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Effect of Cortisone and other Steroids upon *in vitro* Synthesis of Chondroitin Sulfate. (21164)

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It is known that cortisone inhibits the formation of granulation tissue(1) and that this tissue has a capacity to synthesize chondroitin sulfate(2,3). Layton(4) has further demonstrated that cortisone alcohol inhibits the *in vitro* synthesis of chondroitin sulfate by embryonic and wound tissues. From related physiological information regarding the effects of cortisone in the whole organism(5,6), its action by way of inhibiting chondroitin sulfate formation might well be capable of explaining a variety of observations. While the data presented by Layton(4) provided a reasonable inference that the material being measured was chondroitin sulfate, subsequent studies(7-11) have markedly increased the probability that the measurements are those representing chondroitin sulfate, and confirmation of the inhibition by cortisone alcohol has been provided(9,10).

One of the difficulties inherent in the study of the mode of action of the steroid hormones is the lack of an *in vitro* activity which bears any relation to the action of the hormone in the whole animal. It appeared possible that this inhibition of chondroitin sulfate synthesis by the addition of cortisone alcohol *in vitro* might be sufficiently related to its action in the whole animal, where such inhibition is also observed(12), to provide the *in vitro* tool which is so necessary for this type of problem. However, even the system employed by Layton(4) was somewhat too complex as an initial *in vitro* approach, since it was dependent upon embryonic (granulation) tissue, under conditions essentially comparable to those of tissue culture and required incubation periods of 45 hours. It would be desirable to obtain a somewhat similar system which would employ essentially a tissue slice. This was done by Boström and Mansson(8,9) but a more rapid system was still necessary. This has been achieved as described under methods. Having such a system, which in a period of 5 hours synthesized considerable quantities of chondroitin sulfate, it was found possible to inhibit the chondroitin sulfate synthesis by the *in vitro* addition of cortisone alcohol confirming the results reported by Layton(4) and Boström and coworkers(9,10). However, further studies of this system throw some doubt on the specificity of the effect of the steroid cortisone and on the question of whether this action is really related to the action of cortis-

one in the intact animal.

Methods. The uptake of radioactive sulfur* was used as a measure of chondroitin sulfate synthesis. Two ml of the Tyrodes solution prepared as described by Layton(13) except that carrier sodium sulfate was omitted and the solutions were not autoclaved, plus 0.5 ml of other additions were placed in small flasks or bottles which had been carefully cleaned and soaked in glass distilled water to remove traces of metals. Sulfate was supplied to this solution in the form of carrier-free S^{35}. When steroids were used they were dissolved in chloroform (0.5 mg/ml), the proper aliquot supplied to the flask, and the chloroform removed under vacuum. The Tyrodes solution, etc. were added and the flask shaken in water bath for at least 30 min. before the addition of tissue. This permitted solution of the finely divided steroid in the Tyrodes solution, although the absolute quantity dissolved was not measured.

Rat tissues from normal adult males weighing 250-300 g were employed throughout. The animals were killed by a blow on the head and the tissues removed into ice-cold isotonic KCl. For cartilage, the xiphoid process was employed, trimmed free from bone and cut into slices free-hand. Cartilage slices of approximately 10 mg fresh weight were employed; for tail skin, 100 to 150 mg were used. Incubations with tissue were at 37°C in an atmosphere of 95% O_2 and 5% CO_2. As controls, live tissue was incubated under the same conditions except at 0°C rather than at 37°C. It was felt that this process would permit diffusion, etc., of sulfate ion to take place but should prevent most of the metabolic reactions from proceeding at an appreciable rate.

At the end of the incubation period, the slice was removed from the incubation mixture to a piece of clean blotting paper, the surface dried, and placed in approximately 200 times its weight of 10% trichloracetic acid. This hardened the tissue so that subsequent manipulations were much simpler and the slices did not disintegrate. The slices remained in the trichloracetic acid for 24 hours in the cold. They were then removed, blotted dry, and transferred to fresh trichloracetic acid, where they remained for another 24 hours. They were then extracted in the cold with H_2O and defatted with alcohol and petroleum ether. Analysis of the second trichloracetic extract showed essentially no radioactivity so that at this point it was considered that all of the radioactivity remaining in the slice was not extractable with TCA, and was considered to be chondroitin sulfate. Justification for this procedure is found in previous publications(4,7,8).

Digestion of the cartilage was effected by a micro-Carius combustion. The solutions were made to a known volume and aliquots were taken for sulfur determinations and radioactive measurements. Radioactivity was determined using a thin end-window Geiger-Müller tube with the appropriate corrections made for background, decay and self-absorption. Sulfur was determined by the following procedure. Aliquots of the digestion mixture were made up to 2.5 ml and 0.5 ml of a solution containing 10.0 g $BaCl_2$, 1 ml concentrated HCl and 4.0 g of gum arabic in 100 ml was added. The resulting turbidity was measured in a Beckman Model B spectrophotometer at 620 mμ and compared with known standards of ammonium sulfate precipitated in the same fashion. By this procedure as little as 5 μg of sulfur could be determined.

Initial results with the above system were somewhat low, and means for increasing the incorporation of radioactive sulfur were devised. The Tyrodes solution as prepared contains 1 g per liter of glucose. Increase of this concentration to M/100 (final) seemed to help, but in addition supplementation with

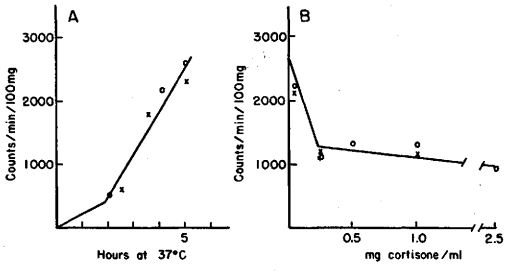

FIG. 1. Uptake of sulfur by rat cartilage *in vitro*. ○ Tissue from adrenalectomized animals. × Tissue from normal animals.

