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Extractable Heparin Level in Rat Tissues Under Normal and Experimental Conditions.* (23262)

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Much of the work on the study of mast cells and their relation to the histamine and heparin content of tissues has been carried out on rats(1,2). There is, however, little available information on normal heparin levels in different rat tisues, or on properties of rat heparin. Obviously there is a need for the establishment of normal levels of extractable heparin in different organs of the rat and a comparison of the product of extraction with an accepted standard. Levels for extractable heparin have been determined for 6 different organs of the rat, and the product has been compared with beef heparin in regard to antithrombic, metachromatic and anticoagulant potencies.

Materials and methods. Rats. Female rats (Wistar strain) of approximately 180 g were fed a standard laboratory diet of Purina Chow. Animals were killed by decapitation, the skin immediately removed and stored at –20°C until extracted. *Cortisone.* A saline suspension of cortone acetate (cortisone acetate Merck), 25 mg/ml was given subcutaneously in daily doses of 10 mg/animal. *Carbutamide* (BZ-55), Eli Lilly and Co., Indianapolis, Ind. A solution was prepared by dissolving 4 g of the material in 100 ml of alkaline solution and adjusting pH to 7.5. Daily subcutaneous injections of 50 mg/animal were given.

Heparin extraction. The method used was that described earlier(3) with the following additional step found desirable, when dealing with rat skin. After first alcohol precipitation the dried product was suspended in 1 ml of buffered saline/g of original tissue. The pH was adjusted to 8.0 and approximately 20 mg of trypsin/g of original tissue were added. This mixture was incubated one hour at 38°C and then treated with 80% phenol. The heparin was precipitated from the aqueous layer with 95% ethanol. For further purification the precipitate was dissolved in saline and the solution treated with benzidine as previously described(3). *Heparin assay.* (a) *Antithrombin Assay.* This was carried out according to the method of Jagues and Charles (4). (b) *Metachromatic Assay.* This was carried out as described by Jagues, Monkhouse and Stewart(5). (c) *Anticoagulant Assay.* Blood was withdrawn from a normal healthy dog by a siliconed syringe and needle and immediately placed in test tubes containing varying amounts of standard and unknown heparin solutions. The clotting times were noted and the potency of the unknown then expressed in terms of the standard heparin. *Heparin standard.* All values are expressed in terms of beef heparin kindly supplied by the Connaught Medical Research Laboratories. Unless otherwise stated the values given in Tables refer to those obtained by antithrombin assay.

Results. Normal extractable heparin levels in rat tissue. In Table I are shown the aver-

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TABLE I. Extractable Heparin Levels in Rat Tissues.

Organ	Animals	Units of heparin/g tissue I	II	III
Heart	22	.4– .8	.4– .7	.4– .5
Spleen	22	.4– .6	.20	.24
Lung	22	.2– .3	1.4–2.4	1.9–2.3
Kidney	22	1.0–1.1	1.7–1.8	1.4
Liver	3		.7– .8	.5
Skin	42			3.9–7.8

I, Assayed after KSCN extraction, phenol treatment and alcohol ppt.

II, Assayed after treatment of product I with trypsin.

III, Assayed after benzidine treatment of II.

age levels of extractable heparin in 6 different organs of the rat. The values for skin represent results from experiments carried out over 3 months. Heart, liver and spleen show extremely low levels, lung and kidney somewhat higher and skin an average level approaching that for beef lung. The relatively high level of extractable heparin in rat skin makes this a useful tissue for the study of changes following hormone therapy or injection of histamine and heparin releasing compounds.

Table II shows results of comparison between antithrombic, metachromatic and anticoagulant activities of partially purified material from a large quantity of rat skin. Judging from preliminary measurements made by Dr. L. B. Jaques (personal communications) on recently crystallized rat heparin, our extract contains considerable amounts of inert material. Nevertheless it is of sufficient purity to indicate that we are working with a potent heparin. The low ratio of anticoagulant to metachromatic activity shows that rat heparin has a lower specific anticoagulant activity than beef or dog heparin.

