THE CHEMICAL NATURE OF THE SUBSTANCES REQUIRED FOR CELL MULTIPLICATION.

II. Action of Glutathione, Hemoglobin, and Ash of Liver on the Growth of Fibroblasts.

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For some time it has been known that fibroblasts and epithelial cells proliferate indefinitely *in vitro* in a medium of plasma and embryo juice.[1] If all the factors that function in this unlimited reproduction of tissues could be known, it seems probable that much could be done to accelerate the processes of repair. Such knowledge might also throw light on the problem of development and growth of tumor. With these ideas in mind, a series of investigations was begun, the object being to ascertain the chemical nature of the substances which are utilized by the tissues for maintenance and growth. It was found that the protein fraction[2] of embryo juice was mainly responsible for the multiplication of the cells. All other proteins tested lacked this growth-promoting power, and even the protein of the embryo juice became inactive on purification by repeated precipitation. However, proteoses prepared from many different proteins stimulated enormously the multiplication of fibroblasts and other cells.[3] Therefore, the hypothesis was advanced that enzymes in the embryonic juice under the influence of the acid produced by the cells hydrolyzed the

[1] Carrel, A., *J. Exp. Med.*, 1913, xvii, 14. Fischer, A., *J. Exp. Med.*, 1922, xxxv, 367. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317; 1923, xxxviii, 487. Carrel, A., *Physiol. Rev.*, 1924, iv, 1; *Compt. rend. Soc. biol.*, 1927, xcvi, 603.

[2] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1926, xliv, 387; *Compt. rend. Soc. biol.*, 1926, xcv, 157.

[3] Carrel, A., and Baker, L. E., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiii, 627; *J. Exp. Med.*, 1926, xliv, 503; *Compt. rend. Soc. biol.*, 1926, xcv, 359.

embryo protein to proteoses which promoted growth. The products of the further hydrolysis of proteins, *i.e.*, the peptones, peptides, and amino acids, were also found to contribute to the nutrition of fibroblasts.[4] Even when these proteolytic products were derived from a chemically pure protein such as egg albumin crystallized six times, they promoted a considerable growth of fibroblasts from fresh embryonic heart tissue.[5] On the other hand, they caused an exceedingly small proliferation of cells from pure cultures of fibroblasts.[5] When glycocoll and nucleic acid were added to the proteolytic products of crystalline egg albumin or of casein, the resulting mixture was found to promote a considerable growth of pure strains of both normal and sarcomatous fibroblasts.[5] It did not, however, cause a growth at all comparable to that produced by embryo juice. After a time, the cells degenerated, showing that the medium lacked some substance or substances essential for their complete nutrition. These experiments indicated, nevertheless, that it might be possible to synthetize an artificial medium which would prove adequate for the maintenance of cell life and multiplication. Therefore, the effect of adding many biologically important substances to this simple mixture of glycocoll, nucleic acid, and the digestion products of either crystalline egg albumin or casein was studied. Many of them, such as vitamines, iron salts and oxides, cysteine, carbohydrates, cholesterol, etc., had no noticeable beneficial action under the conditions of the experiments. Even though no evidence was obtained of the nutritive value of these substances, the negative results must not be accepted as final, for it is conceivable that any one of them might contribute to the functional requirements of the cells, but that its effect could not be observed in experiments on growth when some other substance necessary for complete nutrition was absent.

It is reasonable to suppose that, in addition to the nitrogenous substances required for the synthesis of protoplasm, certain catalysts or substances should be present in the medium for the regulation of the oxidative or respiratory processes of the cell. It is also probable that a definite oxidation-reduction potential of the medium is as important for cell life as a given hydrogen ion concentration or osmotic

[4] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1928, xlviii, 533.

[5] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1928, xlvii, 353.

pressure. For this reason, a study was made of the effect on the growth of fibroblasts of (1) the mineral constituents of liver tissue, (2) of glutathione, and (3) of hemoglobin when added to the incompletely nutritive medium of casein digest, glycocoll, and nucleic acid previously described.[5] The results obtained so far with these substances form the subject of the present communication.

Technique of Cultivating the Tissues.

For this work, a pure strain of sarcomatous fibroblasts from Rat Sarcoma No. 10 of the Crocker Foundation was used. Certain of the experiments were repeated for comparative purposes with a pure strain of normal fibroblasts of the rat. The colonies of fibroblasts were cultivated at 39°C. in flasks 3.5 cm. in diameter, in a coagulum made of 1 cc. of chicken plasma diluted 1:3 with Tyrode solution, and 0.25 cc. of embryo juice. Immediately after coagulation, the serum and embryo juice were removed by washing with Tyrode solution, and 0.5 cc. of the experimental fluid was added. After 24 hours, the coagulum was patched with 0.25 cc. of plasma and 0.25 cc. of embryo juice, and again washed before the addition of the experimental fluid. Every 48 hours, the fluids were removed, the cultures washed in Tyrode solution for 1 hour, and the nutritive media renewed. Every 2 or 3 days, tracings were made under a projectoscope of the growth, the area of which was measured with a planimeter. In each experiment, a colony of cells in active condition was divided into two equal parts. The comparison of the growth-activating action of any two media was made by testing their effect on these two equal parts of the same colony of cells, and each experiment was repeated a number of times. After 8 or 10 days growth, the colonies were removed from the flasks, cut in half, transferred to new flasks, and continued in the same medium, provided the condition of the cells and the area of the growth obtained indicated the desirability of prolonging the experiment.

