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A New Method for Demonstrating Cyto-Antibodies *in vitro*.*

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As observed by Fischer[1] 2 pieces of tissue culture that have been excised and transferred to a test tube containing a physiological solution will coalesce in their growth and form one spherical block of tissue. The same will happen with small fragments of living tissue, for example, pieces from the heart of a young chicken embryo, if they are brought into contact with each other.

This fact may be used to demonstrate the presence of factors which will promote or prevent this coalescence, for example, various drugs and especially antibodies against tissue cells.

The technic employed was as follows: Hearts from embryos 8 to 10 days old were used. They were divided with a cataract-knife into about 50 pieces of approximately equal size. Two of these pieces were placed in each tube. Each piece measured about 0.5 mm in diameter (from 0.35 to 0.65 mm, a majority having a diameter of about 0.5 mm). The glass tubes measured about 8 mm in external and 6 mm in internal diameter. They were 4 cm in height, with a round bottom, provided with a small diverticulum, measuring about 2 to 3 mm in diameter and about 2 mm in depth. The tissue fragments were placed in this small receptacle to secure contact and to prevent their being separated by currents that might arise in the fluid. The tubes were stoppered with corks of proper size. The whole procedure was carried out with aseptic precautions.

The medium here employed was a mixture of equal parts of chicken serum and Ringer's solution. Only 2 or 3 drops are needed for each tube.

The tissue fragments were placed in the tubes with a platinum wire or the delicate point of a cataract-knife. Contact of the fragments was ascertained by means of a magnifying lens (the reverse ocular from a microscope). If they were not in contact, they could easily be moved by tilting and tapping the tube a little. The tubes were then incubated at 39° C, and readings were made after 6, 12, and 24 hours. When the 2 pieces formed one spherical body without

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1 Personal communication.

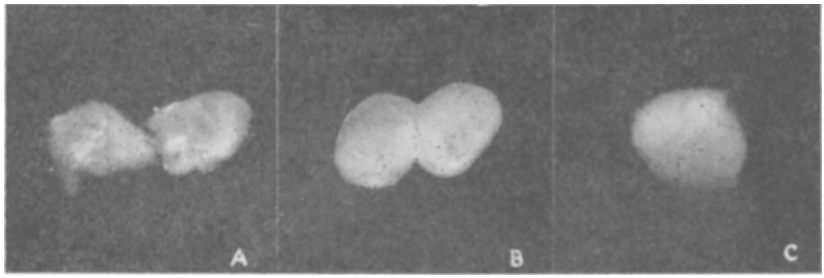

FIG. 1.
A. Tissue pieces before growth has begun. B. Coalescence is well under way (50%). C. Complete coalescence. The pieces were taken out of the tubes for better photographic reproduction.

any depression or indentation whatever it is designated as + or 100% coalescence. When there was substantial coalescence, but a definite groove could be discerned, where the 2 pieces met, it is designated as + —, or 66%. If the pieces were almost separate, but beginning to coalesce at a certain point, the result is called — +, or 33%. At this stage the pieces could not be separated by shaking the tube—a test that could be applied at the last reading only. With some experience in making the readings they may advantageously be graded more precisely, for example, in intermediate degrees too (Fig. 1).

In each experiment from 6 to 12 tubes were used in each series (experiments and control). Pieces from only one heart were used to exclude interference from individual differences in the hearts.

With this technic no visible reaction took place within 6 hours. After 12 hours they began to coalesce. After 24 to 48 hours they were partially or completely fused into one lump. The reaction is not very regular. Some of the tubes in a series may show + coalescence; others + — or — +; and some even —. As a rule the degree of coalescence after 24 hours estimated as described above is between 30 and 66%.

Simms and Stillmann[2] found that the latent period for growth *in vitro* of fibroblasts from the aorta of adult chickens was reduced by treating the tissue with "trypsin." Experiments with such a treatment were therefore carried out, in order to see whether it might influence favorably the inconsistency of my results and their reproducibility.

The "trypsin" used is a commercial preparation from Schering and Kahlbaum. A concentration of 0.2% in Tyrode's solution (pH

[2] Simms, H. S., and Stillmann, N. P., *J. Gen. Physiol.*, 1937, **20**, 603.

8.5 to 9) was used. The tissue fragments were placed in a water-bath at 37°C for from 15 minutes to 2 hours. They were then washed 3 times with Ringer's solution before they were put into the reaction tubes.

In Fig. 2 are shown the results from one of these experiments. The graphs are typical of a series of experiments carried out in the same way. There is a very pronounced difference between the "trypsin"-treated series and the untreated controls. In the former the coalescence has begun definitely within 6 hours and was completed, or almost completed, within 24 hours. Thus modified, the method yields results so uniform that it can be used to demonstrate the growth-inhibiting factors in a medium. It is evident that under the given experimental conditions a digestion period of 15 minutes only was sufficient to secure 100% coalescence in 24 hours. This finding does not permit the conclusion, however, that the optimal digestion period is 15 minutes. Treatment for 1 or 2 hours gave similar results.

A corresponding, preliminary incubation in Tyrode's solution alone did not bring about any difference in the percentage of coalescence.

