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Influence of Hydrocortisone Acetate on the Chemical Composition of Experimental Granulomas.* (32340)

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Although it is well established that certain adrenocortical hormones inhibit granuloma formation (1-6), the mechanism by which this inhibition occurs remains unknown. Many investigators have been concerned with the inhibition of collagen synthesis (7-10), but little attention has been given to the possibility that the observed effects on collagen and other substances are secondary to an alteration in some basic process of protein synthesis. Eichhorn and Sniffen(11) reported a reduced total DNA and protein in cortisol treated granulomas. Yet they found that the amount of labeled amino acids incorporated *in vitro* by the granulomatous cells was not altered and, therefore, concluded that protein biosynthesis was not significantly influenced by *in vivo* administration of cortisol. However, the results were variable, since significant decreases in total amino acid incorporation were obtained in a number of experiments(11). Other investigators have found that certain corticoids have either an inhibitory or stimulatory influence on protein metabolism in various tissues other than granulomas(12-15). In order to explore further the possibility of an effect of cortisol on protein metabolism in granulomatous tissue, studies on the temporal sequence of changes in collagen, DNA, RNA, or protein content and in the incorporation of labeled amino acids in steroid treated granulomas were undertaken.

Methods. Two granulomas were induced in each animal by bilateral implantation of weighed, sterilized cotton sponges (30 ± 1 mg) subcutaneously in the dorsum of male Sherman rats (175-200 g). Penicillin G (2,000 units) was injected intramuscularly. The treated sponges were impregnated with 300 μg of hydrocortisone acetate (Hydrocortone, Merck) and allowed to dry prior to implantation.

On days 1, 3, 5 or 7 after implantation, the granulomas were removed by carefully dissecting the surrounding connective tissue, blotted on filter paper and immediately weighed to the nearest 0.1 mg. Those implants used to determine water content were dried at 98-99°C to constant weight.

For the chemical studies, the granuloma from one side was weighed immediately and used for estimating the total hydroxyproline content by the method of Neuman and Logan (16). The second granuloma was weighed, and total protein and nucleic acids were separated by the Schneider technique(17). Total protein content was determined by the method of Lowry *et al*(18). The diphenylamine and orcinol reactions were used for DNA and RNA estimations, respectively(19,20).

For the study of labeled amino acid uptake, the cotton sponge implanted on one side was impregnated with 300 μg of hydrocortisone acetate while the other was untreated. C^{14}-L-proline (35 μc, uniformly labeled) was injected intraperitoneally at the time of sponge implantation. The implants were removed at 12 or 24 hours. After weighing and homogenization, the total protein was isolated by the method of Schneider(17). An aliquot was then counted in a Packard Tri-Carb Liquid

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TABLE I. Influence of Hydrocortisone Acetate on Weight, Water and Hydroxyproline Content of Experimental Granulomas.†

Day		Wet wt (mg)	Water content (%)	Hydroxyproline	
				μg/mg tissue	Total (μg)
1	Control	505 ± 30	89.8 ± .7	.85 ± .18	478 ±133
	Treated‡	287 ± 13*	94.3 ± .5*	.20 ± .03*	64 ± 35**
2	Control	592 ± 24	91.1 ± .4	.72 ± .08	348 ± 49
	Treated	292 ± 9*	94.9 ± .2*	.10 ± .02*	16 ± 5*
3	Control	589 ± 23	89.9 ± .7	.99 ± .05	572 ± 40
	Treated	302 ± 12*	94.9 ± .5*	.43 ± .04*	153 ± 49*
5	Control	623 ± 33	89.0 ± .2	1.29 ± .10	794 ± 58
	Treated	308 ± 16*	94.7 ± .4*	.53 ± .33***	206 ± 48*
7	Control	484 ± 19	89.9 ± .1	1.35 ± .10	650 ± 36
	Treated	380 ± 19*	92.6 ±1.7**	.58 ± .30*	223 ± 61*

† Each value is the average ± SE (n = 12 for wet wt; 4 for water content and hydroxyproline).
‡ Treated = sponge impregnated with 300 μg hydrocortisone acetate.
* $P < .01$, ** $P < .02$, *** $P < .05$.