Action of the Ash of Liver on the Growth of Sarcomatous Fibroblasts.

Although previous experiments with iron salts at many different dilutions had given unsatisfactory results, it still seemed probable, in view of Warburg's[6] experiments, that iron in some form functioned in the respiratory process of the cells. Moreover, it had been found previously that the proteolytic products obtained by peptic or tryptic digestion of liver, which is very rich in iron, sufficed for the complete nutrition of sarcomatous fibroblasts.[7] The recent work of Hart,

[6] Warburg, O., *Biochem. Z.*, 1924, clii, 479; *Pharm. Monats.* 1925, vi, 105.

[7] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1928, xlvii, 371.

Steenbock, Waddell, and Elvehjem[8] has shown that the ash of liver is effective in curing anemia of rats, and that the copper present in it is as important as the iron. These facts suggested the possibility that both of these elements might play an important rôle in the life of sarcomatous fibroblasts. Experiments were made, therefore, to ascertain whether adding the ash of liver to the mixture of casein digest, glycocoll, and nucleic acid (C. G. N.)[9] increased its nutritive action for sarcomatous fibroblasts.

The ash was prepared by evaporating to dryness 200 cc. of a heated aqueous extract of 120 gm. of liver at pH 5.0, and igniting it in a platinum crucible. When completely oxidized, the ash was dissolved in about 5 cc. of concentrated hydrochloric acid, again evaporated to dryness, and redissolved in sufficient water to make an isotonic solution. Normal sodium hydroxide solution was added to bring the pH approximately to 5.0. If made more alkaline, a precipitate settled out. The solution was, therefore, left acid until after being mixed with the other constituents of the medium, when the whole was adjusted to pH 7.4. Under these conditions, no visible precipitate was formed, except on long standing.

The experimental and control media for cultivating the tissues were made as follows:

Experimental medium	Control medium
1 cc. casein digest[10]	1 cc. casein digest
1.5 cc. glycocoll solution[11]	1.5 cc. glycocoll solution
1.5 cc. nucleic acid solution[12]	1.5 cc. nucleic acid solution
2 cc. ash of liver solution	2 cc. Tyrode solution

The ash caused no significant difference in the area of growth of the tissues (Text-fig. 1). However, the cells in the second passage in the medium containing the ash appeared to be in somewhat better

[8] Hart, E. B., Steenbock, H., Waddell, J., and Elvehjem, C. A., *J. Biol. Chem.*, 1928, lxxvii, 797.

[9] The abbreviation C.G.N. stands in the present paper for a mixture of casein digest, glycocoll, and nucleic acid.

[10] The casein digest was made by digesting at 37°C. 10 gm. of casein with 200 cc. of 0.5 per cent pepsin in N/20 HCl, for 24 hours, boiling, making it isotonic, and adjusting to pH 7.4. It contained 0.7 per cent of nitrogen, 13.5 per cent of which was present as amino nitrogen.

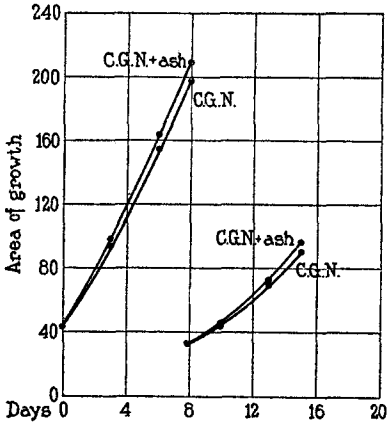
[11] The glycocoll solution contained 50 mg. of glycocoll in 100 cc. of Tyrode solution. It was sterilized by filtering through a Berkefeld filter.

[12] This was a 0.1 per cent solution of thymus nucleic acid in Tyrode solution. It was adjusted to pH 7.4, and sterilized by filtering through a Berkefeld filter.

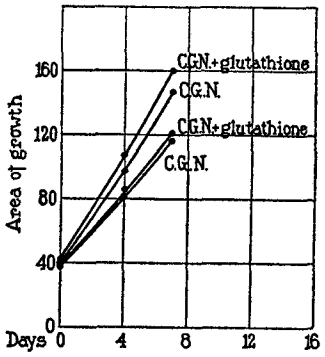
condition than those in the control, and the growth was also slightly thicker. This condition indicated that the mineral constituents of liver were probably beneficial, but if so, other essential substances were still lacking in the medium without which their action could not be definitely demonstrated.

Action of Glutathione on the Growth of Sarcomatous Fibroblasts.

The presence of glutathione in all tissues and its importance in relation to the respiratory processes of the cells have been pointed out



TEXT-FIG. 1.



TEXT-FIG. 2.

TEXT-FIG. 1. Experiments 4612 A and 4639 A. Effect of the ash of liver when added to a mixture of casein digest, glycocoll, and nucleic acid (C.G.N.), on the growth of sarcomatous fibroblasts of the rat.

