and can be eluted with chloroform. However, its red fluorescence is not in agreement with the properties of benzpyrene-5:8-quinone, according to Berenblum & Schoental.

(1) *In vivo*, local production in the skin, kidney, lung and liver (excreted with the bile).

(2) *In vivo* in the large intestine and occasionally in the lung of rabbits and mice.

(3) *In vitro* by post-mortem autolysis.

(4) *In vitro* by adsorption on to alumina.

(5) *In vitro* at elevated temperature *in vacuo*.

(6) *In vitro* by sublimation *in vacuo*.

Of the various steps in the transformation of benzpyrene into 8-hydroxybenzpyrene outlined above, step (1) is the only one concerned in the

problem of benzpyrene carcinogenesis, because it alone occurs in the living cell. The later steps which eventually lead to hydroxybenzpyrene are merely concerned with the removal of the hydrocarbon from the body.

SUMMARY

It has been shown by fluorescence chromatography of quite fresh extracts from organs of mice which had been treated with benzpyrene that the hydrocarbon is not metabolized directly to 8-hydroxybenzpyrene but that the transformation passes through three intermediate stages which have been studied separately. The suggested relationship of these stages has been discussed.

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REFERENCES

- Berenblum, I., Crowfoot, D., Holiday, E. R. & Schoental, R. [1943]. *Cancer Res.* 3, 151.
 — & Schoental, R. [1943]. *Cancer Res.* 3, 145.
 Chalmers, J. G. [1938]. *Biochem. J.* 32, 271.
 — & Crowfoot, D. [1941]. *Biochem. J.* 35, 1270.
 Doniach, I., Mottram, J. C. & Weigert, F. [1943a]. *Brit. J. exp. Path.* 24, 1.
 Doniach, I., Mottram, J. C. & Weigert, F. [1943b]. *Brit. J. exp. Path.* 24, 9.
 Goldberg, E. [1910]. *Brit. J. Photogr.* 57, 649.
 Peacock, P. R. [1936]. *Brit. J. exp. Path.* 17, 164.
 — [1940]. *Amer. J. Cancer*, 40, 251.
 Weigert, F. & Mottram, J. C. [1940]. *Nature, Lond.*, 145, 895.

Effect of Vitamin B on the Growth of Fibroblasts

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Components of the vitamin B complex have long been considered of importance in growth. In particular vitamin B₁ has been assayed by animal growth methods. The experiments described were designed to test whether such a growth-promoting effect could be demonstrated directly on cells *in vitro*. In essence the problem was approached in two ways: first, by depriving fibroblasts of components of the vitamin B complex by growing them in media obtained from pigeons suffering from acute beriberi; secondly, by adding to such deficient media appropriate amounts of vitamin B (yeast extract) and vitamin B₁ (aneurin).

METHODS

Culture material. Tissue cultures of fibroblasts were obtained from the choroidal and sclerotic layers of the eyes of 9-day chick embryos, and culture by the hanging drop technique was carried out for at least ten passages prior to each experiment. This length of cultivation has been found to produce a uniform outgrowth of fibroblasts, and any initial supply of growth-promoting substance present in the tissue has been largely exhausted [Carrel & Ebeling, 1921]. Cultures which showed excellent outgrowth were then graded for an experiment. The areas of the explants were measured, and the cultures divided into sets in such a way that the explants in any two or more sets to be compared were approximately equal in area. The selection

of sets to be used for each treatment was made at random. The preparations were then mounted for the actual experiment by the double coverslip method of Maximow. Care was taken that the volume of plasma and extract used was constant and equal, that the cavity of the hollow ground slide was of the same size for all cultures, and that the size of the spread drop was the same in all cases. This last was effected by placing under each coverslip a standard circle drawn on paper, and spreading the drop to that size for each preparation.

