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Research Article

THE EFFECT OF HYALURONIDASE ON THE ABSORPTION OF PARENTERALLY ADMINISTERED RADIOACTIVE PLASMA PROTEINS IN THE DOG[1]

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There are numerous circumstances in which it would be desirable to administer substances subcutaneously, intramuscularly or intraperitoneally that cannot or should not be given intravenously. Since the need for parenterally administered protein and fat is one of the most urgent problems in clinical medicine, we felt it worthwhile to explore the possibility of facilitating their absorption from the tissues by the use of hyaluronidase,[2] a non-antigenic "spreading factor" first isolated from the testicle (1–3). Sannella (4) observed that this enzyme hastened the absorption of saline solution from the subcutaneous tissues of the rabbit. Hechter (5) found this to be true also in man, not only for saline solution but for plasma as well. Studies were therefore undertaken for a more precise determination of the facts for plasma by the use of plasma tagged with radioactive iodine. Similar observations with respect to fat will be reported (6).

METHOD

Preparation of radioactive iodo-plasma protein

Plasma was iodinated as previously described (7) with the following new modifications. About 2 millicuries of I^{131} (as NaI) (half life, eight days) [3] were added to 5 mgm. of carrier iodine (as a KI solution) and diluted to 15 cc. with distilled water. One cc. of this solution was diluted 100,000 times with distilled water as standard.

The remaining 14 cc. was mixed with 25 cc. of carbon tetrachloride and to this mixture was added 20 mgm. of potassium iodate and 0.1 cc. of concentrated hydrochloric acid. Free iodine was extracted by the carbon tetrachloride, which was separated and added to 500 cc. of plasma (obtained from fresh heparinized dog's blood) containing 20 cc. of 25% sodium carbonate. The violet color of the carbon tetrachloride solution was rapidly discharged on shaking the mixture, which was then allowed to stand for 20 minutes, after which the carbon tetrachloride was separated. The iodinated plasma proteins were then dialyzed in a cellophane bag against cold running tap water for 72 hours to remove unbound ionic iodine. The plasma, now cloudy, was cleared by adding a few grams of sodium chloride and 5–10 cc. of 25% sodium carbonate solution. The volume was measured and 1 cc. was diluted up to 100 cc. and the radioactivity of 1 cc. of this dilution was determined to calculate the per cent of radioactivity incorporated. The pH of the solution was adjusted to 7.4 (glass electrode), with dilute hydrochloric acid. The plasma was then sterilized by Seitz filtration and stored in lead-shielded sterile bottles at 4° C.

Ten to 14% of the radioactive iodine utilized was incorporated in the plasma proteins. The greater the iodine content of iodinated protein, the greater the rate of disappearance from the circulating blood after injection. The iodo-proteins we produced contained about 0.02–0.03% iodine. This amount is well below the upper limit of iodine content required to avoid exceeding the lowest possible degree of denaturation in a protein molecule so tagged. "If one atom of iodine had been incorporated per average protein molecule, the plasma proteins would have contained about 0.2% of iodine, which is the theoretical upper limit of iodine content for obtaining the slowest possible rate of disappearance of a halogenated protein from the circulating plasma" (7), following intravenous injection of iodinated plasma proteins.

Data concerning the stability of the iodine linkage in iodinated plasma protein have been presented previously (7). Examination of a blood specimen, taken 23 days after a dog had been given an injection of radioactive plasma protein subcutaneously, revealed the total absence of radioactivity in the protein free filtrate of the plasma, although considerable radioactivity was present in the plasma. Apparently the radioactive iodine was still bound to the protein.

Animal experiments

Mongrel dogs were given morphine sulfate (2 mgm. per kgm.). Normal oxalated plasma was obtained for preparation of a standard, and the dog then received a

[1] Aided by a grant from the Patrons of Research of the Beth Israel Hospital, Boston.

[2] The hyaluronidase used in these experiments was obtained as "Hyronase" through the courtesy of Schering Corporation, Bloomfield, New Jersey. The preparation used contained approximately 50 turbidity reducing units per mgm.

[3] Obtained from Monsanto Chemical Corporation, Oak Ridge, Tennessee.

