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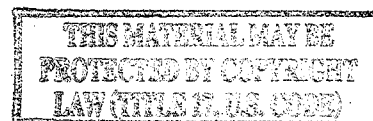
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GROWTH INHIBITORY EFFECTS OF HYDROCORTISONE ON MOUSE LYMPHOMA ML-388 *IN VITRO*^{1, 2}

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The lymphocytolytic effects of adrenal steroids have been known for many years (Dougherty, 1952). Adrenal corticoids and high potency anti-inflammatory steroids have also been used in the medical management of the lymphomas and acute leukemia (Gellhorn, 1959). Certain steroids have been shown to inhibit the growth of transplanted mouse lymphosarcomas *in vivo* (Heilman and Kendall, 1944; Lampkin-Hibbard, 1960). The mechanisms involved in this specific suppressive effect of steroids on lymphatic tissue have been investigated *in vitro* by many workers, and have been reviewed recently by Blecher and White (1959). These studies have shown that certain steroids can suppress respiration, glucose utilization and protein synthesis. Inhibition of phosphorus incorporation into nucleic acids and phospholipids of lymphoid cells has also been demonstrated. In all of these studies steroid concentrations in the range of 10^{-3} to 10^{-5} M were used in nongrowing systems to produce the effects observed.

In this report it will be shown that a mouse lymphoma (ML-388) growing *in vitro* is inhibited by certain adrenal corticoids and high potency anti-inflammatory agents, in concentrations ranging from 10^{-6} to 10^{-10} M. Studies exploring the possible mechanism of action of this growth inhibition are also presented.

METHODS. Cell culture. Mouse lymphoma ML-388 cells (formerly designated P-388) were obtained from L. Herzenberg. The cells were maintained on the defined medium described by Eagle (1955) supplemented with 10^{-4} M serine, 10^{-4} M pyruvate and 5% calf serum (Herzenberg and Roosa, 1960).

Experimental procedures. 1. Dose-response rela-

tionships. Replicate cultures of ML-388 cells were grown in 8-ounce prescription bottles inoculated with $1-3 \times 10^5$ cells. The day after inoculation, 3 or 4 bottles were randomly selected from the group, the supernatant medium gently poured off, and the number of cells in each bottle determined by suspending in 10.0 ml of isotonic saline containing 0.1% trypsin and counting in a Coulter automatic cell counter. All remaining bottles received either drug (0.2 ml) at various concentrations or the appropriate vehicle (as a control). Steroid solutions³ were prepared by dissolving the drug in absolute ethanol and adding distilled water so that the final solution contained 10^{-2} M steroid in 50% alcohol. The solution was then sterilized by filtration and further dilutions made with sterile distilled water as necessary. The final concentration of ethanol in the culture medium never exceeded 0.05%, and this concentration of ethanol had no effect on cellular multiplication. On the fifth day after inoculation the number of cells in each bottle was again determined. A minimum of 3 bottles was used (counted separately) to determine each point.

2. Chemical analysis of steroid toxicity. In these experiments replicate cultures were established in 8-ounce prescription bottles. At various times bottles were randomly selected and analyzed for

³ **Steroids and other drugs.** Compounds used were obtained from the following sources. Hydrocortisone, desoxycorticosterone, cortisone, 17α -hydroxy- 11 -desoxycorticosterone (Reichstein's S) and cholesterol: California Corporation for Biochemical Research. Testosterone, corticosterone and 11 -dehydrocorticosterone (Kendall's A): Merck Sharp & Dohme. Corticosterone: The Upjohn Co. d -Aldosterone and estradiol: Ciba Pharmaceutical Products, Inc. 11 -Epihydrocortisone: Squibb Institute for Medical Research. 6α -Fluoro- 16α -methyl-hydrocortisone, 6α -fluoro- 16α -hydroxyhydrocortisone- $16,17$ -acetonide, 6α -fluoro- 16α -methylprednisolone, 6α - 9α -difluoro- 16α -hydroxyprednisolone- $16,17$ -acetonide: Syntex S.A. 17α -Ethyl- 19 -nortestosterone, spiroactones SC-8911 and SC-9833: G. D. Searle & Co. Diethylstilbestrol: Nutritional Biochemicals Corporation. Insulin (zinc-free): Eli Lilly and Co. Chlorazaniol: Riker Labs., Inc. DL-Leucine- $1-C^{14}$ (10 mc/mM): California Corporation for Biochemical Research.

