SPREADING PROPERTIES OF LEECH EXTRACTS AND THE FORMATION OF LYMPH

By ALBERT CLAUDE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATE 9

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Extracts of certain mammalian organs, notably the testicle, have the power to increase tissue permeability (1). The rapid fading out of the wheal of injection when such an extract has been injected contrasts with the persistence of the bleb which follows the introduction of a fluid not possessed of the spreading property. The way in which tissue permeability comes to be modified is not yet understood. The permeated skin shows minor histological changes, which are restricted to the corium, the epithelial components remaining unaffected. Injection of highly concentrated testicle extracts produces, in addition to the spread, a local edema (2). This fact suggests that the phenomenon of spread may be related in some way to the processes which control the formation and removal of lymph.

Heidenhain endeavored to prove that lymph was a product of secretion of the endothelium, and not the result of mere filtration through the vessel walls. He found that certain substances, for example, peptone, egg albumin, extracts of liver and intestine, but more especially, extracts of mussels, leeches, and crabs, would produce a lasting increase in the lymph flow though they had no effect on the blood pressure. He termed such substances lymphagogues of the first class (3). A second group of lymphagogues included substances which increased the lymph flow from the thoracic duct by raising the pressure of the blood. In view of the possible relationship between the spreading phenomenon and the formation of lymph, some of Heidenhain's lymphagogues have been tested. In a preliminary investigation it was noted that egg albumin failed to produce a spread on injection into the skin and that one make of commercial peptone

had a slight effect, whereas another was completely inactive. A number of lymphagogues of the second class, including concentrated solutions of sodium chloride and sugar, caused no appreciable spread. Leech extracts, on the other hand, had a marked effect and the analysis of this fact has provided the material for the present communication.

Material and Methods

Preparation of Leech Extract.—The medicinal leech was used as the source of the extract. In some tests the crop was partially emptied of the blood previously ingested by leaving the leech in contact with crystals of sodium chloride or with a concentrated salt solution. The bodies were washed thoroughly, first with tap water and then with distilled water. They were then cut into small pieces and passed once or twice through a Latapie masher. The resulting pulp was ground with sand and extracted with a volume of distilled water equal to six times the weight of the pulp. After centrifugation, the supernatant fluid was filtered through coarse paper. The total solid content of such extracts was about 12 to 18 mg. per cc.

The testicle extract used in some tests for comparison was prepared in the manner already described (2). The extraction was made with a volume of distilled water representing six times the weight of the pulp.

Determination of Spreading Power.—The power of an extract to spread was determined by measuring the area of diffusion 24 hours after the rabbit had been injected intradermally with the test solution mixed with India ink. The ink used as indicator was Higgins' India ink diluted 1 : 3 with water and filtered through a Berkefeld candle. The area of spread depends to a large extent on the individual permeability of the rabbit skin, which varies greatly from one animal to another. In an attempt to take these variations into account, the area of spread produced by the extract was divided by the area of spread produced by a control, in this case saline or Ringer's solution, giving an index of diffusion more or less independent of the individual permeability of the skin.

The relation which exists between the extent of the spread and the concentration of the factor in solution is not of a simple order. For instance, the area of spread normally produced by the undiluted extract may be reduced no more than one-half when a tenfold dilution of the extract is injected. This effect may be misleading when one has to compare the relative potency of different preparations obtained from the fractionation of an active extract. The difficulty may be partially obviated by testing repeatedly the solution at various dilutions and comparing the solutions giving equal spread. Such solutions may be assumed to contain an equal quantity of spreading factor.

Spreading Properties of Leech Extract.—Preliminary tests had indicated that leech extract increased the permeability of the skin

in a manner similar to that observed for testicle extract, but more markedly. The following experiments illustrate the comparative spreading power of the two agents.

