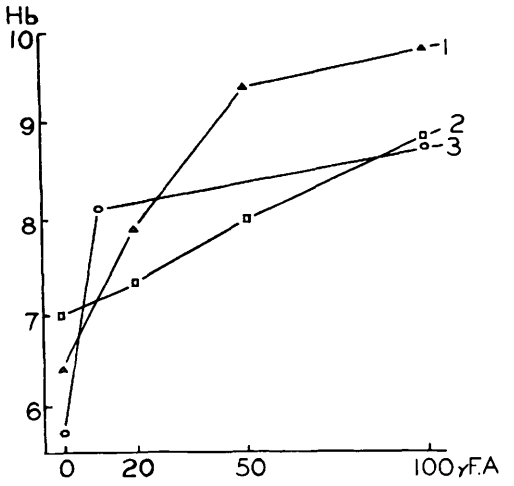

Fig. 4.

Summary of Hemoglobin Production.

The hemoglobin values (in grams per 100 cc of blood) are plotted against the μg of folic acid per 100 g of diet.

1 = High-fat diet (the curves obtained with the corn meal diet or the regular dextrin diet are similar).

2 = Sucrose diet (this is similar to the curve obtained with glucose as the carbohydrate).

3 = High-casein diet (the starch diet produced a similar curve).

is obtained with the corn-meal or dextrin (high or low fat) diets but higher levels of folic acid produce more hemoglobin than is obtained with the other diets. Since low levels of folic acid are effective when fed with high-protein diets and ineffective in diets containing high fat, glucose or sucrose, it seems evident that treatment of this type of anemia should be facilitated by high-protein, low-fat, and low-soluble carbohydrate diets.

The reason for the superior growth and hemoglobin production with a corn-meal diet is not known, but it is evident that corn is, when properly supplemented, a good dietary constituent.

Summary. A study of the effects of dietary constituents upon the activity of synthetic folic acid in chick nutrition shows that no definite requirement for this compound can be established since the response to a given amount of folic acid depends upon the type of ration used. Folic acid produced the least response with high-fat diets or diets containing glucose, sucrose, or starch as the sole carbohydrate, and the best response with diets containing high protein, low fat, or corn-meal and dextrin as the carbohydrates.

Hemoglobin responses were better on diets containing dextrin or corn-meal than diets containing other carbohydrates.

Feather development cannot be correlated with either the amount of folic acid in the diet or the rate of growth of the chicks when low levels of folic acid are included in the diet.

Whole liver substance when added to a diet containing sucrose as the carbohydrate gave an increased growth response that could not be attributed to its folic acid content.

We thank Merck and Company, Inc., Rahway, N.J., for the crystalline vitamins, Wilson Laboratories, Chicago, Ill., for gelatin and whole liver powder, Allied Mills, Peoria, Ill., for soy bean oil, and Lederle Laboratories, Inc., Pearl River, N.Y., for the folic acid.

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Effect of Some of the B Complex Vitamins upon Chick Tissue Cultures.

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In view of the prominence which some of the vitamins of the B Complex are assuming in the role of therapy, especially in some disorders resulting in alterations of the skin and of the nervous system, it was thought of interest to discover whether or not some

of these vitamins had any specific effect upon chick tissues grown *in vitro*.

In the first series of experiments nicotinamide, pyridoxine (B_6), thiamin chloride (B_1), pantothenic acid (as calcium pantothenate), and riboflavin were chosen for use. Skin and brain as ectodermal derivatives and heart as of mesodermal origin were selected from 10-day-chick embryos as the test tissues. Chicken plasma and Ringer-Tyrode solution in equal parts constituted the growth medium for all control cultures. The experimental cultures were planted in equal parts of fresh chicken plasma and Ringer-Tyrode solution to which had been added the various vitamins so that for any one of the above vitamins the series of final dilutions in the cultures were 2000; 1000; 500; 250; 125 and on occasions 62.5 γ per cc of medium.

In the second series of experiments folic acid and biotin* were added to the Ringer-Tyrode solution before the same tissues from 12-day embryos were planted. The cultures were planted similarly to those in the preceding series except that lyophiled plasma[1] was substituted for fresh and that the concentrations of vitamins were very much less. The biotin groups were set up in a concentration series of 5, 2.5, 1.25, 0.625, 0.312, 0.156 γ per cc of medium; the folic acid groups in 3.6, 1.8, 0.9, 0.45, 0.225, 0.112 γ per cc of medium, since assays of these vitamins in chick embryo tissue of 12 days incubation are rather low.[2] Sterilization of all the vitamin solutions was accomplished by passing them through a Seitz filter. They were made up fresh at frequent intervals to avoid the possibility of deterioration.