¹ This work was supported by grants from the American Cancer Society (T38, T39) to Dr. Avram Goldstein.

² A preliminary report of this work has been published (Gabourel and Aronow, 1961; Aronow and Gabourel, 1961).

accumulated cellular constituents. The cellular compounds measured were protein, RNA-ribose, DNA-deoxyribose, and the cold acid-soluble ninhydrin-positive material. At each time interval, 4 bottles were used to determine cell count. Bottles to be chemically analyzed were carefully washed with a buffered balanced salt solution⁴ to remove contaminating protein and amino acids present in the growth medium. Four bottles were selected from this washed group and cell counts again determined to correct for any cell loss due to the washing procedure. The remaining 8 bottles were chemically assayed for cellular constituents.

Chemical procedures. Cellular constituents were analyzed as follows: washed, chilled bottles containing the cells were scraped with ice-cold 8% perchloric acid. After centrifuging, an aliquot of the cold acid-soluble fraction was assayed for ninhydrin-reacting material by the method of Moore and Stein (1948). In some experiments the cold acid-soluble fraction was also assayed for individual amino acids on a Beckman-Spinco automatic amino acid analyzer according to the method of Spackman *et al.* (1958). The cold acid-insoluble residue was extracted with successive 5-ml portions of ethanol-water (4:1), ethanol-ether (3:1) and ether to remove lipids, and then extracted with 4 ml of hot 6% perchloric acid (90°C, 20 minutes). The purified protein precipitate which remained was assayed by the method of Oyama and Eagle (1956). Aliquots of the hot acid extract were analyzed for RNA-ribose by the orcinol reaction of Volkin and Cohn (1954) and for DNA-deoxyribose by the method of Burton (1956). The nutrient medium was assayed for glucose by the glucostat method (Saifer and Gerstenfeld, 1958) and for lactate by the method of Loomis (1961). All assays were done in duplicate.

3. Experiments with C^{14} -leucine. Cellular protein was prelabeled with C^{14} by allowing the cells to grow for 3 days in a medium containing leucine-1- C^{14} (1 μ c/bottle at 1×10^{-5} M). The cells, which remain adherent to the surface of the bottle, were then washed with two 10-ml portions of nonradioactive medium; 20 ml of normal (non-radioactive) medium were then added to each bottle. The following day, steroid (or vehicle) was added and at various times thereafter, a group of bottles was selected randomly and the medium carefully poured off. Some of these bottles were used for cell counts and others were pooled and subjected to the chemical assay procedures, previously outlined. Each chemical constituent so isolated was assayed for its C^{14} content. The

medium from the 4 bottles used for chemical analysis was pooled and assayed for C^{14} content.

Measurement of radioactivity. C^{14} activity was determined in a Packard Tri-Carb Liquid Scintillation Spectrometer. The scintillator solution used contained 7 g 2,5-diphenyloxazole (PPO) per liter, 0.3 g 2,2-*p*-phenylenebis(5-phenyloxazole) (POPOP) per liter and 100 g naphthalene per liter in dioxane (reagent grade). Hyamine was used to facilitate digestion of all solutions containing protein before the scintillator mixture was added. Quenching for all samples was determined and any necessary correction applied.

4. Reversal experiments. All substances or groups of substances used in attempts to reverse the growth inhibitory effects of hydrocortisone were tested at several concentrations up to the limit of toxicity of the "reversing" agent. Steroid and "reversing" agents were added simultaneously, the day after cell inoculation, and cell counts determined on day 5. All agents were tested against a concentration of hydrocortisone that produced between 50% and 80% inhibition of growth in a 5-day period.

RESULTS. The growth inhibitory effects of hydrocortisone on ML-388 cells *in vitro* are shown in figure 1. The concentration required to in-

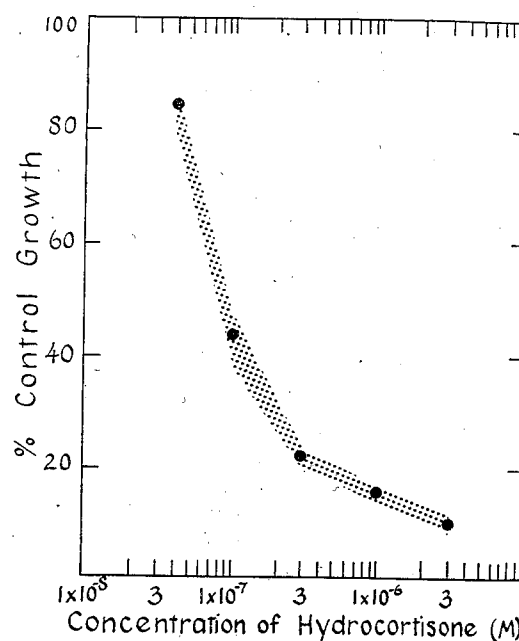


FIG. 1. Effect of hydrocortisone on *in vitro* growth of ML-388 cells.

