mine seems to promote a very rapid "regeneration" of liver tissue, the liver shows relatively little damage even after 28 days on a coramine diet. It is quite possible that necrosis or cirrhosis or both might have been produced by continuing the experiment for several months. Blumberg and Grady[6] found that over 300 days were required to produce fibrosis in rats with fatty livers.

It is surprising that the full effects of coramine on the liver wt/body wt ratio are manifest in 5 days or less and that no obvious progressive change occurs from then up to the 28th day. The growth rate was depressed, but not seriously, and all of the 180 experimental animals appeared to be in good condition at the time of autopsy.

Essentially none of the results presented, either chemical or histological, indicate any serious abnormality in the liver parenchyma of the coramine treated rats. A hypothesis as to the nature of the action of coramine on the liver cannot, however, be advanced. The rapidity and degree of enlargement of the liver superficially resembles the effect produced in organs by their respective trophic hormones. Whether this resemblance is more than superficial remains to be determined.

Summary. (1) Coramine produces a great increase in the liver wt/body wt ratio in growing rats. (2) This increase which is manifest within 5 days or less and which is still apparent for at least 28 days cannot be prevented by the ingestion of choline or methionine. (3) The percentage of water and glycogen in these livers is essentially the same as in the controls. Coramine causes some elevation in the fat content which may be prevented by the administration of choline or methionine. (4) On microscopic examination, aside from the fat globules observed in the livers of rats receiving coramine alone, no marked pathological changes were evident. Binucleate cells and mitoses were present in large numbers.

The authors wish to express sincere thanks to Dr. T. Hernandez for carrying out the microscopic examination of the histological sections.

[6] Blumberg, H., and Grady, H. G., *Arch. Path.*, 1942, **34**, 1035.

16198

Growth of Animal Tissue Cells in Artificial Media.*

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Multiplication and growth of tissue cells *in vitro* depend on the presence of two kinds of substances in the culture media. Substances of a protein nature present in extracts from embryonic tissues were shown by Carrel[1] to act as growth promoting factors. These high molecular weight substances are present only to a very limited extent in blood plasma or serum. Attempts at the isolation of these very labile substances, grouped by us under the name "embryonin",[2] have shown that they can be purified by the extraction methods outlined by Hammarsten[3] for the isolation of a nucleo-protein. Other methods of purification have so far failed. These results have been confirmed by Davidson and Waymouth.[4]

When the culture media (plasma, serum, embryo extract) are dialysed against Ringer-glucose solution, and Tyrode's solution is added (furnishing inorganic phosphate and bicarbonate), the tissue cells disintegrate

* Aided by grants from Rask-Orsteds Fond and from Kong Christian den Tiendes Fond.

[1] Carrel, A., *J. Exp. Med.*, 1913, **17**, 14.

[2] Fischer, A., and Astrup, T., *Pflügers Arch.*, 1943, **247**, 34.

[3] Hammarsten, E., *Z. physiol. Chem.*, 1920, **109**, 141.

[4] Davidson, J. N., and Waymouth, Ch., *Biochem. J.*, 1945, **39**, 188.

within 24 hours, even in the presence of large amounts of dialysed embryo extract containing embryonin as a growth promoting factor.[5] Evidently some low molecular weight substances essential for the metabolism of tissue cells have been removed by the dialysis. The action of these diffusible substances differs from the action of embryonin, for the diffusible factors are able to maintain the cells in good condition, though in the absence of embryonin no growth is obtained. Embryonin, on the other hand, acts only in the presence of the diffusible substances; it does not maintain the cells in dialysed media. For this reason, we call embryonin a growth promoting factor, while for the diffusible substances of low molecular weight we use the term accessory growth factors. These two terms are used throughout in order to distinguish between the two types of substances and between their different modes of action.

