# EFFECT OF THE AMINO ACIDS AND DIALYZABLE CONSTITUENTS OF EMBRYONIC TISSUE JUICE ON THE GROWTH OF FIBROBLASTS.

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It is known that embryonic tissue juice is essential to the continuous growth of connective and epithelial tissues *in vitro*;[1] but the nature of the chemical constituent or constituents present in the proteins of the embryonic tissue extract,[2] which give to it its peculiar growth-promoting properties, has not been discovered. This paper will deal with the separation of the extract into its dialyzable and non-dialyzable constituents and the action of each on the growth of fibroblasts *in vitro* with special reference to the amino acid content of the two fractions. The amino acids were separated from the rest of the extract in order that evidence might be obtained as to whether the nitrogen required for the synthesis of protoplasm is utilized in the form of amino acids.

Since the proteins upon which animals subsist are broken down into amino acids before entering the blood stream and since animals may be maintained in nitrogen equilibrium on enzyme digests of proteins consisting almost entirely of amino acids, it would seem quite probable that the tissues utilize nitrogen in this form. It has been found that the amino acids of serum are incapable of supporting the growth of fibroblasts in pure culture,[3] but whether or not this is true *in vivo*, where the circulating blood brings constantly renewed supplies, has

[1] Carrel, A., *J. Exp. Med.*, 1912, xv, 516; 1913, xvii, 14; 1914, xx, 1. Fischer, A., *J. Exp. Med.*, 1922, xxxv, 367. Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 755. Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, xxxviii, 487. Ebeling, A. H., *J. Exp. Med.*, 1925, xli, 337.

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

[3] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, xxxvii, 759.

not been ascertained by the experiments thus far conducted *in vitro*. Extensive experiments carried out in this laboratory with many individual amino acids and also with mixtures have shown that the acids stimulate the tissues and produce a greater area of migration, but that they do not prolong the life of the tissues and in themselves are not sufficient to bring about continued multiplication of cells.[4] On the other hand, embryonic tissue juice may contain certain specific acids essential to the growth of the tissues which are lacking in serum and in artificial mixtures of amino acids.

The study of the effects of the amino acids and dialyzable constituents of embryonic tissue juice was carried out in several ways.

1. The embryonic tissue extract was dialyzed in very permeable collodion sacks until free from amino acids, and compared with the original extract for its growth-promoting properties.

2. The dialyzed extract was tested to ascertain whether an enzyme were present which produced an increase in the amino acids after those originally present were removed.

3. The amino acids were separated from the extract by ultrafiltration along with other ultrafilterable constituents, and their action was tested for their growth-promoting effect.

4. An artificial mixture of sixteen amino acids was added to extract dialyzed until free from amino acids, and this was compared with the dialyzed extract.

5. The ultrafiltrate was added to extract from which the acids had been removed by dialysis and this was compared with the extract free from amino acids.

6. The amino acids obtained by hydrolyzing embryonic tissue juice, both by acid and by trypsin, were tested at various concentrations for growth-promoting action.

The technique of Carrel and Ebeling[5] for the cultivation of the tissues was used, the two halves of the same culture being carried through several passages, one in the experimental medium and one in the control medium. Measurements of the area of growth were made every 2 days.

### *The Action of Embryonic Extract, Dialyzed until Free from Amino Acids, on the Growth of Fibroblasts.*

In order to remove the amino acids completely as rapidly as possible from the extract, collodion bags of high permeability were used.

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

[5] Carrel, A., *Compt. rend. Soc. biol.*, 1923, lxxxix, 1017. Ebeling, A. H., *J. Exp. Med.*, 1921, xxxix, 231.

The bags were prepared according to the technique of Gates,[6] on gelatin capsules to which a glass neck had been sealed. The capsules were dipped once in a thick collodion solution, drained, and exposed to the air for 2 minutes, then immersed in 95 per cent alcohol for 10 minutes, and hardened in cold water. After the gelatin was washed out with hot water, they were preserved in 95 per cent alcohol until desired for use, at which time the alcohol was washed out with sterile water and the bag itself subjected to dialysis until all traces of alcohol were removed. Tissue extract dialyzed in these sacks became free from amino acids in 20 to 30 hours. Determinations of amino nitrogen were made from time to time in the Van Slyke micro apparatus, until a minimum amount of amino nitrogen remained, *i.e.*, only the quantity characteristic of the protein itself as separated from the extract by precipitation methods. In a number of the experiments, running water was circulated in a narrow coil immersed in ice before coming into contact with the dialysis membrane. In others, tap water at 12–15°C. was used.