The steroid was added the day after inoculation of replicate cultures as described in the text. Each point represents the mean of a group of 3 bottles. The dotted overlay estimates the standard error of the mean.

⁴ Earle's salt solution brought to pH = 7 with CO_2 .

hibit growth to 50% of control growth (IC50) was found to be slightly less than 1×10^{-7} in this experiment. The IC50 for hydrocortisone was found to vary somewhat from experiment to experiment as shown in table 1. The IC50 varies directly with the number of cells present when the steroid is added, i.e., the higher the inoculum the higher the concentration of steroid required.

Dose-response curves similar to that shown for hydrocortisone (fig. 1) were determined for 25 steroids; 12 of these steroids were found to be active to various degrees; 13 were inactive (i.e., $IC_{50} > 10^{-5}$ M). Table 2 lists the active steroids in order of potency. The relative potency was determined by the ratio IC_{50} hydrocortisone/ IC_{50} for each steroid. A dose-response curve for hydrocortisone was always determined simultaneously to minimize possible errors because of fluctuations which might occur between separate experiments due to slight differences in inoculum size or other undetermined factors. The most potent compound tested was 6 α -fluoro-16 α -hydroxyprednisolone-16,17-acetonide, which had an IC_{50} of approximately 2×10^{-10} M.

The following steroids were found to be inactive in this system: testosterone; 17 α -ethyl-19-nortestosterone; dihydroepiandrosterone; estradiol; diethylstilbestrol; prednisone; cortisone; 11-dehydrocorticosterone; 11-epihydrocortisone; pregnenolone; cholesterol; spiro lactone SC-8911; and spiro lactone SC-9833.

In an effort to analyze the mechanism by which hydrocortisone exerts its growth inhibitory effect, protein, RNA-ribose, DNA-deoxyribose and the cold acid-soluble ninhydrin-reacting

TABLE 1
Effect of inoculum size on the median inhibitory concentration (IC50) of hydrocortisone for ML-388 cells

Cell Count at Time of Drug Addition	IC50 Hydrocortisone
0.53×10^5	5.0×10^{-8} M
0.74×10^5	6.8×10^{-8} M
2.00×10^5	9.5×10^{-8} M
3.80×10^5	17.0×10^{-8} M

Hydrocortisone was added in various concentrations to a series of replicate cultures; the inoculum size (cell count) was determined for each series just prior to the addition of drug. The IC_{50} for each series of replicate cultures was determined as shown in figure 1.

TABLE 2
Growth inhibitory effects of various steroids on ML-388 cells in vitro

Steroid	Number of Expts.	Average Relative Potency
6 α -9 α -Difluoro-16 α -hydroxy-prednisolone-16,17-acetonide	1	110.6
6 α -Fluoro-16 α -hydroxyhydrocortisone-16,17-acetonide	1	47.8
6 α -Fluoro-16 α -methylprednisolone	1	15.8
Dexamethasone	3	15.2
6 α -Fluoro-16 α -methylhydrocortisone	1	9.7
9 α -Fluorohydrocortisone	2	3.0
Prednisolone	2	1.4
Hydrocortisone	3	1.0
Corticosterone	2	0.69
Desoxycorticosterone	2	0.31
17 α -Hydroxy-11-desoxycorticosterone	2	0.10
d-Aldosterone	2	0.07

IC_{50} 's were determined for the various steroids as described for hydrocortisone (fig. 1). The IC_{50} 's varied between 10^{-6} M for aldosterone to 2×10^{-10} M for 6 α -9 α -difluoro-16 α -hydroxyprednisolone-16,17-acetonide. Relative potencies were calculated from the ratio IC_{50} hydrocortisone/ IC_{50} steroid.

