THE CHEMICAL NATURE OF SUBSTANCES REQUIRED FOR CELL MULTIPLICATION.

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It is well known that tissue cells live indefinitely when cultivated in plasma and embryonic juice.[1] In such a medium, strains of fibroblasts and epithelium have proliferated without interruption for several years. It appeared that the chemical substances from which the cells synthetize new protoplasm are constituents of embryo juice and not of blood serum.[2] However, it has not been ascertained whether the building up of new cells merely requires nutrient materials present in the embryo juice or whether the juice also contains some specific hormone essential to cell division. Many attempts have been made to determine the nature of the substances used by the tissues, and to isolate the principle responsible for growth. Embryonic tissue extracts were fractionated and the activity of each fraction was tested on fibroblasts. These experiments pointed out that the growth-promoting substance consisted of a protein or was closely associated with a protein.[3] The suggestion was made that the nitrogenous material required by the tissues might be a product obtained from the protein by the cells through some ferment action.[3] It was also found that amino acids and other dialyzable nitrogenous compounds of the embryonic juice caused no increase in the mass of the tissues, although they had the property of stimulating cell migra-

[1] Carrel, A., *J. Exp. Med.*, 1912, xv, 516; 1913, xvii, 14. Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 755; *Compt. rend. Soc. biol.*, 1924, xc, 562; *J. Exp. Med.*, 1925, xli, 337.

[2] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317; 1923, xxxvii, 759.

[3] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1926, xliv, 387.

tion and multiplication.[4,5] The products of tryptic digestion and complete peptic digestion of embryo juice protein were also unable to support cell proliferation, under the conditions of our experiments.

Further investigation of the processes of cell nutrition led to unexpected results: tissue cells were found to utilize the higher cleavage products of the protein molecule for multiplication.[6,7] The purpose of the present paper is to report the experiments in the course of which this important fact was discovered.

Technique for Preparing the Protein Split Products.

The protein split products were obtained by digesting embryonic pulp, egg white, rabbit brain, and commercial fibrin presumably from ox blood, at 40°C. with Fairchild's pepsin in N/20 HCl. The process was carried out for different lengths of time, and the ratios of pepsin to protein and of protein and pepsin to acid were varied in such a manner as to obtain products representing different states of digestion. The degree of digestion was followed by amino nitrogen determinations. Afterwards, the different solutions were brought to a pH of 7.0 by the addition of normal NaOH, and boiled down to somewhat less than half their original volume, in order to destroy the pepsin and remove the toluene which was added in some instances as an antiseptic. The boiling was also designed to remove any unchanged protein or heat-precipitable products and to concentrate the salts. After determination of the cryoscopic point, the solutions were rendered isotonic by the addition of water or Ringer solution of five times its usual concentration. The pH was adjusted at 7.2 to 7.4, and the ratio of total nitrogen to amino nitrogen determined.

Split products were also obtained by autoclaving the protein in water, in N/10 HCl or NaOH in a steam sterilizer at various pressures for different lengths of time. The pH was then adjusted and the solution prepared as described above.

Technique for Testing the Growth-Promoting Action of the Protein Cleavage Products.

The growth-promoting action of the above substances at several concentrations was tested on fibroblasts, according to two techniques which have already been described. In the first procedure,[8] fragments of heart were cultivated on

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[4] Carrel, A., and Ebeling, A. H., *Compt. rend. Soc. biol.*, 1924, xc, 31.

[5] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1926, xliv, 397.

[6] Carrel, A., *Compt. rend. Soc. biol.*, 1926, xciv, 1060.

[7] Carrel, A., and Baker, L. E., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiii, 627.

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

hollow slides in a control medium composed of equal parts of plasma and Ringer solution, and in an experimental medium composed of equal parts of plasma and the protein cleavage products. Two or three cultures were made in each of several different concentrations of the experimental material, and allowed to grow for 48 or 72 hours. The growth was measured by the width of the ring of new tissue, or by the increase in area of the colonies. In the second procedure,[9] heart tissue or fibroblasts were cultivated in flasks containing 0.5 cc. serum, 1 cc. Tyrode

TABLE I.

Rates of Growth of Fibroblasts in the Peptic Digestion Products of Embryo Tissue.

Culture group No.*	Chemical preparation No.	Digestion	Total nitrogen	Amino nitrogen	Ratio: Total N / Amino N	Width† of new tissue					Time of growth	Ratio: Peptic digest / Ringer solution			
						Ringer solution	Peptic digest								
							Pure	Diluted				Pure	Diluted		
								1:2	1:4	1:8			1:2	1:4	1:8
		hrs.	*per cent*	*per cent*							*hrs.*				
3171 A	X–274	32	.378	.168	2.25	1.5	2.0	1.5	2.0		48	1.3	1.0	1.3	
8649 D‡	X–271	16	.298	.135	2.21	5.0				4.0	48				.8
8643 D	X–271	16	.298	.135	2.21	2.0	2.0	2.0	2.0		48	1.0	1.0	1.0	
3178 A§	X–276	3.5	.352	.0525	6.7	1.5	5.0–6.0		4.0	5.0	48	3.6		2.6	3.3
3185 A§	X–276	3.5	.352	.0525	6.7	3.0	7.0		9.0	6.0	72	2.3		3.0	2.0
3192 A	X–276	3.5	.352	.0525	6.7	2.0	2.0		4.0	3.0	48	1.0		2.0	1.5
3201 A‖	X–287	3.5	.358	.0825	4.34	1.5	6.0		2.5	2.0	48	4.0		1.7	1.3
3219 A‖	X–287	3.5	.358	.0825	4.34	3.0	7.0		3.0	2.5	48	2.3		1.0	.83

*Each culture group number represents 6 to 10 experiments.