For the tests 0.5 cc. of leech extract, prepared in the manner described above, and 0.25 cc. India ink indicator were mixed in the syringe and injected intracutaneously into the upper part of the flank of a rabbit. The first indication of the action of the solution was the lack of resistance as the injection proceeded, and the immediate flattening of the bleb thereafter. Dispersion of the ink particles through the dermis, a process which continued for several hours, was relatively rapid, the main direction of the spread being determined by gravity. 20 hours after the injection, the area of spread, calculated from four similar experiments, averaged 120.6 sq. cm. The spreading mixture was found to have reached the lower part of the abdomen, extending to the mid-ventral line, and as far back as the scrotum. The skin of the involved area was thickened and edematous.

TABLE I

Effect of Leech and Testicle Extracts on the Permeability of the Rabbit Skin

Extract tested	Area of spread of 0.5 cc. extract plus 0.25 cc. India ink indicator	Area of spread of 0.5 cc. saline plus 0.25 cc. India ink indicator (control)	Ratio of active spread to spread of control
	sq. cm.	*sq. cm.*	
Leech	120.6	5.0	24.1
Leech (1:50 dilution with saline)	15.7	4.1	3.8
Rat testicle	14.3	5.0	2.9
Bull testicle	18.3	5.0	3.7

Autopsy showed large amounts of a somewhat gelatinous fluid accumulated in the subcutaneous tissue of the lower part of the abdomen and of the testicle.

The results of four experiments are summarized in Table I. For comparison, the effects of the diluted leech factor, and of rat and bull testicle extracts, prepared in the same way and with the same proportion of fluid to tissue, are included in Table I. Fig. 1 illustrates the relative spreading power of leech and of bull testicle extracts. The

accumulation of fluid in the subcutaneous tissue, as a result of the injection of the leech extracts, would suggest that the leech factor, in at least one phase of its activity, affects the exchange of fluids in the living tissues.

The fact that the leech extract, diluted 50 times, is practically as active as the undiluted testicular extract would indicate that the spreading factor is 50 times more concentrated in the standard leech extract, or that the leech factor is a chemically different substance, endowed with a spreading power considerably greater than that of the factor from testicle. With this problem in view, the chemical properties of the leech factor have been investigated.

Chemical Properties of the Leech Spreading Factor

Experiments on Solubility.—The leech spreading factor is readily soluble in water. Aqueous extracts of different parts of the leech (head alone, or body separated from the head) were brought to pH 4.5 by the cautious addition of 0.1 N acetic acid. An abundant precipitate separated, and another precipitate was formed when the acid solution was neutralized by means of N NaOH. The results of the tests in the rabbit skin are given in Table II. It shows that, after removal of the two precipitates, no more than 10 to 20 per cent of the factor originally present was retained in the solution.

In the next experiment, an attempt was made to extract the factor directly with weak acid solutions. The pulp prepared by grinding the whole leech body was extracted with a volume of 0.1 N acetic acid equivalent to six times the weight of the tissue. A second lot was treated in the same way with 0.05 N acetic acid. The reaction of the two extracts after centrifugation and filtration was pH 4.0 and pH 4.4, respectively. The use of acid solution instead of water resulted in a marked reduction in the solid content of the extracts, which was 9.9 mg. per cc. for the 0.05 N acetic acid extract, against 18.2 mg. per cc. for the water extract. The spreading power of these preparations is given in Table II. It shows that the spreading factor is soluble in weak acid, but the water extract was regularly more active. However, the loss in spreading power seemed to be compensated by a parallel loss in inert matter. When the spreading power was compared in terms of dry weight, the acid extract appeared to be at least as active as the neutral extract, giving a spread of 9.6 sq. cm. per mg. of solids as compared to 7.6 sq. cm. for the water control.