Drugs which did not differ significantly ($P > .05$) are connected by brackets. For statistical evaluation the $\log_{10}$ (% control growth) was determined and plotted against the $\log_{10}$ (drug conc.) for each drug. The concentration at $\log_{10}$ (50% control growth) was determined by linear interpolation between the dose above and below and the variances of this estimated drug concentration determined by the method of Cramer (1951, theorem 22.7). Since the variances of the $\log_{10}$ (% control growth) did not vary widely between various concentrations of the same drug, or between different drugs, the variances were averaged and an overall estimate of the variance obtained. The number of degrees of freedom used in this estimate was well over 100 and the test statistic for testing the differences in relative potency between any two drugs can be expected to have a nearly normal distribution. Thus, the hypothesis that the relative potencies of drug x and drug y are the same can be rejected when the average $\log_{10}$ (relative potencies) $\geq$

$1.96 \sqrt{\frac{1}{n_x} + \frac{1}{n_y}}$ where n_x and n_y are the number of separate experiments to determine the average relative potency of each drug.

material were measured in cultures growing in the presence and absence of hydrocortisone. Figure 2 shows the data from a typical experiment obtained when ML-388 cells in log phase growth were treated with 5×10^{-6} M hydrocortisone or the appropriate vehicle. The data show the net

accumulation of cellular constituents on a *per bottle* basis. Assays were performed at 12 hours, 24 hours and 48 hours after the addition of drug. Cell count was depressed slightly in the steroid-treated group as compared to the controls at 12 hours but a marked depression was not

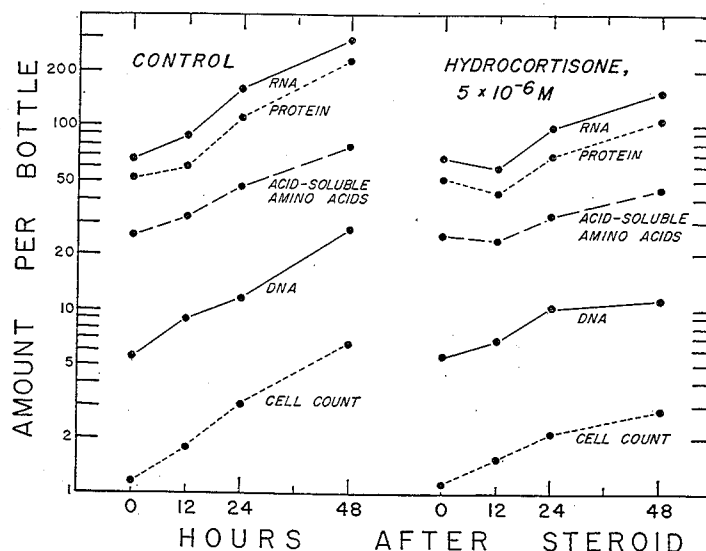


FIG. 2. Effects of hydrocortisone on the accumulation of cellular constituents in cultures of ML-388 cells growing *in vitro*.

Cell counts are the means of groups of 4 bottles at each time. The ribose, deoxyribose, protein and acid-soluble amino acids are values obtained from pooled groups of bottles, as described in the text. The units on a per bottle basis are as follows: cell counts $\times 10^6$; DNA— μmol deoxyribose in hot acid-soluble fraction; RNA— μmol ribose in hot acid-soluble fraction; amino acids— μmol of ninhydrin-reacting material (as leucine-equivalents) in cold acid-soluble fraction; protein— μg protein nitrogen.

TABLE 3

Effect of hydrocortisone (5×10^{-6} M) on glucose utilization, lactate production and cellular composition of ML-388 cells *in vitro*

	Cell Count $\times 10^6$	Glucose (mg %)	Lactate (mg %)	Amount per 10^5 Cells			
				Protein ($\mu\text{g N}$)	Leucine equivalents* (μmol)	Ribose (μmol)	Deoxyribose (μmol)
Control	1.50 ± 0.06	50.3 ± 1.4	91 ± 4	5.03 ± 0.13	60 ± 7.0	5.50 ± 0.12	7.20 ± 0.7
Hydrocortisone treated	1.52 ± 0.20	50.4 ± 0.5	95 ± 3	3.63 ± 0.31	40 ± 3.0	4.83 ± 0.14	5.30 ± 0.3
% Depression				28	33	12	26
P value				<.02	<.01	>.05	<.01

Bottles were inoculated with replicate cultures of ML-388 cells, and 3 days later, hydrocortisone (5×10^{-6} M) or vehicle was added. The cell count per bottle at this time was 1.12×10^6 . After 12 hours, the medium was poured off and 3 groups of treated flasks (6 bottles per group) and 3 groups of control flasks (6 bottles per group) were analyzed as described in the text. Values presented are the means and standard errors. The level of significance was determined using the *t* test.