† Measured under the microscope in 0.1 mm. units.

‡ Experiments were also made at dilutions of 1:12 and 1:16. These showed no increase over the control.

§ Tyrode solution was used as control in place of Ringer solution.

‖ A second control solution, containing pepsin treated in the same way as the experiment, was used. The cultures in the control pepsin solution showed less growth than those in the control Ringer solution.

solution, and 0.5 cc. embryonic tissue extract. After coagulation, 1 cc. of Tyrode solution was added to the controls and 1 cc. of the preparation to the experiments. Every 2 days, the fluid medium was removed, the coagulum washed with 2 cc. Tyrode solution, and fresh fluid added. When digestion of the fibrin occurred, the coagulum of both control and experiment was patched with 0.25 cc. plasma and 0.25 cc. embryonic extract. The tissues were allowed to grow for periods

[9] Carrel, A., *J. Exp. Med.*, 1923, xxxviii, 407.

varying from 1 to 2 weeks. The cultures were placed under the projectoscope daily, and the area of the colonies was traced and measured with the planimeter. The action of the experimental substances was expressed by a graph on which the area of the colonies was plotted as a function of the time.

Action on Fibroblasts of Embryo Tissue Hydrolyzed by Pepsin.

Embryonic tissues reduced to a fine pulp in a Latapie apparatus were digested with pepsin for 32, 16, and 3.5 hours respectively, and a comparison was made of the effects of these products and of Ringer solution on fibroblasts migrating from heart tissue. The products of 32 and 16 hours digestion showed no growth-stimulating action (Table I), and appeared to be somewhat toxic, the cells generally becoming loaded with fat granules and losing their activity. On the contrary, those obtained by 3.5 hours digestion showed a decided stimulating action, the width of the new growth being two and occasionally three times as great as that of the control in Ringer solution (Table I). The ratio of total to amino nitrogen was determined in each of these digests. The 32 and 16 hour digests had a ratio of 2.25 and 2.21 respectively, while the ratios in two different 3.5 hour digests were 4.34 and 6.7. The first two were slightly toxic, while the last ones promoted tissue growth, the greater growth being observed in the last digest. This indicated that the tissues utilized certain intermediate cleavage products for growth, probably some of the larger fragments of the protein molecule.

Action on Fibroblasts of Egg White Hydrolyzed by Pepsin.

The products of partial peptic hydrolysis of egg white were also tested on fibroblasts. The length of the time of digestion varied, but the protein was never completely hydrolyzed and some of the larger cleavage products remained in solution. These substances always determined a large growth of fibroblasts (Table II). It was thus obvious that nutrient substances could be obtained from egg white by pepsin hydrolysis.

Action on Fibroblasts of the Cleavage Products of Commercial Fibrin.

A similar effect on fibroblasts was exhibited by the cleavage products of a protein unrelated to that of chicken embryo or egg white, namely,

commercial fibrin (Table III). The growth-promoting action of some of the hydrolyzed products of fibrin was even greater than that of the embryonic tissue and egg white digests. This phenomenon may be due merely to the state of digestion reached by the protein, rather than to its nature. The split products of fibrin not only brought about

TABLE II.

Rate of Growth of Fibroblasts in the Peptic Digestion Products of Egg White.

Culture group No.*	Chemical preparation No.	Width† of new tissue					Time of growth	Ratio: Peptic digest / Ringer solution			
		Ringer solution	Peptic digest					Pure	Diluted		
			Pure	Diluted							
				1:2	1:4	1:8			1:2	1:4	1:8
							hrs.				
8667 D	X-288	2.3	2.5–4.0	4.0	2.5		48	1.1–1.7	1.7	1.1	
8673 D	X-282	3.0	5.0	4.0	3.0		48	1.7	1.3	1.0	
8680 D	X-288	1.5	2.0				48	1.3			
	X-288 a	1.5	3.0				48	2.0			
	X-291	1.5	3.5				48	2.3			
	X-291 a	1.5	2.5				48	1.7			
8702 D	X-305	3.0	6.0	4.0	3.0		48	2.0	1.3	1.0	
3177 A‡	X-275	1.5	4.0		4.0	3.0	48	2.6		2.6	2.0
3184 A‡	X-275	3.0	11.0		10.0	9.0	72	3.6		3.3	3.0
3191 A‡	X-275	2.0	5.0		3.0	3.0	48	2.5		1.5	1.5
3196 A	X-282	1.5	2.5	2.5	2.0		48	1.6	1.6	1.3	
8686 D	X-288	2.5	2.5				48	1.0			
	X-291	2.5	5.0				48	2.0			
	X-292	2.5	6.0				48	2.4			
	X-282	2.5	6.0				48	2.4			
3209 A	X-291	4.0	8.0		9.0	5.0	72	2.0		2.2	1.2
3212 A	X-292	2.0	4.0	6.0			48	2.0	3.0		

* Each culture group number represents 8 to 10 experiments.