The factor is not soluble in acetone. An aqueous extract was treated with acetone, and the precipitate collected and dried in air. The acetone filtrate was evaporated to dryness. Extracts prepared from the two fractions were tested for spreading power. The acetone soluble fraction was inactive, and no more than 40 per cent of the original activity was recovered from the precipitate. When the

results were calculated on the basis of dry weights, the extract from acetone precipitation was found to represent a purer product in that the spread obtained was 8.5 sq. cm. per mg. of solids, against 2.5 sq. cm. for the watery extract. The absolute loss in spreading power suggests that the factor is partially denatured by acetone.

Experiments on Filtration.—The leech factor readily passes a Berkefeld filter, but is completely held by a collodion membrane which retains proteins. The

TABLE II

Solubility in Weak Acid of the Spreading Factor of Leech Extracts

Material extracted	Solvent	pH of extract	Treatment of extract	Spread in the rabbit skin		
				Area of spread of 0.5 cc. extract plus 0.25 cc. India ink indicator	Area of spread of 0.5 cc. saline plus 0.25 cc. India ink indicator	Ratio of active spread to spread of control
				sq. cm.	*sq. cm.*	
Leech head	Distilled water	6.9	—	96.5	5.3	18.2
	" "	6.9	Brought to pH 4.5 then neutralized	51.0	5.3	9.6
Leech body (head excluded)	Distilled water	7.1	—	37.4	3.4	11.0
	" "	7.1	Brought to pH 4.5 then neutralized	22.1	3.4	6.5
Entire leech	Distilled water	7.0	—	65.8	1.5	43.8
	N:10 acetic acid	4.0	Made neutral	40.7	1.5	27.1
Entire leech	Distilled water	7.0	—	139.7	5.5	25.4
	N:20 acetic acid	4.4	Made neutral	95.0	5.5	17.3

fresh extract of the leech proved to have little diffusible matter, as shown by the fact that the dry weight was reduced only from 18.3 to 15.8 mg. per cc. after 5 hours dialysis in cellophane sacs, impermeable to proteins, on the shaking apparatus of Northrop and Kunitz (4). This treatment caused no reduction in the activity of the solution.

Inactivation by Heat.—A leech extract, prepared in the usual way, and diluted 1:2 with Ringer's solution, was heated by immersion in boiling water for 6 minutes. The resulting precipitate was removed and the clear supernatant fluid tested at various dilutions in the rabbit skin. The results are shown in Table III.

The fact that the area of diffusion of the heated extract at the lowest dilution was not greater than that of the unheated extract, 50 times more diluted, indicates that no more than 2 per cent of the original spreading power was left after the heating.[1]

The fact that 90 to 98 per cent of the spreading power is lost upon heating a crude extract at 95°C. for 5 to 15 minutes, was demonstrated repeatedly in additional experiments. The spread produced by the heated extracts was usually delayed and progressed slowly, as in the case of material of low grade activity.

TABLE III

Effect of Heat on the Spreading Power of Leech Extracts

Test No.	Solutions tested	Dilutions tested	Solids in solution	Area of spread of 0.5 cc. saline plus 0.25 cc. India ink indicator	Non-heated extract		Extract heated at 95°C.		Physical changes on heating
					Area of spread of 0.5 cc. solution plus 0.25 cc. India ink indicator	Ratio of active spread to spread of control	Area of spread of 0.5 cc. solution plus 0.25 cc. India ink indicator	Ratio of active spread to spread of control	
			mg. per cc.	*sq. cm.*	*sq. cm.*		*sq. cm.*		
1	Leech extract (1:6)	1:2	5.90	6.7	66.7	10.0	40.7	6.0	Precipitate formed
	" "	1:20	0.60	6.7	58.8	9.0	23.5	3.8	" "
	" "	1:100	0.12	6.7	39.2	5.8	14.8	2.2	" "
2	Leech heads extract (1:20)		5.0	5.9	41.0	7.0	31.2	5.3	Remains clear

Extracts prepared from the leech head are usually purer than those prepared from the whole body. In the next experiment, leech heads were extracted with a volume of water equivalent to 20 times the weight of the tissue pulp. This extract, after centrifugation and filtration through paper, was immersed in boiling water for 15 minutes. In this case no precipitate developed to complicate the interpretation of the test. The results recorded in Table III show that, even in the absence of flocculation, the spreading power of the solution was considerably reduced by heat, indicating a direct action rather than a secondary inactivation by adsorption on a precipitate.

