EFFECT OF AGE ON SERUM LIPOIDS AND PROTEINS.

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Blood serum is endowed with the property of inhibiting the multiplication of fibroblasts and epithelial cells, as has been shown by the experiments of Carrel and Ebeling.[1] It was also found that this growth-restraining power markedly increases with the age of the animal from which the serum is taken.[2] This phenomenon is due chiefly to the lipoids of the serum and also in some measure to the proteins.[3] However, serum also contains certain principles which precipitate with the euglobulin and possess a slight activating effect on the growth of connective tissue cells.[4] In old age, this property of the serum euglobulin was found to decrease.[5] The growth-restraining power of serum in the senescent period is probably due to some change in its lipoids or its proteins, or in both. The experiments reported in this paper were designed to test this supposition, and to determine the nature of the modifications that occur in the protein and lipoid constituents of serum in the course of life.

EXPERIMENTAL.

Sera were obtained from 6 month old chickens and from 4 to 5 year old hens of the same breed. The lipoid and protein fractions were separated and tested for retarding action on the growth of fibroblasts, the lipoid of the serum of the young animal being compared with

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

[2] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 599; 1922, xxxv, 17.

[3] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1925, xlii, 143; *Compt. rend. Soc. biol.*, 1925, xciii, 79.

[4] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, xxxvii, 653.

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

that of the old hen, and the serum protein of the young chicken with that of the old one. The sera were also analyzed for the concentration of various proteins and lipoids.

Method of Preparing the Lipoid and Protein Fractions.

The sera were poured drop by drop, with constant stirring, into three times their volume of 95 per cent alcohol previously cooled to −10°C., and allowed to stand for 2 hours in a freezing mixture. The precipitated protein was centrifuged and washed twice with 95 per cent alcohol, three times with absolute alcohol, twice with a mixture of absolute alcohol and absolute ether, and finally three times with absolute ether. The alcohol and ether had been previously cooled to −5°C. or below, and the protein was separated from them by centrifuging in a tube surrounded by freezing mixture in a brine-cooled centrifuge, so that the temperature did not rise above zero degree throughout the process. The protein fractions of the sera were immediately dried *in vacuo* over sulfuric acid and redissolved in Tyrode solution. As only a very small amount of the protein became insoluble, solutions could usually be obtained equal in protein concentration to that of the original sera. Nitrogen determinations were made and the protein concentrations of the solutions adjusted to the same ratio as that of the original sera.

The alcohol in which the protein was precipitated and the alcohol and ether washings were mixed, filtered, and evaporated to dryness *in vacuo*. Shortly before the preparation of the cultures, the lipoid residue was emulsified in a volume of water equal to the original volume of serum, the pH adjusted, the cryoscopic point determined, and corrections for salt concentration were made.

Method of Testing the Growth-Inhibiting Action of the Protein and Lipoid Fractions.

The above preparations were tested on fibroblasts from a 14 year old strain, the two halves of the same piece of tissue being cultivated, one in the serum lipoid of the old animal and the other in the serum lipoid of the young animal. Since the two halves of the same culture of an old strain of fibroblasts grow at a uniform rate when the media are uniform,[6] any variation in growth would be due to differences in the media. The original sera were compared with each other in the same way, and also the protein fractions from the sera of the old and young animals. The technique described by Ebeling[6] for measuring the relative growth of the tissues was used. The original area and the area after 48 hours incubation were measured, and the rate of growth was calculated by dividing the latter by the former. The culture media consisted of 1 part of plasma, 1 part of either lipoid emulsion, protein solution, or serum, and 1 part of lipoid emulsion, protein solution, or serum containing 5 per cent embryo juice.

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

Effect of Protein and Lipoid Fractions on the Growth of Fibroblasts.

In every case (Table I), the serum lipoids of the 4 year old hens were more inhibiting to growth than those of the 6 month old chickens. The same is true of the serum proteins (Table II) and also the total

TABLE I.

Comparative Rate of Growth of Fibroblasts in Serum Lipoid from Old and Young Chickens.

