SEROLOGICAL TESTS FOR HOMOLOGOUS SERUM PROTEINS IN TISSUE CULTURES MAINTAINED ON A FOREIGN MEDIUM

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Plate 17

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The important question of the formation of serum proteins has been the subject of a number of investigations. Among these, the extensive studies of Whipple and his collaborators (1–4) should be mentioned especially. The authors brought evidence to show that the liver plays an important part in the production of plasma proteins, in particular albumin. This opinion is based on the results of several sorts of experiments, for example the effect of various types of liver injury such as that obtained by the establishment of Eck fistulae, and feeding experiments with dogs having low plasma proteins. The liver also is held by most investigators to be the organ responsible for the production of fibrinogen. Other workers suggest the participation of the bone marrow in the production of serum proteins. For a more complete analysis of the literature on the subject, the reader is referred to recent reviews by Whipple (5), Juergens (6*a*) and Reimann (6*b*).

Lately, an approach to the question by histological analysis has been made by Sabin (7), who based her thesis upon the relationship between antibodies and serum globulins. The production of antibodies, which are generally considered to be modified serum globulins, is usually attributed to the reticulo-endothelial system (*cf.* 8) and current hypotheses imply an intimate connection between the formation of antibodies and normal serum globulins (see 9, 10). It would follow, then, that normal globulins are likewise formed by this system. And by examination of tissues and exudates of rabbits during immunization with an artificial antigen having intense red color, Sabin obtained histological pictures which led her to conclude that antibodies are formed by the reticulo-endothelial cells.

In the hope of obtaining further information concerning the origin of serum proteins, an attempt was made to grow tissue cells in the plasma of a foreign species and to detect the presence of serum proteins by means of precipitin reactions with immune sera. Interesting experiments to deter-

mine whether tissues would keep their species specificity when cultivated in protein derived from foreign species have already been made by Kimura (11). This was the case in one series of experiments, but the author's results were not consistent, and the cultures were carried for too few generations to guarantee the complete removal of the serum proteins present in the original explants.

Methods and Materials

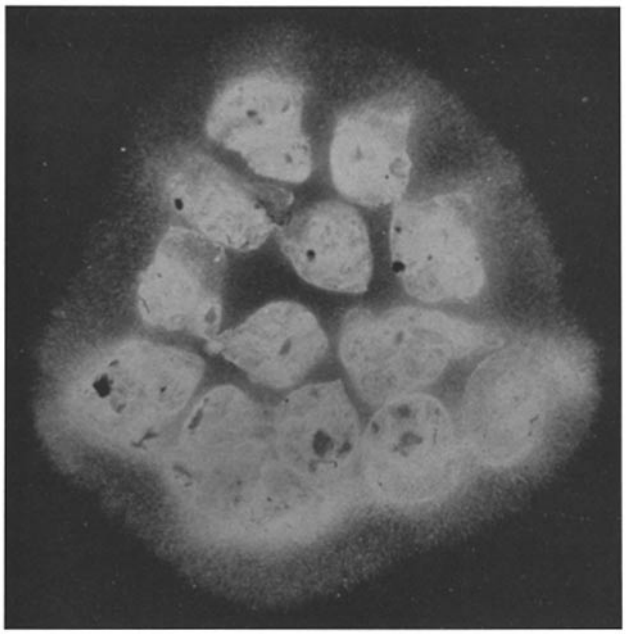
In the experiments to be reported, fragments of skeletal muscle from 12 day old chick embryos were cultivated for one passage (7 days) in chicken plasma and chick embryo tissue juice diluted with Tyrode's solution to which a small amount of phenol red had been added to serve as a pH indicator. On the 7th day, the tissues were transferred to a medium in which the chicken plasma and chick embryo tissue juice were replaced by rabbit plasma and rabbit embryo tissue juice; and once a week thereafter, for more than 7 months in one particular series, the tissues were transferred to fresh flasks and new rabbit medium. This medium was made up as follows: 0.6 cc. of freshly prepared rabbit plasma, 0.4 cc. of Tyrode's solution, 0.2 cc. of Tyrode's solution containing 0.05 per cent phenol red, and, to stimulate growth, 0.2 cc. of a 7.5 per cent solution of Witte peptone, and 0.6 cc. of 25 per cent rabbit embryo tissue juice.