Scintillation spectrometer using Bray's solution. A second aliquot was used for total protein determination(18).

Results. Granuloma formation, as judged by wet weight of the control occurred most rapidly within the first 24 hours. Subsequently, there was a slowly progressive increase to a maximal weight of 623 ± 33 mg (mean ± S.E.) by the 5th day, and then a decline to 484 ± 19 mg by the 7th day (Table I). Hydrocortisone acetate impregnation inhibited granuloma growth by approximately 45% throughout the first 5 days ($P<0.01$).

The water content of the control granulomas remained relatively constant at 89.0 to 91.1% throughout the period of observation. The water content of the treated granulomas was significantly greater (approximately 4%) than that of the controls at all time (Table I). In both the treated and control granulomas there was a progressive increase in the concentration and total hydroxyproline content up to day 5. However, the increase was significantly less in the treated granulomas (Table I).

Both the concentration and total amounts of DNA and RNA in control implants increased progressively to reach a maximal value by day 5, and then either plateaued or decreased by day 7. These were significantly reduced in the treated sponges. The nucleic acids of the treated tissues remained relatively constant during the first 5 days, but then showed a significant increase by day 7 (Table II). Maximal values for total protein and protein per mg of tissue were reached by day 2 (Table II) in both control and treated groups, but total protein synthesis was much less ($P<0.02$) in the hormone treated sponges.

The total protein and C^{14}-L-proline uptake were markedly reduced in the treated sponges at 12 and 24 hours (Table III). The specific activity (cpm/μg protein) of the treated implants averaged 47 and 25 per cent less than that of the controls at 12 and 24 hours, respectively (Table III).

Discussion. These studies reveal that approximately 80% of the increase in granuloma weight occurs within the first 24 hours. This is followed by a progressive increase to a maximal weight at day 5. The total protein content reaches a maximum within 2 days, while increases in hydroxyproline, DNA and RNA are more gradual. These biochemical changes demonstrate that the response to sponge implants represents a true tissue growth characterized by a rapid initial increase in cellularity, protein synthesis and collagen formation.

Impregnation of the sponge with hydrocortisone acetate markedly suppresses the initially rapid growth, and this inhibition persists for approximately 5 days. The inhibition was apparent for wet weight, and total hydroxyproline, protein, DNA and RNA content. The steroid not only decreased the total amounts of these constituents but also their concentration per unit of wet weight. In addition to

confirming the previously reported corticosteroid inhibition of growth and collagen formation in granulomas(1-10,21), these experiments demonstrate that the suppression of growth and collagen formation is paralleled by a reduced DNA, RNA and total protein content.

Eichhorn and Sniffen(11) observed that granulomas treated *in vivo* with cortisol had a decreased DNA and total protein content, but no change in their ability to incorporate amino acids *in vitro*. Thus, it was concluded that cortisol treatment had no effect on protein biosynthesis in granulomatous tissue. Inspection of their data reveals a considerable variability, as indicated by a significant increase or reduction in total amino acid incorporation in several experiments. These variations were obtained despite the use of relatively large doses of cortisol. In the present *in vivo* experiments, using C^{14}-labeled proline, the total amino acid incorporation and specific

TABLE II. Influence of Hydrocortisone Acetate on Nucleic Acid and Protein Content of Experimental Granulomas.†