The dialyzed extracts were made isotonic either by the addition of an equal volume of double strength Tyrode solution or by evaporating to dryness and redissolving in ordinary Tyrode solution. In either case, the concentration of protein nitrogen was determined in the dialyzed extract and in a sample of the original extract saved as a control, and dilutions were made to bring the two to the same per cent of protein. The pH was also adjusted to the same value, approximately 7.6.

All the samples of dialyzed extract, in spite of differences in details of preparation, gave the same result when tested on growing tissues. The growth-stimulating substance was not entirely removed, for the rate of growth was considerably greater in the dialyzed extract than in Tyrode solution (Fig. 1). A very much smaller area of growth was obtained in the dialyzed extract than in the original one (Figs. 2 and 3). This difference, however, may be attributed to two causes: the loss of the amino acids and other dialyzable constituents, or to a denaturation of part of the protein through the long continued action of water. The dialyzed extract often exhibited a greater opalescence than the original one, especially in reflected light, indicating a change in the physicochemical state of the protein. Such preparations gave very poor growth. Others showed a much smaller decrease in growth-promoting properties, both the experiment and control remaining in good condition.

Since it is conceivable that the extract dialyzed until free from

[6] Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 25.

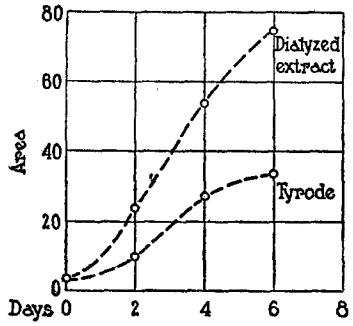


FIG. 1. Rate of growth of fibroblasts from embryo heart in embryo tissue juice dialyzed free from amino acids. Compared with growth in Tyrode solution. Cultures in flasks.

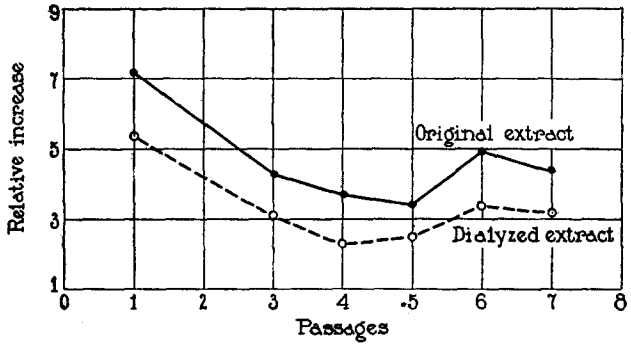


FIG. 2. Comparison of the growth of an old strain of fibroblasts in embryo extract and in the same extract dialyzed free from amino acids. Concentration of nitrogen is the same in each case.

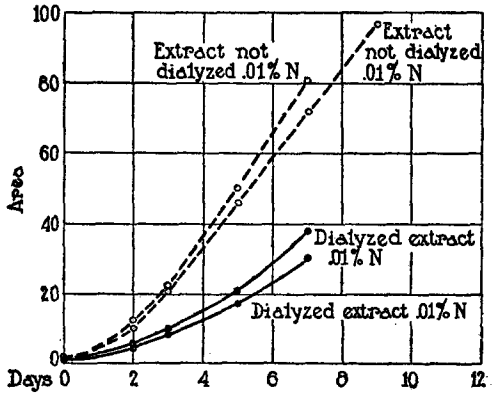


FIG. 3. Comparison of the rate of growth of fibroblasts in embryo tissue extract and in extract dialyzed free from amino acids. Cultures in flasks.

amino acids might not remain so, owing to the action of enzymes on its protein, the dialyzed extract was tested at various pH values for a regeneration of its amino acids. The increase of amino nitrogen was so slow that growth of tissues in this dialyzed extract could not be attributed to that cause. Therefore, it appears that the nitrogenous substances utilized by the fibroblasts growing in dialyzed extract are other than amino acids.