The cultures were all made by the hanging drop method using as far as possible, bits of tissue of comparable size in any one planting. Sterile precautions were used throughout and all cultures were incubated at 37.5°C. The pH of the experimental cultures at the time of planting varied from 7.4-7.6. There were 1369 experimental cultures and 412 controls—one set of controls for approximately each 3 sets of experimental cultures from a single embryo planting. When all experimental cultures in several successive dilutions of a given vitamin showed lack of growth or of any form of migration of cells they were not repeated. If migration and growth were nearly equal to or slightly better than those of the controls, this experiment was repeated once to several times to be sure that the effect might not be due to the idiosyncrasy of a particular planting. Microscopic observations were made daily upon the cultures, but no effort was made to keep planimetric records of growth since cultures of brain tissue particularly, do not yield themselves to this form of observation. As far as could be determined by careful contrasting observations between all control and experimental cultures, no alterations or abnormalities in appearance of the various cell types could be detected in the vitamin cultures.

The outgrowth from both control and experimental cultures consisted of: heart—mostly fibroblasts, macrophages, occasional groups of endothelial- or mesothelial-like cells and very slight or no increase of muscle tissue; brain—myriads of axones from within the explant, some neurones, microglia (macrophages) and cells interpreted as astrocytes from their staining reactions; and skin—many fibroblasts, some capillary sprouts, scanty epithelial sheets, macrophages and a few melanophores.

In all these experiments embryo juice was not employed as a growth stimulant since this is obtained from steeping all the minced tissues of 10-12-day embryos in Ringer-Tyrode solution. The supernatant fluid must contain apart from other cell substances a complement of vitamins. Embryo tissues as sug-

* The author wishes to express his appreciation to Merck & Co., Inc., for supplying all the vitamins used in the first series of experiments and also for their extensive bibliographies relating to them. Thanks are also due to Dr. Philip Handler of the Department of Biochemistry for the use of a folic acid concentrate (approx. 60% pure substance, Lederle) and crystalline biotin (Merck); to Dr. Wm. A. Perlzweig for advice and criticism; and to Jane Stanley Craig for her technical assistance.

1 Hetherington, Duncan C., and Craig, Jane Stanley, PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 831.

2 Williams, Roger J., Taylor, Alfred, and Cheldelin, Vernon, *Univ. of Texas Publ. No.* 4137, 61-66.

gested by the available data on the content of liver, brain, and heart[3] are good sources—particularly the liver—of all the B vitamins in question. The total vitamin content of the liver declines approximately 50% by hatching time even though nicotinic acid is synthesized by the developing chick.[4] To what extent the chicken plasma of the medium itself might have contained B vitamins is not positively known but it was assumed to be a negligible factor throughout the experiments.

Since the growth stimulating effect of embryo juice was absent, it must be kept in mind that all these cultures grew probably by virtue of their own stored growth energy or growth substances and/or perhaps, to a certain extent, by utilizing the plasma and also cell autolysates from within the explant. As a consequence their existence was shortened and degeneration set in usually after the third or fourth day in culture although some lived for more than 12 days.

The literature relating specifically to growth of cultures with vitamins is practically nonexistent. The effects of these substances upon any organ or tissue, derived principally from deficiency states produced within the whole living animal, and later treated by addition of the missing vitamin or vitamins, must needs be evaluated by different standards from effects of these same vitamins upon tissue cultures. In these a mere fragment of tissue is living in an abnormal environment removed from all the modifying factors of the whole living animal. The control cultures might be considered as bits of tissue living in a multiple vitamin deficiency state since the only vitamins present are essentially those carried into the culture by the bit of tissue itself. The addition then of a given vitamin to these cultures should, if alteration of growth occurs, be considered perhaps as evidence of a specific effect for that vitamin within the limits of the experiment.

TABLE I.

Showing Maximal Concentrations of B Vitamins in γ per cc of Medium for Equivalent or Better Growth of Brain, Heart and Skin in Tissue Cultures from Chick Embryos of 10-12 Days Incubation.

Vitamin	Tissues explanted		
	Brain	Heart	Skin
Riboflavin	62.50	62.50	62.50
Nicotinamide	125.00	125.00	250.00
Thiamin	500.00	*	125.00
Pyridoxine	250.00	62.50	125.00
Calcium pantothenate	125.00	500.00	250.00
Biotin	5.00	1.25	0.62
Folic acid	1.50	1.50	3.00

* Growth rate of cardiac cultures was unaffected by any of the concentrations used.