† Measured under the microscope in 0.1 mm. units.

‡ Tyrode solution was used as control in place of Ringer solution.

a great increase in the proliferation of fibroblasts, but they also appeared to supply the tissues with the necessary nutrient material for prolonged growth (Fig. 1). When the digestion of fibrin was carried to the optimum degree, the growth of the tissues exceeded even that produced by embryonic juice. The fibroblasts that grew in the fibrin digestion products outlived those cultivated in embryo juice and, in

some cases, the volume of the tissue became four times as large as that of the controls (Fig. 3). Colonies of fibroblasts 2 cm. in diameter were obtained.

TABLE III.

Rate of Growth of Fibroblasts in the Peptic Digestion Products of Commercial Fibrin.

Culture group No.*	Chemical preparation No.	Width† of new tissue				Time of growth	Ratio: Peptic digest / Ringer solution		
		Ringer solution	Peptic digest						
			Pure	Diluted			Pure	Diluted	
				1:2	1:4			1:2	1:4
						hrs.			
8682 D	X-297	3.0	6.0	10.0	12.0	72	2.0	3.3	4.0
8693 D	X-297	2.5	6.0	5.0	6.0	48	2.4	2.0	2.4
3232 A	X-297	3.0	5.0	5.0		48	1.7	1.7	
3225 A	X-297	2.0	5.0	3.0	3.0	48	2.5	1.5	1.5
8683 D	X-298	2.0	3.0	3.0	3.0	48	1.5	1.5	1.5
8692 D	X-298	2.0	4.0	6.0	6.0	48	2.0	3.0	3.0
8703 D	X-298	2.5		5.0		48		2.0	
	X-299	2.5		6.0		48		2.4	
3233 A	X-298	2.0	4.5	3.5		48	2.25	1.75	
8703 D	X-299	4.0		6.0		48		1.5	
3234 A	X-299	4.0	5.0	5.0	6.0	48	1.25	1.25	1.5
8696 D	X-303	4.0	7.0	8.0	5.0	48	1.75	2.0	1.25
3288 A	X-329	2.0	6.0			48	3.0		
8710 D	X-310	2.0	3.0	3.5	3.0	48	1.5	1.75	1.5
3265 A	X-318	1.5	4.0			48	2.6		
	X-302		4.0			48	2.6		
	X-304		4.0			48	2.6		
3277 A‡	X-324	2.5	4.5			48	1.8		
	X-325		6.0			48	2.4		
	X-326		6.0			48	2.4		
8732 D‡	X-324	1.0	1.5			48	1.5		
	X-325		4.5			48	4.5		
	X-326		6.0			48	6.0		

* Each culture group number represents 8 experiments.

† Measured under the microscope in 0.1 mm. units.

‡ Preparations X-324, X-325, and X-326 varied considerably in the degree of hydrolysis. The ratio of total to amino nitrogen was 5.2, 6.3, and 9.0 respectively.

In order to secure some information concerning the nature of protein cleavage products utilized by the tissues, three different digests were prepared by varying the ratio of the weight of fibrin to the weight

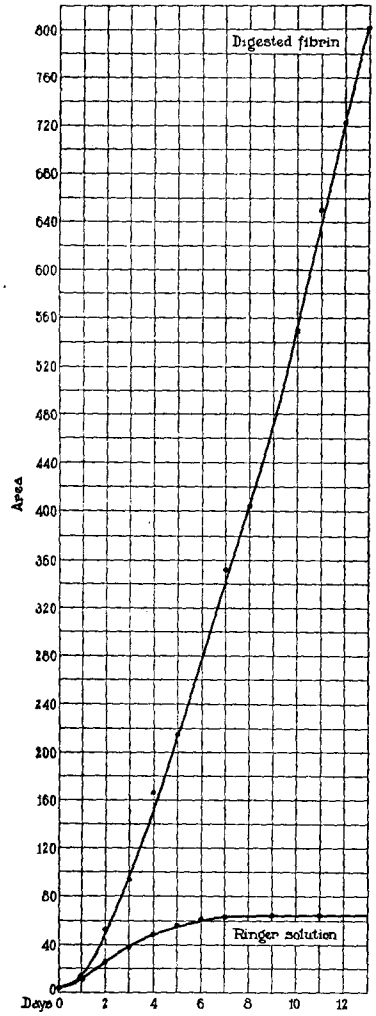

FIG. 1.

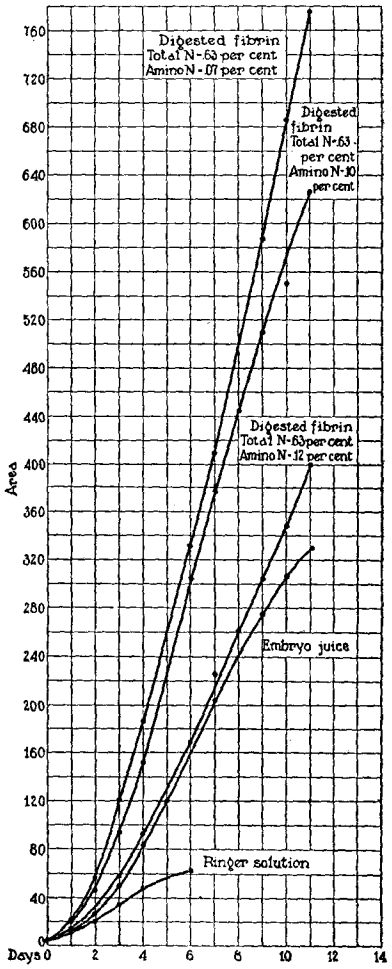

FIG. 2.