TEXT-FIG. 2. Experiment 4609 A. Effect of glutathione when added to a mixture of casein digest, glycocoll, and nucleic acid (C.G.N.), on the growth of sarcomatous fibroblasts of the rat.

by Hopkins.[13] What its effect is on the growth of tissues, either through its being used as a food substance, or through its action in regulating the oxidation-reduction potential of the medium, has not been ascertained. Its universal presence in cells indicates either that it must be synthetized by the cell or supplied as such in its nutritive medium. That it probably is an important factor in the multiplica-

[13] Hopkins, F. G., *Biochem. J.*, 1921, xv, 286.

tion of cells is indicated by the fact that it is very abundant in embryonic tissue[14,15] and also in liver,[14] both of which give extracts with marked growth-promoting properties. Therefore, an attempt was made to see if it would increase the growth-promoting power of the mixture of casein digest, glycocoll, and nucleic acid (C. G. N.).

The glutathione in this and the other experiments reported in the present paper was very kindly furnished for this purpose by Dr. Carl Voegtlin of the U. S. Public Health Service at Washington, D. C., and had been shown to have the same composition as the glutathione prepared by Hopkins,[13] namely that of a dipeptide of glutamic acid and cysteine.[16] The solution used contained 20 mg. of glutathione in 10 cc. of Ringer solution and was sterilized by filtering through a Berkefeld filter. Only 10 cc. of solution were prepared at a time and this was kept in a refrigerator until completely used to avoid oxidation changes as far as possible.

The following media were used for the experiment:

Experimental medium	Control medium
1.0 cc. casein digest	1.0 cc. casein digest
1.5 cc. glycocoll solution	1.5 cc. glycocoll solution
1.5 cc. nucleic acid solution	1.5 cc. nucleic acid solution
1.0 cc. glutathione solution	1.0 cc. Tyrode solution

In two out of three experiments, there was a slightly larger growth in the medium containing glutathione (Text-fig. 2). The difference was too small to be very significant, but here again microscopical examination of the colonies showed that the growth was thicker in the medium containing glutathione, and the cells at the edge of the colony were also in somewhat better condition than those in the control medium.

Combined Action of Glutathione and Ash of Liver on the Growth of Sarcomatous Fibroblasts.

Since both the ash of liver and glutathione seemed to increase slightly the nutritive value of the mixture of casein digest, glycocoll, and nucleic acid, the action of the two together was tested.

[14] Thompson, J. W., and Voegtlin, C., *J. Biol. Chem.*, 1926, lxx, 793. Hopkins, F. G., *Biochem. J.*, 1921, xv, 286. Tunnicliffe, H. E., *Biochem. J.*, 1925, xix, 194. Murray, H. A., *J. Gen. Physiol.*, 1926, ix, 621.

[15] It has also been shown by Murray that the glutathione content of embryos varies with age. He suggests that this may be associated with the different rates of growth of embryonic tissue taken from embryos of different ages. Murray, H. A., *J. Gen. Physiol.*, 1926, ix, 621.

[16] Johnson, J. M., and Voegtlin, C., *J. Biol. Chem.*, 1927, lxxv, 703.

The media were made as follows:

Experimental medium	Control medium
1.0 cc. casein digest	1.0 cc. casein digest
1.5 cc. glycocoll solution	1.5 cc. glycocoll solution
1.5 cc. nucleic acid solution	1.5 cc. nucleic acid solution
1.5 cc. glutathione solution	3.0 cc. Tyrode solution
1.5 cc. ash of liver solution	

In this case, there was a very noticeable difference in the nutritive value of the two media. At the end of the first 7 days, there was a slightly larger growth in the colonies cultivated in the medium contain-

TABLE I.

Effect of Glutathione and Ash of Liver When Both Are Added to a Mixture of Casein Digest, Glycocoll, and Nucleic Acid, on the Growth of Sarcomatous Fibroblasts.

Experiment No.	Time of growth	Area of growth* in C. G. N. Control	Area of growth* in C. G. N., ash, and glutathione. Experiment	Ratio: $\frac{E}{C}$	Remarks
	days				
4617 A	7	125	150	1.2	First passage
4618 A	7	110	122	1.1	" "
4637 A-1	7	90	98	1.1	Second " ; thicker growth in experiment
4637 A-2	7	91	113	1.2	Second passage; thicker growth in experiment
4638 A-1	9	80	100	1.25	Second passage; thicker growth in experiment
4638 A-2	9	102	143	1.4	Second passage; thicker growth in experiment

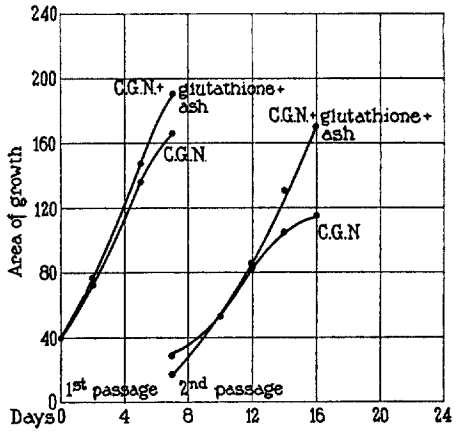
* This is the area in square centimeters of the image of the new growth as drawn with a projectoscope, which magnifies the size approximately 250 times.