Effect of cortisone and carbutamide on extractable heparin levels in rat skin. In 2 experiments the skin was obtained from rats which had been treated with agents affecting carbohydrate metabolism. In the first series the animals were divided into 4 groups. One group received saline injections, a second group received 10 mg cortisone daily, a third group received 50 mg carbutamide/day and a fourth group received 10 mg of cortisone plus 50 mg of carbutamide daily. On 6th, 9th, 13th and 15th days of treatment 5 animals from each of the first 2 groups were sacrificed. In addition 5 animals from each of the other 2 groups were sacrificed on 13th and 15th days. Skin from rats of each group was chopped up and thoroughly mixed. Duplicate samples of 6 g each were then taken from these pools for heparin extraction. The results are shown in Table III. The extractable heparin levels were lower in samples of skin from the cortisone treated animals than in samples from controls on the 9th and 13th days of treatment but were within the control range on the 6th and 15th days. The heparin level in samples of skin from animals injected with carbutamide only, was within the control range on both the 13th and 15th days of treatment. It was significantly lower than the control level, in the skin of rats treated with cortisone plus carbutamide, on the 13th and 15th days.

TABLE III. Extractable Heparin Level in Rat Skin following Cortisone and BZ-55 Treatment.

Treatment	Heparin, units/g tissue* Days of treatment 6	9	13	15
Normal	7.1	7.0	6.5	7.8
Cortisone	7.1	3.0	5.8	7.5
Carbutamide			8.3	7.8
Cortisone + carbutamide			3.1	3.7

* Each value given is the mean of duplicate samples taken from a pool of skin from 5 rats.

In the second experiment all animals were sacrificed after 15 days of treatment. The

TABLE II. Heparin Activity/mg of Extract from Rat Skin at Different Stages of Purification.

Stage purification	Wt of ppt (mg)	Units of heparin: Antithrombin Total	U/mg	Metachromatic Total	U/mg	Anticoagulant Total	U/mg
*Benzidine precipitation	300	1200	4.0				
2nd trypsin digest	130	1000	7.7				
2nd benzidine precipitation	49.8	650	13.0	1500	30	600	12

* This represents extract from 260 g of skin from 45 different rats.

TABLE IV. Extractable Heparin Level in Rat Skin after Cortisone and Cortisone plus Carbutamide. Six animals/series.

Treatment	No. of determination	Heparin, units/g tissue
Controls	12	4.2 S = .4
Cortisone	12	2.7 S = .4
Cortisone + carbutamide	12	1.4 S = .8

$S = \sqrt{\frac{(x-\bar{x})^2}{n-1}}$, where n = No. determinations.

heparin level was determined in duplicate for each animal. Three groups of 6 rats to a group were used. One group served as controls and received saline injection, a second group received cortisone and a third group received cortisone plus carbutamide. A summary of the results is shown in Table IV. The extractable heparin level in rats receiving both cortisone and carbutamide is in turn significantly lower than for the animals receiving cortisone only.

The results of these experiments clearly indicate that, while carbutamide alone has no effect on the extractable heparin level in rat skin, it has a significant lowering effect when given in combination with cortisone. Cortisone treatment alone caused a decrease in the extractable heparin level in both instances. However, in the first experiment the level appeared to return to normal by 15th day of treatment while in the second experiment it was significantly lower than the controls at this time.

A third experiment was therefore carried out. Twelve rats served as controls and 12 were given daily injections of cortisone. On each of the 3rd, 6th, 9th and 15th days of treatment, 3 animals from each group were sacrificed. The extractable heparin level was determined in duplicate on the skin of each animal. A summary of results is shown in Table V. It can be seen that the heparin level in the skin of animals receiving cortisone is significantly less than that of controls as early as the third day of treatment and remains low throughout the fifteen day period.