In the first series of experiments, concentrations of the vitamins of 2000 and 1000 γ per cc of medium were definitely detrimental to the growth of the skin and brain and with the exception of thiamin, to the heart also. Migration of cells did not take place and the explants rapidly became opaque and disintegrated—a sign of death. Until growth became nearly equal to or better than that of the controls the vitamins were considered to be toxic or in some instances suppressive.

The heart cultures consisting principally of fibroblasts were wholly unaffected in growth rate by thiamin in all dilutions used. This is in accord with the work of Hengstman[5] using the same vitamin on cultures of chick heart and also that of Paterson and Thompson[6] employing pure cultures of chick embryo fibroblasts with aneurin chloride hydrochloride (thiamin) even in greater concentrations than in the present experiment.

In the second series of experiments with folic acid and biotin none of the concentrations tested was suppressive for any of the tissues and in general growth was a little better throughout than with the vitamins of the first series.

The maximal concentrations for equivalent or better growth of all the experimental cultures appear in Table I. It must be stated here that the differential between growth "equal to" and "better than" was so slight

3 Taylor, Alfred, Mitchell, Herschel K., and Pollock, Maxwell, A., *Univ. of Texas Publ. No.* 4137, 67-80.

4 Dann, W. J., and Handler, Philip, *J. B. C.*, 1941, **140**, 935.

5 Hengstman, Von H., *Z. g. Vitamin Forschng.*, 1938-39, **8**, 208.

6 Paterson, Edith, and Thompson, Mary V., *Biochem. J.*, 1943, **37**, 501.

that the general conclusion must be drawn from the experiments that, riboflavin, nicotinamide, thiamin chloride, pyridoxine, calcium pantothenate, folic acid and biotin produced a negligible effect upon growth; that is, proliferation of cells was not enhanced.

A much more interesting observation noted was the effect of the B vitamins upon *survival* of the outgrowths. Many cultures, regardless of the rate of proliferation, outlived their controls for some time after all growth was static. In other words, they did not degenerate as rapidly even though active growth had ceased. This was most noticeable in brain cultures with biotin and folic acid in which the controls were badly degenerated after 5 days whereas the vitamin cultures, especially those containing folic acid, survived an additional 7 days.

It is common knowledge among tissue culturists that to maintain growth and multiplication of cells in culture, one must supply a medium containing some stimulating substance, the best of which is embryo tissue extract.[7] Cultures grown in media without embryo extract grow only to a limited extent and degenerate rapidly as compared with cultures of the same tissues grown with embryo juice. Since the B vitamins investigated here in certain concentrations produced equivalent growth in cultures of brain, skin, and heart where embryo juice was not employed it seems probable that they may, in part at least, be responsible for supplying some of the growth factors of tissue extracts.

It is interesting that data from the Texas group of investigators[8] on the analysis of beef heart and beef heart nuclei for all of the B vitamins indicate that the content of nuclei for each vitamin was 3 to 4 fold greater than for the cytoplasm except for pyridoxine which was slightly less and biotin which was about half as much. If such analyses hold true for other tissues in general it might be suggested that, with the exception of biotin and pyridoxine, the B vitamins might be essential for maintaining the integrity of the nuclei; biotin and pyridoxine, for cytoplasmic effects. Since the nucleus is considered paramount for the maintenance of the cell as a living unit, might the longevity noted in some of the vitamin cultures over their controls be due to an effect principally upon the nuclei?

Summary. Cultures of skin and brain (ectodermal derivatives) and heart (mesodermal derivative) in the presence of the B complex vitamins in certain concentrations, grew as well as, or slightly better than their own controls without vitamins. From the results of all the experiments the conclusion must be drawn that the added B complex vitamins have a negligible growth stimulating effect upon tissue cultures of skin, brain and heart. However, they did seem to affect favorably the longevity of the cultures, particularly those of brain, with biotin or folic acid. These outlived the majority of their controls in good (biotin) and excellent (folic acid) condition by a span of days more than once again as long.

[7] Ebeling, A. H., *J. Exp. Med.*, 1913, **17**, 273.

[8] Isbell, Edith R., Mitchel, Herschel K., Taylor, Alfred, and Williams, Roger, *Univ. of Texas Publ. No.* 4237, 81-83.

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Production of Atheromatosis in the Aorta of the Chicken by Administration of Diethylstilbestrol.*

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The experimental production of a hyperlipemic atheromatosis by methods other than the feeding of cholesterol has not hitherto been reported. It is shown here that atheromatosis can be induced in the bird by the subcutaneous implantation of diethyl-

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