ON THE PHYSICOCHEMICAL REGULATION OF THE GROWTH OF TISSUES.

The Effects of the Dilution of the Medium on the Growth of the Spleen.*

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Plates LXXV and LXXVI.

INTRODUCTION.

Since the appearance of the work of Jacques Loeb, it is well known that the rate of growth of certain marine organisms is markedly influenced by the physicochemical conditions of the water. In alkaline sea-water, the fertilized eggs of the sea-urchin develop more quickly than in normal sea-water. When tubularia are deposited in hypotonic sea-water, their growth is considerably increased. The experiments of Loeb have demonstrated the extreme sensitiveness of living cells to variations in concentration of the hydroxyl and hydrogen ions, and the importance of the osmotic tension of the water for the growth of the organisms.

The growth of the tissues of mammals is probably controlled by the conditions of the interstitial lymph in the same way that the growth of the egg of the sea-urchin is influenced by the conditions of the water. The organic and inorganic components of the blood are doubtless the most important of the physicochemical mechanisms which regulate the growth of the body. Therefore it may be assumed that the facts discovered by Loeb in the lower marine organisms are the expressions of general laws which control the development of the tissues and organs of the higher animals as well.

Since the tissues, in their development, must adapt themselves to the morphological plan of the organism, their growth must be con-

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stantly regulated by some unknown factors. This regulation may be caused by certain chemical compounds contained in the blood and the interstitial lymph. Until this year, there was no method for studying the action of the changes in the composition of the blood on the rate of growth. It became possible by the development of the method of cultivation of tissues *in vitro*.

We assumed that the growth of tissues in normal plasma could be compared to the growth of the same tissue in the organism. Then we attempted to determine what physicochemical factors could activate the rate of growth. It was logical to suppose that a medium more efficient than normal plasma could be found, as the tissues in the organism certainly do not meet with the best possible conditions of growth. Thus, if the blood were the best possible medium, the cells would grow without restraint, the organs and tissues would lose their relative size and morphology, and the whole body would become monstrous. Therefore, it may be assumed that the power of growth is kept under constant restraint, that every organ is compelled to follow the morphological plan of the organism, and that normal plasma is far from being the optimum medium for the culture of normal cells. A better medium can probably be obtained easily by modifying in many ways the conditions of the plasma.

We began to study the variations of growth of a few tissues in plasma, the conditions of which had been modified. In this article, we shall describe only the modifications of the rate of the growth of the spleen, brought about by the modification of the dilution of the culture medium, and of its concentration in sodium chlorid.

METHOD.

Our method consisted in cultivating the spleen of adult chickens or of fourteen or sixteen day chick embryos in normal plasma and in plasma of which the osmotic tension had been modified. Although the growth of a culture may be influenced by many causes, nevertheless the results of a given series must be accurately compared. Therefore the details of the technique must be carefully established, as it is of great importance not to consider a merely accidental variation of growth as due to the composition of the plasma.

The rate of growth of a fragment of tissue, cultivated *in vitro,* may be influenced by causes that are inherent to tissue, or to the preparation and preservation of the cultures. The condition of the tissue, its size and thickness, the manner in which it has been cut, the period which elapses between the interruption of the circulation and the imbedding in plasma, the duration of the exposure to air, the degree and duration of the chilling, etc., may have an influence on the rate and extent of growth. The preparation and the preservation of the cultures, and some details of the technique, often affect the growth. The nature of the plasma, the size and thickness of the drop, the dimensions of the hollow slides, the hygrometric condition of the air, the time elapsing between the imbedding of the tissue in plasma and the sealing of the hollow slides, the temperature of the incubator, and the variations of temperature according to the different parts of the incubator, etc., may cause important variations. Unless great care is taken to eliminate, as far as possible, these sources of error, the results of the cultures in different media can not be accurately compared. It was, therefore, necessary to give greater precision to the technique, in order that the growth of the cultures in a given medium should be rendered uniform.