Day		No. of animals	DNA μg/mg tissue	DNA Total (μg)	RNA μg/mg tissue	RNA Total (μg)	Protein μg/mg tissue	Protein Total (mg)
1	C‡	4	2.4 ± .2	1024 ± 53	.7 ± .1	324 ± 35	27.3 ± 1.7	11.9 ± .6
	T§	4	.8 ± .0*	248 ± 78*	.4 ± .1*	133 ± 37*	19.2 ± .7*	5.6 ± .9*
2	C	4	2.6 ± .2	1407 ± 234	.9 ± .1	460 ± 53	32.9 ± 2.3	17.9 ± 3.2
	T	4	.6 ± .1*	185 ± 84*	.4 ± .1*	132 ± 32*	21.0 ± 2.7**	6.3 ± 1.6**
3	C	4	3.0 ± .3	1750 ± 91	1.1 ± .2	619 ± 91	26.4 ± 2.0	16.0 ± 1.9
	T	4	.7 ± .1*	223 ± 33*	.4 ± .1**	108 ± 7*	14.1 ± 1.8*	4.8 ± .7*
5	C	4	3.7 ± .2	2379 ± 156	2.1 ± .1	1329 ±133	26.3 ± 2.2	16.4 ± 1.1
	T	4	.7 ± .1*	255 ± 39*	.4 ± .1*	119 ± 38*	14.4 ± 1.5*	5.0 ± .4*
7	C	4	2.9 ± .3	1599 ± 258	1.9 ± .5	978 ±309	29.6 ± .1	15.6 ± .5
	T	4	1.0 ± .1*	390 ± 50*	.6 ± .1**	298 ± 43**	16.6 ± 1.1*	6.1 ± .6*

† Each value = average ± SE.
‡ C = Control.
§ T = Treated (sponge impregnated with 300 μg hydrocortisone acetate).
* $P < .01$, ** $P < .02$.

TABLE III. Influence of Hydrocortisone Acetate on the Weight, Total Protein Content, Total C^{14}-L-Proline Uptake and Specific Activity in Granulomas.

Animal No.		Wet wt (mg)	Total protein (μg)	Total counts (cpm)	Specific activity (cpm/μg prot)	% Change
24 hr						
1	C*	387.3	11,250	15,250	1.36	
	T†	319.2	6,000	5,648	.94	—30.8
2	C	536.1	18,400	50,298	2.73	
	T	367.8	8,000	17,463	2.18	—20.1
3	C	584.1	22,000	33,383	1.52	
	T	284.0	9,625	10,790	1.12	—26.4
4	C	542.2	19,000	38,713	2.04	
	T	288.8	6,250	9,748	1.56	—23.6
12 hr						
1	C	410.2	12,600	39,950	3.17	
	T	354.0	5,200	8,420	1.62	—48.9
2	C	426.2	16,100	44,640	2.77	
	T	369.2	10,300	15,605	1.52	—45.2
3	C	471.9	14,875	71,810	4.83	
	T	392.5	7,700	19,590	2.54	—47.4

* C = Untreated sponge.
† T = Treated (sponge impregnated with 300 μg hydrocortisone acetate).

activities of steroid treated implants were consistently decreased in all instances. The experiments cited above(11) are not truly comparable with those reported herein since the conditions were quite different. In particular, their use of 7 day granulomas may have allowed spontaneous regression to influence the activity of cells in the control implant. Furthermore, the steroid was administered subcutaneously in large doses, and an *in vitro* method for measuring amino acid incorporation was used. The determination of specific activity may have been influenced by the difference in the amounts of protein formed in the control and treated sponges prior to the amino acid uptake measurements. In the present experiments, the injection of the radioactive amino acid at time zero obviated this complication. The decrease in specific activity and total amino acid incorporation seen in these initial studies represents an effect of the steroid on granulomatous tissue which warrants further investigation.

The marked reduction in DNA content of the treated granulomas observed in this study is in accord with histological evidence that 17-OH steroids reduce the number of cells present in granulomatous tissue(1,2,22,23). The steroid induced decrease in cellularity probably contributes to, and may account entirely for, the reduction in the total amounts of the chemical constituents. In addition, a change in cell metabolism is suggested by the reduced amino acid uptake and the decreased concentrations of collagen, DNA, RNA and protein. Analysis of this system is complicated by the fact that the steroid may affect differently the various cell types in granulomas. Published data clearly indicate a steroid induced decrease in total collagen concentration per unit wet weight of tissue (7-10,21,24). Yet on a dry weight basis, conflicting results have been reported(7,21,24).