TABLE I

Radioactivity in the circulating blood plasma in per cent of the total radioactivity injected *

Time in hours	Dog 1			Dog 2			Dog 3			Dog 4			Dog 5		
	Subcutaneous injection	Subcutaneous injection and hyaluronidase	Intravenous injection	Subcutaneous injection	Subcutaneous injection and hyaluronidase	Intravenous injection	Subcutaneous injection	Subcutaneous injection and hyaluronidase	Intravenous injection	Subcutaneous injection	Subcutaneous injection and hyaluronidase	Intravenous injection	Subcutaneous injection	Subcutaneous injection and hyaluronidase	Intravenous injection
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
1	0.3	1.0	50	2.0	1.0	49	1.0	2.0	53	1.0	2.6	63	1.0	0.9	49
3	0.7	1.4	46	3.1	2.0	48	1.5	3.2	46	1.0	2.8	52	1.2	1.6	47
5	0.9	1.8	43	4.1	4.1	46	2.0	4.0	41	1.1	3.5	49	1.3	2.2	45
8	1.5	2.5	37	5.0	14	43	4.5	6.0	38	2.0	6.0	46	1.6	3.9	41
12	2.0	4.0	30	7.1	16	39	11	19	33	4.0	9.5	44	3.8	7.0	36
24	4.0	9.0	25	17	27	28	18	32	23	14	27	32	10	15	28
36	8.3	14	22	19	27	26	19	35	19	19	31	29	14	22	21
48	10	17	20	20	25	24	19	33	16	20	30	24	16	26	21
56	12	18	18	18	24	22	17	22	16	22	29	21	17	24	20
72	11	15	16	17	20	19	13	21	15	18	26	17	14	23	18
96	10	12	12	15	17	17	11	19	12	15	21	13	13	21	16
120	9.0	9.2	11	13	16	14	8.5	15	11	13	19	12	12	19	15
144	8.2	9.2	9.9	12	14	13	7.5	13	10	11	17	11	9.0	17	13
168	7.5	9.0	9.1	10	9.9	14	6.5	12	8.5	9.5	16	10	6.5	15	13
192	6.8	7.2	8.2	9.0	8.0	11	5.9	10	7.5	8.5	14	9.5	6.5	13	12

* About six weeks elapsed between each injection in order to allow the radioactivity in the blood plasma to disappear completely.

subcutaneous injection in the thigh of 10 cc. per kgm. of sterile radioactive plasma proteins. Blood specimens for radioactivity determinations were then taken from the opposite leg at or about the following intervals, in hours: 1, 3, 5, 8, 12, 24, 36, 48, 72, 96, 120, 144 and 168. When the radioactivity of the plasma reached zero, usually at the end of about six weeks, the same animal received a subcutaneous injection in the opposite thigh of 12 TRU [4] of hyaluronidase in 10 cc. of sterile normal saline followed at once by 10 cc. per kgm. of sterile radioactive plasma proteins.[5] The injection was given slowly and gently into the subcutaneous space and without producing undue tissue pressure. The time required for the mass of subcutaneous fluid to disappear was observed. The thigh injected was immobilized for 12 hours to prevent the introduction of error due to motion or pressure.

When the radioactivity of the plasma again reached zero, the dog was given 10 cc. per kgm. of sterile radioactive plasma protein intravenously in order to observe its rate of disappearance from the blood. For this purpose, samples were drawn at about the same intervals mentioned previously.

[4] TRU—Turbidity reducing units.

[5] It has been observed that the spreading response induced by hyaluronidase is influenced by the enzyme concentration and the volume of injection (8). To employ the best conditions for the action of hyaluronidase, 240 μg. of the enzyme or at least 10 times that ordinarily recommended and a volume of plasma protein larger than the potential volume of the injection site were used. The thigh was used because of the tightness of the tissues in this area.

Because of the variation in the rate of absorption from dog to dog, it was found necessary to use the same animal for all three parts of the experiment. Five such experiments were completed. Similar experiments were carried out on two dogs in which radioactive plasma was injected intraperitoneally instead of subcutaneously.

Measurement of radioactivity

Plasma (1 cc.) was evaporated in an aluminum cup to dryness at 37° C. for 24 hours. The radioactivity of this preparation was compared with a standard which was similarly prepared after adding 0.2 cc. of the original radioactive plasma used for the injection to 1 cc. of non-radioactive plasma prepared from the blood sample taken from each dog just before it received the radioactive plasma.

All specimens were measured for radioactivity with a Tracerlab Autoscaler and bell type G.M. tube with mica window (4 mgm. per sq. cm.), shielded with 1.5 inches of lead. All readings were at least five times background. The percentage of injected radioactivity circulating in the blood plasma was calculated by the following formula:

$$\%\ \text{circulating radioactivity} = \frac{\text{activity in counts per minute of 1 cc. of plasma} \times \text{estimated plasma volume} \times 100}{\text{activity of 0.2 cc. of injected radioactive plasma} \times 5 \times \text{dilution factor}^{6} \times \text{number cc. injected}}$$

[6] In some cases it was necessary to dilute the radioactive plasma 100 times before preparing the standard with 0.2 cc. and 1 cc. of non-radioactive plasma.