ing glutathione and ash of liver than in the controls. The new cells around the edge of the colony were in better condition than those of the controls in the C.G.N. mixture,[9] although there were a number of dead cells at the center of the colonies. The center was then removed, and the colonies were transferred to new flasks to continue the experiment. At the end of 16 days, there was a very decided difference in their appearance. Those in the medium containing the glutathione and ash were not only larger in area (Text-fig. 3, Table I), but con-

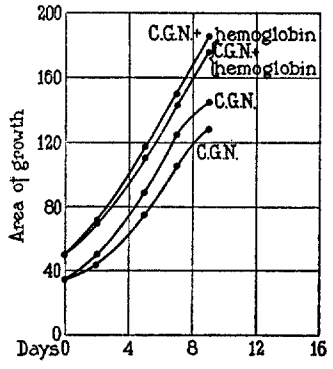
tained a much thicker growth and the cells were in far better condition than those of the colonies of the controls. Their condition was not as good, however, as those of the same strain which were cultivated in embryo juice.

Action of Hemoglobin on the Growth of Sarcomatous Fibroblasts.

The hemoglobin for the experiments was obtained from the red cells of heparinized rat blood.



TEXT-FIG. 3.



TEXT-FIG. 4.

TEXT-FIG. 3. Experiments 4617 A and 4638 A. Effect of ash of liver and glutathione when added to a mixture of casein digest, glycocoll, and nucleic acid (C.G.N.), on the growth of sarcomatous fibroblasts of the rat. The growth in the C.G.N., glutathione, and ash was much thicker than that in C.G.N. alone.

TEXT-FIG. 4. Experiment 4685 A. Effect of rat hemoglobin when added to a mixture of casein digest, glycocoll, and nucleic acid (C.G.N.), on the growth of sarcomatous fibroblasts of the rat.

The cells were separated from the plasma by centrifugation, washed three times in Ringer solution, and centrifuged. 1.5 cc. from the lower layer of cells was removed by a capillary pipette and hemolyzed by the addition of 9 cc. of water, and again centrifuged. 1 cc. of the clear supernatant fluid was diluted with 9 cc. of Ringer solution. The final preparation contained 0.029 per cent of nitrogen.

When this hemoglobin was added to the mixture of casein digest, glycocoll, and nucleic acid, a very small but decided increase in the area of growth was obtained (Text-fig. 4, Table II). In one experi-

ment, after five passages a small cell proliferation was still taking place in the medium containing the hemoglobin, although the colonies in the control had died. Here also it seemed probable that hemoglobin had some beneficial effect, but that the full extent of its action was not demonstrated because some other essential substance was lacking.

Combined Action of Ash of Liver, Glutathione, and Hemoglobin on the Growth of Sarcomatous Fibroblasts.

Since the preceding experiments indicated that each of these substances had a slightly beneficial effect on the growth of fibroblasts,

TABLE II.

Effect of Hemoglobin When Added to Casein Digest, Glycocoll, and Nucleic Acid, on the Growth of Sarcomatous Fibroblasts.

Experiment No.	Time of growth	Area of growth* in C. G. N. Control.	Area of growth* in C. G. N. and hemoglobin. Experiment	Ratio: $\frac{E}{C}$	Remarks
	days				
4685 A-1	10	91	126	1.4	First passage
4685 A-2	10	107	140	1.3	" "
4697 A-1	10	38	57	1.5	" "
4697 A-2	10	26	28	1.1	" "
107 J	7	94	139	1.5	" "
131 J	9	69	96	1.4	Second "
155 J	6	30	65	2.2	Third "
178 J	9	15	75	5.0	Fourth "
208 J	6	0	14	∞	Fifth "

their combined action was tested by adding all three of them to the mixture of casein digest, glycocoll, and nucleic acid, and comparing the growth-promoting power of this mixture[17] with that of casein digest, glycocoll, and nucleic acid alone.

The media used were made as follows:

Experimental medium	Control medium
1.0 cc. casein digest	1.0 cc. casein digest
1.5 cc. glycocoll solution	1.5 cc. glycocoll solution
1.5 cc. nucleic acid solution	1.5 cc. nucleic acid solution
1.0 cc. ash of liver solution	3.0 cc. Tyrode solution
1.5 cc. glutathione solution	
0.5 cc. hemoglobin solution	

[17] This mixture will be designated by the abbreviation C.G.N.A.G.H.

Very striking results were obtained. In the first passage, the rate of growth of the tissues in the mixture containing hemoglobin, ash, and glutathione greatly exceeded that in the C.G.N. mixture (Text-fig. 5). In some cases, the difference was as great as 90 and 100 per cent (Table III). In the second and third passages, still greater differences were observed. The rate of growth in the mixture of casein digest,

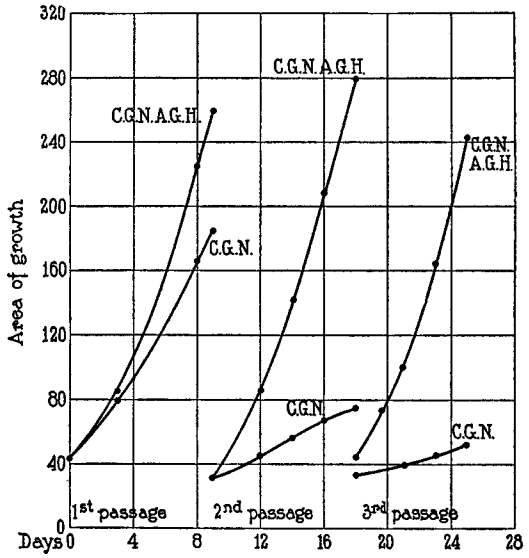
TABLE III.