Group No.	Culture No.	Chemical preparation No.	Serum No.		Rate of growth in lipoid emulsion		Ratio of growth: Young / Old	Average ratio
			Young	Old	Young	Old		
1	2414 A	X-144	1431	1430	6.30	5.80	1.10	
					7.20	5.50	1.30	1.25
					3.80	2.80	1.35	
2	2781 A	X-232	2230	2229	3.48	2.64	1.30	
					5.40	2.90	1.85	1.65
					3.85	2.15	1.80	
3	2799 A	X-236	2402	2401	3.45	3.00	1.15	
					3.08	2.47	1.25	1.22
					2.90	2.30	1.25	
4	2823 A	X-241	2431	2432	4.00	3.20	1.25	1.28
					4.10	3.20	1.30	
5	2841 A	X-246	2494	2495	8.30	5.70	1.45	
					5.60	4.10	1.35	1.33
					8.40	7.00	1.20	
6	2857 A	X-249	2528	2529	5.90	4.70	1.25	
					7.20	4.35	1.65	1.53
					10.20	6.01	1.70	
Average.....								1.38

sera (Table III). Hence it is evident that changes take place with age in both the lipoid and protein fractions of the serum of an animal, which cause its increased inhibiting action on the growth of fibroblasts. The average ratio of the growth in the serum lipoid of the young animal to that in the serum lipoid of the old animal was 1.38, while the

ratio of the growth in the young serum to that in the old was 1.56. The ratio for the protein fractions was 1.50. While there is a wide variation in the figures from which these average ratios were obtained, due to individual differences in the animals and to the inevitable ex-

TABLE II.

Comparative Rate of Growth of Fibroblasts in Serum Protein from Old and Young Chickens.

Group No.	Culture No.	Chemical preparation No.	Serum No.		Rate of growth in serum protein		Ratio of growth: Young / Old	Average ratio
			Young	Old	Young	Old		
1	2413 A	X-145	1431	1430	7.60	4.40	1.70	
					6.60	5.10	1.30	1.43
					10.00	7.60	1.30	
2	2774 A	X-233	2230	2229	6.65	5.00	1.30	
					8.60	6.90	1.25	1.25
					7.00	5.70	1.20	
3	2800 A	X-237	2402	2401	4.90	3.00	1.60	
					7.00	3.40	2.10	1.83
					6.70	3.70	1.80	
4	2822 A	X-243	2431	2432	4.50	2.60	1.70	
					4.00	3.20	1.25	1.45
					5.40	3.90	1.40	
5	2851 A	X-247	2494	2495	4.25	3.10	1.35	
					6.50	2.70	2.40	1.85
					8.27	4.65	1.80	
6	2863 A	X-250	2528	2529	4.20	3.25	1.30	
					6.00	4.90	1.20	1.20
					6.20	5.50	1.10	
Average								1.50

perimental error, the average ratio for the lipoid differs from that of the sera and protein fractions in a manner to be logically expected. It is known[5] that the sera of the young animals contain in addition to inhibiting substances a small amount of growth-stimulating substance precipitated with the euglobulin by CO_2. This stimulating substance

is absent from the sera of the older animals.[5] Thus, in Ebeling's experiments, the difference in rate of growth of fibroblasts in the CO_2 precipitate of sera of old and young chickens was 0.18.[5] The ratio 1.56 for the sera is due, then, to two factors, the smaller quantity of inhibiting substance in the sera of the younger animals, and the pres-

TABLE III.

Comparative Rate of Growth of Fibroblasts in Serum of Old and Young Chickens.

