Effect of Phosphorylated Hesperidin and Hyaluronidase on Absorption From Peritoneal Cavity of Rats.* (23818)

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During experiments unrelated to the study here presented, we observed that an intraperitoneally administered solution of phosphorylated hesperidin, a hyaluronidase inhibitor (1), was not absorbed from the peritoneal cavity for a prolonged period of time. This observation prompted an investigation of the effect of phosphorylated hesperidin and hyaluronidase on absorption of fluid from the peritoneal cavity of rats.

Materials and methods. Hooded female rats (185 ± 15 g) raised at this Institute were employed throughout this study. For determination of fluid absorption from the peritoneal cavity, the following procedure was employed. Rats, under light ether anesthesia, were injected intraperitoneally (I.P.) with 15 ml of a 0.9% NaCl solution employing a 27-gauge hypodermic needle ⅔ inches long. Subsequently, groups of animals were sacrificed at various time intervals following the injection. The peritoneal cavity was exposed surgically, the free fluid recovered by suction and drainage, and then measured. The hyaluronidase† and phosphorylated hesperidin‡ were prepared before each experiment in a 0.9% NaCl solution. All injections were made intraperitoneally. Adrenalectomy, employing the lumbar approach, was performed under ether anesthesia 48 hours before an experiment. The drinking water was replaced after operation by a solution of 0.9% NaCl. All other animals received tap water *ad lib.* during the experiment. Statistical analysis of the data was performed employing the Student's t test.

Results. Reliability of fluid recovery method. To evaluate reliability of fluid recovery technic 16 animals were injected I.P. with 15 ml of 0.9% NaCl solution (normal saline). Animals were sacrificed from 2 to 4 minutes after injection and the fluid recovered and measured. From 93 to 96% of total injected volume could be recovered.

Hesperidin series. Animals were divided into 2 groups. One group received saline solution only, the second received hesperidin and saline, as indicated in Table I (Groups 1 and 2, respectively). Groups of animals were sacrificed 2 minutes to 24 hours following last injection. The results of this experiment are summarized in Fig. 1. For the first 2 hours after 15 ml injection, hesperidin treated animals absorbed the fluid at same rate as untreated animals, but after the second hour absorption rate decreased markedly.

Hyaluronidase series. The effect of hyaluronidase was determined in a similar manner (Table I, Groups 3 and 4, respectively). The animals were sacrificed 2, 3, 6 and 8 hours after 15 ml injection. Data from this experiment, (Fig. 2), show that volume of fluid recoverable from animals treated with hyaluronidase was significantly less than that for saline treated controls.

Hesperidin-hyaluronidase series. It was of interest to determine whether hyaluronidase would counteract the effect of hesperidin. A small but effective dose of hesperidin was considered most practical for this study. A dose response curve was experimentally constructed with this in mind (Fig. 3). From this curve a dose of 5 mg per injection was selected. Animals were divided into 4 groups for this experiment, one group received saline only, the second received hesperidin and saline and the third received hyaluronidase, hesperidin and saline, these two substances being administered with saline in separate injec-

* The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting views of Navy Department or naval service at large.

† Hyaluronidase was purchased from Worthington Laboratories. The enzyme was of testicular origin supplied in powder form with activity of 175 U.S.P. units/mg.

‡ Phosphorylated hesperidin was donated generously by Dr. G. J. Martin of National Drug Co.

TABLE I. Treatment Schedule for Hesperidin and Hyaluronidase Experiments.

Group	Treatment	No. of animals	Treatment schedule and dosages: Day 1 — 9 a.m.	Day 1 — 4 p.m.	Day 2 — 9 a.m.
1	Saline	95		.25 cc saline	15 cc saline
2	Hesperidin and saline	99		15 mg hesperidin in .25 cc saline	15 mg hesperidin in 15 cc saline
3	Saline	29	.25 cc saline	.25 cc saline	15 cc saline
4	Hyaluronidase saline	29	3000 U.S.P. units hyaluronidase in .25 cc saline	3000 U.S.P. units hyaluronidase in .25 cc saline	3000 U.S.P. units hyaluronidase in 15 cc saline

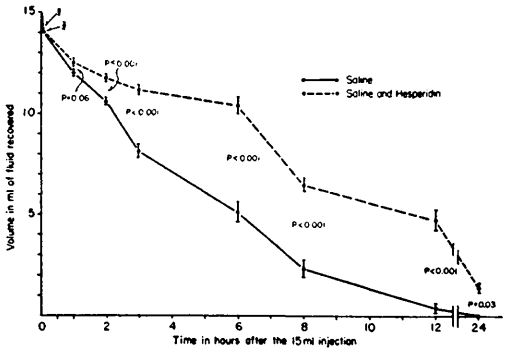

FIG. 1. Effect of hesperidin on fluid absorption from peritoneal cavity of rats.

tions. The fourth group was set up in the same manner as the third, except that a higher dose of hesperidin was employed. Table II shows details of the experimental design and results indicating that hyaluronidase abolished completely the effect of low and almost completely that of high doses of hesperidin.