Effect of Hemoglobin, Glutathione, and Ash of Liver When Added to a Mixture of Casein Digest, Glycocoll, and Nucleic Acid, on the Growth of Sarcomatous Fibroblasts.

Experiment No.	Time of growth	Area of growth* in C. G. N. Control	Area of growth* in C. G. N. A. G. H. Experiment	Ratio: $\frac{E}{C}$	Remarks
	days				
4621 A-1	9	138	255	1.8	First passage; cells better in experiment
4621 A-2	9	126	213	1.7	" "
4654 A-1	9	69	138	2.0	" "
4654 A-2	9	70	117	1.7	" "
4651 A-1	9	36	195	5.4	Second passage; cells better in experiment
4651 A-2	9	34	191	5.6	" "
4651 A-3	9	49	246	5.0	" "
4651 A-4	9	35	219	6.2	" "
4676 A	7	19	190	10.0	Third passage; cells better in experiment
4679 A	7	57	133	2.3	Second passage; cells better in experiment
4680 A	7	35	160	4.6	Third passage; cells better in experiment

glycocoll, and nucleic acid decreased progressively as the time increased, and the cells gradually degenerated. In the mixture containing the ash, glutathione, and hemoglobin, the initial rapid rate of growth continued in the second and third passages, giving large colonies with thick growth and very active cells in good condition (Text-fig. 5). In fact, the growth appeared to be fully as great as that which usually takes place in embryo juice. Therefore, after the second passage, some of the colonies which had been growing for 18

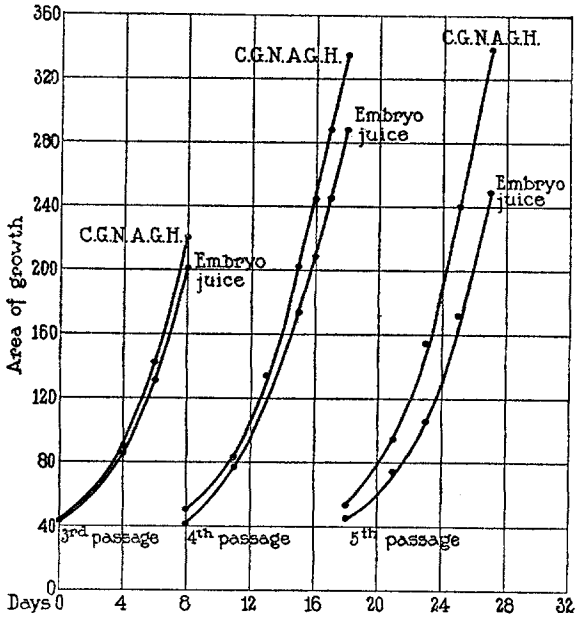
days in this mixture (C.G.N.A.G.H.) were divided. One half of each was cultivated in the same medium while the other half was cultivated as a control in embryo juice. The area of growth in the third, fourth, and fifth passages, or for 27 days more, proved to be fully as great in this artificial medium as in embryo juice (Text-fig. 6, Table IV). The nutritive value of this artificial medium is, however, not as great as that of embryo juice. The colonies in the latter were composed of a fine network of cells with apparently clear cytoplasm,



TEXT-FIG. 5. Experiments 4621 A, 4651 A, and 4676 A. Effect of ash of liver, glutathione, and rat hemoglobin (A.G.H.) when added to a mixture of casein digest, glycocoll, and nucleic acid (C.G.N.), on the growth of sarcomatous fibroblasts of the rat.

all in healthy condition. Those in the C.G.N.A.G.H. mixture, although larger than those in embryo juice, were composed of cells that showed from the beginning many tiny fat droplets and numerous cytoplasmic granules. This seems to be a characteristic appearance of cells cultivated in proteolytic products and is not necessarily a condition of degeneration, but is often associated with rapid proliferation. For the first four passages, the cells were small, oval, grew compactly, and were very similar to the sarcomatous fibroblasts

cultivated in tryptic digests of liver. After the fourth passage, the growth was much thinner, and a considerable number of dead cells were scattered throughout the culture. In any passage after 8 days of growth in one flask, the central or original fragment of the colony was surrounded with dead cells. The cells further out, however, appeared to be in a healthy condition and continued in an active state



TEXT-FIG. 6. Experiments 4671 A and 4690 A. Comparison of the growth of sarcomatous fibroblasts of the rat in a mixture of casein digest, glycocoll, nucleic acid, ash of liver, glutathione, and rat hemoglobin (C.G.N.A.G.H.) during the third, fourth, and fifth passages in this mixture, with that in embryo juice.

of proliferation. The presence of dead cells within these colonies seems to be associated with the presence of glutathione, as this condition prevailed in the other glutathione experiments in which hemoglobin and ash were not used. When transplanted to a new flask in a new plasma coagulum, a rapid growth of the colonies occurred in which no dead cells appeared for many days. The exact cause of this phenomenon has not been ascertained. It is evident that this artificial medium either still lacks some substance essential for the

proper functioning of the cells or else contains some toxic substances. It is also highly probable that the proportion of the various constit-

TABLE IV.