Preparations of accessory growth factors that induce the growth of tissue cells placed in dialysed media (containing embryonin) have been obtained from blood, serum, kidney, heart, yeast, and barley malt.[6] The partial purification of malt extracts[7] showed that it is possible to remove considerable quantities of inactive material from these mixtures without any significant interference with the biological properties of the extract, which suggests that only a few of the substances present may be responsible for its action on tissue cells. Recently, we were able, by a treatment with living bakers' yeast, to remove from such an extract all the fermentable carbohydrates. With this product, we were able to show the importance of glucose, mannose, and fructose in cell metabolism.[8] The further purification was only partially successful, but from the diffusible substances present in calf embryo muscle we obtained very potent preparations.[9]

Accessory Growth Substances in Dialysates from Embryo Juice. In these experiments, the test material consisted of chicken heart fibroblasts cultivated for 5-8 passages in the usual manner before being placed in the dialysed media in Carrel flasks. The technic has been described previously.[5,6,7] The crude material is prepared as follows:

The muscles are removed from 5 calf embryos, passed through a meat chopper, and an equal volume of Ringer's solution is added. After standing for about one hour at room temperature, with occasional stirring, the mixture is pressed through gauze. The resulting extract (8 liters), corresponding to 4 liters of muscle, is dialysed in cellophane tubings against 2 portions of 8 liters each of distilled water for 2 days. Toluene or xylene must be added in sufficient quantities as preservatives, otherwise inactive products are obtained. The combined dialysates containing about 75% of the dialysable substances of the extract are evaporated almost to dryness *in vacuo* in a water bath at 40-50°. The residue is treated with 120-150 ml of a mixture of 1 vol. glacial acetic acid and 2 vol. of methanol (possibly with slight heating) and, as a result, most of the inorganic salts (chlorides) are left undissolved, while all the active substance is found in the solution. After centrifugation, it is precipitated with 3 vol. of absolute ethyl alcohol, and the precipitate is isolated by centrifugation or filtration on a sintered glass filter and treated with absolute alcohol and dry ether. The product is very hygroscopic and must be treated with utmost care at this point. It is immediately dried *in vacuo* over H_2SO_4 and solid NaOH in a desiccator. When dry, it can be kept indefinitely in a stoppered bottle. Preparation V-521 yielded 32.4 g, corresponding to 10.8 mg substance per ml of embryo muscle. It contained 3.44% N, 1.37% inorganic P, and 1.41% organic P. Addition of 0.2 ml of a solution containing 10-33 mg of this product per ml (in physiological saline, neutralized and sterile-filtered) to a Carrel flask containing dialysed media restores the ability of the media to promote the normal appearance and growth of the tissue cells.

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5 Fischer, A., *Acta Physiol. Scand.*, 1941, **2**, 143.

6 Fischer, A., and Astrup, T., *Pflügers Arch.*, 1942, **245**, 633; Astrup, T., Fischer, A., and Volkert, M., *Acta Physiol. Scand.*, 1945, **9**, 134.

7 Astrup, T., and Fischer, A., *Acta Physiol. Scand.*, 1945, **9**, 183; 1946, **11**, 187.

8 Astrup, T., Fischer, A., and Oehlenschläger, V., *Acta Physiol. Scand.*, 1947, **13**, 267.

9 Astrup, T., Fischer, A., Ehrensvärd, G., and Oehlenschläger, V., *Acta Physiol. Scand.*, in press.

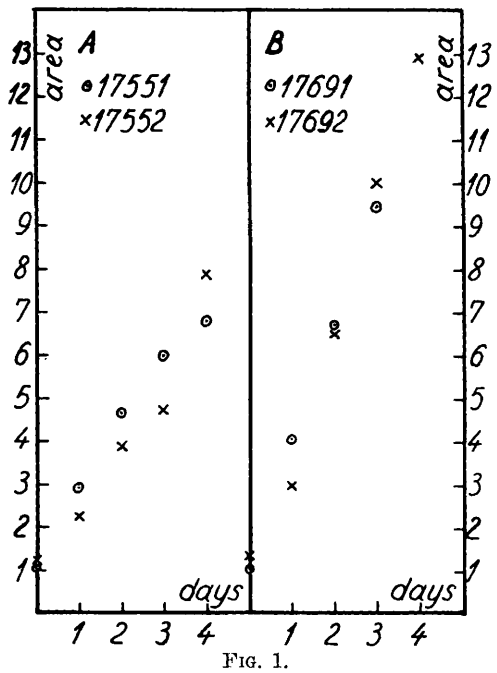

FIG. 1.