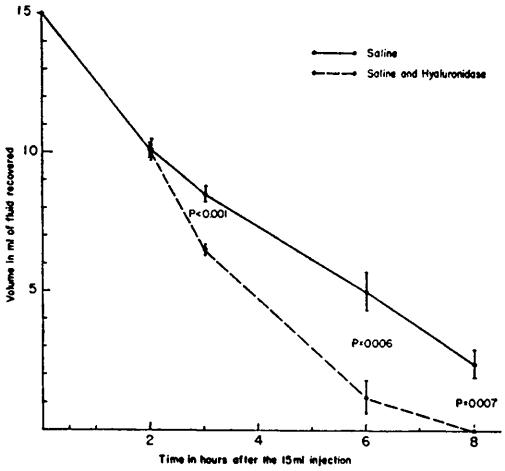

FIG. 2. Effect of hyaluronidase on fluid absorption from peritoneal cavity of rats.

Adrenalectomy series. Four groups of animals were used to test the possibility that hesperidin exerts its effect, *via* the adrenal-pituitary axis, directly or indirectly. One group was adrenalectomized, the second was sham-adrenalectomized and the third and fourth groups were left intact. The first 3 groups were treated with hesperidin in saline, the fourth group received saline only. All animals were sacrificed 6 hours after the 15 ml injection. The schedule of injection dosages and results are summarized in Table III which shows that the effect of hesperidin was not modified by adrenalectomy nor by sham-adrenalectomy.

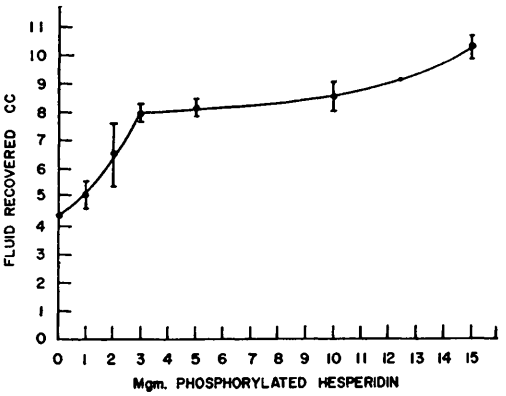

FIG. 3. Effect of various doses of phosphorylated hesperidin on the volume of fluid recovered from the peritoneal cavity of rats 6 hr after an inj. of 15 cc of 0.90% NaCl in distilled water.

Discussion. The mechanism of fluid absorption from the peritoneal cavity presents a multifaceted problem. This problem is magnified by the consideration that the net transfer of an injected solution through the peritoneum and into the circulation probably represents a sum of 2 processes, namely, an in-

TABLE II. Modification by Hyaluronidase of Effect of Hesperidin on Fluid Absorption from the Peritoneal Cavity of Rats.

Group	Treatment schedule and dosages — Day 1 — 9 a.m.	Day 1 — 4 p.m.	Day 2 — 9 a.m.	Vol of fluid recovered, ml — 4 hr‡	6 hr‡	8 hr‡
1	.25 ml Sal.	.5 ml Sal.	15 ml Sal.	(12)*7.7 ± .35†	(12) 5.2 ± .4	(6) 3.6 ± .64
2	*Idem*	5 mg P.H. in .5 ml Sal.	5 mg P.H. in 15 ml Sal.	(11) 9.6 ± .25 P = .02 Group 1 *vs* 2	(18) 8.8 ± .3 P <.001 Group 1 *vs* 2	(6) 5.5 ± .56 P <.05 Group 1 *vs* 2
3	3000 U.S.P. units HY in .25 ml Sal.	5 mg P.H. in .25 ml Sal. 3000 U.S.P. units HY in .25 ml Sal.	5 mg P.H. in .25 ml Sal. 3000 U.S.P. units HY in 15 ml Sal.	(6) 6.4 ± .31 P <.001 Group 2 *vs* 3	(12) 2.3 ± .4 P <.001 Group 2 *vs* 3	(6) .4 ± .02 P <.001 Group 2 *vs* 3
4	*Idem*	15 mg P.H. in .25 ml Sal. 3000 U.S.P. units HY in .25 ml Sal.	15 mg P.H. in .25 ml Sal. 3000 U.S.P. units HY in 15 ml Sal.		(5) 6.5 ± .52 P <.001 Group 2 *vs* 4	

* No. of animals. † Mean ± stand. error of mean. ‡ Hr after 15 ml inj.
P.H. = Phosphorylated hesperidin. HY = Hyaluronidase. Sal. = .9% NaCl sol.

flux of water and solutes into the tissue spaces and blood stream and a return flux in the reverse direction.