FIG. 1. Experiment 3284 A. Growth of fibroblasts in the products of partial peptic digestion of commercial fibrin. The control in Ringer solution died in 7 days. At the end of 2 weeks, the cells cultivated in the digested fibrin were still proliferating rapidly.

FIG. 2. Experiment 3287 A. Comparison of the rate of growth of fibroblasts in three fibrin digests of different degrees of hydrolysis, and in embryo juice and Ringer solution. The three fibrin digests contained the same concentration of total nitrogen, but the ratios of total to amino nitrogen were 9.0, 6.3, and 5.2 respectively. The growth of the cultures in the fibrin digests continued far beyond the points shown in this chart.

of pepsin. 5, 10, and 15 gm. of fibrin were digested in 200 cc. of N/20 HCl with 1 gm. each of Fairchild's pepsin. In the first case, all the fibrin was transformed into soluble products which remained in solution on neutralizing and boiling. In the other two cases, some fibrin was left and much protein and meta-protein were precipitated

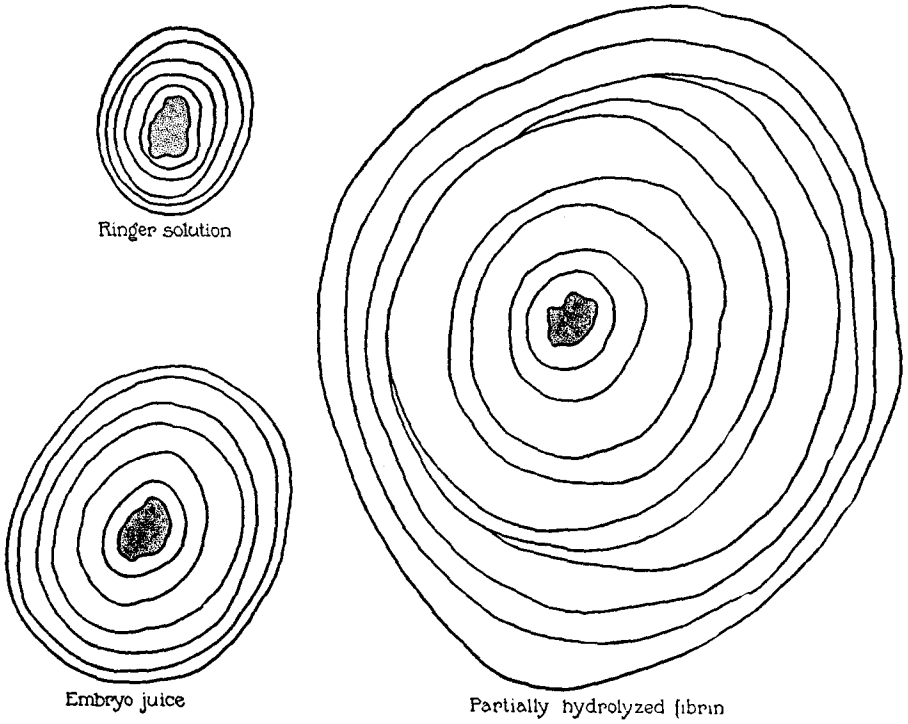

FIG. 3. Experiment 3287 A. Daily tracings under the projectoscope of areas of colonies of fibroblasts cultivated in Ringer solution, embryo juice, and partially digested fibrin. Some contraction of the clot took place on one side of the colony, resulting in a deformation. The growth in partially digested fibrin yielded the largest single colony of fibroblasts ever obtained *in vitro* in this laboratory.

when the solutions were neutralized and boiled. As a basis of comparison, the solutions were diluted to the same concentration of total nitrogen, 0.63 per cent, before being tested for growth-stimulating action on fibroblasts. The amino nitrogen concentration in these solutions was 0.12, 0.10, and 0.07 gm. per 100 cc., giving a ratio of

total to amino nitrogen of 5.2, 6.3, and 9.0, respectively. These solutions were compared with each other and with Ringer solution and embryonic tissue extract, with respect to their growth-promoting power on fibroblasts (Fig. 2). The results clearly indicate that the higher cleavage products are the ones utilized by the tissues. All of these digests produced more rapid and prolonged growth than the embryo juice did. Another pair of digests was compared in which the ratio of total to amino nitrogen was 7.7 and 8.6. In this case, a similar result was obtained. A series having the ratios 8.4, 8.6, and 10.3 showed very little difference, the rate of growth being exceptionally large in all three.