[1] Incidentally, these results illustrate the point, discussed above, that the area of spread is not directly proportional to the concentration of the active factor.

Separation by Copper Sulfate.—In the repeated attempts to find a simple method for the purification of the leech factor, it was found that copper sulfate would precipitate large amounts of inert material without reducing the spreading power of the extract. A 0.5 per cent copper sulfate solution was added drop by drop to a water extract of leech heads until the reagent no longer produced further precipitation. After removal of the precipitate by filtration on paper, the clear solution was dialyzed against cold, distilled water on the shaking machine for 8 hours. Test showed that precipitation and dialysis had removed as much as 89.2 per cent of the solids of the original extract, the precipitation with copper having accounted for 60 per cent of the reduction. The final product, tested in the rabbit skin, showed a spreading power greater than that of the original extract. The relative

TABLE IV

Effect of Copper Sulfate on the Spreading Power of Leech Extracts

Solutions tested	Characters of extracts	Total solids	Spread in the rabbit skin		
			Area of spread of 0.5 cc. solution plus 0.25 cc. India ink indicator	Ratio of active spread to spread of control	Area of spread per mg. solids in solution
		mg. per cc.	*sq. cm.*		*sq. cm.*
Leech heads extract.....	Dark brown, turbid	14.7	93.0	17.0	6.3
Copper sulfate filtrate.....	Clear, colorless	5.5	110.4	20.0	20.0
Copper sulfate filtrate, dialyzed.	" "	1.6	94.6	17.1	59.0
Copper sulfate, 0.5 per cent solution (control).....	—	—	5.5	—	—

spreading power, expressed in terms of dry matter in solution, was 6.3 sq. cm. per mg. solids for the untreated extract against 59.0 sq. cm. for the purified fraction. The results are shown in Table IV.

The increase in spreading power, after treatment with copper sulfate, is difficult to explain unless we assume that the action of the reagent dissociated inhibiting elements from the spreading factor, or that copper sulfate reacted with it to form a more active compound. A 0.5 per cent solution of copper sulfate had no apparent effect on skin permeability.

In the foregoing tests the leech factor was found to be soluble in water and in weak acid, but was precipitated by acetone. It did not

pass collodion or cellophane filters, which retain proteins. There is evidence that the factor is inactivated by heat. All active fractions from the leech gave a positive diazo reaction, similar in this respect to the active fractions separated from testicle (5). In these general properties the leech factor resembles the testicular factor which, in a recent study, was shown to present the characters of proteins. Elucidation of the chemical relationship which may exist between the spreading factor from the leech and that found in mammalian organs must await further experiments.

Relation between the Leech Anticoagulating and Spreading Factors

The anticoagulating properties of leech extracts have long been recognized (6). The occurrence of a spreading agent in the same extracts brought up the question of the possible identity of the two principles. The following experiments represent an attempt to dissociate the spreading from the anticoagulating factor.

As the anticoagulating factor is assumed to originate in the buccal cavity and the pharynx (7), extracts of the different parts of the leech body were prepared and tested for the two factors. The head, taking in the first 10 segments, was the source of one extract and the rest of the body of the other. In some tests the body was prepared before extraction by opening the crop and washing out the contents. Tests were also made of extracts prepared from the latter and from the gonads. The ovaries, spermatophores, and the nine pairs of testicles were extracted together. Before testing, the crop content was diluted up to six times its volume with water. In all cases the tissue extracts were prepared as described above with a volume of water representing six times the weight of the tissue. The results of the tests in the skin of rabbits are shown in Table V.