A. Effect on tissue cells of soluble calcium salts prepared from calf embryo dialysate after removal of inorganic phosphates. Culture No. 17551: Dry preparation isolated from dialysate (V-521-1). Culture No. 17552: Soluble Ca-salts (V-521-61). (Abscissa: Days. Ordinate: Relative growth area.)

B. Effect on tissue cells of a calf embryo dialysate after treatment with yeast and removal of inorganic P. Culture No. 17691: Soluble Ca-salts before yeast treatment (V-521-61). Culture No. 17692: Soluble Ca-salts after yeast treatment (V-521-74).

With barium acetate it is possible to precipitate all the active substances together with all the phosphorus. After dissolving this precipitate and removing the Ba^{++}, all inorganic P may be precipitated with Ca^{++} without significantly decreasing the activity.

A dry preparation, V-537-1, prepared as described, contained 0.227 mg N, 0.1290 mg inorganic P, 0.0406 mg organic P, and 2.50 mg sugar per ml solution.

1.48 g of V-537-1 (corresponding to 200 ml original solution) are dissolved in 20 ml water, and 20 ml ethanol is added. After making the solution just alkaline to phenolphthalein with 2N NaOH, 2 ml 30% Ba-acetate solution are added. The precipitate is removed by centrifugation, dissolved in water by means of diluted HCl, Ba^{++} is removed with sodium sulphate, the centrifugate diluted to 200 ml, made alkaline to phenolpththalein with 2N NaOH, and 4 ml 10% $CaCl_2$ solution, saturated with $Ca(OH)_2$, is added. The solution is neutralized, concentrated *in vacuo* to 50 ml (4 times the original concentration), sterile-filtered and tested as usual. The solution is almost as active as the original product, and contains, per ml of the original concentration, 0.053 mg N, no inorg. P, 0.0246 mg org. P, and 0.56 mg sugar (preparation V-537-11).

The mother liquor from the Ba-acetate precipitation and the calcium precipitate were both almost inactive (Fig. 1 A).

By treatment with yeast, it is possible to remove part of the sugar in the preparations, and, at the same time, the organic phosphates are almost completely transformed into inorganic phosphates and may be removed as such by treatment with $CaCl_2$. These operations do not interfere significantly with the biological action of the preparations. Thus, preparation V-521-74 contained originally, per ml, 3.70 mg sugar, 0.190 mg inorg. P, and 0.161 mg org. P. After treatment of 200 ml with 10 g of bakers' yeast, overnight at room temperature, and removal of inorg. P with $CaCl_2$-solution at pH 8-9, there remained 1.34 mg sugar per ml but no inorg. or org. P. Fig. 1 B shows the activity tested in 4 times the original concentration.

Synthetic Accessory Growth Substances. The first attempt of any significance to devise a synthetic medium was made by Baker and Ebeling.[10] But because their results were based on the use of a digest of whole blood, they are of little use in disclosing the mechanism of cell nutrition, even though a medium made in this manner may be useful for other purposes.

The inability of embryo juice alone to furnish the substances necessary for normal growth of tissue cells was clearly demonstrated by Baker[11] who showed that serum is needed to furnish additional nutriment. Work on a synthetic medium must proceed, therefore, along 2 lines, for substances of both high and low molecular weight are involved.

[10] Baker, L. E., and Ebeling, A. H., *J. Exp. Med.*, 1939, **69**, 365.

[11] Baker, L. E., *J. Exp. Med.*, 1939, **69**, 625.

TABLE I.
Mixture V-605 of Biologically Active Substances Tested on Tissue Cultures.
(Mg of substances contained in 1 liter solution.)