Comparison of the Growth of Sarcomatous Fibroblasts in a Mixture of Casein Digest, Glycocoll, Nucleic Acid, Ash of Liver, Glutathione, and Hemoglobin, with That in Embryo Juice.

Experiment No.	Time of growth	Area of growth* in embryo juice	Area of growth* in C. G. N. A. G. H. Experiment	Ratio: $\frac{E}{C}$	Remarks
	days				
4679 A	7	98	133	1.4	Second passage in C. G. N. A. G. H.
4671 A	8	160	158	0.99	Third " " "
4690 A	10	248	277	1.1	Fourth " " "
4690 A	10	186	278	1.5	" " " "
4711 A	9	161	283	1.8	Fifth " " "
4711 A	9	205	307	1.5	" " " "
4712 A	7	146	186	1.3	" " " "

TABLE V.

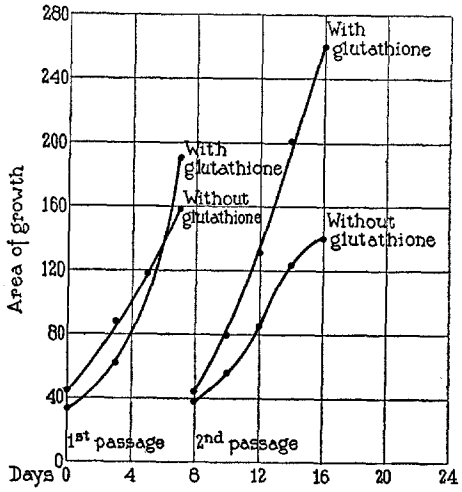
Comparison of the Growth of Sarcomatous Fibroblasts in a Mixture of Casein Digest, Glycocoll, Nucleic Acid, Ash of Liver, and Hemoblogin, with and without the Addition of Glutathione.

Experiment No.	Time of growth	Area of growth* without glutathione. Control	Area of growth* with glutathione. Experiment	Ratio: $\frac{E}{C}$	Remarks
	days				
4672 A	8	96	223	2.3	First passage without glutathione; cells better in experiment
4680 A	7	113	160	1.4	First passage without glutathione
4693 A-1	7	100	174	1.7	Second " " " cells poor in control
4693 A-2	7	92	214	2.3	Second passage without glutathione; cells poor in control
4702 A	9	64	169	2.6	Second passage without glutathione

uents to each other is not the optimum for maintaining the cells in the best condition.

The Rôle Played by the Ash and the Glutathione in the Artificial Medium.

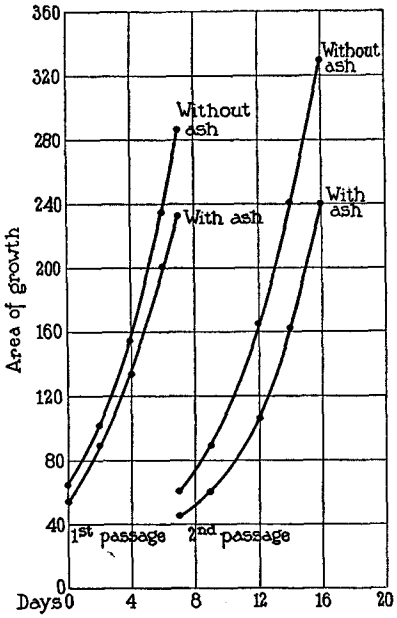
Although the combined action of the ash of liver, glutathione, and hemoglobin, when added to casein digest, glycocoll, and nucleic acid, caused a rapid proliferation of fibroblasts, it does not necessarily follow that all three of these contributed to the result. The above experiments left no doubt regarding the beneficial action of hemoglobin. In order to ascertain conclusively whether both the ash and glutathione were utilized, this artificial medium was tested for its growth-promoting power on sarcomatous fibroblasts, with and without the



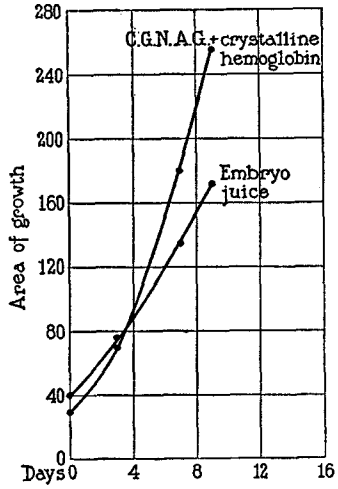
TEXT-FIG. 7. Experiments 4680 A and 4693 A. Comparison of the growth of sarcomatous fibroblasts of the rat in a mixture of casein digest, glycocoll, nucleic acid, ash of liver, and rat hemoglobin, with and without the addition of glutathione.

glutathione, and also with and without the ash. The omission of the glutathione from the medium resulted in a marked decrease of growth, especially in the second passage (Text-fig. 7, Table V). A rapid degeneration of the cells also took place. On the other hand, the colonies cultivated without the ash grew fully as large as those with it, and their cells seemed to have fewer cytoplasmic granules (Text-fig. 8, Table VI). Since the ash had caused a slight increase in the nutritive value of the C.G.N. mixture; and better results were obtained with glutathione and ash than with only the glutathione, it must be con-

cluded that some mineral constituent of the ash, probably the iron, does play some part in the functioning of the cells, but that the hemoglobin furnishes the necessary iron in a more utilizable form. There is also the possibility that the quantity of mineral constituents



TEXT-FIG. 8.