It will be seen from Table V that the greatest spread was obtained from the isolated head and gonads, these two fractions being about equal in effect. The spreading power exhibited by the crude body extract was practically lost when, prior to mincing and extraction, the organs were removed and the internal wall was washed with a stream of water. Washing the surface of the sex organs before extraction removed also much of the spreading factor. It can be inferred from this that the muscular sheet of the leech body contains no appreciable amount of the spreading factor, and that even an important part of that present in the extract of the unwashed gonads

may come from another source. It seems unlikely that surface washing would remove the factor if it existed in the tissue. Body cavities and the sex organs located within the adjoining segments may conceivably be contaminated by the content of the pharynx when the head is removed. According to these results, the anterior digestive tract would appear to be the main source of the spreading factor as well

TABLE V

Effects of Extracts from Different Parts of the Leech on Skin Permeability

Tissues extracted	Characters of the extracts	pH	Total solids	Area of spread of 0.5 cc. extract plus 0.25 cc. India ink indicator	Area of spread of 0.5 cc. saline plus 0.25 cc. India ink indicator (control)	Ratio of active spread to spread of control
			mg. per cc.	*sq. cm.*		
Leech head	Light brown or greenish in color; clear or opalescent	6.9–7.1	13.1	91.2	5.0	18.2
Leech gonads	Light brown; opalescent	6.9–7.1	—	80.7	4.9	16.4
Leech gonads (washed)	Light brown; opalescent	6.9–7.1	7.9	41.6	7.4	5.6
Leech body	Dark brown to red in color; turbid	7.1	14.7	66.9	4.9	13.6
Leech body (washed)	Dark brown to red in color; opalescent	7.1	8.0	21.0	7.4	2.8
Crop content (mainly ingested blood)	Dark red; clear	—	—	20.9	4.0	5.2

as that of the anticoagulating factor. Other methods have been sought to separate the two.

Spreading and Anticoagulating Power of Leech Extracts.—In the next series of experiments the leech extracts were tested for both spreading and anticoagulating properties. The anticoagulating power was determined by adding 0.5 cc. of leech extract to 2 cc. of fresh rabbit blood and recording the time of clotting.

As shown in Table VI, the extracts most active in retarding blood coagulation were also those endowed with the greatest spreading power. This parallelism was maintained when the leech extracts were heated by immersion in boiling water for 6 minutes. Purification by precipitation with copper sulfate and dialysis enhanced the spreading and anticoagulating properties of the extract. These results would

TABLE VI

Spreading and Anticoagulating Power of Leech Extracts

Test No.	Solutions tested	Dilution of original extract with saline	Area of spread of 0.5 cc. solution plus 0.25 cc. India ink indicator	Clotting time of 2 cc. fresh rabbit blood plus 0.5 cc. test solution
			sq. cm.	*min.*
1	*Extracts from various sources*			
	Leech head	1:20	61.6	100
	Leech body	1:20	44.0	14
	Leech gonads	1:20	32.3	9
	Saline (control)	—	6.6	5
2	Leech head	1:50	33.0	20
	Leech gonads (washed)	1:50	11.4	2
	Leech body (washed)	1:50	10.6	1
	Saline (control)	—	5.5	1
3	*Effect of heat on activity*			
	Leech head extract	1:600	39.2	33
	Leech head extract, heated	1:600	14.8	16
	Ringer's (control)	—	6.7	4
4	*Effect of copper purification*			
	Leech body extract	—	31.6	24
	Leech body extract after copper precipitation, and dialysis	—	37.0	Fluid after 12 hrs.
	Saline (control)	—		

seem to favor the view that both effects are produced by a single factor. This opinion is not supported by the results given in Table VII.

In this experiment, commercial hirudin (6, 7) and fresh leech extracts were compared, the stock solutions being adjusted to contain 5 mg. matter per cc. The anticoagulating power of the hirudin preparation appeared to be even superior to that of the fresh extract of the

same concentration, whereas its spreading power was low and was practically abolished by 1:10 dilution. This indicates that the spreading and anticoagulating properties may exist independently in the leech extracts.

