LIPOIDS AS THE GROWTH-INHIBITING FACTOR IN SERUM.

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INTRODUCTION.

Experiments of Carrel and Ebeling have shown that serum has a marked inhibiting action on the growth of fibroblasts *in vitro*, and that this inhibiting action increases with the age of the animal from which the serum is taken.[1] The chemical nature of the inhibiting substance is not known. Carrel and Ebeling have shown[2] that it is not euglobulin or associated with euglobulin, for the CO_2 precipitate from the serum of young animals is somewhat stimulating to the growth of tissues and the filtrate more inhibiting than the original serum. The present work was undertaken to throw light on the chemical nature of the substance. Since experiments on embryonic tissue juice have indicated that its lipoid is somewhat inhibiting to growth, a study of serum lipoids was undertaken. The lipoids of serum and the protein after extraction of the lipoids were tested for their growth-inhibiting properties, each being compared to the original serum and also to Tyrode solution as control.

The Method of Testing the Serum Protein and Lipoids.

The substances were tested with fibroblasts from a 12 year old strain, the two halves of the same tissue being cultivated, one in the experimental medium and one in the control medium, since it has been

[1] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317, 599; 1922, xxxv, 17, 647; xxxvi, 399; 1923, xxxvii, 759.

[2] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, xxxvii, 653; xxxviii, 419; *Compt. rend. Soc. biol.*, 1924, xc, 170, 172. Carrel, A., *Compt. rend. Soc. biol.*, 1924, xc, 1005.

demonstrated[3] that the two halves of the same culture of an old strain of fibroblasts grow at a uniform rate when the media are uniform. This is not true of embryonic tissue taken directly from an animal.

The technique of Ebeling[3] for measuring the relative growth of the tissues was used, the original area and the area after 48 hours being measured.

The media used were as follows:

For Serum Protein Compared with Serum or Tyrode.

Experimental medium.	Control medium.
1 drop plasma.	1 drop plasma.
1 " serum protein solution.	1 " serum or Tyrode.
1 " " " " containing 5 per cent tissue juice.	1 " " " " containing 5 per cent tissue juice.

The protein concentration of the serum and of the serum protein was determined by the micro Kjeldahl method and the two adjusted to contain the same per cent of protein nitrogen.

For Serum Lipoid Compared with Tyrode Solution or Serum.

Experimental medium.	Control medium.
1 drop plasma.	1 drop plasma.
1 " lipoid suspension.	1 " Tyrode or serum.
1 " " " containing 5 per cent embryonic tissue juice.	1 " " " " containing 5 cent embryonic tissue juice.

Action of Serum Extracted by Ether on the Growth of Fibroblasts.

Experiments were carried out in which the serum was extracted by ether and also by both alcohol and ether, care being taken to alter the protein as little as possible in order to insure that any change in the growth-inhibiting properties of the serum should be due only to the removal of the lipoid.

The method of extracting by ether found to be the most suitable was as follows:

The serum in a layer about 1/2 cm. thick was evaporated very rapidly in a vacuum desiccator over sulfuric acid by means of a Cenco Hyvac pump. All conditions of the evaporation were so adjusted that the serum supercooled and froze instantaneously. The evaporation was continued until the serum became anhydrous. The instantaneous freezing and drying in the frozen state left the protein of the serum in a very finely divided flaky condition admirably suited to

[3] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

extraction. It was then transferred to a Soxhlet extractor and extracted continuously for 48 to 60 hours with absolute ether freshly distilled over sodium. The extracted serum was finally freed from ether by evaporation *in vacuo* and dissolved in water. The per cent of nitrogen was determined; the proper adjustments of protein and salt concentration were made and the pH brought to 7.6, the reaction of the original serum.

The experiments on the growing tissues showed that in all cases the serum extracted with ether was less inhibiting to the growth of fibroblasts than the original serum, averaging 28 per cent less (see Table I), although it still retained inhibiting properties.

TABLE I.

Rate of Growth of Fibroblasts in Serum Extracted with Ether. Serum Used as Control.