NaCl	7500	*l*(—)-Lysine, 2HCl	15
KCl	200	*l*(—)-Histidine, HCl	5
$CaCl_2$	200	*l*(—)-Arginine	2
$MgCl_2$	100	*d,l*-Valine	14
Na_2HPO_4	50	*l*(—)-Leucine	9
$NaHCO_3$	1000	*d,l*-Isoleucine	10
		d,l-Threonine	12
$FeCl_2$	0.6	*d,l*-Phenylalanine	7
$CuCl_2$	0.2	*l*(—)-Tryptophan	2
$MnCl_2$	0.3		
$ZnCl_2$	1.0	*d,l*-Methionine	6
$CoCl_2$	0.01	Choline (as hydrochloride)	10
		Creatine	10
Glucose	800	Nicotinic acid	0.3
Mannose	100		
Galactose	100	Cystine	5
Inositol	20	Glutathione	5
Adenosinetriphosphate	200	Pantothenic acid	0.07
Fructose-diphosphate	100	Biotin	0.007
β-Glycerophosphate	100		
Inosinic acid	30	*p*-Aminobenzoic acid	1
		Hypoxantine	100
Cozymase	5		
Thiamine	3	Sodium succinate	10
Riboflavine	0.2	" fumarate	10
Pyridoxine	0.3	" malate	10
		" oxaloacetate	10
Glutamine	250		
		Ascorbic acid	2
		Methylnaphtohydroquinone-sulphate	0.005

Among the substances of high molecular weight are the growth-promoting substances of protein nature contained in the embryo tissue juice, the presence of which are necessary for cell multiplication. Cell survival, however, is possible without this addition. Further, the proteins of serum may play a role that has not yet been clarified. And then there are the substances of low molecular weight contained both in the embryo tissue juice and in the serum.

By dialysing the media, as we do, the problems are simplified, for we retain all the high molecular weight substances and have only to substitute the low molecular weight, dialysable substances by a synthetic mixture. We feel that it is necessary to distinguish clearly between the effects of the dialysable and of the non-dialysable substances and to solve these two questions separately. Further, we always add to our media a surplus of dialysed embryo tissue juice in order to furnish the growth-promoting factor and thus to obtain active growth of the cells. In this manner, we have a much more rigid proof of the ability of the added mixture to provide accessory growth substances than is the case when only the life of the cells is maintained. In this respect, our investigations differ from those of most previous authors.

We next proceeded to devise a mixture of all the substances of biological importance known to be present in animal tissue, and undertook to use them in concentrations comparable with those found in the living organism. In this manner, we devised Mixture V-605, described in Table I. The mixture was made from neutralized concentrated sterile solutions of the various groups of components, and 1 ml was used in the medium in a Carrel flask. Tested in the manner described, very intense growth and normal appearance of the cells were obtained even for cultures of small size. In the absence of glutamine, the mixture was rather active when tested on large cultures, but almost inactive on small cultures. Glutamine alone was quite inactive. Removal of the group containing the organic phos-

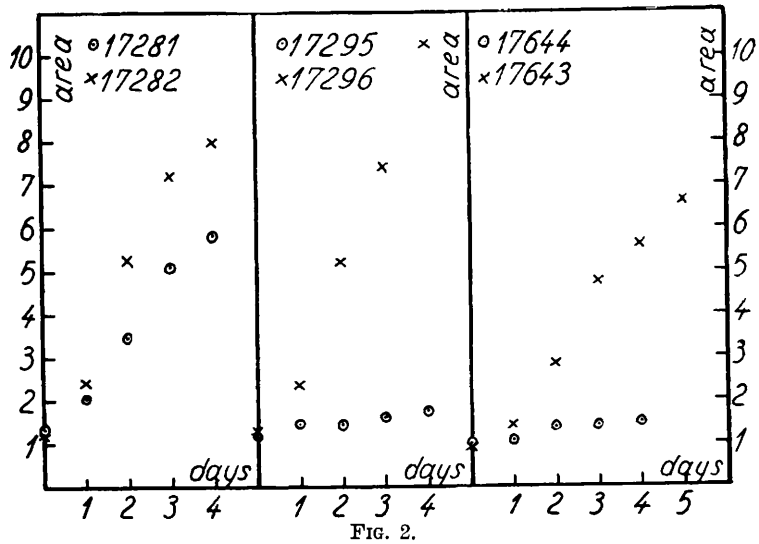

FIG. 2.