From the foregoing experiments it appears that the spreading and anticoagulating factors have practically the same distribution in the body of the leech, originating for the most part in the anterior digestive tract.[2] It is still uncertain whether a single substance is endowed with both anticoagulating and spreading properties, although the

TABLE VII

Spreading and Anticoagulating Properties of Leech Extracts and of Commercial Hirudin

Solutions tested	Total solids in solution	Area of spread of 0.5 cc. solution plus 0.25 cc. India ink	Clotting time of 2 cc. fresh rabbit blood plus 0.5 cc. test solution				
			10 min.	15 min.	2 hrs.	10 hrs.	20 hrs.
	mg. per cc.	*sq. cm.*					
Hirudin	5.0	19.5	−	−	−	−	+ Clot not contracted
Leech head extract	5.0	54.5	−	−	−	−	+
Leech body extract	5.0	47.0	−	−	+		
Hirudin (1:10)	0.5	6.5	−	−	+		
Leech head extract (1:10)	0.5	27.1	−	+			
Leech body extract (1:10)	0.5	16.3	−	+	Clot contracted		
Saline (control)	−	5.8	+				

existence of two factors appears to be probable. If two different factors are involved, they may be closely related chemically, a fact which would explain the difficulties encountered in their separation.

DISCUSSION

The equilibrium between tissue fluid and the blood is maintained by the capillary wall, acting as a semipermeable membrane, and the

[2] It is conceivable that the spreading factor is released together with the anticoagulating factor at the moment of the bite. Professor L. Delrez has brought to our attention the fact that the therapeutic application of leeches is sometimes attended by extensive suffusion of blood in the subcutaneous tissue.

interplay of physical forces which give rise to filtration, osmosis, and diffusion. The theory which would refer the formation of lymph to these factors, first formulated by Ludwig and Starling, has received the support of modern investigators, and the observations that have accumulated on the subject are best interpreted in terms of this hypothesis (8–10). Heidenhain made the observation that certain substances, when introduced into the circulation, had the power to increase the lymph flow from the thoracic duct without exerting any definite action on the blood pressure (3). At the same time he noted that the content of organic matter in the lymph was augmented. He held these observations to demonstrate a secretory function of the capillary wall. The interpretation of Heidenhain is not accepted now and the facts are taken to indicate that the permeability of the endothelium of the vessels has been increased. The intimate changes induced in the capillary wall by Heidenhain's lymphagogues of the first group, especially by extracts of leech and mussels, seem not to have been investigated. The effect of leech extract on lymph formation brings up the question whether the phenomenon of spread may be caused, at least in part, by a sudden increase in vascular permeability. An excess of fluid filtering through the capillary wall and flooding the tissue spaces may separate widely the components of the connective tissue where solutes or particles, if present, would be dispersed passively. Against the view that a local increase in the permeability of the vessels is the only factor conditioning the spread, is the observation that testicular (11) and leech extracts will spread, although to a lesser extent, in a fragment of skin separated from the body.

Azoproteins have been shown to spread when introduced into the dermis (12). Although the area of spread produced by the injection of leech extracts or azoproteins may be ultimately the same, there is evidence that the phenomenon is induced by a different mechanism in the two instances. During the active spread the skin injected with leech extract remains smooth and relatively flaccid, whereas that receiving azoproteins becomes thickened and appears to be under tension. The osmotic pressure of the azoproteins tested for spreading power was found to be considerably greater than that of the uncoupled proteins, which under the conditions of the experiments was negligible (12). The presence in the subcutaneous tissue of azoprotein solutions

of high osmotic pressure may cause large quantities of fluid to filter from the vessels into the tissue spaces, until an equilibrium is reached, without affecting necessarily the normal permeability of the blood capillary. As a result the tissue spaces would become distended temporarily by the excess of fluid, allowing a passive dispersion of the substances which may have been introduced along with the azoproteins. Further experiments may be necessary to test this possible mode of action of azoproteins.