Group* No.	Culture No.	Rate of growth in.		Ratio *E/C.*	Remarks.
		Serum (*C*).	Extracted serum (*E*).		
1	1814 A	2.53	3.09	1.22	48 hrs. growth.
2	479 C	5.95	6.7	1.12	48 " "
3	494 C	2.73	4.07	1.49	24 " "
4	"	5.52	8.59	1.55	48 " "
5	1824 A	2.29	2.52	1.10	24 " "
6	"	4.58	5.6	1.22	48 " "
Average				1.28	

* Each group constitutes three or four experiments.

Action of Serum Extracted with Both Alcohol and Ether on the Growth of Fibroblasts.

The serum extracted with ether alone still contained a considerable quantity of lipoid material no matter how long the extraction continued or how the serum was dried, whether alone or on absorbent material. A subsequent extraction with alcohol always removed a large quantity of lipoid which, after evaporation of the alcohol, was soluble in ether and of quite different character from the material extracted by ether alone. This was also true when the serum was continuously extracted without drying in an apparatus designed for

the ether extraction of liquids. Extraction with both alcohol and ether was therefore undertaken, the serum, ether, and alcohol all being kept at about −10°C. during the entire process. This is a modification of the method used by Young[4] in preparing crystalline serum albumin.

The serum at zero degrees was poured drop by drop with constant stirring into 3 times its volume of 95 per cent alcohol at −10°C., the alcohol being surrounded by a freezing mixture. Only alcohol freshly distilled over KOH to remove aldehydes was used. The mixture was allowed to stand from 1 to 3 hours at a temperature of from −10° to −14°C. It was then packed in a freezing mixture and centrifuged in a centrifuge cooled to 0°C. by circulating brine. The precipitate was washed four times with absolute alcohol, twice with a mixture of absolute alcohol and absolute ether, freshly distilled over sodium, and finally three times with absolute ether. Then after drying in a vacuum the protein was ground to a powder and dissolved in Tyrode solution. If the process had been carefully carried out without rise of temperature until the protein was anhydrous the resulting product was a fine white powder readily soluble in Tyrode solution in a concentration equal to that of the original serum. A very small quantity of the protein remained insoluble but this was negligible in comparison with the whole. The protein concentration was determined by the micro Kjeldahl method and adjusted to be equal to that of the original serum.

The protein so prepared, when tested for growth-inhibiting properties, was found to be much less inhibiting than the original serum, the cultures in the lipoid-free protein growing 78 per cent larger than in the serum (see Table II). These preparations were also less inhibiting than the serum extracted with ether alone in which the cultures grew 28 per cent larger than in the control serum.

It is evident that the removal of lipoid from serum decreases its inhibiting action to a very large extent. In order to ascertain whether the inhibiting action was entirely eliminated, the extracted sera were tested on growing fibroblasts, with Tyrode solution as the control (see Table III). In all cases there was found to be some inhibiting action, the growth averaging 19 per cent less in the serum protein solution. In order to learn whether this was due to the protein itself or perhaps to a small quantity of lipoid contained in it, several preparations of protein were made in which, after the final washing with anhy-

[4] Young, E. G., *Proc. Roy. Soc. London, Series B*, 1922, xciii, 15.

drous ether at −10°C., the protein was transferred to a Soxhlet extractor and extracted for 48 to 60 hours with anhydrous ether (see Table III). This treatment did not lessen the inhibiting effect. One of these preparations seemed to have no inhibiting action in the first passage but the inhibiting effect was quite marked in the second passage and the average of all results even in the first passage gave a figure almost exactly corresponding to that for the protein extracted only at low temperature. It would seem, therefore, that the protein

TABLE II.

Rate of Growth of Fibroblasts in a Solution of Serum Protein Which Had Been Extracted by Alcohol and Ether.
Serum Used as Control.

Group* No.	Culture No.	Preparation No.	Rate of growth in.		Ratio *E/C.*	Remarks.
			Serum (C).	Serum protein (*E*).		
1	2211 A	x − 105	4.57	8.54	1.87	
2	2275 A	x − 110	3.82	7.77	2.02	
3	1254 C	x − 119	3.74	6.6	1.76	
4	1273 C	x − 119	3.6	6.03	1.67	2nd passage.
5	1253 C	x − 120	4.36	6.9	1.58	
Average					**1.78**	

* Each group constitutes three or four experiments.

of the serum has some retarding action on growth, but it is not as marked as the retarding effect of the lipoid. The possibility still remains that a small amount of lipoid is chemically combined with the protein. If so, it is exceedingly small in amount. Reprecipitation of the protein in alcohol and ether has not extracted any measurable quantity.

