

THE INFLUENCE OF TESTICLE EXTRACT ON THE INTRA-
DERMAL SPREAD OF INJECTED FLUIDS
AND PARTICLES*

BY D. C. HOFFMAN, M.D. AND F. DURAN-REYNALS, M.D.

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

PLATE 18

(Received for publication, November 13, 1930)

It has been shown by one of us (1, 2, 3) that extracts of certain organs of the body have an enhancing action on the development of vaccine virus and staphylococcus infections and that all organs studied can be placed in three groups according to the effect of their extracts. Group I comprises the testicle, kidney, brain and skin, the extracts of which always enhance the infections. Group II contains the adrenal, retina, ovary and the entire embryo, the extracts of which do not modify the infections. Group III includes blood, spleen, lymph nodes and bone marrow, the extracts of which tend to lessen or in some instances even to suppress the infections.

Of all the organs of the first group the testicle is by far the most active. The enhancing power of its extracts has been shown to extend to other infections, besides those above mentioned as demonstrated by Hoffman (4) with the viruses of herpes, vesicular stomatitis and Borna disease, by Thompson (5) in one observation with poliomyelitis virus and Pijoan (6) with 20 different bacteria. The active principle responsible for the enhancing effect has been referred to by Ledingham and Barratt (7) as the Reynals factor and for convenience the term will be used in this paper.

The nature of the action of testicle extract has not been understood, although there are observations in the literature which have a bearing on the interpretation of the enhancement phenomenon. Thus the stimulation of cell growth *in vitro* by testicle as well as by other organ or tissue extracts has been well established (8, 9, 10, 11). It

* A preliminary report of this work appeared in *Science*, 1930, 72, 508.

has been claimed that testicle or kidney pulp makes an excellent medium for the multiplication of viruses *in vitro* (12, 13), but later work has shown that the methods of preparation of the pulps employed did not eliminate living cells (14). The activity of testicle extract *in vivo* is demonstrated by its stimulating action on the healing of chronic refractory ulcers (15, 16). The lymphoid tissue, which seems to have the opposite effect from testicle extract on the virus infections is known to exert an inhibitory influence on cell growth (17).

Previous work (2) from this laboratory has shown that if testicle extract is injected intracutaneously and vaccine virus intravenously, the virus infection will be sharply localized to the skin area in which testicle extract has been injected. The phenomenon will be referred to later in the present paper, but it may be said in this connection that localization of various intravenously injected dyestuffs, notably methylene blue and trypan blue, also occurs in several pathological processes, as *e.g.* pneumonia, encephalitis, experimental tuberculosis and inflammation brought about by thermal agents, mustard oil, aleuronat, turpentine, etc. (Menkin, Friedheim and others (18, 19)).

An experimental observation has provided a clue for the investigation of the mode of action of the Reynals factor. The wheal resulting from the intracutaneous injection of mixtures containing testicle extract disappeared much more rapidly than those caused by mixtures not containing the extract. This suggested that the testicle extract may produce its effect by influencing tissue permeability. The present study was undertaken to test this possibility.

Methods and Materials

Organ extracts were prepared by grinding the tissue with sand and its own volume of Ringer's solution. The pulp was centrifuged and the supernatant fluid used.

In order to study the rate of spread after intracutaneous injection of testicle extract, or any other organ extract, it was essential to render the injected mass visible through the superficial epidermal layers.

For this purpose we employed in the preliminary tests a mixture of equal parts of iron ammonium citrate and potassium ferrocyanide, each in a 0.5 per cent aqueous solution. Injections of 0.5 cc. of the mixture plus an equal volume of rat or rabbit testicle extract, or of Ringer's solution as control, were made in different areas of the shaved skin of the rabbit. Thereafter, at intervals of 10 minutes, small pieces of skin were removed and fixed in a 10 per cent formalin solution which con-

tained 5 per cent HCl.* This precipitated the Prussian blue. Microscopic sections were prepared in the customary manner and counterstained very lightly with hematoxylin. In the later experiments recourse was had to India ink diluted 1:2 with Ringer's solution for with this material the rate of spread could be more accurately followed.