The chicken tissue introduced into each flask consisted of from 15 to 30 small fragments that were placed in rather close proximity to one another in mosaic fashion. After a few days, living cells grew out from the individual fragments and filled in the spaces that had been left between them, thereby forming a plaque of living tissue with denser areas representing the tissue fragments that had been placed in the culture (Text-fig. 1). If, as occasionally happened, coagulation set in before all of the fragments could be properly arranged, those which became enmeshed in the coagulating plasma at too great a distance from the others were ordinarily lost to the experiment; these fragments usually digested the fibrin of the coagulum or else showed so little growth that they could not be transplanted. The cultures were gassed every day with a mixture of 80 per cent O_2, 3 per cent CO_2, and 17 per cent N_2.

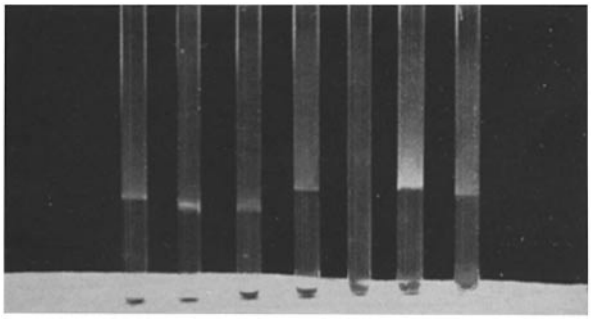
At the time of each weekly transfer, the plasma coagulum was loosened in its entirety from the flask and spread out as a disc on a glass plate. Then, with a cataract knife, the plaque of tissue was separated from the surrounding medium and cut into strips which, in turn, were divided into small fragments. These small fragments were now washed by placing them in a "glucosol"[1] bath for the short time prior to the preparation of the new cultures. Ordinarily, the amount of outgrowth obtained in 7 days from the transplanted fragments was just sufficient to fill the spaces between them, and to provide a narrow fringe of new tissue at the outer margin of the plaque. Due to several factors—the inevitable loss of tissue at transfers, the small amount of outgrowth obtained in 7 days, and the constant process of cell death and cell disintegration going on even in very active cultures—the volume of tissue handled from week to week did not alter to any great extent. As a result, the tissues were usually transferred serially from flask to flask without the preparation of plural subcultures.

From time to time, material was taken for the serological tests. As soon as the tissue

[1] Tyrode's solution with omission of sodium bicarbonate (12).



TEXT-FIG. 1. 4 day culture of connective tissue cells derived from the skeletal muscle of a 12 day chick embryo and cultivated for 16 passages (weeks) in rabbit plasma. The denser areas represent fragments cut from a culture in the preceding passage; these fragments are completely surrounded by new outgrowth. × $7\frac{1}{2}$.



TEXT-FIG. 2. Precipitin ring tests set up with culture fluids and rabbit immune serum for chicken serum. Tubes 1 to 4 show reactions with the following passages: 16th, 33rd, 31st, 9th, from four different strains. In the fifth tube, the fluid was obtained from 17th passage tissue killed by freezing. The sixth and seventh tubes show the reactions against chicken serum diluted 1:2000 and 1:8000 respectively.

fragments had been cut from the coagulum and placed in the glucosol bath, the remainder of the coagulum was chopped very fine, added to the fluid portion of the medium, and centrifuged. After centrifugation the supernatant fluid was saved for examination.

When the fibrin of the plasma coagulum became badly digested during the weekly intervals between passages, it was necessary to transfer the cultures on the 6th day in order to save them. As a rule, however, the cultures were left undisturbed for the full 7 day period.

New strains were started at various intervals after beginning the main series. Towards the end of the experiment, the various cultures represented strains that had been carried for 10, 16, 31, and 35 passages (weeks), respectively.

RESULTS

Histological examination of cultures after 9, 26, 30, and 32 passages showed the actively growing tissues to consist of a meshwork of fibroblasts, dense in some places, loose in others (Figs. 1 to 3). Scattered in these were foci of dense fibrous connective tissue containing few nuclei and occasional lime salt deposits. These areas constituted either degenerated remnants of some of the original tissue or of the cell growth of former generations.

Beginning with the 5th weekly passage, the fluids obtained from the cultures were examined by means of ring tests with immune sera obtained from rabbits after injections with chicken serum. These tests regularly gave definitely positive results, namely, sharp rings of turbidity which usually appeared soon and were well developed in the course of one hour. This is demonstrated in Text-fig. 2, which shows the reactions observed with various culture fluids. The more pronounced reactions corresponded in intensity to those made with chicken serum in dilutions of 1:5000 to 1:20,000, or were even stronger. On titration of the fluid, reactions were seen up to dilutions of 1:16 to 1:32.