* Cold acid-soluble ninhydrin-reacting material is expressed in terms of leucine equivalents.

evident until 24 hours. The cold acid-soluble ninhydrin-reacting material, protein, RNA-ribose and DNA-deoxyribose are depressed to a greater extent than the cell count at 12 hours so that the amount of these cellular constituents per cell is reduced. This reduction on a per cell basis is more apparent at 12 hours than at subsequent times. In order to examine with more precision the effects of the steroid on cellular composition 12 hours after addition of the steroid, an experiment was done utilizing 3 separate groups of control flasks and 3 separate groups of treated flasks. The results (table 3) indicate that the cellular content of protein, ninhydrin-reacting cold acid-soluble material, and DNA-deoxyribose were all significantly lower on a per cell basis in the steroid treated groups than in the control groups. The cell count was identical in both groups at this time, and there were no differences in the glucose or lactic acid concentrations in the medium. Cellular RNA-ribose was also slightly depressed at this time in the steroid-treated group, but this depression was not significant at the 5% level.

To investigate further the early effect on protein synthesis, experiments were carried out

TABLE 4
Effect of 5×10^{-6} M hydrocortisone on protein synthesis in ML-388 cells

Time (hr)	Cellular Protein Specific Activity (cpm/ μ g Protein Nitrogen)				C ¹⁴ -Leucine cpm/0.2 ml of Culture Medium			
	Normal		+ Steroid		Normal		+ Steroid	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
0	1080	1042			366	475		
12	707	788	757	774	449	610	478	814
24	447	506	578	583	489	699	581	836
48	245	325	409	481	496	800	707	1054

Cell protein was prelabeled with C¹⁴-leucine and the cells allowed to grow in the presence or absence of hydrocortisone as described in the text. Protein was isolated and an aliquot used to determine the total amount per bottle. Another aliquot of the same sample was used to determine C¹⁴ activity. The medium from these cell cultures was pooled and 0.2-ml aliquots were assayed for C¹⁴ activity. All assays were done in duplicate. The expected growth-inhibition was observed in the steroid treated groups of both experiments over the 48-hour period. The average decrease in specific activity was 41.2 units/12 hours greater in the control groups than in the steroid treated groups. This figure has a standard error of 11.5 (method of Bowker and Lieberman, 1959) and is significant at P < .02 by a t test with 5 degrees of freedom.

TABLE 5
Loss of C¹⁴-leucine from prelabeled cellular protein in the presence or absence of hydrocortisone

Time after Hydrocortisone (5×10^{-6} M)	Counts per Minute per 0.2 ml Medium $\pm$ S.E.			
	Normal leucine (1.5×10^{-4} M)		High leucine (5×10^{-4} M)	
	Control	+ Steroid	Control	+ Steroid
hr				
0	200 $\pm$ 1.5		210 $\pm$ 5.3	
12	279 $\pm$ 5.5	304 $\pm$ 5.4	290 $\pm$ 4.6	321 $\pm$ 6.9
24	320 $\pm$ 4.9	367 $\pm$ 4.2	385 $\pm$ 5.3	393 $\pm$ 14
48	416 $\pm$ 3.8	492 $\pm$ 5.9	521 $\pm$ 5.9	516 $\pm$ 23

The experimental design was identical to that described in table 4 except that the medium of some cultures contained higher than normal leucine concentrations. 0.2-ml aliquots of medium from each bottle were assayed separately for C¹⁴ activity; all assays were done in duplicate. Each value above represents the mean activity from 3 bottles. Hydrocortisone produced a similar growth inhibition of about 50% in both high and low leucine mediums. However, the difference in the rate of appearance of C¹⁴ in the medium between control and steroid treated groups was significantly reduced (P < .01) in the presence of a high leucine medium. Significance was calculated by the t test where variances are heterogeneous using the method of Cochran (1951).

using C¹⁴-leucine to prelabel the cellular protein.⁵ The results of these experiments (table 4) show that the dilution of cellular radioactive protein by newly formed nonradioactive protein was substantially reduced in the hydrocortisone treated cells as compared to cells treated with the vehicle. At the same time radioactivity measurements of the medium⁶ showed that the control cells were better able to retain their C¹⁴ than the cells treated with hydrocortisone. However, when this experiment was repeated, but placing the prelabeled cells in a nutrient medium containing 3.3-fold the usual concentration of leucine, this differential effect on C¹⁴-leucine appearance into the medium could be eliminated; the usual growth inhibition still occurred (table 5). In the presence of high leucine concentrations in the external medium, the loss of radioactivity from

⁵ Analysis of various cell fractions after exposure to C¹⁴-leucine showed that virtually all of the cellular C¹⁴ was associated with the protein fraction. Only trace amounts were found in the cold and hot acid-soluble fractions; none was found in the lipid fraction.