The diluted ink in quantities of 0.25 cc. was mixed immediately before injection with an equal amount of organ extract or with Ringer's solution as control, and injected into a number of different spots in the shaved skin of the same rabbit. 1 hour later the areas in which the ink had spread were measured and recorded. They could still be discerned after 24 hours or even later, and indeed a gray spot was well defined for weeks after, but no essential increase in the area involved occurred after 1 hour. In several cases the rabbit was killed at this time and the various injected areas were excised and fixed for histological examination. At first ordinary staining with eosin and methylene blue was used. Later we found that diffuse coloration with a 1:100 picric acid solution gave a background on which the ink particles contrasted sharply.

Influence of Testicle Extract on the Spreading of Iron Ammonium Citrate and Potassium Ferrocyanide

Five experiments were performed as described. In each the wheal caused by the injected mixture containing testicle extract flattened out and disappeared very promptly, and after a few minutes a blue discoloration of the skin alone remained. The wheal caused by the injection of the dye alone persisted in many instances for more than 1 hour. Microscopic examination of it showed a rather diffuse blue color throughout the intracellular spaces. There was much edema and at the edge of the section the Prussian blue had been precipitated in large quantities. In only a very few instances was there any suggestion of the dye having penetrated cells. On the other hand, the sections taken from the areas which had received the mixture with testicle extract showed a much fainter blue color, and most of them had granules or masses of the dye within the cells of the connective tissue (see Fig. 1). Only in one instance were the blue particles seen within epithelial cells, but not infrequently endothelial cells of the blood vessels were full of them. Sections of skin removed 30 to 40 minutes after the injection showed the greatest amount of Prussian blue within the cells. It was also noted that the dye very commonly was seen adhering to the cell membranes.

* All animals subjected to operation were etherized.

Influence of Testicle Extract on the Spreading of India Ink

Eleven experiments were performed with rat and rabbit testicle extract plus India ink. As shown in Chart 1, the mixtures of India