Group No.	Culture No.	Chemical preparation No.	Serum No.		Rate of growth in serum		Ratio of growth: Young / Old	Average ratio
			Young	Old	Young	Old		
1	1448 C	X-142	1431	1430	8.40	7.60	1.10	
					6.95	5.10	1.35	1.28
					9.30	6.50	1.40	
2	2773 A	X-231	2230	2229	7.10	3.50	2.00	
					5.70	3.35	1.70	1.80
					6.00	3.55	1.70	
3	2813 A	X-235	2402	2401	4.50	2.88	1.55	
					8.90	4.25	2.10	1.62
					7.95	6.70	1.20	
4	2802 A	X-242	2431	2432	5.30	3.90	1.35	
					6.00	3.80	1.60	1.40
					8.60	6.90	1.25	
5	2842 A	X-245	2494	2495	13.90	7.20	1.90	
					9.40	6.30	1.50	1.65
					7.90	5.10	1.55	
6	2873 A	X-252	2528	2529	4.80	3.54	1.35	
					9.75	4.58	2.10	1.63
					5.40	3.74	1.45	
Average								1.56

ence of a stimulating substance. Since the latter would be absent in the lipoid fractions, a lower ratio for these would be expected and was actually found.

The data given here do not show how much greater is the inhibiting action of the lipoid than that of the protein fraction, since the lipoid

and the protein were not tested on the two halves of the same tissue, and the growth of two different pieces of tissue cannot be compared, because of the marked effect of their previous culture medium and their inherent growth activity. The relative effect of the lipoid and protein was demonstrated in previous experiments,[3] in which the lipoids proved even a little more inhibiting than the sera from which they were derived, while the protein fractions were much less inhibiting than the original sera. It is interesting to note that although the protein is less inhibiting than the lipoid, the rates at which their inhibiting action increases with age seem approximately equal.

TABLE IV.

Concentration of Protein in Sera of 6 Month and 4 to 5 Year Old Chickens.

6 mos. old			4 to 5 yrs. old		
Serum No.	Per cent nitrogen	Per cent protein	Serum No.	Per cent nitrogen	Per cent protein
1431	0.455	2.85	1430	0.885	5.53
2230	0.462	2.89	2229	0.690	4.32
2402	0.621	3.88	2401	0.912	5.70
2431	0.634	3.96	2432	0.707	4.42
2494	0.670	4.18	2495	0.547	3.42
2528	0.532	3.33	2529	0.709	4.44
Average...	0.562	3.52		0.742	4.64

Concentration of Protein and Various Protein Fractions in Sera of Old and Young Animals.

The sera used for the growth experiments were tested in the course of the work for their concentration of protein (Table IV). Here again, the individual variations are large, but it is evident that in general the sera from the older animals contain a higher concentration of protein, the average being 0.742 per cent nitrogen for the 4 to 5 year old hens, and 0.562 per cent nitrogen for the 6 month old chickens. The ratio of the average concentrations was 1.32. This corresponds fairly closely to the ratio that represents the inhibiting action on growth, *i.e.*, the rate of growth in the protein fractions is approximately inversely proportional to the protein concentration. Since serum contains a growth-stimulating substance which precipi-

tates with CO_2, a determination of the concentration of protein not precipitated by CO_2 was made on sera from old and young chickens. Here again, the concentration was found to be greater in the sera from the older animals (Table V). Hence, it is evident that the

TABLE V.

Concentration of Various Protein Fractions of the Sera of Old and Young Chickens.

Serum No.	Per cent total protein	Per cent protein not precipitated by CO_2	Per cent protein precipitated by CO_2. By difference	Per cent globulin in CO_2 filtrate	Per cent albumin
3 to 5 mos. old					
3007	3.13	2.92	0.21	1.45	1.47
3020	3.22	2.50	0.72	1.21	1.29
3029	2.29	1.87	0.42	0.56	1.31
3036	3.04	2.53	0.51	0.94	1.59
3040	2.52	2.06	0.46	0.90	1.16
3046	3.56	2.26	1.30	1.13	1.13
3053	3.28	2.76	0.52	1.42	1.34
3064	3.31	2.66	0.65	1.35	1.31
Average.....	3.04	2.45	0.60	1.12	1.33
4 to 5 yrs. old					
3006	4.89	3.19	1.70	1.58	1.61
3021	2.99	2.41	0.58	1.00	1.41
3030	4.89	3.69	1.20	2.37	1.32
3035	4.12	3.15	0.97	1.31	1.84
3039	4.55	2.20	2.35	1.17	1.03
3045	3.63	2.53	1.10	1.25	1.28
3052	3.94	3.14	0.80	1.45	1.69
Average.....	4.14	2.90	1.24	1.45	1.45
Ratio: $\frac{\text{Old}}{\text{Young}}$	1.36	1.18	2.06	1.29	1.09

increase in the inhibiting action of the protein with age is due not only to the disappearance of the small quantity of stimulating substance in the CO_2 precipitate, but also to a change in the concentration of inhibiting protein.