⁶ It was found that 80 to 85% of the C¹⁴ found in the medium of both hydrocortisone treated and control groups was in a cold acid-soluble form (presumably as free C¹⁴-leucine or small peptides containing C¹⁴-leucine).

control cultures was increased, and became very similar to the values observed in the presence of hydrocortisone.

Table 6 shows the amounts ($\mu\text{mol}/10^5$ cells) of individual amino acids found in the cold acid-soluble fraction of hydrocortisone treated and control cells. The levels of three of these amino acids, aspartate, glutamate and alanine, were markedly altered in the presence of hydrocortisone; most other amino acids were unaffected. The two dicarboxylic acids, aspartate and glutamate, were depressed, while alanine was increased in the steroid treated group as compared to controls. In three similar experiments, the depression of aspartate and glutamate was regularly observed; however, the alanine level was not elevated in two of these experiments.

Many attempts were made to reverse the growth inhibition produced by hydrocortisone by the addition of various metabolites, hormones or drugs to the culture medium. These compounds were generally added at concentrations up to the limit of toxicity of the agent itself, as shown by the effects of these agents in control cultures,

in the absence of steroid. None of the agents tested had any effect whatsoever on the steroid-induced growth inhibition of ML-388. The agents tested, and the highest concentrations employed, were as follows: 1. Added metabolites: alanine (10^{-3} M); α -ketoglutarate (10^{-3} M); glutamine (4×10^{-3} M); glutamate (10^{-3} M); aspartate (10^{-3} M); uridine (2×10^{-5} M); taurine (10^{-4} M); a mixture of the nonessential amino acids alanine, glycine, proline, hydroxyproline and aspartate (10^{-4} M each); taurine plus the nonessential amino acids listed above (10^{-4} M each); and Eagle's amino acid mixture at $4.0 \times$ normal concentration with or without nonessential amino acids (3×10^{-5} M each) listed above; thymidine (10^{-4} M); hypoxanthine (10^{-4} M); a mixture of glycine, thymidine, and hypoxanthine (10^{-4} M each). 2. Drugs and hormones: spirolactone SC-8911 (2×10^{-6} M); cortisone (3×10^{-6} M); estradiol (2×10^{-6} M); insulin ($5 \mu\text{g}/\text{ml}$); testosterone (2×10^{-6} M); chlorazaniol (10^{-4} M); salicylate (10^{-3} M); and pilocarpine ($0.2 \mu\text{g}/\text{ml}$). 3. Minerals: Mg^{++} and Zn^{++} (2×10^{-5} M each); and Ca^{++} , Fe^{++} and Mn^{++} (2×10^{-5} M each). In addition, attempts were made to reverse the growth inhibiting effects of hydrocortisone by withdrawing pyruvate, serine or both from the culture medium.

DISCUSSION. That the lymphocyte cell *in vivo* is a target cell for certain steroid hormones is well established (Dougherty, 1952). The work described here shows that hydrocortisone, in concentrations as low as 10^{-7} M, can sharply inhibit the growth of a mouse lymphoma cell line growing *in vitro*. Other cell lines in tissue culture systems do not exhibit this sensitivity to hydrocortisone (Johnson and Gabourel, 1961; Kline *et al.*, 1957). Certain synthetic anti-inflammatory steroids of high potency are also exceedingly active in inhibiting the growth of ML-388 cells *in vitro* (table 2).