Preparation of the Plasma.—A large quantity of blood was taken from a chicken in order that several series of experiments could be made with the same plasma. The plasma was prepared by the ordinary method, and part of it was used for the control cultures. The other part was rendered hypotonic or hypertonic by the addition of distilled water or sodium chlorid. The hypotonic plasma was composed of normal plasma diluted with one fifth, two fifths, one half, three fifths, and four fifths distilled water. The hypertonic plasma was obtained by adding four volumes of normal plasma to one volume of a solution of 0.015, 0.02, 0.03, and 0.04 of sodium chlorid in distilled water. If we suppose that normal chicken plasma contained 0.008 sodium chlorid, the hypertonic plasma contained 0.0094, 0.0104, 0.0124, and 0.0144 sodium chlorid, respectively.

Preparation of the Spleen.—The spleen was taken either from an adult chicken, or, as was more generally the case, from a fourteen or sixteen day old chick embryo. Great care was taken to dissect

the tissue very rapidly as soon as the circulation was interrupted, or the egg opened, in order to diminish, as much as possible, the period of exposure of the tissue. A small fragment of spleen was divided into eight or ten smaller pieces of equal size. The pieces were rapidly deposited on the cover glass, and covered with the hypertonic or hypotonic plasma. One control culture in normal plasma was made at the beginning and at the end of each group. The conditions and the preparation of the tissues of the same group of cultures were almost identical, and the results could therefore be legitimately compared.

After the fragments of tissues had been covered with plasma, the cover glasses were quickly placed on hollow slides and sealed. If some of the cover glasses remained exposed to the air for a longer time than the others, the plasma evaporated and became more concentrated, and the results were modified. The amount of plasma and the dimensions of the confined atmosphere of the hollow slides must be the same for each culture of a group.

The cultures, divided into several groups of eight or ten slides, were deposited in the incubator. The location of the slides near the wall or near the door of the incubator may have an influence on the rate of growth. It is necessary that the temperature should remain exactly the same for each slide of a group.

The results were examined a few hours after the preparation of the culture. Cells of the fetal spleen started to migrate immediately, without any latent period, and after two hours a large area of densely packed cells could be seen around the original tissue. Often it covered all the culture medium in twenty-four or thirty-six hours. The rate of growth was appreciated by the changes of the dimensions of the ring of new tissue surrounding the original fragments. It is important to cut the tissue into as regular fragments as possible, in order to obtain an equal growth all around it. The peripheral part of the new tissue appeared then as a regular circumference, and it was easy to calculate the area covered by the cells which had wandered out from the tissue or which multiplied in the culture medium. It was, then, possible to know the rate of growth in the different media. Often several series of camera lucida drawings were made of the growing specimens.

The main source of error was changes in growth produced by an accident of technique. When the control cultures showed widely different conditions of growth, or when some of the fragments of tissues did not grow at all, the technique was considered defective, the variations of growth were interpreted as due to an accident, and the group which presented the irregularities was discarded. Although the different groups of a series can sometimes be compared, it is preferable to compare only the different cultures of the same group and their controls. Even by using these precautions, all sources of error are not eliminated, but they are greatly reduced. They can be suppressed only by comparing many experiments and discarding the exceptional results. As the technique is complicated, there is always the possibility of an accident. Conclusions must not be based on one group of experiments only; they must be controlled by the results of another group, for the cultures of tissues *in vitro* give comparable results only when the greatest care is taken to eliminate all technical errors.

EXPERIMENTS AND RESULTS.

Small fragments of the spleens of adult chickens and chick embryos were cultivated in normal, hypertonic, and hypotonic plasma.

1. *Cultures in Normal Plasma.*—The fetal spleen grew without a latent period. After two hours, the tissue was already surrounded by a thick crown of cells. The growth of adult spleen was very much slower and became apparent ordinarily after twelve hours. The cultures in normal plasma were used as controls for the cultures in hypertonic and hypotonic plasma.

2. *Cultures in Hypertonic Plasma.*—The spleen was very sensitive to the action of hypertonic plasma. The fetal spleen still grew in plasma containing 0.0094 sodium chlorid, but the growth was much less extensive than in normal plasma. In one case it was sixteen times smaller. In plasma containing 0.0104 sodium chlorid, it grew slightly, and not at all in the more hypertonic plasma. The adult spleen showed slightly different variations (figure 1, *a, b, c, d, e*). For instance, in series S_5 the growth in normal plasma was three hundred and fifty and three hundred and thirty, while the growth was three hundred and ninety-two in plasma containing

0.0094 sodium chlorid, sixty-three in plasma containing 0.0104 sodium chlorid, and nothing at all in the still more hypertonic plasma. The results observed in the other groups of cultures showed also that hypertonic plasma diminishes greatly the growth of the spleen. Nevertheless, some activation of the growth may be observed in cultures in slightly hypertonic plasma.