TEXT-FIG. 9.

TEXT-FIG. 8. Experiments 4694 A and 4709 A. Comparison of the growth of sarcomatous fibroblasts of the rat in a mixture of casein digest, glycocoll, nucleic acid, glutathione, and rat hemoglobin, with and without the addition of ash of liver.

TEXT-FIG. 9. Experiment 4607 A. Comparison of the growth of sarcomatous fibroblasts of the rat in embryo juice and in a mixture of casein digest, glycocoll, nucleic acid, ash of liver, glutathione, and hemoglobin recrystallized three times.

of the ash required by the cells is so small that they have absorbed from their previous medium a sufficient supply to function for a considerable period.

The Effect of Using Crystalline Hemoglobin in the Artificial Medium.

The same rapid proliferation of cells took place when a solution of rat hemoglobin crystallized three times was substituted for the

original solution of hemoglobin (Text-fig. 9). Therefore, it is the hemoglobin itself, and not any other substance derived from the hemolyzed red cells, that brings about this action. However, the cells in these experiments were not in quite as good condition as in the previous ones. Whether the crystallization removed some substance which accounts for this has not been ascertained as yet.

Action of Glutathione, Ash of Liver, and Hemoglobin on the Proliferation of Normal Fibroblasts.

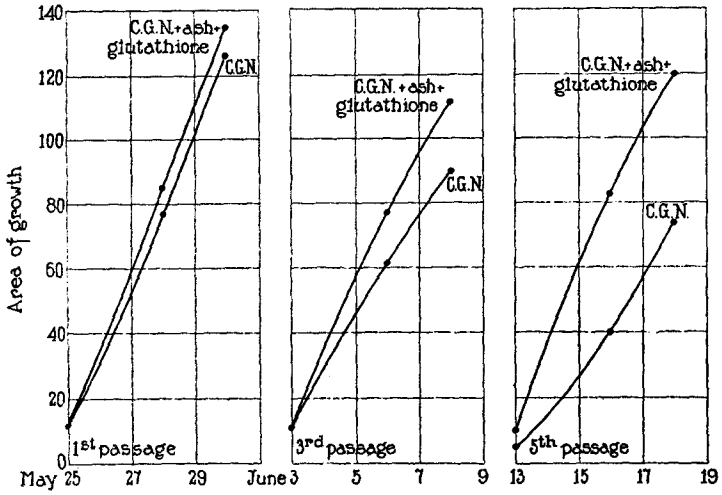
In some cases, experiments parallel with the above were carried out simultaneously on normal fibroblasts of the rat. For these cells

TABLE VI.

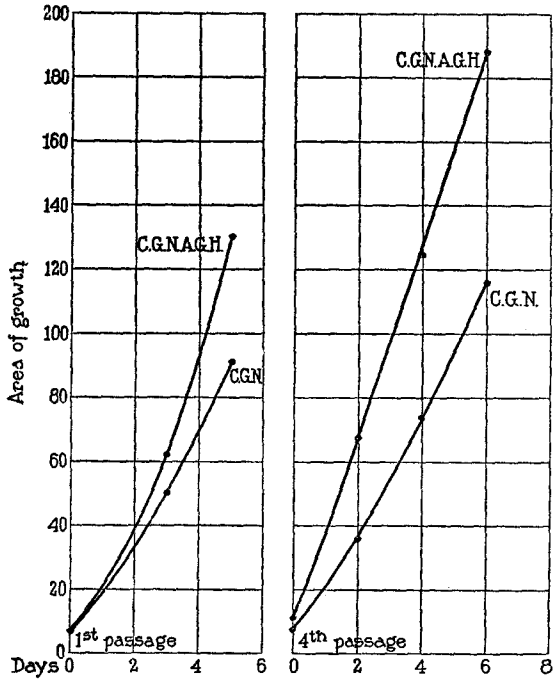
Comparison of the Growth of Sarcomatous Fibroblasts in a Mixture of Casein Digest, Glycocoll, Nucleic Acid, Glutathione, and Hemoglobin, with and without the Addition of Ash of Liver.

Experiment No.	Time of growth	Area of growth* without ash. Control.	Area of growth* with ash. Experiment	Ratio: $\frac{E}{C}$	Remarks
	days				
4677 A	7	116	121	1.0	First passage without ash
4694 A	7	220	178	0.81	Second " " "
4701 A	9	221	205	0.93	First " " "
4709 A-1	9	270	145	0.54	Second " " "
4709 A-2	9	194	195	1.0	" " " "
4713 A-1	8	138	186	1.3	" " " "
4713 A-2	8	149	188	1.3	" " " "

also, the addition of ash of liver, glutathione, and hemoglobin to the mixture of casein digest, glycocoll, and nucleic acid increased the nutritive value of the mixture (Text-figs. 10 and 11). This artificial medium is not as well adapted to the cultivation of the normal as of the sarcomatous fibroblasts. The cells in the experiments and also those in the controls filled with fat, and the colonies presented a much more opaque appearance than those cultivated in embryo juice. This difference between normal and sarcomatous fibroblasts has been observed in all cases where attempts have been made to cultivate them in media containing hydrolyzed proteins, as for instance in the peptic and tryptic digests of liver.[4,7] Nevertheless, the normal fibroblasts proliferated for several passages in this medium.