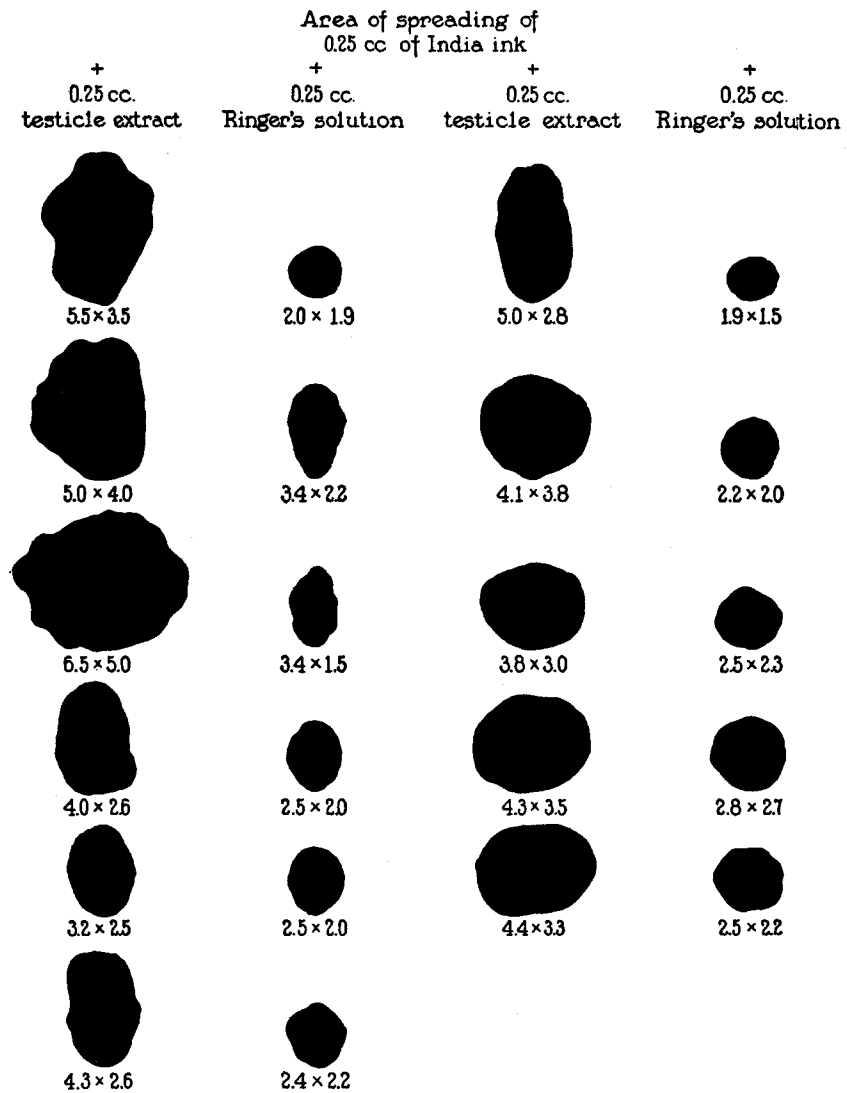


CHART 1

ink plus testicle extract had spread over a much larger area than was the case with the control material of India ink in Ringer's solution.

Sections were taken of both areas. In those receiving India ink plus testicle extract the ink particles were widely distributed, gradually thinning out towards the edges, and they could be seen far from the point of inoculation. The ink particles outlined the contours of the cells, sometimes indeed appearing to be within the cell protoplasm, but this can not be definitely affirmed without more refined histological methods (see Fig. 2). In the control areas the spreading of the ink stopped suddenly at a short distance from the injected locus and the particles were in general independent of the cells. In both cases, the ink spread only through the connective tissue spaces, the Malphigian layers, muscle bundles and hair follicles being free from it. In only rare cases, and where testicle extract had been injected, could some particles be seen between the muscle bundles, but none were observed to have penetrated the muscle sheath.*

Influence of Kidney and Spleen Extracts and Blood Serum on the Spreading of India Ink

The foregoing experiments reveal that the Reynals factor increases the spread through the tissues of both soluble and particulated matter. This property may be responsible at least in part for the enhancement of infections. The parallelism between the spreading and the enhancement phenomena was next tested by a study of the influence of other organ extracts. The results of the experiments are shown in Chart 2.

One can note in Chart 2 that extracts of organs belonging to Group I, the enhancing group, have an effect similar to that of testicle extract, but kidney extracts cause a less degree of spread and the results are not so consistent. Rat or rabbit blood serum, which has an inhibiting influence on infections, does not increase the spreading of India ink, but indeed sometimes slightly interferes with it. Spleen extract, which is also an inhibitor, in one test gave a slightly increased spread,

* In this connection it is of interest to note that testicle extract does not render healthy conjunctiva more permeable to methylene blue, when the mixture of dye and extract is instilled into the eye; nor is the wall of the digestive tract rendered more permeable to proteins, for animals fail to become sensitized when fed foreign proteins with testicle extract.

but in the others proved inactive in this respect. The comparative results are better seen in Chart 3 where the averages of the areas of spreading induced by different organ extracts are shown.