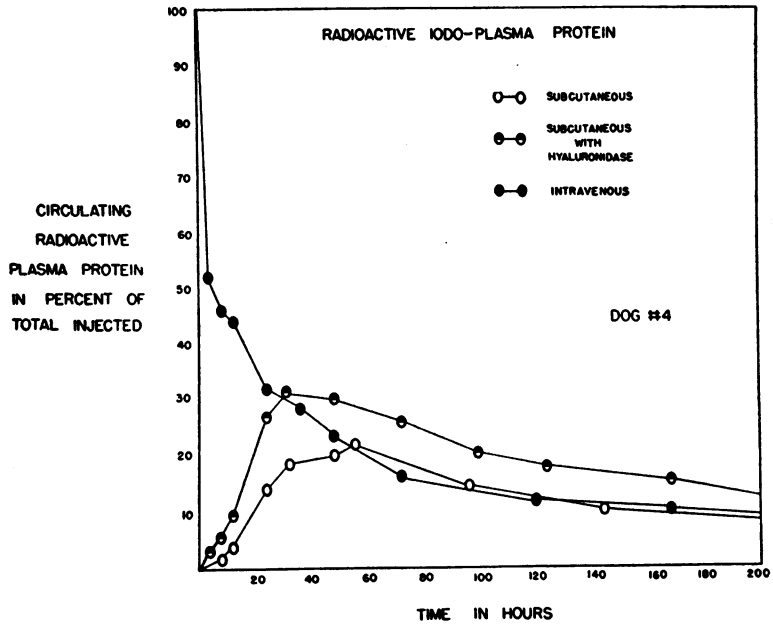

FIG. 1. RATE OF ABSORPTION INTO BLOOD OF INJECTED RADIOACTIVE IODO-PLASMA PROTEIN

The hematocrits of several dogs averaged 50%. Assuming the blood volume to be 10% of the body weight, the plasma volume was estimated to be 5% of the body weight. Table I shows the results of these calculations for all experiments. The results of the three series of observations in each experiment were plotted for comparison. In Figure 1 the disappearance of radioactivity from the plasma after administration by three techniques is shown for one dog.

It will be noted that the disappearance rate of radioactivity after an intravenous injection of radioactive iodo plasma proteins is faster than that observed by Fine and Seligman (7). This may be due to the fact that more denaturation of the plasma protein may have been produced with sodium carbonate treatment or by the use of HCl for the adjustment of the pH to 7.4.

In order to compare the quantity of radioactive plasma, with and without hyaluronidase, absorbed into the circu-

TABLE II

A comparison of the quantity of circulating radioactivity at various time intervals from zero after the subcutaneous injection of radioactive iodo plasma proteins (with and without hyaluronidase) with the quantity of the circulating radioactivity at similar intervals after the intravenous injection of radioactive iodo plasma proteins in the same dog * †

Time in hours	Dog 1		Dog 2		Dog 3		Dog 4		Dog 5	
	Subcutaneous injection	Subcutaneous injection with hyaluronidase	Subcutaneous injection	Subcutaneous injection with hyaluronidase	Subcutaneous injection	Subcutaneous injection with hyaluronidase	Subcutaneous injection	Subcutaneous injection with hyaluronidase	Subcutaneous injection	Subcutaneous injection with hyaluronidase
	%	%	%	%	%	%	%	%	%	%
24	6	12	22	41	17	30	12	25	11	16
48	18	36	40	65	40	62	30	58	29	44
72	28	46	53	74	57	75	47	75	41	63
100	36	56	59	80	65	89	56	88	50	75
120	41	58	69	88	68	95	60	90	53	80

* The data are given in per cent and were calculated by a planimeter determination of the area under the blood plasma concentration curve for each experiment at various time intervals from zero. The area under the curve after the intravenous injection is taken as 100% for each interval. See Figure 1 for an example of these blood curves in experiment 4.

† Several weeks were required between each injection in order to allow the radioactivity in the blood plasma to disappear.

lation from the tissues at any given time with the amount remaining in the circulation after an equal interval following an intravenous injection, the area under each curve for a given time interval was measured by means of a planimeter. The area under the curve of the intravenous injection was taken as 100% for each time interval. Table II presents these data, comparing the results among the various dogs.

RESULTS

The results tabulated in Table I show that in three dogs out of five the effect of hyaluronidase is noted within one hour after the subcutaneous administration of radioactive iodo-plasma proteins. As the concentration levels indicate, the presence of hyaluronidase increases the absorption of the radioactive plasma proteins into the circulation two to three times that without it. Within five hours, similar results are observed in four out of five dogs; within eight hours, similar results are observed in all dogs. Within 36 hours, the rising concentration of circulating radioactivity tapers off. Within 96 hours, the concentration of circulating radioactivity is the same as without the enzyme in two dogs, but is one and one-half to two times that without the enzyme in three dogs.