Similar results have been obtained in the cultivation of other tissues in hypertonic plasma. The growth was also retarded. The

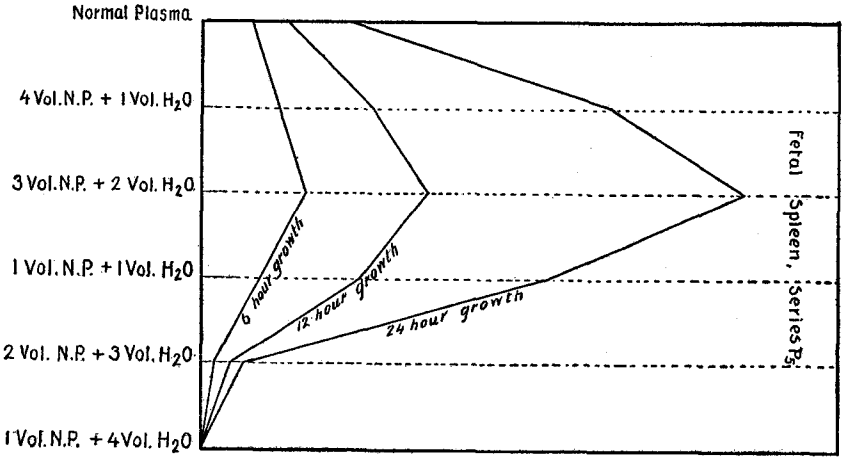

CHART I shows the variations in growth, at different periods after the preparation of the culture, of fragments of fetal spleen cultivated in normal and in diluted plasma.

skin reacted in a different manner and was considerably activated in slightly hypertonic plasma. It is probable that the sensitiveness of tissues to a hypertonic medium varies according to their nature.

3. *Cultures in Hypotonic Plasma.*—A slight dilution of the plasma always produced acceleration of growth. The growth of the spleen in plasma containing one fifth distilled water was very much greater than in normal plasma (figure 2, *a* and *b*). It was still greater in plasma containing two fifths distilled water (figure 2, *c*), and when the plasma was diluted still more, the area of growth diminished progressively; but in plasma containing one half distilled water, it was still larger than in normal plasma (figure 2, *d*). In plasma containing three fifths distilled water, fetal spleen

could still grow, although very much less than in normal plasma (figure 2, *e*). Adult spleen did not grow at all in the plasma containing three fifths and four fifths distilled water.

In the drawings (figures 1 and 2), in the chart, and in the table are summarized the action of hypotonic and hypertonic plasma on fetal and adult spleen.

Tissue.	Time of cultivation.	Normal plasma.	+ H_2O $\frac{1}{5}$	+ H_2O $\frac{2}{5}$	+ H_2O $\frac{3}{5}$	+ H_2O $\frac{4}{5}$	+ H_2O $\frac{5}{5}$
Fetal spleen	6 hours	1130	1760	2488	1318	329	Few cells
Series P_5	12 hours	2010	4005	5356	3732	716	Few cells
	24 hours	3540	9676	12741	7050	990	Few cells
Fetal spleen	4 hours	120 $\times\pi$	229 $\times\pi$	483 $\times\pi$	440 $\times\pi$	350 $\times\pi$	0
Series S_6	9 hours	960 $\times\pi$	2600 $\times\pi$	3430 $\times\pi$	2208 $\times\pi$	2115 $\times\pi$	0
Adult spleen							
Series S_5	24 hours	255 $\times\pi$	960 $\times\pi$	1520 $\times\pi$	168 $\times\pi$	0	0

Analogous results were observed when the skin, heart, and liver of chickens were cultivated in hypotonic plasma. In the experiments made in this laboratory by Dr. Ruth on the cicatrization *in vitro* of cutaneous wounds of adult frogs, it was also found that plasma containing one half distilled water accelerated verv much the epithelial growth.

CONCLUSIONS.

It may be concluded that the degree of dilution of the culture medium has a marked influence on the rate of growth of splenic tissue. The maximum acceleration was obtained in a medium composed of three volumes of normal plasma and two volumes of distilled water. The growth in this hypotonic plasma was very much larger than in normal plasma. On the contrary, the growth of the spleen in hypertonic plasma was always less than in normal plasma.