The steroid treated implants had a constant and significantly higher water content than did the controls. This was unexpected in view of the marked inhibition of vascularization, obvious upon gross inspection, and the well known effect of glucocorticoids to suppress the influx of fluid into an inflammatory site. Robertson and Sanborn did not find a change in water content in steroid treated granulomas; this may be accounted for by the different methods of granuloma induction and treatment which they employed(7).

Summary. Granulomas were induced by the bilateral implantation of weighed sterile cotton sponges in the dorsum of male Sherman rats. Hydrocortisone acetate (300 μg) impregnation significantly inhibited granuloma growth for approximately 5 days. In addition, steroid treatment significantly reduced the total amount and concentration of collagen, total protein, DNA and RNA. Water content was increased by about 4% in the steroid treated granulomas. Hydrocortisone acetate (300 μg) impregnation resulted in significant reduction of both total C^{14}-L-proline incorporation and specific activity (cpm/μg protein), measured at 12 and 24 hours after implantation.

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Partial Replacement of CO_2 in Strain L and HeLa Cultures.* (32341)

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It has been reported that HeLa or conjunctival cells of human origin would not multiply under conditions of CO_2 deficiency (1) but that net multiplication could be restored partially by extracts of normal cells (2) or completely by a combination of ribonucleosides and oxalacetate(3). In the latter study, over 90% of the label fixed from $NaHC^{14}O_3$ by the cells was found in the acid soluble and nucleic acid fractions. It was concluded that, under the experimental conditions used, the chief function of CO_2 was to provide specific precursors for synthesis of purines, pyrimidines, and oxalacetate. Reported here is a study of CO_2 substitution in fibroblast-like mouse cells.

Materials and methods. The cells were a subline of NCTC clone 929 (strain L). The basal medium was Waymouth's MB752/1(4) modified as follows: $MgSO_4 \cdot 7H_2O$(100), Na_2HPO_4(150), nicotinamide(0.5), choline (124), ascorbic acid(8.75), cysteine HCl(45), glutathione(7.5), folic acid(0.5), $FeSO_4$(0.5) (all mg/l) (personal communication, C. J. Waymouth). Horse serum(5%) that had been heated at 52°C for one hour was added; $NaHCO_3$ was omitted. Compounds(all from Nutritional Biochemicals Corp., Cleveland, Ohio) used in the experiments were added to the basal medium which then was filtered through a sterile fritted-glass or membrane filter. Oxalacetate(OA) created a problem when added to media because of a rise in pH due to spontaneous decarboxylation and concomitant loss of an acidic group. This was compounded by the presence of alkaline traps since the CO_2 resulting from the decarboxylation was removed from the medium and did not form H_2CO_3 which ordinarily would offset partially the loss of the carboxyl group from OA. In such cases, the pH was kept in a range suitable for the cells by addition of dilute HCl as frequently as necessary.

After inoculation into 250 ml milk dilution bottles, the cells were allowed to grow in monolayers for 24 hours before establishment of experimental conditions. Deficiency in CO_2 was established by use of an alkaline trap similar to one previously described(1) except that the glass tubing extended completely through the stopper and had its exterior end closed by a sleeve-type serum bottle stopper. Four-tenths ml of 15% NaOH was introduced onto a 1.5 × 2 inch Whatman No. 1 filter paper fan contained in the glass tubing. Media and alkaline traps were changed every 48 hours unless otherwise noted. Cells were scraped from the glass and counted with a Model B Coulter Counter (Coulter Electronics, Hialeah, Fla.). Cultures were periodically tested for PPLO contamination by Madoff's modification of Diene's procedure (5).

Results. Cultures of strain L cells deficient in CO_2 showed no increase in number if the

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