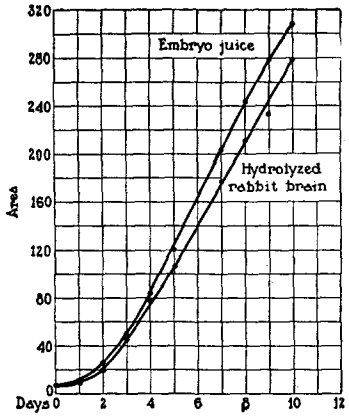

FIG. 4. Experiment 3304 A. Growth of fibroblasts in rabbit brain partially hydrolyzed by pepsin. The growth is comparable to that in embryo juice.

Action on Fibroblasts of Other Proteolytic Products.

Various proteins when partially hydrolyzed appeared to be nutrient for fibroblasts. Two pepsin digests of rabbit brain showed a nutrient action almost equal to that of embryo juice (Fig. 4). These digests gave a strong biuret reaction and contained protein split products having a ratio of total to amino nitrogen of 5.3. The rate of growth produced was comparable to, though less than, that of the fibrin digest having a ratio of 5.2. Hydrolytic products obtained by autoclaving egg white and fibrin in an acid medium at 15 pounds pressure for 1.5 hours proved stimulating to growth (Table IV). More

complete hydrolysis of these substances produced by autoclaving at 25 pounds pressure for 4 hours yielded toxic or inactive products, thus affording a further indication that the higher cleavage products of protein determine growth.

Action on Fibroblasts of Commercial Peptones.

Commercial "peptones" in solutions from 20 to 1 per cent, sterilized by boiling, were used in the culture medium of fibroblasts.

TABLE IV.

*Rate of Growth of Fibroblasts in Protein Cleavage Products Produced by Autoclaving in Acid.**

Culture group No.†	Chemical preparation No.	Substance autoclaved	Width‡ of new tissue				Time of growth	Ratio: Experiment / Control		
			Ringer solution	Experiment						
				Pure	Diluted			Pure	Diluted	
					1:2	1:4			1:2	1:4
							hrs.			
3218 A	X-295	Fibrin	1.5	3.0			48	2.0		
3227 A	X-296	"	3.5	5.0			48	1.4		
3235 A	X-296	"	3.0	6.0			48	2.0		
3237 A	X-296	"	1.0	4.0			48	4.0		
3245 A	X-296	"	1.0	4.0			48	4.0		
8689 D	X-296	"	2.0	4.0			48	2.0		
3205 A	X-290	Egg white	2.0	4.0	3.0	3.0	48	2.0	1.5	1.5
3205 A	X-290	" "	2.0	8.0	8.0	9.0	72	4.0	4.0	4.5

* 15 pounds pressure for 1.5 hours.

† Each culture group number represents 4 to 8 experiments.

‡ Measured under the microscope in 0.1 mm. units.

Armour's peptone was almost inactive. Parke Davis' and Fairchild's peptones exhibited a small growth-promoting action, while Witte's peptone determined a large growth of fibroblasts (Fig. 5). At a concentration of 5 to 10 per cent, it was toxic. Even in the cultures containing a low concentration of the peptone, the cells showed many fat globules. In spite of this, the rate of growth of the tissues treated with Witte's peptone was as great as that cultivated in embryo juice (Fig. 6). This difference in activity of the commercial peptones is undoubtedly due to the very large concentration of proteoses and the

smaller amount of the lower degradation products present in Witte's peptone in comparison with the others.

Action on Fibroblasts of the Proteose Fractions of Witte's Peptone.

The foregoing experiments indicated that proteoses are probably responsible for the effect of Witte's peptone and other protein digests on the proliferation of fibroblasts. A few preliminary experiments on the fractionation of Witte's peptone confirmed this idea. The meta-

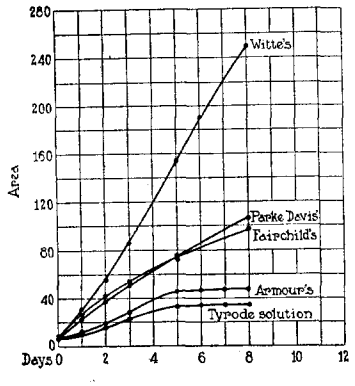

FIG. 5.

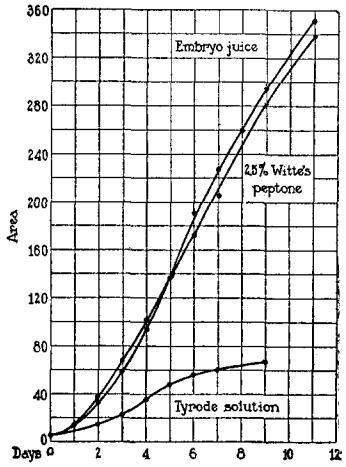

FIG. 6.

FIG. 5. Experiment 3349 A. Action of 2 per cent solutions of commercial "peptones" on the growth of fibroblasts *in vitro*.

FIG. 6. Experiment 3133 A. Growth of fibroblasts in 2.5 per cent Witte's peptone, the control being cultivated in embryo juice.

protein precipitated at pH 6.0 and the substances precipitated by 2.5 per cent trichloracetic acid had some growth-stimulating action on fibroblasts, but not as much as the remaining fractions. A fraction containing only proteoses was, therefore, prepared by precipitating the higher products, including a part of the proteoses, in 2.5 per cent trichloracetic acid. The remaining proteoses were separated from the peptones and lower degradation products by saturating at 33°C. with sodium sulfate, after removal of the trichloracetic acid. This process was repeated four times to insure removal of the other frac-

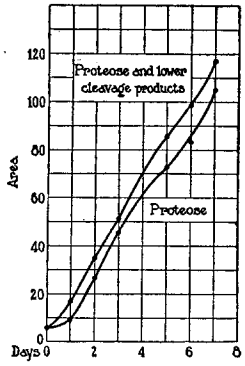

FIG. 7.