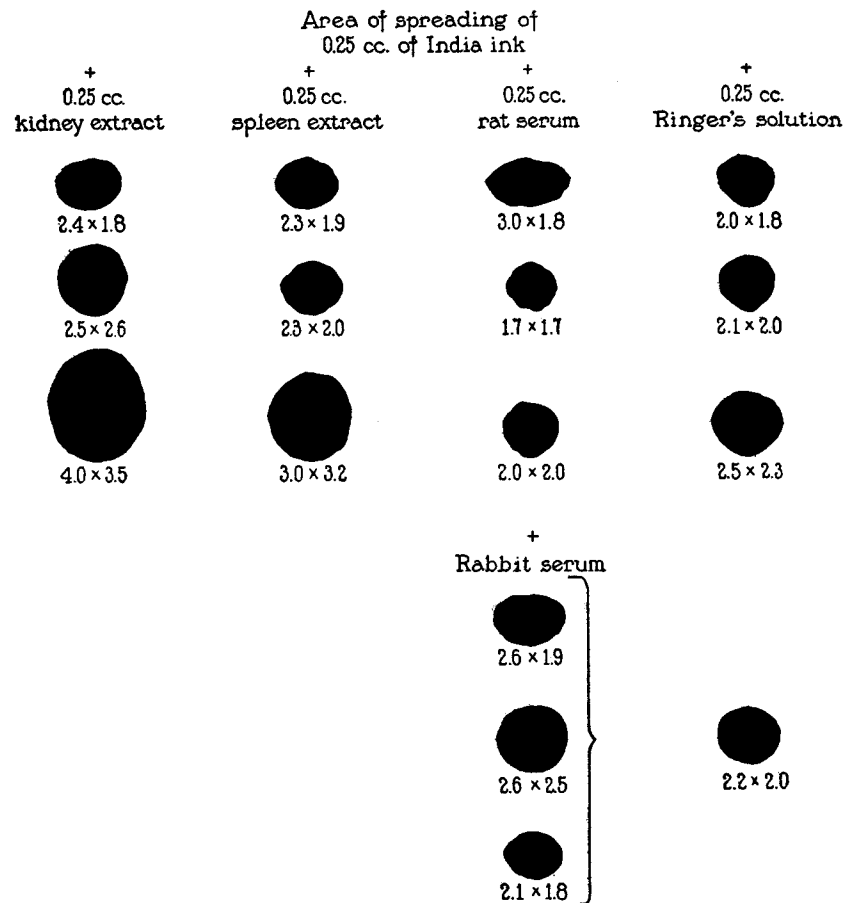


CHART 2

Influence of Testicle Extract on the Localization of Methylene Blue

As already remarked, methylene and trypan blue injected intravenously tend to come out especially into areas affected by pathological processes. The fact has a possible relation to the localization of vaccine virus in the skin area which has received an injection of testicle ex-

tract. For the next experiment dyes were injected intravenously into rabbits having testicle extract in the shaved skin.

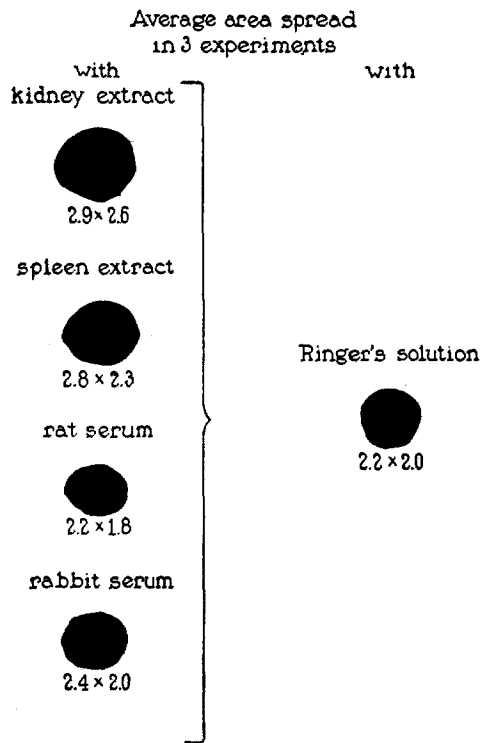
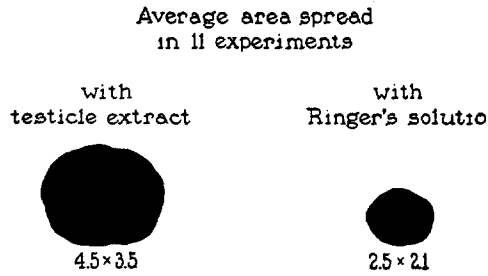