The structure-activity relationship explored in this paper demonstrates that the growth inhibitory activity of steroids in ML-388 cells cultured *in vitro* is very similar to the anti-inflammatory spectrum of activity recently reviewed by Liddle (1961), or the ability of steroids to cause thymic involution (Stephenson, 1956). The most obvious difference is the inactivity of 11-keto compounds (cortisone, 11-dehydrocorticosterone, prednisone) in ML-388 growth inhibition, despite the fact that these compounds

TABLE 6
Amino acid analysis of cold acid-soluble fraction of ML-388 cells grown in presence and absence of hydrocortisone

Amino Acid	12 hr after Vehicle ($\mu\text{mol}/10^5$ cells)	12 hr after 3×10^{-6} M Hydrocortisone ($\mu\text{mol}/10^5$ cells)
Aspartic acid	2.35	1.22
Threonine	0.36	0.37
Serine	0.53	0.65
Glutamic acid	2.69	1.87
Proline	0.55	0.44
Glycine	0.74	0.74
Alanine	1.21	1.67
Valine	0.10	0.12
Isoleucine	0.10	0.09
Leucine	0.09	0.13
Tyrosine	0.17	0.15
Phenylalanine	0.13	0.14

The cold acid-soluble material was obtained from ML-388 cultures 12 hours after addition of drug or vehicle as described in the text. Similar results were obtained in an additional experiment, but two other experiments failed to show an elevated alanine content after steroid treatment. All experiments showed a pronounced depression of aspartic and glutamic acids.

are active anti-inflammatory agents in the intact animal. However, it has been shown by Petersen *et al.* (1957) that the anti-inflammatory activity of cortisone is probably due to its metabolism *in vivo* to hydrocortisone; apparently such metabolism cannot occur in the *in vitro* culture system employed here.

Although complete reduction at the 11-position enables the molecule to demonstrate some slight activity (as in DOC and Reichstein's "S"), a hydroxyl function in the β configuration greatly enhances activity (hydrocortisone, corticosterone, table 2). An 11-hydroxyl group in the α orientation (11-epihydrocortisone), or a keto function in the 11-position (cortisone, 11-dehydrocorticosterone and prednisone) abolishes activity.

The very close correspondence of the structure-activity relationship of the thymic-involution and growth inhibition of ML-388, coupled with the exquisite sensitivity of these cells growing *in vitro* to low concentrations of active steroids, strongly suggests that we are studying a physiological effect of certain steroids on a target cell. The advantages of working with an *in vitro* system, in a defined growth medium, would seem to render this system most attractive for the study of the intimate biochemical mechanism whereby the steroid exerts its effect.

Despite the advantages of the *in vitro* culture system, it is not yet possible to describe with assurance the primary biochemical lesion responsible for the growth inhibition. However, chemical assay of cellular composition of ML-388 cells grown in the presence and absence of hydrocortisone indicates that one of the early effects of this steroid is to inhibit protein accumulation (fig. 1 and table 3). Since this effect could be due to either an accelerated rate of protein breakdown or to an inhibition of protein synthesis, experiments on cells containing C^{14} -leucine in their cellular protein were performed. In these experiments dilution of the protein specific activity with newly synthesized nonradioactive protein was used as a measure of protein synthesis. The results showed that the specific activity of such prelabeled cells falls more slowly in the presence of hydrocortisone than in control cultures and demonstrates that protein synthesis has been inhibited. It was also shown that the presence of hydrocortisone caused a more rapid loss of radioactivity from the cells (tables 4 and 5).

This last effect of hydrocortisone could be

explained in either one of two ways: 1) an increased rate of protein breakdown, or 2) a decreased re-utilization of labeled leucine from the intracellular pool as a result of decreased protein synthesis. If the first hypothesis were correct, the differential effect should be maintained when cells were grown on a medium containing a large excess of nonradioactive leucine. If the second hypothesis were true, the differential effect should be reduced or abolished under these conditions; the control (vehicle-treated) cells would now be expected to lose C^{14} activity almost as rapidly as hydrocortisone-treated cells. The results of such an experiment (table 5) indicate that the differential effect could be abolished by increasing the concentration of nonradioactive leucine in the nutrient medium. Thus there can be no doubt that one of the early effects of hydrocortisone is an inhibition of protein synthesis with little or no change in the rate of protein breakdown.

Data showing that the percentage of radioactivity appearing as free amino acids or small peptides⁶ (*i.e.*, the cold acid-soluble fraction) in the culture medium was the same for both control (vehicle-treated) and hydrocortisone-treated cells argue against cell lysis as a mechanism for the growth inhibitory effects of hydrocortisone. Had cell lysis occurred as a result of hydrocortisone treatment, substantial amounts of radioactivity would have been released into the medium as protein. Further, alterations of cellular protein specific activity described above could not be explained by cell lysis.