In other experiments, we found that in diluted plasma there was also an acceleration of the growth of the skin, the heart, and the liver of chickens. The skin of adult frogs also grew more actively in this plasma.

The optimum degree of dilution varied according to the nature of the tissues and to the species of the animals. While the plasma containing two fifths distilled water produced the largest growth of splenic tissue, a slightly less diluted medium was more favorable for the liver and the heart, and generally for the skin also. The

action of hypertonic plasma varied also in a large measure. While the spleen did not grow at all in the medium containing 0.0124 and 0.0144 sodium chlorid, the skin, on the other hand, could stand a high concentration of the sodium chlorid. Even its growth was activated in media containing 0.0094 and 0.0124 sodium chlorid and was greater than with normal plasma. The spleen of kittens was very easily affected by the changes of the dilution of the plasma, while the skin of the frog presented its best growth in plasma containing one half distilled water. Marked variations in the sensitiveness of tissues to hypertonic and hypotonic media will probably be observed in animals of different species.

From these experiments, three conclusions can be drawn: namely, that certain laws of growth, discovered by Loeb, in lower organisms are true also for higher organisms; that normal plasma is not the optimum medium for the growth of tissue; and that each tissue has probably its optimum medium.

The growth of the spleen is, without doubt, considerably modified by the variations of the dilution and perhaps of the osmotic tension of the plasma. It is possible then that the influence of osmotic tension, discovered by Loeb, in the growth of certain organisms, is a general law applicable as well to higher forms of life—frogs, cats, and chickens—as to lower organisms—tubularia and sea-urchins. In placing tubularia in different dilutions of sea-water and distilled water, Loeb found that the greatest rate of regeneration was observed when two volumes of distilled water were added to three volumes of sea-water. But fertilized eggs of sea-urchins were more sensitive to the action of hypertonic plasma, and they all died in a dilution of sea-water with two fifths distilled water. If only one fifth distilled water was added to the sea-water they developed normally. We found that the cells of certain tissues of the chicken follow a similar rule, since the maximal growth of the spleen is obtained in plasma containing two fifths distilled water, while other tissues grow better in a less hypotonic medium.

Normal plasma is certainly not the ideal medium for the growth of tissues, since slight modifications of the tension, the alkalinity, or the addition of certain inorganic salts to normal plasma, increase the rate of the growth of tissues.

It is possible, also, that the composition of an optimum medium would be different for each kind of tissue, and that no tissue meets inside of the organism with the best possible conditions for its development. If a tissue or an organ found in the body the best possible medium, it would grow indefinitely, reach an enormous size, and become a source of danger to the organism itself. Nevertheless, it would be very important to determine the composition of the medium that each organ and each tissue requires for its maximal development. Thus favorable conditions could possibly be given to a tissue temporarily, without interfering greatly with the nutrition of the other tissues of the organism. For instance, the peripheral part of a cut nerve often does not regenerate because a fibrous scar prevents the outgrowth of the axis cylinders from the central end. If the conditions of the interstitial lymph or of the culture medium which activate the growth of the nervous cells were known, we might accelerate artificially the rate of growth of the axis cylinders, and cause them to penetrate the peripheral end of the nerve before the formation of a scar, and thus promote regeneration.

It would, therefore, be of great value to determine for each tissue the medium which permits its maximal growth. Even if the accomplishment of this does not lead to any immediate practical application, the knowledge of the optimum conditions may lead to the discovery of some of the physicochemical mechanisms which regulate the development of the organs and compel them to comply with the morphological plan of the organism.

EXPLANATION OF PLATES.

PLATE LXXV.

FIG. 1. Camera lucida drawings showing the variations in growth, twenty-four hours after the preparation of the cultures, of fragments (*a, b, c, d, e*) of adult spleen cultivated in normal plasma and in plasma of which the concentration in sodium chlorid was increased.

PLATE LXXVI.

FIG. 2. Camera lucida drawings showing the variations in growth, twelve hours after the preparation of the cultures, of fragments (*a, b, c, d, e, f*) of fetal spleen cultivated in normal and in diluted plasma.