*The Action of the Amino Acid Fraction of Tissue Juice Obtained by Ultrafiltration.*

It was found that the amino acids of the tissue extract could be separated from the protein by a process of ultrafiltration under pres-

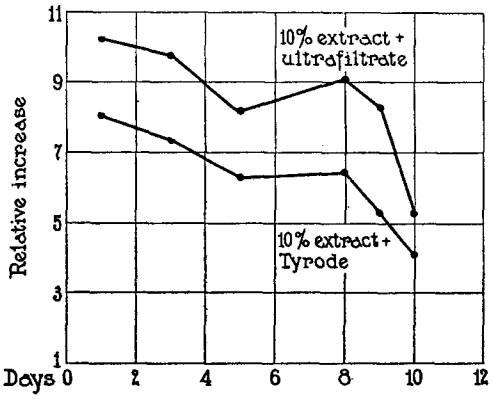


FIG. 4. Effect of the ultrafilterable constituents of embryo tissue juice on the rate of growth of fibroblasts.

sure through a collodion sack prepared exactly as those used for the dialysis. No protein passed through these sacks, *i.e.*, the ultrafiltrate gave none of the tests for protein, and there was no increase in amino nitrogen after hydrolysis by acid. The amino nitrogen content of the ultrafiltrate was found to be almost equal to that of the original tissue juice, the difference being due to amino groups attached to the protein molecule. Experiments on growing fibroblasts, with this ultrafiltrate, demonstrated that the tissues lived no longer in it than in Tyrode solution, although the area of growth at each passage was

slightly larger (Table I). The behavior was quite analogous to that of tissues in artificial mixtures of amino acids reported by Carrel and Ebeling.[4] When 10 per cent of fresh embryonic extract was added to the ultrafiltrate and also to the Tyrode solution, in order to prolong the life of the tissues, it was found that a larger area of growth was obtained in the presence of the ultrafiltrate at every passage (Fig. 4), although the difference was not sufficient to increase the mass of the tissues in one case more than in the other. It is, therefore, only an increase in the area of migration and not in the mass of tissue that

TABLE I.

*Rate of Growth of Fibroblasts in the Ultrafiltrate of Embryo Juice. Tyrode Solution Used as Control.*

| Group No.* | Preparation No. | Rate of growth in | | Ratio: *E/C.* | Remarks. |
|---|---|---|---|---|---|
| | | Tyrode (*C*). | Ultrafiltrate (*E*). | | |
| 6304 | X-3 | 5.93 | 8.02 | 1.35 | 1st passage. |
| 6312 | X-3 | 5.85 | 7.90 | 1.35 | 2nd " |
| 6327 | X-3 | 5.57 | 8.20 | 1.47 | 3rd " |
| 6276 | X-3 | 4.03 | 5.85 | 1.45 | 1st " |
| 6314 | X-6 | 8.8 | 9.6 | 1.09 | 1st " |
| 6328 | X-6 | 5.27 | 7.76 | 1.47 | 2nd " |
| 33268 | X-26 | 4.88 | 5.2 | 1.06 | 1st " |
| 33286 | X-26 | 5.0 | 5.0 | 1.0 | 2nd " |
| 33297 | X-26 | 4.71 | 5.95 | 1.26 | 3rd " |
| Average..... | | | | 1.28 | |

* Each group consists of three or four experiments.

is produced by the amino acids and other ultrafilterable constituents of embryo tissue juice.

The residue from the ultrafiltrate retained some growth-stimulating action, but so much of the protein became insoluble in the process that it was not a valuable experimental material.

### *Effect of Adding the Ultrafiltrate or an Artificial Mixture of Amino Acids to Dialyzed Extract.*

Since the removal of amino acids from tissue extract by dialysis diminishes its growth-promoting action, an experiment was tried to

see whether the addition of the amino acids and other dialyzable constituents obtained by ultrafiltration would restore this action. Fig. 5 illustrates the rate of growth of tissues in the dialyzed extract,

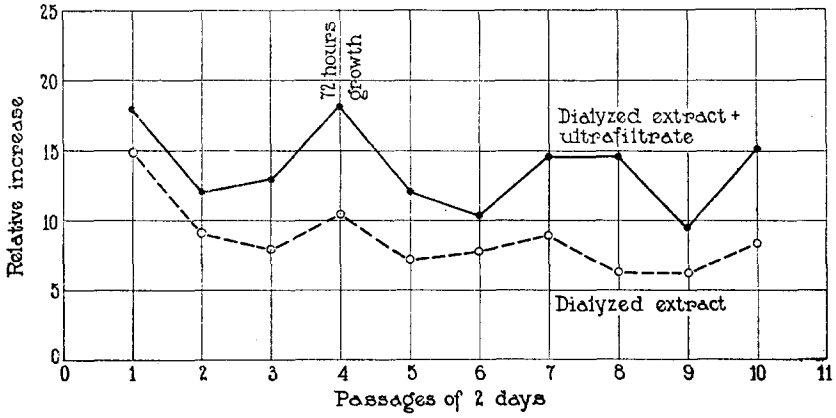


FIG. 5. Effect of replacing the constituents removed by dialysis with the ultrafiltrate of embryo tissue juice on the rate of growth of fibroblasts.