Determinations were also made of the concentration of albumin

and of pseudoglobulin[7] remaining in the filtrates from the CO_2 precipitation. Both of these proteins were found to occur in greater concentration in the sera of the old animals (Table V). Therefore, it is not possible to attribute the increased inhibiting action of the protein to either one of these proteins by itself. It is more probably due to their joint action. Even the quantity of euglobulin precipitated by CO_2 is greater in the sera of the older animals (Table V), indicating the probability that the euglobulin itself is not the stimulating substance, but rather some substance carried down with it. No rigid conclusion should be drawn here, however, since it is possible that this precipitate might contain some of the fibrin of the plasma from which the serum was made.

There is considerable variation among the sera of the individual animals of the same age in the concentration of the different protein fractions. Duplicate determinations on any one serum agreed closely, however, showing that this phenomenon is due to actual differences in the animals, and not to the experimental technique. The same variation is noted in the growth of fibroblasts in the sera of animals of the same age. Therefore, although the average results are obtained from figures varying to quite an extent, they indicate without any doubt greater inhibiting action in the serum protein of the older animals, and also a higher concentration of protein in the sera as the age of the animals increases. It seems probable that this inhibiting action is not due to any one protein, but rather to the protein as a whole.

Concentration of the Total Lipoid, Lecithin, and Cholesterol in Sera of Old and Young Animals.

An examination of the changes taking place in the lipoid of the serum with increasing age is even more important than that of the protein, since the lipoid is chiefly responsible for the inhibiting action. The total lipoid was determined in the sera of chickens 6 months old and 4 to 5 years old, the lecithin for 3 month, 6 month, and 4 to 5 year old chickens, and cholesterol for 3 month and 4 to 5 year old chickens. The total lipoid was determined as follows:

5 cc. of serum were poured drop by drop into 75 cc. of a mixture of alcohol and ether (3:1). The solution was heated to boiling, cooled, made up to 100 cc.,

[7] This fractionation was made by the use of sodium sulfate.

and filtered. An aliquot portion of the filtrate (75 cc.) was taken for analysis. Three times its volume of absolute ether was added. The precipitate obtained had no inhibiting action on fibroblasts, while the lipoids remaining in solution showed the usual retarding action. After centrifugation, a large aliquot (250 cc.) was evaporated to dryness in a tared flask, and the residue weighed. To ascertain whether the residue contained salts that would invalidate the results, the residue was redissolved, transferred to a platinum crucible, and ashed. A negligible amount of ash remained (0.0014 gm.), showing that the salts had been satisfactorily removed. The lipoid itself would, of course, yield a small quantity of ash. Therefore, no error from this source needs to be considered. In developing the above procedure, it seemed desirable to use a method resembling as nearly as possible that employed in preparing the lipoid fraction for the culture medium, especially since the usual methods of determining total lipoid are not very precise. Sufficiently large quantities of serum were used (5 cc.) to diminish the experimental error to less than 1 per cent.

TABLE VI.

Concentration of Total Lipoid in Sera of 6 Month Old and 4 to 5 Year Old Chickens.

6 mos. old		4 to 5 yrs. old	
Serum No.	Weight per 100 cc. gm.	Serum No.	Weight per 100 cc. gm.
2755	0.800	2754	1.133
2774	0.815	2773	1.055
333C	0.992	332C	0.993
2835	0.895	2834	1.042
		2848	1.072
Average.....	0.876		1.059

Lecithin in the sera was determined at first by the method of Randles and Knudson,[8] and later by Whitehorn's[9] modification of that method. The Whitehorn modification gave more uniform results than the technique of Randles and Knudson on duplicate determinations. The method of Bloor[10] was found to be the most suitable for determining cholesterol. As other experimenters have found, the colors did not always match the standard. Both a cholesterol standard and the inorganic standard of copper sulfate and sodium dichromate of

[8] Randles, F. S., and Knudson, A., *J. Biol. Chem.*, 1922, liii, 53.
[9] Whitehorn, J. C., *J. Biol. Chem.*, 1924, lxii, 133.
[10] Bloor, W. R., *J. Biol. Chem.*, 1916, xxiv, 227.