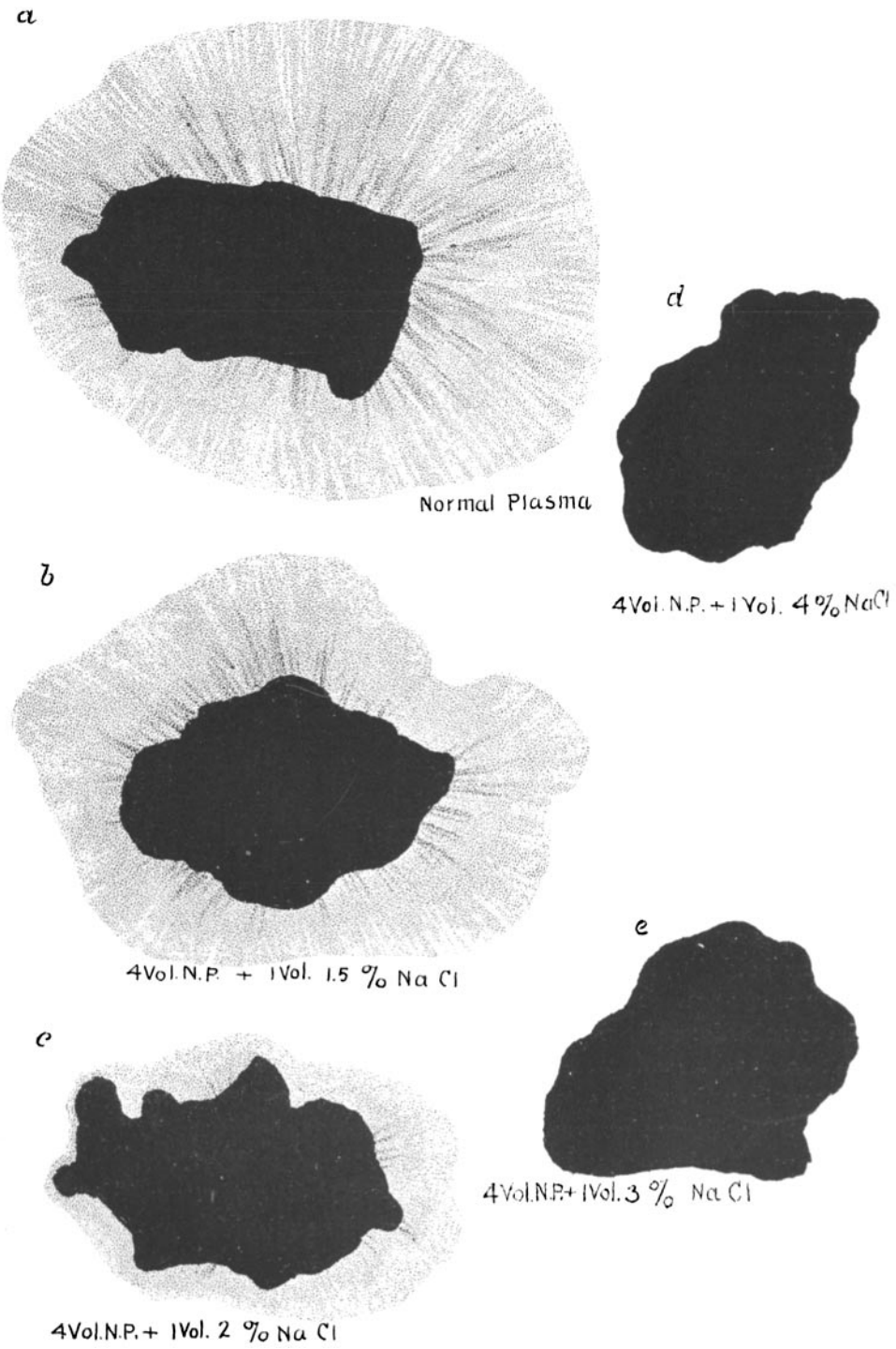
a
Normal Plasma
d
4Vol. N.P. + 1 Vol. 4 % NaCl
b
4Vol. N.P. + 1Vol. 1.5 % Na Cl
e
4Vol.N.P.+ 1Vol. 3 % Na Cl
c
4Vol.N.P.+ 1Vol. 2 % Na Cl

FIG. 1.