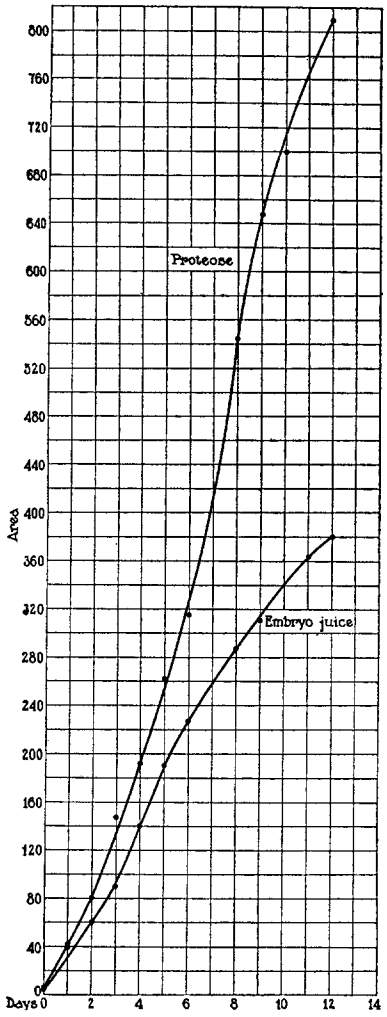

FIG. 8.

FIG. 7. Experiment 3292 A. Comparison of the rate of growth of fibroblasts cultivated in the proteose fraction of Witte's peptone and in the fraction containing proteoses, peptones, and lower degradation products. The cells in the proteose were in better condition than those in the fraction containing the lower split products, and they multiplied at about the same rate. Concentration of nitrogen equaled 0.398 per cent in both media.

FIG. 8. Experiment 8758 D. Growth of fibroblasts in the pure proteose fraction of Witte's peptone (0.2 per cent N). The control culture in embryo juice died on the 13th day. The colonies in the proteose were still growing after 30 days cultivation.

tions. The sulfate was precipitated by barium chloride and the solution was finally dialyzed. The effects on fibroblasts of this preparation at 0.398 per cent nitrogen, and of the trichloracetic acid filtrate containing the proteoses, peptones, and smaller split products, at the same nitrogen concentration, were compared. The rates of growth in the two media did not differ appreciably (Fig. 7), but the cells cultivated in the purified proteose solution showed fewer fat globules in their cytoplasm than those kept in the solution where peptones were still present. In another experiment, a comparison was made of the tissues growing in the purified proteose solution at 0.2 per cent N and in embryonic juice (Fig. 8). The colonies kept in the purified

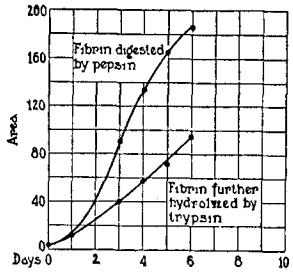

FIG. 9. Experiment 3326 A. Growth of fibroblasts in fibrin partially digested by pepsin and in the same substance further digested by trypsin at equal nitrogen concentration.

proteose grew twice as rapidly as those cultivated in embryonic tissue extract, and the cells contained a smaller number of fat globules.

Evidently, the tissues utilize proteose for building new protoplasm. The concentration of nitrogen in the proteose solutions and in the various protein digests is larger than that of embryonic tissue extract. This undoubtedly means that a part of these nitrogenous substances is not utilized by the tissues. Further fractionation of the proteose is being carried out in order to ascertain whether a more active moiety can be obtained.

Action on Fibroblasts of the Tryptic Digest of the Protein Cleavage Products.

A comparison was made of the rates of growth of fibroblasts in the most active peptic digest of fibrin and in the same solution further

hydrolyzed by trypsin. The results (Fig. 9) demonstrated that hydrolysis by trypsin greatly reduced the growth-promoting power of the solution, although it did not destroy it completely. The tryptic hydrolysis was not complete, however. It reduced the ratio of total to amino nitrogen from 9.0 to 2.5. Analysis of this tryptic digest showed the absence of proteose except in the smallest traces, and the presence of a considerable amount of peptones and peptides. Therefore, it would seem that the peptones and possibly some of the lower degradation products are nutrient to a certain extent, although they

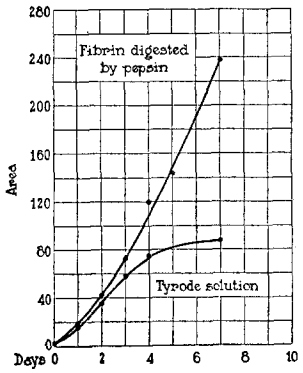

FIG. 10.

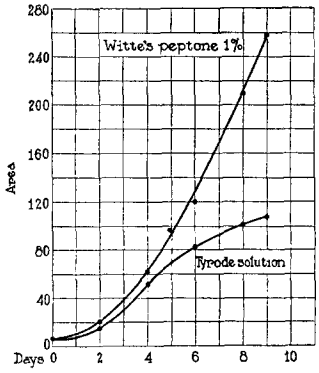

FIG. 11.