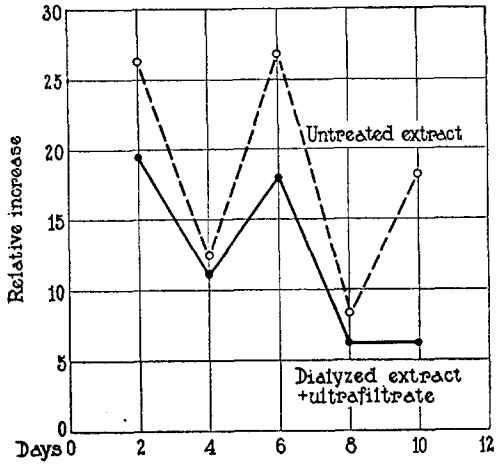


FIG. 6. Comparison of the rate of growth of fibroblasts in dialyzed extract plus the ultrafiltrate with that in the original extract.

and in the dialyzed extract to which the ultrafiltrate has been added. It appears that some of the lost activity is restored, for there is a slight increase in the migration of the cells. It is not all restored however,

for when this same dialyzed extract containing the ultrafiltrate was compared with a sample of the original extract not treated in any way, the growth in the original extract was somewhat better (Fig. 6). This phenomenon is probably not due to any difference in the amino acids of the embryonic extract, but to a denaturing of part of the protein by the action of water, and by changes in temperature and concentration.

Finally, in order to ascertain whether the amino acids or some other ultrafilterable and dialyzable constituents were responsible for the effect observed, experiments were made in which an artificial mixture of sixteen amino acids was added to the dialyzed extract. The results

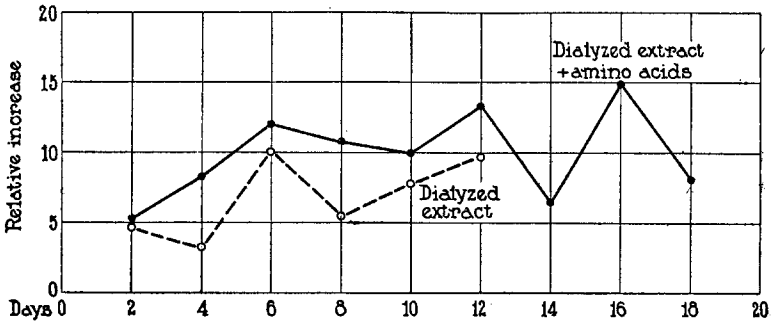


FIG. 7. Effect of the addition of an artificial mixture of amino acids to dialyzed embryo extract on the rate of growth of fibroblasts.

were quite analogous to those obtained on adding the ultrafiltrate (Fig. 7). In all cases, the presence of the amino acids or ultrafiltrate gave a larger area of migration at each passage, but this did not bring about any increase in the mass of the tissue.

### *Action of Amino Acids Obtained by Hydrolyzing Embryonic Tissue Juice by Acid and by Trypsin.*

Since the concentration of amino acids in the embryonic extract and in the ultrafiltrate is quite small (6 to 12 mg. per 100 cc.), it was thought that a higher concentration of the acids obtained by the hydrolysis of the tissue juice protein with acid or trypsin might produce decided growth, although those already present had failed to do so. These digests, however, proved toxic even when diluted to such an

extent that they contained very little more amino nitrogen than an ordinary embryonic extract (Table II).

TABLE II.

*Rate of Growth of Fibroblasts in Amino Acids Obtained by Hydrolysis of Embryo Juice Protein.*