SUMMARY

1. The injection of leech extracts into the skin increases its permeability, as shown both by the spread of fluid and of foreign particles through the dermis. The spread is followed some hours after the injection by more or less edema of the subcutaneous tissue.

2. A preliminary study of the chemical properties of the leech spreading factor indicates a similarity with the spreading factor prepared from testicle.

3. Attempts to separate the leech spreading and anticoagulating factors showed that the two have practically the same distribution in the leech body, extracts from the separated head being the most active.

4. It is undetermined whether two distinct factors are responsible for the spreading and anticoagulating properties of leech extracts. A chemical similarity is suggested by the fact that agents which affect the activity of one factor have a parallel effect on the other.

5. The mechanism of the spread produced by leech extracts and by other spreading agents is discussed.

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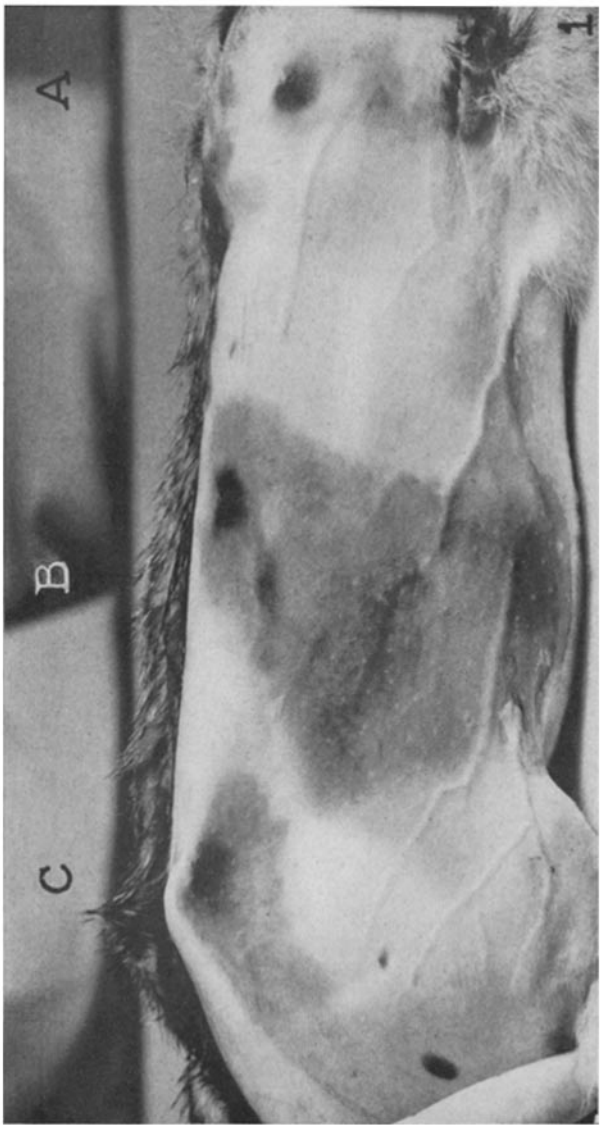
EXPLANATION OF PLATE 9

FIG. 1. Rabbit 9-99 (right side). The spread of India ink as influenced by: A, saline control; B, leech extract; C, bull testicle extract. The final measurements of the areas of spread were 6.6, 112.2, and 17.0 sq. cm., respectively. Under the action of the leech extract the ink particles spread extensively through the cutaneous tissue. Less than 24 hours after the injection, the mixtures of leech extract inoculated respectively on the two sides of the back had merged under the abdomen at the mid-ventral line. Ink particles were found accumulated along the abdomen as far back as the connective tissue of the scrotum. The subcutaneous tissue of the skin over the ventral surface of the abdomen was edematous.



Photographed by Joseph B. Haulenbeek

(Claude: Spreading properties of leech extracts)