FIG. 10. Experiment 3283 A. Growth of a 14 year old strain of fibroblasts in the products of partial peptic digestion of fibrin. Control tissue in Tyrode solution.

FIG. 11. Experiment 8722 D. Growth of a 14 year old strain of fibroblasts in 1 per cent Witte's peptone. Control tissue in Tyrode solution.

do not promote the rapid growth which is produced by the proteoses. All of the amino acids were not present in this tryptic digest in the same concentration as in the peptic digest containing proteoses, for some insoluble products were formed as a result of the hydrolysis. This suggests the hypothesis that the function of the proteose is to furnish a higher concentration of certain amino acids than could be obtained even from their saturated solutions, and to supply them to the tissue cells in a soluble and diffusible form.

Action of the Protein Split Products on Pure Strains of Fibroblasts, Leucocytes, Epithelium, and Thyroid Cells.

The foregoing experiments were generally made on fibroblasts that had migrated from fragments of embryonic heart. Similar experiments were repeated on a 14 year old strain of fibroblasts and on pure cultures of pavement epithelium and thyroid cells. Fibroblasts obtained from the old strain were cultivated in various digestion products of fibrin, in Witte's peptone, and in fractions of Witte's peptone (Figs. 10 and 11). The results were similar to those reported above.

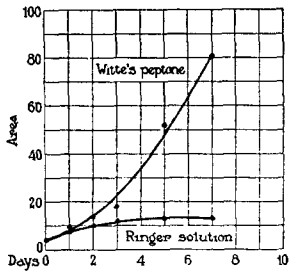

FIG. 12.

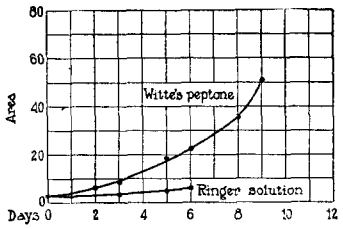

FIG. 13.

FIG. 12. Experiment 3337 C. Growth of iris epithelium in Witte's peptone. Control tissue in Ringer solution.

FIG. 13. Experiment 3216 A. Growth of guinea pig skin in Witte's peptone. Control tissue in Ringer solution.

Epithelial and thyroid cells as well as fibroblasts were found to multiply with great speed in the presence of the protein split products (Figs. 12 to 14). Leucocytes died when kept in Witte's peptone at a concentration of 2.5 per cent. They utilized it, however, for their nutrition when its concentration in the medium was much lower.

DISCUSSION AND CONCLUSIONS.

These results indicate the nature of the nitrogenous substances required by fibroblasts and epithelial cells for the building up of protoplasm. Tissue cells obtain their nitrogen from proteoses and possibly from some of the other split products of the proteins. The smaller split products do not induce the rapid proliferation of the cells that the

proteoses do, although they appear to be utilized to a slight extent as nutrient materials. The rôle of amino acids, peptones, and proteoses in cell nutrition has not been studied extensively. Burrows[10] found that certain amino acids, even at a low dilution, were toxic for tissues growing *in vitro*. Philippson and Mendeleef[11] hydrolyzed serum by sulfuric acid and observed that the tissues displayed more activity in hydrolyzed than in normal serum. Unfortunately, the technique of those experimenters was imperfect, and their results did not indicate whether the medium was nutrient or merely stimulating, and whether the effect could be attributed to a loss of the natural inhibiting power of the serum or to the production in this serum of nutrient or stimulat-

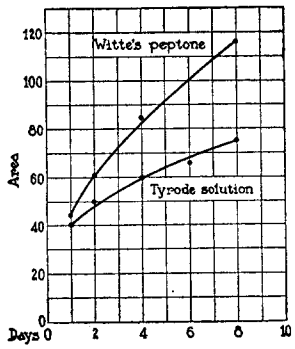

FIG. 14. Experiment 3526 C. Growth of thyroid cells in Witte's peptone. Control tissue in Tyrode solution.

ing substances. Carrel and Ebeling[4] studied in a quantitative manner the action of artificial mixtures of amino acids on fibroblasts in pure culture, and found that these acids were not toxic and brought about some acceleration of the cell migration without increasing, however, the mass of the tissues. The ultrafiltrate of embryonic juice yielded a similar result, when added to the medium of fibroblasts. These facts have been confirmed lately by Wright,[12] who observed the presence in

[10] Burrows, M. T., and Neymann, C. A., *J. Exp. Med.*, 1917, xxv, 93.

[11] Philippson, M., and Mendeleef, P., *Soc. roy. Sc. méd. et nat. Bruxelles, Vol. Jubilaire*, 1822–1922, 713.

[12] Wright, G. P., *J. Exp. Med.*, 1926, xliii, 591.

embryonic juice of substances which pass through a collodion membrane and stimulate cell division. So far, no increase in the mass of a tissue in pure culture has been determined by a mixture of amino acids, or by the ultrafiltrate of embryonic juice. On the contrary, the larger fragments of the protein molecules can be used as food by tissue cells.