*Tyrode Solution Used as Control.*

| Group No.* | Preparation No. | Rate of growth in | | Ratio: *E/C.* | Remarks. | |
|---|---|---|---|---|---|---|
| | | Tyrode (*C*). | Ultrafiltrate (*E*). | | Passage. | |
| 34–144 C | X-69 | 9.1 | 7.5 | 0.82 | 1st | Hydrolyzed by acid. |
| 9 C | X-69 | 12.0 | 9.7 | 0.81 | 2nd | Tryptophane and |
| 20 C | X-69 | 10.7 | 8.0 | 0.75 | 3rd | cysteine added. |
| 34 C | X-69 | 2.8 | 1.0 | 0.36 | 4th | |
| 8 C | X-70 | 11.1 | 7.2 | 0.65 | 1st | |
| 21 C | X-70 | 8.45 | 4.71 | 0.56 | 2nd | Hydrolyzed by acid. |
| 35 C | X-70 | 4.0 | 1.95 | 0.49 | 3rd | |
| 212 C | X-83 | 12.0 | 15.0 | 1.25 | 1st | Hydrolyzed by tryp- |
| 224 C | X-83 | 9.2 | 8.8 | 0.96 | 2nd | sin. 11.5 mg. |
| 235 C | X-83 | 10.5 | 3.9 | 0.37 | 3rd | amino N per 100 cc. |
| 23 C | X-72 | 10.9 | 9.8 | 0.90 | 1st | Hydrolyzed by tryp- |
| 36 C | X-72 | 4.13 | 2.8 | 0.68 | 2nd | sin. 62 mg. amino |
| 47 C | X-72 | 12.2 | 5.1 | 0.42 | 3rd | N per 100 cc. |
| 205 C | X-82 | 15.2 | 18.3 | 1.2 | 1st | Hydrolyzed by tryp- |
| 217 C | X-82 | 10.5 | 11.4 | 1.08 | 2nd | sin. 6 mg. amino |
| 225 C | X-82 | 12.6 | 8.78 | 0.70 | 3rd | N per 100 cc. |
| 236 C | X-82 | 5.1 | 3.1 | 0.61 | 4th | |

* Each group consists of three or four experiments.

## DISCUSSION AND CONCLUSIONS.

The above evidence indicates that the substance or substances present in embryonic tissue juice which give it its peculiar growth-promoting action are not removed by dialysis or by ultrafiltration in collodion bags of high permeability, and that they are not of the nature of amino acids.

It is probable that the amino acids are not the form in which tissues absorb the nitrogen which they build into protoplasm. These conclusions are in accord with previous experiments in which the growth of tissues, in protein precipitates of embryonic extract containing no uncombined amino acids, was studied.[2] The amino acids of the extract together with other ultrafilterable substances are unable to maintain the life of tissues for a longer time than does Tyrode solution. They do stimulate cell migration and possibly multiplication. This effect is probably not due to some other diffusible substance which might be mixed with the amino acids, because a similar stimulation was observed when a solution of sixteen amino acids (given to us by Dr. P. A. Levene, or obtained from Hoffmann La Roche and Pfanstiehl) was added to the medium of fibroblasts. The stimulation of mitosis, observed by Wright[7] in embryonic cells cultivated in a diffusate of embryonic tissue juice, is probably a phenomenon of the same nature. Amino acids presumably play an important rôle in cell life since a greater area of tissue was invariably obtained in their presence, although its mass was not noticeably increased. Even if they cause only a temporary activation of cell metabolism, this may be a very important factor in normal and pathological phenomena. A possible explanation of these phenomena may rest on the hypothesis that some of these acids are utilized as food when other necessary substances with which they can unite are supplied by the protein fraction of the embryo juice, although alone they cannot be utilized. It is also conceivable that, with a different method of cultivating tissues, the cumulative effect of this greater migration might be observed over a longer period of time. It must be remembered that these experiments do not mean that tissues can grow in the complete absence of amino acids, since some amino acids are always present in the plasma which forms part of the medium, but merely that the growth-promoting substances, which distinguish embryonic juice from other fluids in its capacity to maintain the life of fibroblasts and epithelial cells indefinitely *in vitro*, are not to be found among its dialyzable or ultrafilterable components.

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

SUMMARY.

The ultrafilterable constituents of embryonic tissue extract are unable to support cell life *in vitro*. They stimulate cell migration and possibly multiplication, without increasing the mass of the tissue.

Embryonic tissue extract freed from amino acids by dialysis still retains a considerable part of its growth-promoting properties.

The area of growth of tissues in embryonic tissue extract free from amino acids is appreciably less than that with the whole extract, probably owing to the denaturation of part of the protein, or perhaps to the inactivation or loss of an enzyme.

The addition of either the ultrafilterable components or an artificial mixture of amino acids to this dialyzed extract increases the area of cell migration but does not restore all the activity lost on dialysis.

The observed differences in growth of tissue, due to the addition or removal of dialyzable and ultrafilterable constituents of the extract, prove that the amino acids produce a more active cell migration and possibly multiplication, but no building up of new protoplasm.

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