min in the case of ML-388 cells and for 10 min in the case of rat thymus (see Fig. 1 and legend for Table 2). Various combinations of microsomes and cell sap were tested for their ability to incorporate ^{3}H-leucine as indicated above.

M_V = microsomes isolated from control (vehicle-treated) cells.

M_F = microsomes isolated from steroid-treated cells.

S_V = cell sap isolated from control (vehicle-treated) cells.

S_F = cell sap isolated from steroid-treated cells.

TABLE 4. EFFECT OF HYDROCORTISONE ON ^{3}H-LEUCINE UPTAKE BY RAT THYMUS AND ML-388 MICROSOMES

Final conc. of Steroid	^{3}H incorporated by microsomes in the presence of hydrocortisone expressed as % of control	
	Rat thymus	ML-388
5×10^{-6} M	107	87·5, 102, 99·5
5×10^{-5} M	109	
5×10^{-4} M	122	

Microsomes were obtained from a 25% homogenate of cellular material by centrifuging at 15,000 *g* for 20 min as described under Methods; 0·3 ml of the 15,000-*g* supernatant was added to the complete incubation system (see Methods) which contained either hydrocortisone or the appropriate vehicle (vehicle had no measurable effect on ^{3}H-leucine uptake). Each result above is the average of triplicate assays.

Total counts/min incorporated by the control system in the 20-min incubation period was 442 for rat thymus microsomes and 1,800–2,100 for ML-388 microsomes.

DISCUSSION

The inhibitory effect of hydrocortisone on protein synthesis in lymphoid tissue has been studied *in vitro* in the microsomal systems isolated from a mouse lymphoma ML-388 growing *in vitro* and from the thymus gland of rats. The results presented in this report demonstrate that microsomes isolated from cells (ML-388 or rat thymus) pretreated with hydrocortisone have a depressed ability to incorporate a labeled amino acid into an acid-insoluble form, as compared with microsomes isolated from control cells. This effect was not seen if the steroid was added directly to the microsomal amino acid-incorporating system in concentrations up to 5×10^{-4} M. It can be assumed, therefore, that the normal protein-synthesizing apparatus, once formed, is not susceptible to the inhibitory action of hydrocortisone. These data suggest that the steroid acts by interfering with the formation of the microsomal protein-synthesizing system—perhaps by decreasing the availability of some limiting component.

Crossover-type experiments in which microsomes from steroid-treated cells were incubated with cell sap from control (vehicle-treated) cells showed that ^{3}H-leucine incorporation was depressed to the same extent as when these microsomes were incubated with cell sap from steroid-treated cells. Similarly, when microsomes from control cells were incubated with cell sap from steroid-treated cells, ^{3}H-leucine incorporation did not vary significantly from control. The data suggest that the particulate fraction is in some way altered by pretreating the lymphoid cells with hydrocortisone.

These data do not rule out the possibility that additional changes have also occurred in the activity of cell sap enzymes which were not detected by the *in-vitro* system used because they were not limiting. It is also possible, of course, that changes occurred in the cellular metabolism, which limit energy or the cofactors required for activation of amino acids, since these factors were supplied exogenously to the *in-vitro* system.

Recent work on growth hormone,[6] estogens,[7-9] testosterone,[10, 11] and thyroid hormone[12] suggest that various hormones are characterized by their ability to alter the rate of synthesis of messenger RNA prior to any effects on protein synthesis. In line with this concept and the current theories of protein synthesis,[13] it is tempting to speculate that hydrocortisone may have an effect on the integrity of polysomes (aggregates of ribosomes held together by strands of messenger RNA). Such an effect might be brought about either by an inhibition of messenger RNA synthesis or a facilitation of messenger RNA breakdown; corticosteroids have also been shown to stimulate RNase in a lymphosarcoma in rats.[14] It should be remembered however, that the data presented in this report do not rule out other mechanisms for the effect of hydrocortisone on this protein-synthesizing system.

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