One preparation (see Table IV) gave results differing from the others. Instead of being inhibiting it had a very slight stimulating action. So far it has not been possible to duplicate this preparation, although exactly the same process has been carried out in the attempt to do so. The explanation probably lies in the fact that the serum of young animals contains a growth-stimulating substance precipitated with euglobulin by CO_2. Experiments on embryonic tissue juice have

shown that its growth-stimulating substance is precipitated by CO_2 but is very unstable and easily destroyed. Probably in this one preparation the growth-stimulating substance escaped complete

TABLE III.

Rate of Growth of Fibroblasts in a Solution of Serum Protein Which Had Been Extracted with Alcohol and Ether. Tyrode Solution Used as Control.

Group* No.	Culture No.	Preparation No.	Rate of growth in.		Ratio *E/C.*	Remarks.
			Tyrode (*C*).	Serum protein (*E*).		
1	1433 C	x − 110	7.53	5.9	0.78	Serum kept at −10° during extraction.
2	1434 C	x − 120	13.9	11.2	0.81	
3	1388 C	x − 140	9.6	8.8	0.92	1st passage.
4	7797 D	x − 145	6.37	5.49	0.86	
Average of 1st passage in serum extracted only at low temperature.....					**0.84**	
5	1498 C	x − 150	9.4	7.9	0.84	Serum extracted at −10° and also by ether at 37°C. for 48 hrs. in Soxhlet extractor.
6	2430 A	x − 150	12.5	8.4	0.67	
7	7846 D	x − 158	6.18	6.35	1.03	
8	2494 A	x − 163	10.50	8.43	0.80	1st passage.
9	1631 C	x − 163	5.03	4.21	0.83	
Average of 1st passage in serum protein extracted with ether at 37°C. after extraction at −10°C.....					**0.83**	
10	1406 C	x − 140	4.92	3.49	0.71	2nd passage.
11	1515 C	x − 150	9.5	6.27	0.66	
12	2441 A	x − 150	9.8	6.3	0.64	
13	7839 D	x − 158	12.97	8.86	0.68	
14	7860 D	x − 158	5.20	4.06	0.78	
Average of 2nd passage.....					**0.70**	

* Each group constitutes three or four experiments.

destruction while in the other preparations it disappeared, or it may be that this particular serum contained more of the growth-stimulating substance than the others. Experiments are now being carried on to determine whether any growth-stimulating substance can be obtained in the CO_2-precipitated fraction of the extracted sera.

The question of whether the protein is altered by such a strenuous treatment as that used in these experiments is not easy to answer. All evidence which has been obtained so far, however, indicates that there is no alteration except in a very small portion that becomes insoluble. Practically all the protein goes back into solution readily and remains in solution permanently. The protein also contains, as shown by chemical precipitation tests, euglobulin, pseudoglobulins I and II, and albumin, each of which is precipitated as usual and redissolves readily. When plasma is used for the experiment even the fibrinogen is unchanged by the treatment and coagulates on adding a

TABLE IV.

Stimulating Action of Serum Protein Extracted by Alcohol and Ether.

Group* No.	Culture No.	Rate of growth in.		Ratio *E/C.*	Remarks.
		Tyrode (*C*).	Serum protein (*E*).		
1	2246 A	6.2	6.5	1.05	1st passage.
2	2254 A	6.25	7.7	1.23	2nd "
3	2264 A	3.57	4.39	1.23	3rd "
4	1552 C	7.3	9.2	1.26	1st "
5	1574 C	5.9	6.6	1.12	2nd "
6	1589 C	10.9	10.4	0.95	3rd "

* Each group constitutes three or four experiments.

little embryonic tissue juice. Moreover, crystalline serum albumin has been made from the protein of horse serum after this treatment and it is the same in properties as that crystallized from the whole serum.[4] All of these facts point to the conclusion that the protein is not altered.