As is well known, immune serum against tissue cells will inhibit the growth of the corresponding cells *in vitro*. (For references see

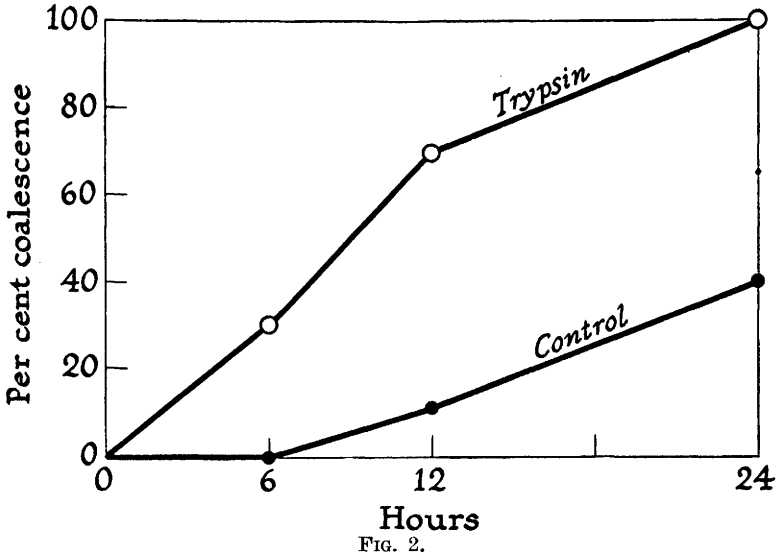

FIG. 2.

Shows the result of one experiment where the pieces in one series (marked Trypsin) were treated with "trypsin" for 15 minutes. The control series was treated with Tyrode's solution for the same length of time. For explanation of estimation of growth, see text.

Sigurdsson.[3]) Experiments were made to see whether the presence of such immune serum in the medium would prevent the coalescence of the tissue fragments. The immune serum produced in rabbits by injecting minced chick embryo inhibited the coalescence rather strongly, and in considerably lower concentrations than it inhibited growth in ordinary tissue cultures.[3] Normal rabbit serum did not have such an effect. The results from experiments with different concentrations of immune serum are shown in Fig. 3. It will be noted that immune serum in 10% concentration inhibits the coalescence completely; a concentration of 5% almost completely; and even 2% immune serum gives considerable inhibition.

This seems to be a simple and relatively reliable method for demonstration of cyto-antibodies and possibly other growth-inhibiting factors in a chosen medium.

Further it may be mentioned that heart fragments from 21-day-old chick embryos showed only a very poor tendency to coalesce. A brief treatment with "trypsin" (15 min.) alters this fact very little.

Many aspects of the method need further investigation: for example, the optimal intensity of "trypsin" treatment, the possible influence of embryonal extract in the medium, its applicability to pharmacological purposes, etc., but the work had to be discontinued.

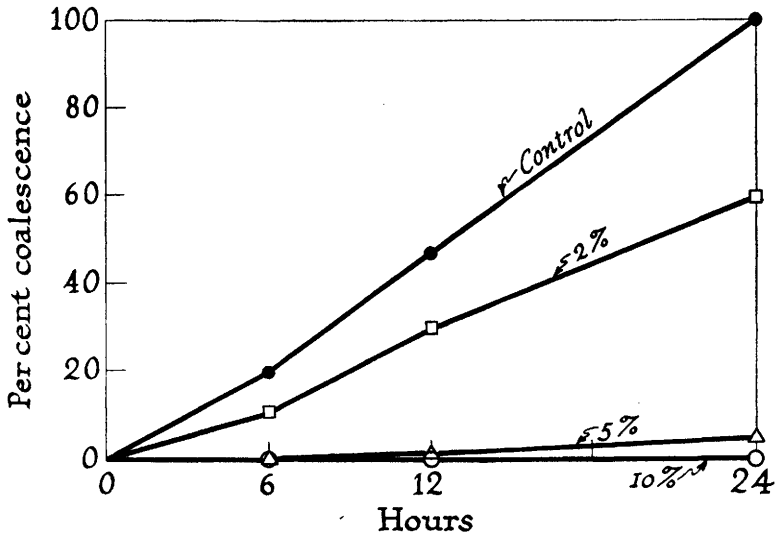

FIG. 3.

Rabbit immune serum against chicken tissue was added to the medium in the percentage indicated. All the tissue pieces had been treated with "trypsin." Control does not contain antiserum.

[3] Sigurdsson, B., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 237.

Summary. Small pieces of heart tissue from young chick embryos coalesce to form a spherical body when brought into actual contact with each other in a mixture of chicken serum and Ringer's solution. The pieces must touch each other. A preliminary treatment of these fragments with "trypsin" increases the rate and percentage of coalescence. The method is serviceable for titration of cyto-antibodies in a medium, as these inhibit the coalescence in low concentrations.

The method is described, and its use is demonstrated by examples.

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Effect of a Single Dose of 3,3′-Methylenebis (4-Hydroxycoumarin) upon Blood Coagulation in Humans.*†

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The pharmacological and chemical activity of 3,3′-methylenebis (4-hydroxycoumarin) has been reported from agricultural experiment stations.[1,2] The most recent publication[3] by the Wisconsin workers describes the effect of a single dose of this dicoumarin upon the prothrombin time of the plasma of various animals. In this communication we describe the effect of single doses of this drug on blood coagulation in humans. Prandoni and Wright[6] have reported on the effect of daily doses in man. However, the few re-

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5 Butt, H. R., Allen, E. V., and Bollman, J. L., *Proc. Staff Meet. Mayo Clinic*, 1941, **16**, 388.

6 Prandoni, A. G., and Wright, I. S., presented before the N. Y. section of the Soc. for Exp. Biol and Med., Nov. 20, 1941.