TEXT-FIG. 10. Experiments 4615 A, 4646 A, and 4666 A. Effect of ash of liver and glutathione when added to a mixture of casein digest, glycocoll, and nucleic acid (C.G.N.), on the growth of *normal* fibroblasts of the rat.



TEXT-FIG. 11. Experiments 4659 A and 4665 A. Effect of ash of liver, glutathione, and rat hemoglobin when added to a mixture of casein digest, glycocoll, and nucleic acid, on the growth of *normal* fibroblasts of the rat.

DISCUSSION.

The hypothesis was suggested above that a definite oxidation-reduction potential in the medium might be as important for the continuous proliferation of fibroblasts as a given hydrogen ion concentration or osmotic pressure. It is quite possible that the glutathione and hemoglobin act not only by regulating the respiration and oxidation-reduction reactions within the cell, but also by creating a desirable oxidation-reduction potential of the medium. They also furnish necessary materials for the synthesis of protoplasm. A study is being made of the oxidation-reduction potential of this medium and of the others in which cell proliferation takes place. It does not necessarily follow from these experiments that hemoglobin as such is essential for the proliferation of sarcomatous fibroblasts. It has already been shown that they proliferate indefinitely in the tryptic and peptic digests[4,7] of liver in which the protein part of the molecule has been destroyed by enzymatic action. Experiments will be made to ascertain whether an iron pyrrol compound such as hemin can replace the hemoglobin in this medium. The growth-promoting action of glutathione is interesting in connection with the data recently published on the nitrogen metabolism of fibroblasts, in which it was shown that a certain amount of peptide nitrogen was required for their continuous proliferation.[4] The growth-promoting action of liver digests was destroyed by complete hydrolysis with hydrochloric acid.[4] It is quite probable that the deleterious action of acid hydrolysis was due in part at least to its destruction of glutathione, and that this substance cannot be resynthetized by these cells. It has been suggested by Voegtlin and Thompson,[18] as a result of their work on the glutathione content of tissues of tumor-bearing animals, that the rapid growth of tumor, especially in sarcomatous rats, deprives other tissues of their normal content of glutathione and brings about a state of malnutrition. They could not ascertain whether this effect was due to the utilization of glutathione and other nutrients by the growing tumor or to the toxic action of necrotic material. These experiments prove that glutathione is required as a nutrient substance by sarcoma cells. There is, however, no evidence as yet as to whether

[18] Voegtlin, C., and Thompson, J. W., *J. Biol. Chem.*, 1926, lxx, 801.

the requirement of sarcomatous fibroblasts exceeds that of normal fibroblasts. Even though their requirement should be identical, it is probable that rapid multiplication of tumor tissue could produce this effect.

The experiments cited above are interesting in connection with the question of respiration or oxidation within the cell. The continuous life and multiplication of cells *in vitro* would most certainly indicate that a respiration normal for the tissue in question, and oxidations essential to life processes were occurring. For the life of fibroblasts *in vitro*, both glutathione and an iron compound are necessary. Iron in the form of hemoglobin, or hemoglobin hydrolyzed by enzymes, suffices, while iron in the inorganic form has failed to do so in all experiments tried thus far. These experiments, therefore, are in accord with the observations of Meyerhof,[19] and of Hopkins [20] on the importance of compounds containing the SH groups to cell oxidations, and also with those of Warburg[21] on the importance of iron or an iron-containing compound as a respiratory ferment.

SUMMARY.

1. It has been shown that the ash of liver, hemoglobin, and glutathione each exerts a very slight beneficial effect on the growth of sarcomatous fibroblasts of the rat, or on the condition of their cells when cultivated in a synthetic medium.

2. The addition of all three of these substances, or of only glutathione and hemoglobin, to a mixture of casein digest, glycocoll, and nucleic acid gives a medium in which sarcomatous fibroblasts of the rat proliferate for a considerable time as rapidly as in embryo juice.

3. The mixture is not as adequate a nutritive medium as embryo juice, for after a time dead cells are found surrounding the central fragment of the culture, and after several passages the growth becomes thinner.

4. The hypothesis is suggested that glutathione and hemoglobin may function not only by regulating the respiration and oxidation-

[19] Meyerhof, O., *Arch. ges. Physiol.*, 1923, cxcix, 531.

[20] Hopkins, F. G., *Biochem. J.*, 1925, xix, 787.

[21] Warburg, O., *Biochem. Z.*, 1924, clii, 479; *Naturwissensch.*, 1928, xvi, 345.

reduction reactions within the cell, but also by regulating the oxidation-reduction potential of the medium.

5. It is suggested that the failure to obtain growth of fibroblasts in mixtures of amino acids or of the products of complete acid hydrolysis of proteins is in part due to the absence of glutathione, and that this substance is not synthetized by fibroblasts.

6. The growth of normal fibroblasts of the rat is also increased by the addition of the above mentioned substances to a synthetic medium.