Reports in literature as well as findings related in this paper are consistent with the idea that permeability changes of the peritoneum *per se* probably play an important role in the process of absorption. Hyaluronidase produces a marked increase of the permeability of calf peritoneum *in vitro* to normal saline solution(2). Amante and Mittino(3) showed that hyaluronidase increased the absorption of trypan blue from the peritoneal cavity of guinea pigs.

In the present study we have demonstrated, using the net absorption rate as our criterion of fluid absorption, that fluid is absorbed more rapidly in hyaluronidase treated animals. On the other hand treatment with phosphorylated hesperidin markedly retarded the net absorption. That the hesperidin effect is not dependent on presence of adrenals has been shown above (Table III).

The effects of hesperidin were completely abolished by a concomitant administration of hyaluronidase and when an excess of hyaluronidase was employed the absorption was faster than in the saline controls.

Our *in vivo* observations are paralleled by *in vitro* study of the effect of hyaluronidase and phosphorylated hesperidin on the permeability of a mouse connective tissue membrane(4), showing that hyaluronidase increases the permeability and hesperidin reduces it to the level found before exposure to hyaluronidase. Furthermore it was demon-

TABLE III. Effect of Hesperidin on Fluid Absorption from the Peritoneal Cavity of Adrenalectomized Rats.

Group	No. animals	Treatment schedule and dosages — Day 1—9 a.m.	Day 2—4 p.m.	Day 3—9 a.m.	Fluid recovered*(ml)	P-values
1	10	Adrenalectomy	5 mg P.H. in .25 ml Sal.	5 mg P.H. in 15 ml Sal.	8.3 ± .4†	P = .82 Group 1 *vs* 3
2	5	Sham-adrenalectomy	*Idem*	*Idem*	7.9 ± .5	P = .55 Group 2 *vs* 3
3	10		"	"	8.2 ± .3	P <.001 Group 3 *vs* 4
4	12		.25 ml Sal.	15 ml Sal.	4.4 ± .4	

* Six hr after 15 ml inj. † Mean ± stand. error of mean.
P.H. = Phosphorylated hesperidin. Sal. = .9% NaCl sol.

strated in these studies that hesperidin alone reduces permeability of the connective tissue membrane. In view of the quoted evidence the possibility that hesperidin alters the absorption process by a direct effect on the membranes rather than by inhibition of hyaluronidase deserves serious consideration. Also, sight should not be lost of the possibility that hesperidin decreases the net absorption not by blocking nor diminishing the transfer of the fluid out of the peritoneal cavity, but by increasing the return flux from the circulatory system and tissue spaces into the peritoneal cavity.

Summary. 1. The effect of hyaluronidase and phosphorylated hesperidin on absorption of 0.9% NaCl solution from the peritoneal cavity of female rats was studied. 2. Intraperitoneal administration of hyaluronidase increased and administration of phosphorylated hesperidin decreased the net fluid absorption. 3. Hyaluronidase abolished the effect of hesperidin when administered to hesperidin treated animals. 4. Adrenalectomy had no effect on the action of hesperidin. 5. The possibility that the hesperidin decreases the net absorption in untreated animals by direct effect on permeability of the membranes rather than by inhibition of hyaluronidase is discussed.

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Utilization of Vit. A and Carotene by Normal and Deficient Sheep.* (23819)

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For many years, the utilization of Vit. A and carotene has been extensively investigated in monogastric animals but to a limited degree in ruminants. It is well known that there is a marked specie difference regarding the relative values of carotene and types of Vit. A. Results recently reported(9) have indicated a depressed carotene utilization by the Vit. A deficient bovine. Furthermore, unpublished results from our laboratory have shown that the value of carotene for the bovine, as measured by hepatic Vit. A storage, was greatly influenced by the initial Vit. A content of the liver. Therefore, it was of interest to elucidate the suggested impairment of carotene utilization by the deficient animal as well as to establish relative biological values of types of Vit. A and carotene for sheep.

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Method. Nineteen crossbred yearling wethers were divided into 2 groups of 9 and 10 animals each and pretreated with diets adequate and deficient in carotene respectively for 232 days. Four days prior to and during the experimental period all animals were fed a carotene deficient ration of barley straw and cottonseed meal. Aqueous solutions of beta carotene, Vit. A alcohol and Vit. A acetate were prepared, immediately preceding administration by the following method: One gram of the respective materials was dissolved in 20 ml chloroform; 25 ml Tween 80 were added and the chloroform was removed under vacuum at 62°C. Each remaining solution was diluted with distilled water to give a final concentration of 2 mg/ml. Trial A: Within 6 hours following liver biopsy, half of the normal and half of the deficient sheep were injected, intraruminally, with 0.6 mg of beta carotene/lb body weight. The re-