McMaster[11] were used. While these figures involve quite a large experimental error, there was no difficulty in distinguishing between

TABLE VII.

Concentration of Lecithin Phosphorus in Sera of Chickens of Different Ages.

3 mos. old			6 mos. old			4 to 5 yrs. old		
Serum No.	Mg. phosphorus per 100 cc. serum	Average	Serum No.	Mg. phosphorus per 100 cc. serum	Average	Serum No.	Mg. phosphorus per 100 cc. serum	Average
2871	4.80 5.40	5.10	2817	5.90	5.90	2754	6.70 6.50	6.60
2870	5.90 4.95	5.43	2333	5.00 5.10	5.05	2773	7.20 7.50	7.35
2859	4.50 4.60	4.55	2774	6.40 6.30	6.35	332C	6.90 7.50	7.20
2849	3.45 4.85	4.15	2755	6.10 5.40 5.20	5.57	2818	6.70 6.70	6.70
2835	4.80 4.60	4.70				2834	7.80 7.50	7.65
						2848	7.50 7.60	7.55
						2857	9.30	9.30
						2869	7.30 6.70	7.00
						2868	7.40 7.30	7.35
Average.....		4.78			5.72			7.41

the quantity of cholesterol present in the sera of the old and young chickens.

The results (Tables VI to VIII) show that there is an increase in

[11] McMaster, P. D., *J. Exp. Med.*, 1924, xl, 25.

TABLE VIII.

Concentration of Cholesterol in Sera from 3 Month and 4 to 5 Year Old Chickens.

3 mos. old			4 to 5 yrs. old		
Serum No.	Mg. cholesterol per 100 cc. serum	Average	Serum No.	Mg. cholesterol per 100 cc. serum	Average
2870	282 226 338	282	2869	166 188 200	185
2871	200 165 100	155	2868	83.0 95.3 95.3	91
2891	200 198	199	2889	75 68	71
2892	273 250	261	2904	187 220 250	219
2892	273 238	255	2905	107 107 99	104
2859	157 144 144	148	2857	170 200 200	190
2907	340 340 322	334	2754	192 205 196	198
2906	191 178 166	178	2818	161 134 128	141
2870	215 201 213	210	2834	87 80 86	84
Average.....		225			143

the total lipoid and also in the quantity of lecithin as the age of the chicken increases, but a decrease in the cholesterol.[12]

The total lipoid in the sera of the 4 to 5 year old chickens was 1.06 per cent and in the 6 month old, 0.876 per cent, giving a ratio of 1.21. The concentration of phosphorus due to lecithin was 4.78 mg. per 100 cc. in the sera of the 3 month old chickens, 5.72 mg. per 100 cc. in the sera of the 6 month old chickens, and 7.41 mg. per 100 cc. in the sera of the 4 to 5 year old chickens. The ratio of the 4 to 5 year old to the 6 month old animals was 1.29. These figures are in general accord with the results on growth in the serum lipoids of animals of these ages, and indicate that the inhibiting action is due, at least partly, to the lecithin which increases in concentration in old age. The fact that the concentration of cholesterol decreases as age increases, 225 mg. per 100 cc. at 3 months to 143 mg. per 100 cc. at 4 to 5 years, shows the necessity of further investigation in this field. The differences with age are so small, in comparison with the individual variations between animals of the same age, that it is impossible as yet to draw any conclusions concerning the part cholesterol plays in the increased inhibiting action of serum lipoids with age. As yet, the separate lecithin and cholesterol fractions of the sera have not been investigated in relation to their action on growing fibroblasts. A study of the action of pure cholesterol is now being undertaken.