CHART 3

Four rabbits were injected intracutaneously with 1 cc. of testicle extract in two separate areas and immediately thereafter 10 or 20 cc.

of 1 per cent methylene blue was injected intravenously. Shortly after the dye injection, the treated skin areas turned faintly blue, but the color soon faded. When 20 cc. of the dye was injected the whole shaved skin together with the nose and lips of the animal exhibited a faint bluish color which was more marked, however, in the injected cutaneous areas. In some instances, after a few minutes, these areas became more blanched than the surrounding skin, owing probably to a reduction of the dye by the living cells. The basis for this

TABLE I

No. of guinea pigs	Amount tetanus toxin <i>cc.</i>	Amount rat testicle extract <i>cc.</i>	Ringer's solution <i>cc.</i>	Resultant local tetanus
1	2.64	0.5	—	++++ (Died in 5 days)
2	1.98	0.5	—	+++
3	1.32	0.5	—	+++
4	1.32	0.5	—	+++
5	0.66	0.5	—	+++
6	0.33	0.5	—	+
7	2.64	—	0.5	++++ (Died in 4 days)
8	1.98	—	0.5	++++ (Died in 6 days)
9	1.32	—	0.5	+++
10	1.32	—	0.5	+++ (Died in 24 hours)
11	0.66	—	0.5	+++
12	0.33	—	0.5	+++

supposition lies in the fact that when the blanched area of skin was removed and immersed in a solution of a hydrogen peroxide it showed a reappearance of the blue color. Control spots injected with serum or spleen extract did not show any greater coloration than the surrounding skin.

The Effect of the Reynals Factor on Toxins and Enzymes

We next attempted to determine the effect *in vivo* of the Reynals factor on toxins and enzymes.

Trypsin.—Two rabbits were injected into the shaved skin with 1.5 cc. of a mixture made by diluting Fairchild-Foster's trypsin to 1:100 and adding to it the same volume of rabbit testicle extract. Two other control areas received the same amount of the trypsin solution diluted with its own volume of Ringer's solution. The mixtures of trypsin and testicle extract spread immediately after the injection through a much larger area than the control material as shown by the disappearance of the wheal. The resultant lesion, however, was of the same extent in each of the four areas.

Tetanus Toxin.—In two experiments involving 12 guinea pigs, weighing from 200 to 300 gm., tetanus toxin was employed with an M.L.D. of 0.000066 cc. This toxin was diluted to 1:10,000. It was then injected under the skin in the inner part of the hind leg in the amounts shown in Table I, together with rat testicle

TABLE II

Experiment	<i>B. coli</i> toxin	Testicle extract	Ringer's solution	Size of resultant lesion
	cc.	cc.	cc.	cm.
1	0.5	0.5	—	5 x 3.5
	0.5	—	0.5	5 x 4
	1.0	1.0	—	5.5 x 3.8
	1.0	—	1.0	7.3 x 3.7
2	0.5	0.5	—	7.2 x 4.5
	0.5	—	0.5	8 x 3
3	0.5	0.5	—	6 x 6
	0.5	—	0.5	6 x 5.5
	1.0	1.0	—	9 x 6
	1.0	—	1.0	5.5 x 4

extract or Ringer's solution as a control. As Table I shows, not only was the effect of the toxin unenhanced but to a certain degree its action was inhibited.

B. coli Endotoxin.—A two weeks' old culture of *B. coli* in broth was filtered through a Berkefeld V filter and the filtrate was injected into the shaved skin of 3 rabbits, together with rat testicle extract. Control mixtures of the endotoxins with Ringer's solution were also injected. This endotoxin brought about in from 12 to 24 hours a reddish, elevated lesion which had clear-cut edges, easily measured, and which disappeared in from 3 to 4 days. That testicle extract did not increase the size of the lesion is shown in Table II.