Discussion. Estimation of extractable heparin has revealed that, with the exception of skin, most rat tissues have a relatively low heparin level. Rat skin contains several times as much heparin as any other rat tissue. This is not surprising since it also contains an abundance of mast cells. Marx *et al.*(6) recently reported values for some rat tissues which were much higher than those presented here. They suggested that their method probably gives a more complete extraction of heparin. This is certainly possible but they may also be measuring something which is not heparin. Their values for rat kidney are approximately equal to those found for dog liver and beef lung by the method of extraction used here. It would be interesting to know what levels they would obtain from these tissues with their method. They found that rat kidney had a very low mast cell count, despite the high heparin content. It is quite consistent with present-day knowledge to find a tissue with a high concentration of metachromatic staining cells and a low heparin content, since other sulphonated polysaccharides will stain similarly. It remains to be explained, however, how a compound such as heparin can be present in large amounts without showing the characteristic metachromatic reaction. These authors do not report values for rat skin.

It was recently shown that the amount of extractable heparin in the liver of hypophysectomized dogs was greater than in control animals(3). This finding, coupled with reports(7) that the injection of cortisone or ACTH causes a decrease in the number of mast cells in dermal connective tissue of the rat suggests that a metabolic pathway involving heparin is influenced by the pituitary gland through the adrenal cortex. The reduc-

TABLE V. Extractable Heparin Level in Rat Skin during Cortisone Treatment.

Treatment	Heparin units/g tissue, Days after treatment 3	6	9	15
Control	(3) 5.0 S = .65	(3) 4.6 S = .60	(3) 4.9 S = .65	(3) 3.9 S = .50
Cortisone	(3) 2.6 S = .30	(2) 3.4 S = 1.25	(3) 2.2 S = .82	(3) 2.7 S = .35

( ) = No. of animals; duplicate determination carried out on each animal.

$S = \sqrt{\frac{(x-\bar{x})^2}{n-1}}$, where n = No. determinations.

tion in mast cells by cortisone and ACTH injections has been reported for other species (8).

The results of experiments reported here show that the extractable heparin level in rat skin is decreased by the injection of cortisone. Presumably this decreased level is due to an inhibition of heparin formation, since Layton (9) showed that cortisone inhibited mucopolysaccharide synthesis in the intact rat and Böström and Odeblad(10) have shown that cortisone decreases the rate of sulphate exchange in chondroitinsulphuric acid.

The chance finding that carbutamide augments the depressing action of cortisone on heparin level is of immediate interest. It is worth noting that Dr. D. W. Clarke of this department found, that compared to controls, the cortisone treated animals had significantly higher per cent liver fat and, that the animals treated with both cortisone and carbutamide had a higher per cent liver fat than those given cortisone only. An increase in liver fat is thus found under conditions which also produce a decreased heparin level in the skin. Further experiments will be required to determine if these are merely coincidental happenings or if they are indeed related.

Summary. The extractable heparin level in different rat tissues has been measured. The skin of the rat is relatively high in heparin but the content of the other tissues measured is low. The heparin extracted has a lower specific anticoagulant activity than either beef or dog heparin. The skin of rats receiving daily cortisone injections has a significantly lower heparin level than that of controls. Injection of Carbutamide (BZ-55) alone into rats does not affect the extractable heparin level but it greatly augments the depressing action of cortisone.

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Effect of Intravenous Soy Bean Phosphatides on Blood Coagulation in Rabbits.* (23263)

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Inosithin® is a commercial preparation of alcohol-insoluble phosphatides derived from soy beans. This material has coagulant properties resembling those of platelets, and it functions as an effective platelet substitute in the thromboplastin generation test and in the partial thromboplastin time.† Moreover, like platelets, Inosithin® is anticoagulant *in vitro* when concentrations above the optimal range are used(1,2). The following study was performed to determine the *in vivo* effects of Inosithin® on blood coagulation.

Methods and materials. Experimental subjects were male rabbits weighing between 2.5 and 3.5 kg. Inosithin® was administered as a 5% emulsion suspended in 5% glucose solution.‡ This emulsion gave optimal activity

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† Partial thromboplastin studies were performed by Dr. John Graham of Univ. of North Carolina.

‡ Inosithin was kindly provided by Mr. J. Eichberg of Associated Concentrates, Woodside, L. I., and prepared as a sterile emulsion by Don Baxter, Inc., Glendale, Calif.