DISCUSSION.

It is evident from the foregoing experiments that the changes in serum accompanying an increase in age, which render it more inhibiting to the growth of fibroblasts, occur in both the lipoids and the proteins of the serum. Although the serum protein is less inhibiting than the serum lipoid, the rate at which their retarding actions augment with age is approximately the same and also the same as the rate at which the retarding action of the serum itself augments with age. The change taking place in the protein fraction is due to the disappearance of a small quantity of growth-stimulating substance and also

[12] Roffo found an increase in both lecithin and cholesterol in rats between the ages of 3 and 5 months, but he made no examination of the sera of older rats. Roffo, A. H., *Compt. rend. Acad.*, 1925, clxxx, 1529.

an increase in the quantity of inhibiting protein. The concentration of each protein fraction of the serum is also greater as age advances. The increased inhibiting action of the serum lipoid in old age is associated with a higher concentration of total lipoid and of lecithin, and a smaller content of cholesterol than is present in the sera of young animals. An investigation of the action on fibroblasts of the lecithin and cholesterol fractions of the lipoid and of pure lecithin and cholesterol is necessary.

It would be interesting to know the mechanism by which these chemical substances act upon the cells and restrain their multiplication. Under normal conditions, the humors of the animal acquire a delicate balance between the growth-stimulating substances and those retarding growth, an understanding of which might give some information concerning abnormal conditions in which this balance is not maintained.

Probably there is also a delicate balance between conditions producing growth and those under which functioning of cells takes place. It is known that heart tissue kept in a medium unsuitable for growth will continue its muscular functioning, but this stops when nutritive substances are added to the tissue and growth takes place. In connection with this, it is interesting to note that the lipoids retard growth of tissues, but that an isolated heart perfused continuously with Ringer solution will cease beating, due to the loss of some substance removed by the Ringer solution, but will have that function restored on the addition of a small amount of the ether extract of serum.[13] Lecithin produces the same effect. As yet these phenomena are far from being understood.

The data at hand are also too meager to venture on any explanation of how lipoid and protein inhibit growth. However, since it has been discovered[14] that fibroblasts proliferate rapidly in proteose solutions and peptic digests of proteins, and that they live and multiply in the protein of embryo juice, it seems probable that this protein is first hydrolyzed to the proteose stage by means of enzymes either in the

[13] Clark, A. J., *J. Physiol.*, 1913–14, xlvii, 66.

[14] Carrel, A., and Baker, L. E., *J. Exp. Med.*, 1926, xliv, 503. Carrel, A., *Compt. rend. Soc. biol.*, 1926, xciv, 1060. Carrel, A., and Baker, L. E., *Compt. rend. Soc. biol.*, 1926, xcv, 359.

embryo juice or within the cells. It is known that serum exerts an antienzymatic action. It is possible, therefore, that the inhibiting action of serum on growth may be associated with its antienzymatic action, which would retard the production of nutritive hydrolytic products.

SUMMARY.

The increased inhibiting action of serum with age on the growth of fibroblasts has been shown to be due to changes in both the lipoids and the proteins.

Although the serum protein is less inhibiting than the serum lipoid, the rate at which their inhibiting action augments with age is approximately the same.

The change in the serum protein with age is due to the disappearance of a small quantity of growth-stimulating substance, and also to an increase in concentration of inhibiting protein. The concentration of all protein fractions becomes larger as age advances.[15]

The greater inhibiting action of the lipoid is associated with a higher concentration of total lipoid and lecithin, and a smaller content of cholesterol as the animal grows older.

The hypothesis is suggested that the inhibiting action of the serum is associated with its antienzymatic action.

[15] It is well known that the protein of serum increases as age advances. Investigators differ in their findings on the relations between age and the different protein fractions. Wells, C. E., *J. Biol. Chem.*, 1913, xv, 37. Hatai, S., *J. Biol. Chem.*, 1918, xxxv, 527. Toyama, I., *J. Biol. Chem.*, 1919, xxxviii, 161.