These facts agree with the results previously obtained by the fractionation of embryo juice.[3] In those experiments, it was found that fibroblasts do not feed upon the amino acids and other ultrafilterable constituents of the embryo juice, but on the protein fraction. It might be supposed that the protein was utilized as such by the cells, or was

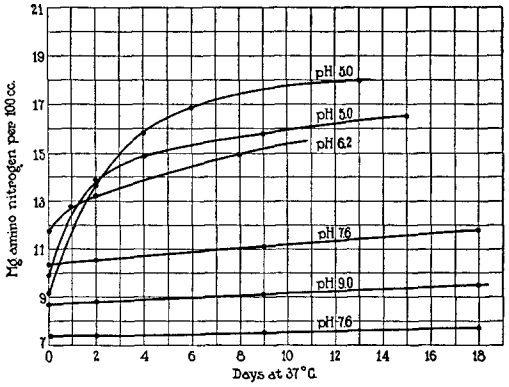

FIG. 15. Increase of amino nitrogen at pH 5.0 and 6.2 in embryo juice incubated at 37°C., showing the presence of a proteolytic enzyme.

hydrolyzed before being absorbed. Many attempts were made to obtain a fraction more active than the original embryo juice. The results of these experiments were negative, probably because the growth-activating substances are not preformed in the embryo juice but are continuously made from its proteins by the cells themselves or by an enzyme contained in the medium. It is a plausible hypothesis that an enzyme contained in the tissue juice becomes active in the presence of living cells. When embryonic tissue juice is incubated at 37°C., at a pH varying from 7.6 to 9.0, no increase in amino nitrogen is observed. But the quantity of amino nitrogen increases rapidly as the medium is made more acid (Fig. 15). At pH 6.2, some hydrolysis

takes place and an increasing amount at pH 5.0. It must be remembered that the fluid contained in the flasks is at a pH of 7.6 and so buffered that it does not change to any great extent during the period of incubation. Under these conditions, the embryo juice enzymes do not manifest their activity, and no proteoses are produced. The pH of the coagulum immediately adjacent to the tissue, however, is modified by the mere presence of living cells. Long ago, Rous observed that tissues growing in a medium containing litmus became surrounded by a pink area.[13] When phenol red was added to the coagulum in the flasks, the medium as a whole remained alkaline, but a considerable acid production took place around the colonies as shown by the brilliant yellow crown surrounding them. A change to a pH of at least 6.0 occurred at the surface of the cells, which is probably sufficient to enable the tissue juice enzymes to hydrolyze proteins into the proteoses which are absorbed and utilized by the tissues. The objection may be made to this hypothesis that serum is not nutrient for fibroblasts, although it contains enzymes which hydrolyze the proteins in an acid medium. However, it is possible that the removal of the antiproteolytic and growth-inhibiting lipoids would cause serum proteins to be used by the cells, provided a sufficient concentration of enzyme remained. Or it may be that the configuration of the molecules of the protein of embryonic juice is such that they are attacked by cell enzymes which have no effect on plasma proteins.

It has been considered as probable that embryonic juice contains, together with nutrient substances, a specific hormone essential to cell division. The fractionation of the juice did not give any evidence of the existence of such a hormone. Although amino acids or dialysate products had a stimulating effect on cell migration and multiplication,[4, 12] the substances found to support continuous growth consisted of proteins. The experiments described in the present article render it probable that for cell multiplication a specific hormone is not required and is not contained in embryonic juice, since growth-promoting substances can be obtained from proteins of many sources. While proteoses undoubtedly furnish the nitrogenous materials required for cell multiplication, the fact remains that these products

[13] Rous, P., *J. Exp. Med.*, 1913, xviii, 183.

have been obtained from impure proteins and, therefore, other substances may be present which have some action on tissue growth. The study now under way of the digests of pure proteins may show what other products, if any, beside the proteoses are essential to the proliferation of epithelial cells and fibroblasts.[14]

SUMMARY.

1. Fibroblasts and epithelial cells in pure culture obtain the nitrogen, which they build into protoplasm, from proteoses and possibly other primary derivatives of proteins. These proteoses have been prepared from embryo tissues, egg white, commercial fibrin, rabbit brain, Witte's peptone, etc.

2. The presence in embryo juice of a hormone that stimulates cell division is improbable.

3. Proteoses separated from peptic digests of fibrin by sodium sulfate determine a more abundant and prolonged multiplication of the fibroblasts than is produced by embryo juice. Peptones and the smaller split products appear to furnish some nutrient material, but do not cause the rapid proliferation characteristic of proteoses, and are sometimes toxic for tissue cells.

4. Possibly the effect of embryo juice on fibroblasts and epithelium is due to the splitting of the protein of the juice into proteoses by the cell enzymes, or by other enzymes activated by the presence of living cells.

The authors wish to express their thanks to Dr. Michael Heidelberger for his criticisms and suggestions in connection with this work.

[14] Since this paper was written, a study has been begun of the products of the partial peptic digestion of crystalline egg albumin. The albumin was recrystallized three times to insure removal of all other substances. A peptic digest of this material, having a ratio of total to amino nitrogen of 4.7, was found to promote the growth of fibroblasts in the same manner as the proteolytic products of the impure proteins. This fact demonstrates that the action of the peptic digests reported above is due to the higher protein split products, and not to any accompanying impurity.