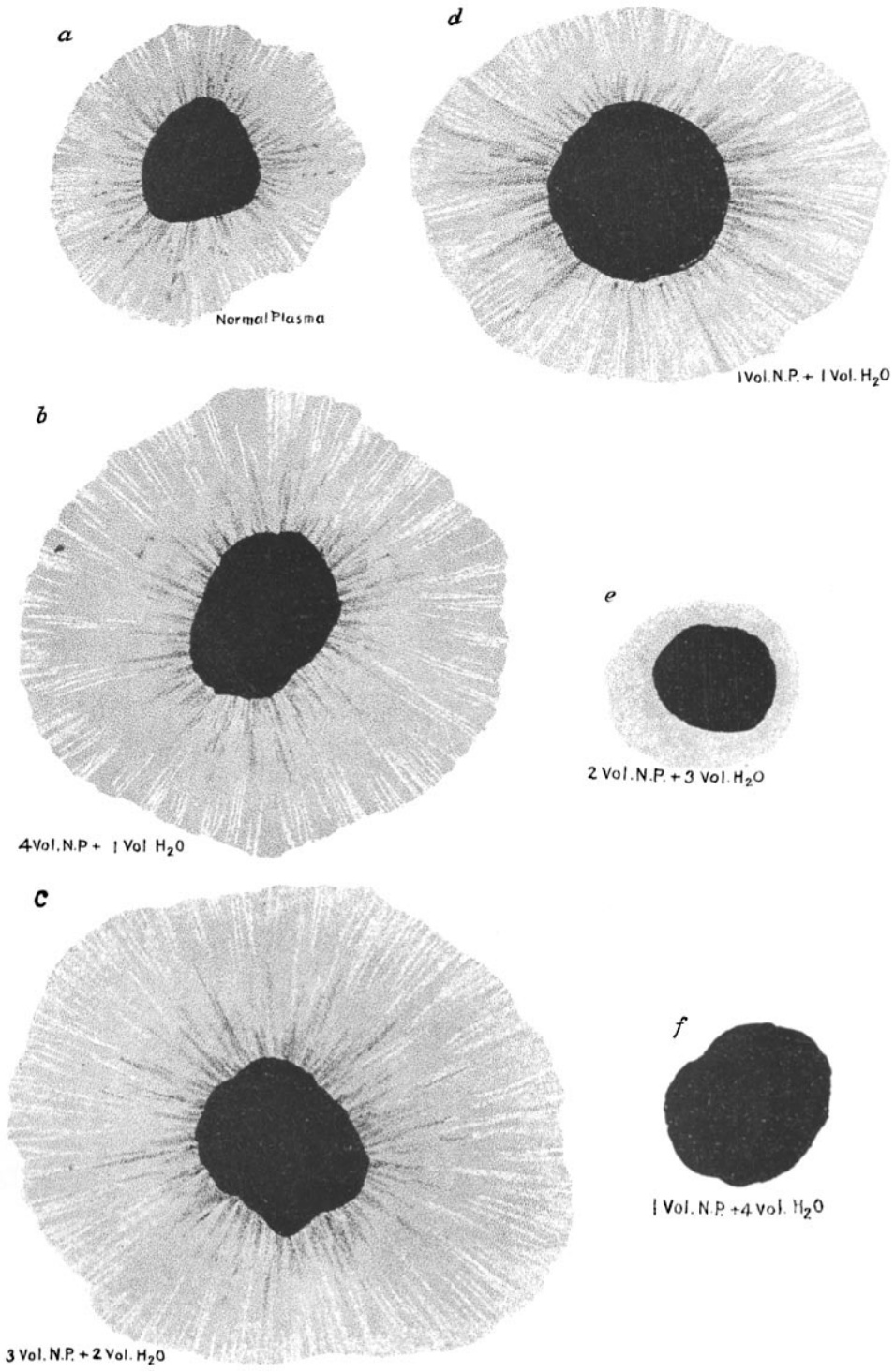
a
Normal Plasma
d
1 Vol. N.P. + 1 Vol. H_2O
b
4 Vol. N.P. + 1 Vol H_2O
e
2 Vol. N.P. + 3 Vol. H_2O
c
3 Vol. N.P. + 2 Vol. H_2O
f
1 Vol. N.P. + 4 Vol. H_2O

FIG. 2.