Further evidence that the protein is unchanged is obtained from experiments on the surface tension of the lipoid-free serum and the normal serum. Du Noüy[5] has shown that a notable change in the time-drop of the surface tension of serum occurs at certain high dilutions, at which a monolayer of the serum molecules is formed. Experiments on the surface tension of the serum protein solutions gave curves at these high dilutions (1:9,000 to 1:12,000) practically duplicating those of the normal serum. Such a concordance of results could

[5] du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv, 575, 707; xxxvi, 547;1924, xxxix, 37.

hardly be obtained if the protein of the serum were altered chemically or physically.

As a further test immunization experiments were made on rabbits, using as antigens whole chicken serum and chicken serum protein prepared as above. The serum of the rabbits immunized with the serum protein reacted in the precipitin test to the whole serum and to the serum protein with equal intensity, no difference being observable in the quantity of precipitate obtained when the antigens were diluted from 1 in 5 parts to 1 in 2,000 parts. The serum of the rabbit immunized with the whole serum reacted to both antigens, the reaction to the extracted protein being but very slightly less than to the whole serum even at a dilution of 1 in 2,000. Such closely agreeing quantitative results show that no marked change had occurred in the configuration of the protein molecule.

From all the above evidence it seems safe to conclude that the decrease in growth-inhibiting action of the serum can be attributed to the removal of the lipoid.

Action of the Lipoid of Serum on the Growth of Fibroblasts.

The above results which indicate that in removing the lipoid from serum one removes the growth-inhibiting factor are confirmed by tests on the lipoid itself. The problem of obtaining a solution of the lipoid in such a state that it will be utilized by the tissues is difficult. Obviously after once removing it from the serum where in all probability it is held in a chemical or physical union with protein, it cannot be again distributed through an aqueous solution in as suitable a condition for utilization. In the attempt so to do it is important that the lipoid should be kept chemically unaltered if possible. The following technique was chosen as the most suitable for the purpose.

The alcohol which was used for the precipitation and also the first washing, was filtered and evaporated to dryness in a vacuum desiccator at room temperature. It was found best to leave the residue of lipoid dry and in vacuum until just before it was to be used on the cultures of fibroblasts. It was then taken up in a volume of water slightly less than that of the original serum. This was done by flowing the water over the surface of the dry lipoid and rocking the dish gently back and forth. In this way the lipoid and salts were taken up in a uniform emulsion which was very finely divided. If the lipoid was scraped from the dish, such an

even emulsification did not occur and the inhibiting effect on the fibroblasts was not so marked. A sample of the emulsion was tested for its salt concentration by determining the freezing point and enough salt or water added to the remainder to make it isotonic. Also the volume was finally adjusted so that the percentage of lipoid would equal that in the serum from which it was taken.

The lipoid so prepared proved inhibiting to the growth of fibroblasts in comparison with Tyrode solution used as control (see Table V).

TABLE V.

Rate of Growth of Fibroblasts in a Serum Lipoid Emulsion. Tyrode Used as Control.

Group* No.	Culture No.	Preparation No.	Rate of growth in.		Ratio *E/C*.
			Tyrode (*C*).	Lipoid (*E*).	
1	2223 A	x — 106	7.62	4.1	0.54
2	1407 C	x — 106	8.66	7.53	0.87
3	2326 A	x — 128	7.7	6.6	0.86
4	1389 C	x — 137	8.0	3.65	0.46
5	2374 A	x — 141	12.3	6.4	0.52
6	2403 A	x — 141	8.4	4.65	0.55
7	7803 D	x — 149	7.39	6.44	0.87
8	1486 C	x — 147	11.65	9.54	0.82
9	2423 A	x — 147	8.5	5.2	0.61
10	7834 D	x — 157	6.7	6.37	0.95
11	7829 D	x — 157	6.88	4.38	0.63
Average					**0.70**

* Each group constitutes three or four experiments.

Not only was the area of growth much less than in Tyrode, averaging 70 per cent, but the cells became fatty and the tissue died after a short time, the lipoid acting as a toxic substance.