The finding of altered amino acid concentrations in the cold acid-soluble pool of free amino acids (table 6) seems worthy of further investigation. The decreased aspartate and glutamate levels, coupled with increased alanine levels (in two of four experiments) implicate the transaminase enzymes as playing a role in this response. It has been shown by Blecher and White (1958) that the activity of transaminase enzymes in rat thymic lymphocytes could be altered by incubating the cells with certain steroids. Rosen and co-workers (1961) have studied this response in more detail, using lymphosarcomas carried in rats. Their studies clearly show a hydrocortisone-induced stimulation of glutamic-pyruvic transaminase in this tumor. It is conceivable that steroids exert their growth inhibitory effect by stimulating intracellular transaminase sufficiently

to lower the intracellular pool of aspartate and glutamate below the levels needed for continued rapid protein and nucleic acid synthesis, but much more work is needed to establish this hypothesis. Indeed, the failure of externally supplied glutamate and aspartate to reverse steroid inhibition immediately renders it questionable.

Finally, we have observed (table 3) that 12 hours after steroid addition, there is a reduced amount of DNA per cell in the treated groups as compared to control groups. The most ready explanation of this finding might be that the steroid blocked DNA synthesis, while cells which had previously completed DNA replication proceeded to divide normally. The result might be a partial degree of synchrony of the cell culture, with an accumulation of cells containing the diploid amount of DNA, and very few cells containing the pre-mitotic, twice-diploid amount of DNA. However, at the moment these are purely speculative matters.

There have been many previous studies by other workers on *in vitro* effects of steroid hormones. Perhaps the most interesting in this connection are the studies of Tomkins *et al.* (1961) who have shown that steroids can affect (by disaggregation of sub-units) the activity of purified glutamic dehydrogenase. Their studies show that purified beef liver glutamic dehydrogenase can be disaggregated (and this is accelerated by steroids) to sub-units, which have increased activity for alanine dehydrogenation, and less activity as glutamic dehydrogenase. The relation of this work to the present studies is obscure at the moment, since our findings of intracellular amino acid levels are in just the opposite direction than one would predict from their work, namely, an increased alanine level and a decreased glutamic acid level. Further, structure-activity relationships are not in harmony, since the most active steroids (diethylstilbestrol and estradiol) used by these workers are completely inactive in inhibiting the growth of ML-388. Similar considerations also indicate that the extensive studies of Blecher and White (1959, 1960a, b) showing alterations in carbohydrate metabolism with high steroid concentrations (17 α -ethyl-19-nortestosterone and testosterone being the most active) in a nongrowing system,

may have little to do with growth inhibition of lymphoid tissue.

The experiments seeking reversal of steroid-induced growth inhibition have proved disappointing. These experiments were predicated on the hypothesis that the steroid inhibited or blocked some metabolic process, resulting in the depletion of an essential metabolite. However, no alteration of the growth medium has as yet resulted in modifying the steroid effect in any way.

Although we have concentrated our attention in this study on the inhibition of protein synthesis which occurs in these cells soon after steroid addition, it is by no means clear that this is in any sense a primary effect, responsible for the slower growth rate. Other early effects remaining to be explored include an altered amino acid pattern in the intracellular pool, and a depression in the deoxynucleic acid content of cells 12 hours after steroid addition.

SUMMARY

A new bioassay for steroids is described, involving the growth inhibition of a mouse lymphoma cell line cultured *in vitro*. Structure-activity relationships reveal the response in general parallels the anti-inflammatory activity of corticoids. Under the conditions of our assay, hydrocortisone can inhibit growth of these cells by 50% at a concentration of less than 10^{-7} M. Several synthetic compounds are many times as active as hydrocortisone, while other steroids (estrogens, androgens, cholesterol and others) are devoid of activity.

The mechanism of this effect of hydrocortisone was studied by attempting to reverse the growth inhibition by a great variety of alterations and additions to the culture medium, all of which were uniformly ineffective. Studies on the accumulation of cellular constituents revealed a diminution of the cellular protein, deoxynucleic acid, and amino acid pool size as early as 12 hours after steroid addition. Experiments with radioactive leucine conclusively demonstrated an inhibition of protein synthesis as early as 12 hours, with no effect on protein catabolism. Examination of the amino acid pattern in the cold acid-soluble, intracellular pool revealed that at 12 hours, the intracellular pools of aspar-

tate and glutamate were depressed, while alanine was occasionally increased in the steroid-treated cell as compared to controls.

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