* Radioactive sulfur was obtained on allocation from the Atomic Energy Commission, Oak Ridge, Tenn.

TABLE I. Uptake of Radioactive Sulfur by Cartilage.

Treatment	Wet wt, mg	Total sulfur, μg	Specific activity, c/m/mg S	% uptake of controls†
0°C	15	15.9	10	<.2
	15	5.8	3	
Control	21	10.6	4,113	100
	30	17.2	4,056	
Cortisone	14	16.4	2,998	63
	15	18.1	2,129	
Cortisone acetate	19	*	*	
	13	9.2	2,727	67
Hydrocortisone	21	11.4	11,387	221
	28	11.4	6,658	
Hydrocortisone acetate	25	*	*	
	17	14.9	12,868	730
Desoxycorticosterone	23	16.4	1,385	38
	18	8.2	1,762	
Substance S	22	16.3	4,358	106
	25	*	*	
Cholesterol	17	12.8	5,174	113
	16	16.2	4,049	

* Samples exploded during combustion.
† Avg of the 2 samples.

glutamate (to M/100 final concentration) markedly increased the sulfur incorporation in the case of cartilage. For example, cartilage supplemented with glucose or with glutamate gave 750 to 780 counts/min./100 mg wet weight of tissue, but supplementation with both increased the count to 1870 during a 6-hour incubation period. When both glutamate and glucose were present, but the tissue was incubated at 0° rather than at 37° for the 6-hour period, only 8.7 counts/min./100 mg were incorporated.

Fig. 1A illustrates that after a lag period, the rate of sulfate uptake, and thus presumably chondroitin sulfate synthesis, is a straight line function of time. There is no difference observed between tissues from a normal or from an adrenalectomized animal, the latter being included on the presumption that if cortisone inhibited chondroitin sulfate synthesis, the tissue from adrenalectomized rat (maintained on saline for several weeks), being free from adrenal steroids, should show enhanced chondroitin sulfate synthesis. It is obvious that this is not the case. Nevertheless, Fig. 1B illustrates that sulfate uptake is inhibited by cortisone as reported by Layton (4) and Boström and Odeblad(10).

We therefore chose to regard this system as comparable to that of Layton(4) and with it we confirmed his results. The problem remaining, however, was the relationship of this *in vitro* response to the action of cortisone in the whole intact animal. It had been demonstrated, of course, that there were changes in the skin(5,6) and even in the chondroitin sulfate synthesis(12) on treatment of the whole animal with cortisone, but these observations might be secondary to other changes, and not due to a direct inhibition of chondroitin sulfate synthesis by cortisone. All such changes *in vivo* are observed with cortisone alcohol, cortisone acetate, and other esters thereof, hydrocortisone alcohol, hydrocortisone acetate, but are not observed with desoxycorticosterone or 11-desoxy-17-hydroxycorticosterone (substance S). We therefore reasoned that if the effect observed *in vitro* were related to the *in vivo* observations, the same specificity should be shown.

It can be seen (Table I) that although cortisone and hydrocortisone have identical activity *in vivo,* they have completely opposite effects *in vitro.* Moreover, cortisone and desoxycorticosterone, which are quite unlike *in vivo,* act the same *in vitro.* These con-

tradictory effects observed throw considerable doubt upon the relation of the *in vitro* response observed to the action of these agents in the intact animal. The *in vitro* response, therefore, seems to be related to some property of the steroid which bears no discernible relationship to its physiological action. This conclusion seems to be substantiated by the observation of Jones and Gerarde(14) that cortisone acetate has no effect in essentially the same system used by Layton(4) where the effect of cortisone alcohol was observed.

Summary. An *in vitro* system for the synthesis of acid insoluble sulfate (presumably chondroitin sulfate) has been devised. The synthesis may be influenced (inhibited or stimulated) by the *in vitro* addition of cortical steroids. The influence of the steroids, however, seems to bear no relation to their physiological action. There was no difference in the rate of *in vitro* sulfate synthesis by cartilage from normal or adrenalectomized rats.

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Assay of Influenza Virus Infectivity with Chicken Embryonic Membranes.* (21165)

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(Introduced by Max A. Lauffer.)
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The purpose of this study was to develop an improved assay technic for influenza virus infectivity. The method proposed has several advantages over the titration in chicken embryos in that it requires less labor, less space, and one-twentieth the usual number of eggs. The principal disadvantage is that this new method is not as sensitive. Two animals have proven satisfactory for measuring influenza virus infectivity. These are the mouse(1) and the chicken embryo(2). Bernkopf(3) demonstrated that the virus could be grown on the chorioallantoic membranes of eggs from which the fetus had been removed. There have been references(4-9) to the growth of the virus in cells growing in tissue culture. In view of these findings it was considered likely that small pieces of the chorioallantoic membrane would support influenza virus growth and could, therefore, be used as the host in an assay system for the virus.

Materials and methods. The PR8 strain of influenza virus was used. Stocks had undergone from 17 to 19 egg passages from a pool of preparations A, B, and C described in a paper by Lauffer and Wheatley(10). The individual assay unit consisted of a half-inch test tube containing one ml of nutrient broth, one membrane section, and the appropriate virus inoculum. Membrane sections were prepared by first removing everything except the chorioallantoic membrane from the inside of

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