There are several reasons for concluding that the precipitable substance demonstrated by the reactions was not carried over from the chicken plasma and tissue used in setting up the original cultures, but has been formed during the cultivation. The contrary assumption appears to be ruled out, first, by the high dilution resulting from the weekly transfer of a small quantity of tissue (with some adherent coagulum) into a relatively large quantity of new medium free from chicken protein. In fact, the ratio of tissue to medium in each culture was roughly 1:150, or more. Secondly, the reactions, although varying in intensity with different strains and passages—possibly due to variations in the rate of growth—did not diminish with increasing number of culture generations. Finally, when tissues (*i.e.* from various passages, beginning with the 9th) were killed by freezing or heating, and transferred to new medium in the same manner as the

living tissues, the fluid collected after a week's incubation gave negative or at most very faint reactions. In the experiments just mentioned, the fragments were alternately frozen and thawed three or four times with solid CO_2, or else heated for ½ hour at 55°C. From these results it is difficult to escape the conclusion that the tissues during their cultivation continued to produce substances which, according to their serological reactions, behaved like chicken serum protein. It should also be mentioned that the culture fluids did not react with anti-chicken-ovalbumin sera.[2]

Tests for Forssman antigen with anti-horse kidney immune sera gave faint precipitation with some, but not with all of these sera; the reactions were not proportional to the hemolytic activity of the sera for sheep blood. Thus, one strongly hemolytic serum gave negative reactions with the culture fluids. Furthermore, fluids from killed cultures showed the same faint reactions with horse kidney sera as did those from live cultures. These phenomena were clarified by the observation that the Witte peptone present in the medium used gave, with the anti-kidney sera, precipitation of the same (weak) intensity as the culture fluids. The reactions of the latter were evidently due to the Witte peptone.

It has been stated that the growing tissues in the cultures consisted of young fibroblasts; and while it may be unexpected that these cells should have the capacity of producing serum proteins, one may recall that fibroblasts are related to macrophages. In fact, macrophages have been observed to change into fibroblasts, and *vice versa* (12), and, as already remarked, there is evidence to indicate that the macrophages of the reticuloendothelial system are involved in the production of certain serum proteins. The present results, it would appear, are compatible with this hypothesis.

It has still to be considered whether the substances demonstrated in the culture fluids can be identified as serum proteins, as seems indicated by the sharp serological distinction commonly observed between diverse proteins from the same animal species. Non-proteins which would react with the immune sera employed are not known. If protein constituents of the cells are responsible for the reactions described, these must be very similar to serum proteins. As already mentioned, negative results have been obtained with fluids from cultures whose tissues had been killed by freezing or by heating. In order to carry further the investigation as to the nature of the substances involved, it will be expedient to prepare antisera for isolated serum proteins, as globulins and albumins.

In conclusion, it may be added that, in spite of cultivation of chicken tissues in a foreign plasma over an extended period, there was no apparent loss of species specificity.

[2] These sera were used after previous absorption with chicken serum with which they originally gave faint precipitin reactions.

The authors are indebted to Miss E. H. Tetschner, who assisted in the serological work, and to Miss A. J. Hollender for her aid in cultivating the tissues.

SUMMARY

Connective tissue cells (fibroblasts) derived from skeletal muscle of 12 day old chick embryos were cultivated for almost 8 months (35 weekly passages) in rabbit plasma and rabbit embryo tissue juice diluted with Tyrode's solution. When fluids separated from these cultures were tested with immune precipitins developed against chicken serum, they gave positive reactions which showed no tendency to diminish with an increasing number of culture generations. Barring the intervention of unknown precipitable substances, these results indicate that connective tissue can produce proteins which are identical with, or closely related to, serum proteins. The experiments further demonstrated that tissues cultivated in a foreign plasma do not lose their species specificity.