Thus the three agents, trypsin, tetanus toxin and *B. coli* endotoxin, which were active by themselves but which are inanimate substances, were not enhanced in their activity by the Reynals factor despite the

fact that their spreading, as shown by the rate of disappearance of the initial bleb, was affected.

The Influence of Heat on the Spreading Power of the Reynals Factor

We have previously determined that the enhancing influence of testicle extract on vaccine virus is lost when the extract is heated to 60° for half an hour. A test was not made of whether heating would interfere with the spreading of India ink. A rabbit was injected in two areas with each of the following mixtures: India ink plus its volume of fresh testicle extract, India ink with the extract which had been heated to 60°C., the ink with extract heated to 80°C. for 30 minutes, and as control the ink diluted with Ringer's solution. A spreading over four times the area of the control resulted from the use of fresh testicle extract, whereas mixtures containing the heated extracts resulted in no greater spread than in the case of the control. Evidently heating to 60° destroys not only the power to enhance a virus but that to increase the spread of India ink.

Relation of the Reynals Factor to the Spermatogenic Cells of the Testes

Some earlier observations have shown that only a very feeble enhancement of infections if any results from extracts prepared from cryptorchic testicles in which the spermatogenic function is practically absent, but which are known to be rich in interstitial tissue. Later experiments, comprising four tests, have revealed that extracts of the epididymis from rat or rabbit have as great an enhancing effect as testicle extract. In one test rabbit sperm as such was found to contain a considerable amount of the enhancing factor. We believe that this is evidence in favor of correlating the Reynals factor in the testicle with the sperm and with actively dividing spermatogenic cells. That this factor is not the same as the male sex hormone is shown by the absence of any virus enhancing power in lipid testicle fractions highly active in provoking the growth of the comb and wattles in castrated roosters.* Work is now under way by one of us to ascertain the nature and location in the tissues of the enhancing material.

* We wish to express our indebtedness to Dr. Koch of the University of Chicago for supplying us with his highly active lipid preparation.

DISCUSSION AND CONCLUSIONS

The experiments in this paper show that testicle extract causes India ink particles and those of Prussian blue to spread much more extensively through the intercellular spaces than similar suspensions made with Ringer's solution. Methylene blue inoculated intravenously localizes more extensively in areas previously injected with testicle extracts than in control areas receiving injections of tissue extracts without enhancing power. Kidney extracts have this property to a less degree, whereas spleen extracts and blood serum are devoid of it. The spreading power of extracts is destroyed by heating at 60°C. for 30 minutes, as is also the power to enhance infections. The precise mode of action of the Reynals factor is not known, but the results of the experiments here presented suggest that it may depend at least in part on the property whereby testicle extract increases the spread of injected material and alters the permeability of tissue cells. It is not inconceivable that changes in permeability facilitate the passage of vaccine virus through the endothelial cells of the blood and lymph vessels, and lead to the generalized vaccinia which is of frequent occurrence in the reported results (20).

It has been shown that fluids and suspensions of inert particles are spread by the extract. *B. tetanus* and *B. coli* exotoxins and trypsin were not enhanced at all in their action despite the fact that they were spread through a more extensive area in the tissues. Viruses, on the other hand, are markedly influenced and in this respect resemble bacteria, not toxins and enzymes. It appears probable that a definite capacity for multiplication on the part of an injected substance is required if its pathogenic effects are to be enhanced. It may be concluded tentatively that the enhancing power of the testicle extract may depend on that property which not only spreads the injected material through a larger area but renders the tissue cells more easily penetrable by the agents.