Effect of synthetic mixture V-605 on tissue cultures. Culture No. 17281: Mixture V-605 without glutamine. Culture No. 17282: Mixture V-605. Culture No. 17295: Glutamine alone. Culture No. 17296: Mixture V-605. Culture No. 17643: Mixture V-605. Culture No. 17644: Mixture V-605 without organic phosphates.

phates yielded a quite inactive mixture. Examples are shown in Fig. 2. Most of the other substances, however, could be discarded without influencing the action of the mixture on the cells, and in this manner we arrived at mixture V-612 (Table II), in which we have further increased the concentrations of organic constituents, except glutamine, with good results. Fig. 3 shows the action of this mixture with and without glutamine, tested on comparatively large cultures. The effect of glutamine is evident. The concentration of glutamine could be lowered to one-tenth without significantly interfering with its supplementary effect on the mixture. Further experiments show that it is possible also to discard β-glycerophosphate and inosinic acid, thus retaining only fructose-diphosphate (Fig. 3). Removal of the amino acids significantly decreases the activity. The importance of each individual amino acid has yet to be studied.

Discussion. A comparison of Tables I and II shows the substances not essential for growth of tissue cells under the conditions of the experiments. They include the heavy metals, known for their catalytic action on certain enzyme reactions, adenosine-triphosphate, cozymase, the different vitamins tested, choline, creatine, and the C_4-acids (functioning in the Krebs

TABLE II.

Simplified Mixtures V-612 and V-614 for Use with Tissue Cultures.
(Mg of substances contained in 1-liter solution.)
(β-glycerophosphate and inosinic acid were omitted in mixture V-614.)

	Mg
NaCl	7500
KCl	200
$CaCl_2$	200
$MgCl_2$	100
Na_2HPO_4	50
$NaHCo_3$	1000
Glucose	2000
Fructose-diphosphate	200
(β-Glycerophosphate	200)
(Inosinic acid	60)
l(—)-Lysine, 2HCl	30
l(—)-Histidine, HCl	10
l(—)-Arginine	4
d,l-Valine	28
l(—)-Leucine	18
d,l-Isoleucine	20
d,l-Threonine	24
d,l-Phenylalanine	14
l(—)-Tryptophan	4
Cystine	10
Glutathione	10
Glutamine	250

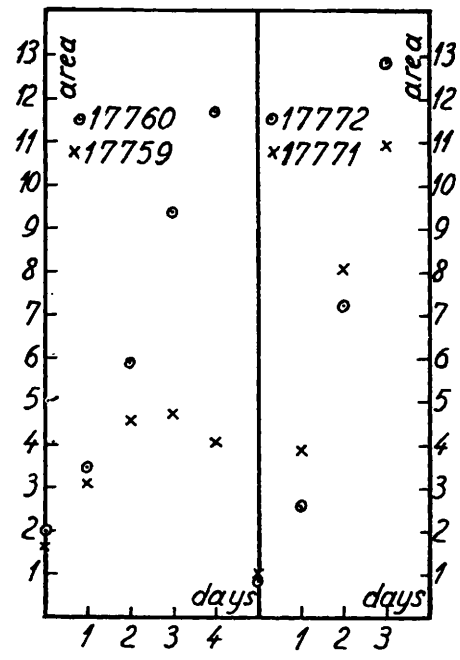

FIG. 3.
Effect of synthetic mixtures V-612 and V-614 on tissue cells. Culture No. 17759: Mixture V-612 without glutamine. Culture No. 17760: Mixture V-612. Culture No. 17771: Mixture V-612. Culture No. 17772: Mixture V-614.

cycle). It was surprising to us that the tissue cells would show normal growth in the absence of several of these substances. It may be that during the relatively short growth period (5-8 days) the constituents of the original tissue fragment suffice for the metabolism of the tissue cells, or that some of the components otherwise considered to be low molecular weight substances may be retained in the dialyzed plasma in combination with proteins. It is known, for example, that the heavy metal ions and certain organic components, *e. g.*, vitamin B_6, may be separated from proteins only with difficulty.

The vital importance of glutamine was expected in view of the relation between its function as a growth factor for certain streptococci and its role in streptococcal glycolysis, as found by McIlwain.[12] The importance of fructose-diphosphate is well understood in relation to its action in fermentation, and it may furnish a sufficient amount of hexose-diphosphate for the glycolytic system during the first hours after transplantation of the tissue fragment and before the cells themselves are able to regain all their normal enzymatic functions. It is important in this connection, however, to remember that it seems possible to remove all organic phosphorus from the previously described accessory growth substances contained in dialysates from embryo tissue juice without interfering with the biological activity. These products contain no glutamine and their mode of action may be different from that of the synthetic mixture described here.