For comparing the lipoid with the serum, not only the alcohol used for the first washing but all the wash alcohol and ether were mixed and evaporated. The residue was prepared as above. These total lipoids proved to be 20 per cent more inhibiting than the original serum and were also toxic (see Table VI). The fact that the lipoid was more inhibiting to growth than the original serum would not necessarily mean that it contained all the inhibiting substance of the serum.

As we have seen above, the protein left was still somewhat inhibiting. The smaller growth in the lipoid fraction is probably due to the fact that it has different properties, after its union with the protein of the serum is broken, than it has in its original combination. Also the emulsion of lipoid, although originally adjusted to the same pH as the serum, possesses less buffer action than serum to changes of pH produced by the growth of the tissues.

The question naturally arises as to whether the incease of the inhibiting action of serum with the age of the animal is due to a change in the quantity or character of the lipoid of the serum. A large amount of work will be necessary to answer this question. One preliminary

TABLE VI.

Rate of Growth of Fibroblasts in an Emulsion of Lipoid from Serum. Serum Used as Control.

Group* No.	Culture No.	Preparation No.	Rate of growth in.		Ratio *E/C.*
			Serum (*C*).	Lipoid (*E*).	
1	2375 A	x − 141	7.12	5.72	0.80
2	2404 A	x − 141	5.52	5.05	0.91
3	2424 A	x − 147	8.7	7.2	0.83
4	2432 A	x − 152	7.1	5.3	0.74
5	1499 C	x − 152	5.5	4.65	0.84
6	1595 C	x − 162	8.2	6.0	0.73
Average					**0.81**

* Each group constitutes three or four experiments.

experiment is interesting in its results. The ratio of the growth in the serum of the old animal to that in the serum of a young animal was 0.81. Exactly the same ratio was obtained for the lipoid fractions derived from these sera respectively and also for the two protein fractions free from lipoid.

Since alcohol extracts other substances than lipoid, a few experiments were carried out to ascertain whether it is the lipoid itself or some other alcohol-extractable material that is responsible for the inhibiting action. The alcoholic solution was evaporated to small volume and a large excess of anhydrous ether added (4 or 5 times its

volume). This precipitated most of the salts, amino acids, and substances insoluble in ether. The mixture was then centrifuged, and both the ether-soluble and ether-insoluble fractions tested on the cultures. The lipoid soluble in ether proved to be the inhibiting substance, while the solution of the salts, amino acids, etc., was found to be either inactive or slightly stimulating. Hence it would appear that lipoid or some substance closely bound to lipoid is the inhibiting substance. Further experiments are being carried out on fractionating this lipoid to ascertain whether the inhibiting substance is one particular substance, or whether all the serum lipoids are inhibiting.

TABLE VII.

Rate of Growth of Fibroblasts in Lipoid Emulsions from Various Sources.

Group* No.	Culture No.	Source of lipoid.	Rate of growth in.		Ratio *E/C*.
			Tyrode (*C*).	Lipoid (*E*).	
1	7804 D	Chicken liver.	10.95	7.02	0.64
2	2363 A	" "	3.3	1.98	0.60
3	2347 A	" "	5.3	3.87	0.73
4	2252 A	Egg lecithin.	13.5	6.76	0.50
5	2364 A	Chicken brain.	5.3	3.13	0.59
6	2348 A	" "	4.2	2.8	0.67
7	2845 A	Embryonic tissue extract.	7.2	5.9	0.82
8	2493 A	" " "	4.55	3.4	0.75 (2nd passage).

* Each group constitutes three or four experiments.

That lipoids, as a class, are inhibiting, is suggested by the results of experiments in which lipoids from egg, chicken brain, chicken liver, and chicken embryonic extract were used. These were prepared in a manner similar to the serum lipoid. As is seen from Table VII, all have proved inhibiting to growth. Further experiments should, however, be carried out, involving the use of these substances in many different concentrations.

SUMMARY.

The growth-inhibiting action of serum has been shown to be due largely to the lipoids.

Serum from which the lipoids have been removed is much less inhib-

iting to the growth of fibroblasts *in vitro* than is the original serum, and only slightly more inhibiting than Tyrode solution.

The lipoids extracted from the serum are toxic and more inhibiting to the growth of fibroblasts than the original serum.

Lipoids extracted from chicken brain, chicken liver, egg, and embryonic tissue have likewise an inhibiting action.