Similar conclusions are derived from a comparison of the quantity of circulating radioactivity at various time intervals from zero after the subcutaneous injection of radioactive iodo plasma proteins (with and without hyaluronidase) with the quantity of circulating radioactivity at similar intervals after the intravenous injection of the same amount in the same dog (Table II). In 24 hours after subcutaneous injections, the amount of circulating radioactive plasma proteins were twice as much in three, and one and one-half times as much in two dogs receiving hyaluronidase, as compared to five dogs not receiving the enzyme. This difference persists to a somewhat lesser degree for the duration of the experiment. By comparing these quantities of circulating radioactive plasma with that following an intravenous administration, it appears that within 120 hours, 90% of an injection of radioactive plasma protein was absorbed in four out of five dogs receiving hyaluronidase, whereas less than 70 % was absorbed after the same period when no hyaluronidase was given.

It was observed by Hechter (5) that the addition of hyaluronidase to a saline clysis allowed four

TABLE III

A comparison of the effect of pressure or hyaluronidase on circulating radioactivity at intervals after intravenous or subcutaneous injections *

Dog 2

Time in hours	Subcutaneous injection†	Subcutaneous injection with pressure†	Subcutaneous injection with hyaluronidase†	Intravenous injection†
1	2.0	0.4	1.0	49
3	3.1	0.7	2.0	48
5	4.1	1.5	4.1	46
8	5.0	2.2	14	43
12	7.1	7.0	16	39
24	17	15	27	28
36	19	21	27	26
48	20	26	25	24
56	18	27	24	22
72	17	25	20	19
96	15	23	17	17
120	13	16	16	14
144	12	16	14	13
168	10	13	9.9	14
192	9.0	12	8.0	11

* Six weeks between each experiment.
† Percentage of total radioactivity injected.

and one-half times as much fluid to run into the tissues in a given interval, while a given volume of fluid was taken up 12 times faster. These data do not necessarily reflect accurately the rate of absorption into the circulation. Figure 1 and Tables I and II show that hyaluronidase increases the rate of absorption into the blood of radioactive iodo plasma proteins given subcutaneously, one and one-half to three times. The increased rate for saline presumably would be greater. The increased rate of absorption is an indirect effect of the spreading action of hyaluronidase.

Without hyaluronidase the tension in the dog's thigh at the site of the injected plasma remains high for at least one hour and then diminishes slowly. The fluid mass disappears within four to seven hours. When hyaluronidase is added to the plasma proteins, the fluid mass from the beginning is not tense, but soft, and completely disappears within one hour. As expected, the discomfort due to tension is therefore avoided.

It may be conjectured that pressure may facilitate the spreading out and disappearance of a fluid mass just as hyaluronidase does. The data of such an experiment (Table III) were obtained when a dog turned itself so as to lie on the injected site. It will be observed that the peak of circulating radioactivity was not reached until 48 hours after that observed in a hyaluronidase experiment in the

TABLE IV

Circulating radioactivity after an intraperitoneal injection of radioactive iodo plasma protein

Time in hours	Dog IP. 1*		Dog IP. 2*	
	Intraperitoneal injection†	Intraperitoneal injection with hyaluronidase†	Intraperitoneal injection†	Intraperitoneal injection with hyaluronidase†
1	1.0	4.0	1.4	2.0
3	10	10	4.0	10
5	22	16	10	19
8	38	23	22	25
12	36	36	26	28
24	32	33	26	26
36	27	30	23	22
48	23	26	21	20
56	22	25	20	18
72	20	22	16	15
96	17	19	13	15
120	15	17	13	12
144	14	15	10	9.5
168	12	13	8.5	9.0

* Six weeks between each injection.
† Percentage of total radioactivity injected.

same dog. The peak level in both experiments was about equal but considerably greater than in the control. Pressure, therefore, by virtue of dissemination, increases the rate of absorption. The rate of increase presumably is related to the degree of pressure applied, but pressure seems to be less efficient than hyaluronidase as a spreading agent.

In three preliminary experiments, which are not included in this series, abscesses developed at the site of injection, probably because unsterile radioactive plasma proteins were used at that time. The repeated injection of hyaluronidase into these areas did not seem to increase the extent of infection.

Inasmuch as hyaluronidase is considered to be a protein-like substance, it might be expected to be antigenic. The repeated injection of 240 μg. in all dogs used in these experiments resulted in no evidence of anaphylaxis.

In two experiments, hyaluronidase did not increase the rate of absorption of radioactive iodo plasma proteins administered intraperitoneally (Table IV). This is consistent with the assumption that hyaluronidase acts by depolymerizing the intercellular cement substance.

CONCLUSIONS

1. Hyaluronidase increases the rate of absorption of plasma proteins injected subcutaneously, one and one-half to three times.
2. It is not antigenic in the doses used herein.
3. It acts more efficiently than pressure.
4. It does not increase the rate of absorption of plasma proteins administered intraperitoneally.

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