BIBLIOGRAPHY

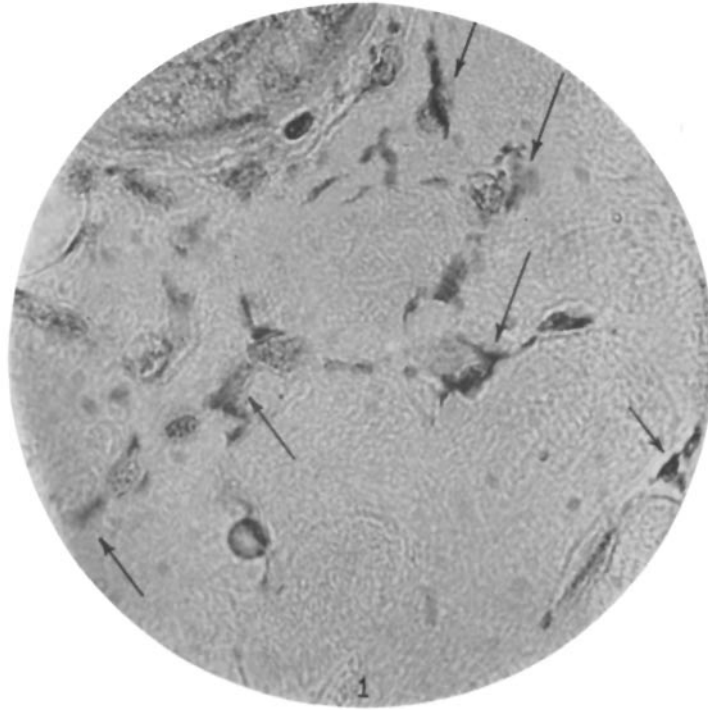
1. Duran-Reynals, F., *Compt. rend. Soc. biol.*, 1928, 99, 6.
2. Duran-Reynals, F., *J. Exp. Med.*, 1929, 50, 327.
3. Duran-Reynals, F., and Suñer-Pi, J., *Compt. rend. Soc. biol.*, 1928, 99, 1908.
4. Hoffman, D. C., *J. Exp. Med.*, 1931, 53, 43.
5. Thompson, R., *J. Exp. Med.*, 1930, 51, 777.

6. Pijoan, M., *J. Exp. Med.*, 1931, 53, 37.
7. Ledingham J. C. Y., and Barratt, M. M., *Lancet*, 1929, 2, 515.
8. Carrel, A., *J. Exp. Med.*, 1913, 17, 14.
9. Carrel, A., and Ebeling, A. H., *Compt. rend. Soc. biol.*, 1923, 89, 1142.
10. Drew, A. H., *Brit. J. Exp. Path.*, 1922, 3, 20; 1923, 4, 46; *Lancet*, 1916, 2, 204, 785.
11. Zakrewski, Z., *Z. Krebsforsch.*, 1929, 30, 106.
12. Maitland, H. B., and Maitland, M. C., *Lancet*, 1928, 2, 596.
13. Maitland, H. B., and Laing, A. W., *Brit. J. Exp. Path.*, 1930, 11, 119.
14. Rivers, T. M., Haagen, E., and Muckenfuss, R. S., *J. Exp. Med.*, 1930, 50, 181.
15. Rho, F., *J. Trop. Med.*, 1930, 33, 103.
16. Nagell, H., and Langhaus, J., *Münch. med. Woch.*, 1930, 77, 450.
17. Murphy, J. B., The lymphocyte in resistance to tissue grafting, malignant disease, and tuberculous infection. An experimental study. Monograph of The Rockefeller Institute for Medical Research, No. 21, New York, 1926.
18. Menkin, V., *J. Exp. Med.*, 1929, 50, 171; 1930, 51, 285.
19. Friendheim, E. A. H., *Frankf. Z. Path.*, 1927, 35, 549.
20. Stewart, F. W., and Duran-Reynals, F., *J. Exp. Med.*, 1929, 50, 341.

EXPLANATION OF PLATE 18

FIG. 1. Subcutaneous area of skin. Testicle extract plus Prussian blue with light hemotoxylin counterstain. Showing cells which contain numerous granules of the blue as indicated by small arrows.

FIG. 2. Subcutaneous area of skin. Same magnification. India ink plus testicle extract with picric acid counterstain. Showing much the same phenomenon as Fig. 1, but in more marked form.



(Hoffman and Duran-Reynals: Testicle extract)