Recently, White[13] has attempted to devise a completely synthetic medium. Our results are not in accordance with those of previous authors, and especially not with those of White, and though we consider it too early to discuss these differences a few points may be stressed. First of all we use a different technic, which in our opinion simplifies the problems in question by making possible a stepwise solution of the very complicated phenomenon of tissue growth. It is of special importance that while previous authors have preferred to work with media intended for maintenance of the cells for long periods, we use the active growth of cultures over short periods as a measure of the ability of the media to furnish the necessary substances. In this respect, it is of interest to note that two of our most important substances, *i. e.*, glutamine and fructose-diphosphate, are absent from the mixture described by White. Also, though White found that fibroblast migration is very much more active during the first few days in dextrose than in sucrose, he considers it probable that sucrose is superior to dextrose if the nutrient is not to be renewed frequently. In our experience, the tissue cells are completely unable to metabolize sucrose (saccharose).[8] We suppose, therefore, that the tissue cells in White's experiments use the carbohydrates (dextrose) contained in the tissue pulp used for cultivation, and that the cells of the pulp would survive equally well if no sucrose at all were added. White seeks to avoid unknown constituents in the culture media (*e. g.*, serum, peptone, fibrin-digest, tissue extract, and sim-

[12] McIlwain, H., *Biochem. J.*, 1946, **40**, 67, 460.

[13] White, P. R., *Growth*, 1946, **10**, 231.

ilar products), but in the pulp of macerated tissues used for cultivation he introduces large amounts of cell constituents of both high and low molecular weight. They may be removed by repeated washings either before or after they are placed in the culture tubes, and thus the difference between his results with dextrose and sucrose may be explained. The active growth of his cultures of tissue cells indicates the presence of the growth-promoting substance "embryonin" in his media, this substance being present in the macerated cells. A comparison with controls consisting of cultures made from washed tissue pulp would have been desirable.

Summary. 1. The difference between growth-promoting substances and accessory growth substances and between their different modes of action is pointed out. 2. From dialysates from embryo juice, preparations may be obtained which completely restore the ability of the dialysed culture media to induce normal growth of the tissue cultures. They may be prepared free from organic phosphates and glutamine. 3. A synthetic mixture is described which has the same properties of restoring the dialysed media. The most important of its components seem to be glutamine and fructose-diphosphate.

16199

Hereditary Dwarfism in the Descendants of Mice Receiving Methylcholanthrene—Parallel Induction.

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The following biological variants have been obtained in the descendants of mice whose ancestry have been injected with methylcholanthrene for many generations. (1) Several germinal mutations changing susceptibility to tumors induced at the site of the injection of the carcinogen. (2) Germinal mutations altering the incidence of specific types of tumors, following the injection of the methylcholanthrene and subsequently under spontaneous conditions in their untreated descendants—such as bronchiogenic carcinoma, leiomyo-sarcoma of the uterus, and gastric lesions of several histological types. (3) Many embryological disturbances such as dextrocardia, dextroversion, situs inversus, anovariae, absence of the cervix and vagina, absence of one kidney together with the absence of the horn of the uterus on the same side, craniorachichisis, several eye lesions, and possibly (?) twins. (Twenty-two pairs of twins have been obtained in mice which have been injected with methylcholanthrene. In view of the reported rarity of this condition in mice (only three cases could be found in the literature) it would seem that twinning in these methylcholanthrene-treated mice is highly significant. However, in more recent work especially by W. F. Hollander,[1] it has been found that twinning or placental fusions in mice are of very frequent occurrence. It is doubtful, therefore, whether the injected methylcholanthrene had much influence on the development of this biological variant.) The above biological variants are considered to be induced by methylcholanthrene when they have fulfilled one of the following criteria: either (a) they have occurred only among the injected mice or their immediate descendants and never among a comparable group of controls, or (b) they have occurred among the methylcholanthrene-treated mice at an increased frequency over the controls. (4) Several physiological and morphological charac-

* This experiment has been made possible by grants from The Jane Coffin Childs Memorial Fund for Medical Research, The Anna Fuller Fund, and The American Cancer Society, Committee on Growth.

[1] Hollander, W. F., unpublished data.