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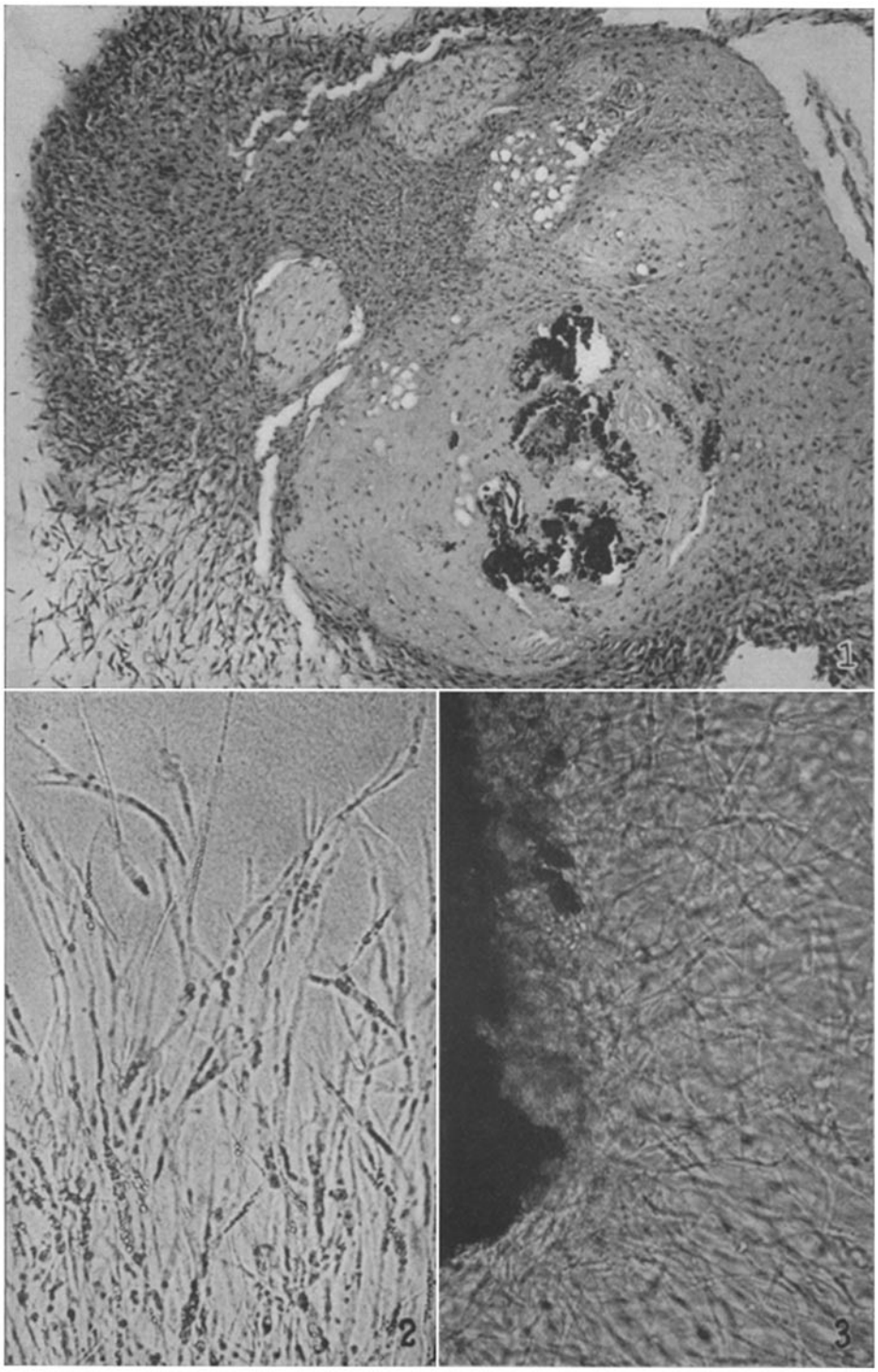
EXPLANATION OF PLATE 17

FIG. 1. Portion of a 6 day culture of connective tissue cells derived from the skeletal muscle of a 12 day chick embryo and cultivated for 30 passages (weeks) in rabbit plasma. Hematoxylin-eosin after Ringer-formol. × 115.

FIG. 2. Marginal outgrowth from a 3 day culture of connective tissue cells derived from the skeletal muscle of a 12 day chick embryo and cultivated for 29 passages (weeks) in rabbit plasma. × 200.

FIG. 3. New cellular outgrowth between neighboring fragments of a 3 day culture of connective tissue cells derived from the skeletal muscle of a 12 day chick embryo and cultivated for 31 passages (weeks) in rabbit plasma. The dense, marginal area represents part of a fragment cut from a culture in the preceding passage. × 200.



(Landsteiner and Parker: Homologous serum proteins in tissue cultures)