Culture medium. The medium consisted of equal parts of the tissue extract to be tested and plasma obtained from pigeons suffering from acute beriberi ('beriberi plasma'). This plasma was used in all the experiments both for the experimental and for the control groups of cultures. In any one experiment the plasma for both experimental and control cultures was obtained from the same pigeon. Since the plasma was constant and the technique the same for all cultures of any experiment, the only varying factors which might influence growth were the tissue extracts or the vitamin preparations which were added.

Brain extracts ('beriberi brain extract') for the experimental cultures were obtained from pigeons with the classical symptoms of acute beriberi as described by Peters [1936]. These symptoms included opisthotonus in all cases except one which showed emprosthotonus (Exp. 5, pigeon used for refeeding cultures at 48 hr.). Defective vision not amounting to blindness was present; this symptom was not ascertained in the case of two birds which had typical convulsions. Weight loss averaged about 30% of the original weight of the bird. In the pigeons used the time taken to produce these effects was 24–29 days. The methods used to produce beriberi were those recommended by Kinnersley, Peters & Reader [1928]. The brain was removed as soon as possible after decapitation, and extracts were made immediately. Extracts for the control cultures were similarly prepared from the brains of normal pigeons (normal brain extract). In experiments requiring extracts of normal embryos, these were prepared by mincing fowl chicks of 8–9 days' incubation.

It was found by W. Dale and one of us that the water content of adult avian brain tissue was less than that of avian embryo, the latter containing 2.03 times the water of the former. To standardize our extracts, therefore, 2.03 cc. of Ringer-Compton-Pannett solution were added to each gram of freshly prepared brain pulp. After standing for 30 min., the diluted brain pulp or the undiluted embryo pulp was centrifuged at 1500 r.p.m. for 10 min. The supernatant fluid was regarded as 100% extract and further dilutions were made on this basis. Extracts of normal brain have been used successfully in this laboratory for some years, and have been found to provide a rich source of growth-promoting substances.

In Exp. 1, for convenience, dried extracts of brain were used throughout. An apparatus similar to that described by Flosdorf & Mudd [1935] was employed. Such dried extracts can be diluted to any required strength and are satisfactory and convenient.

Vitamin sources. The vitamin B₁ used throughout was synthetic (aneurin chloride hydrochloride) and was supplied by Prof. A. R. Todd. The concentration used was such that 0.04 mg. was present in 100 ml. of the medium in which the cultures were grown. This calculation was based on the curative dose of aneurin for a pigeon weighing 300 g. If this amount is regarded as being 3 µg./day, or 6 µg. in 2 days, then the amount in the culture medium

(changed every 48 hr.) was five times the curative concentration for a pigeon.

For vitamin B an extract of brewer's yeast was used. This had been prepared for experimental purposes by Glaxo Laboratories Ltd. It was at first thought to include all the members of the B complex of vitamins found in brewer's yeast and to contain 133 µg./g. of vitamin B₁ (as aneurin chloride hydrochloride) and 154 µg./g. of riboflavin (as hydrochloride). It was later learned that most of the biotin of this preparation had remained in the residue, and that the vitamin B₁ content was unstable, and probably negligible at the time of the experiment. The amount of the extract used was based on the stated content of aneurin, and was calculated as given above for vitamin B₁. Neither the aneurin nor the yeast extract was sterilized. Both were weighed with aseptic precautions and were dissolved in the sterile distilled water used in making the Ringer solution with which the tissue extract was diluted.

The measurement of outgrowth. Experimental and control groups of cultures were incubated at 39°. After 48 hr. the outline of each explant and its outgrowth was drawn by projection. The cultures were then washed with the Ringer solution and fed with fresh extracts in the same dilution as was used in the first instance. After incubation for a further 48 hr., the new outlines were drawn. The areas obtained were measured with a planimeter, and the relative increase in size was calculated after the method described by Ebeling [1921]. The difference in the mean relative increase in two groups of cultures differently treated was examined for significance using the 't' test and extensions of this test.

RESULTS

The influence of beriberi brain extracts on the growth of fibroblasts in vitro

(1) The influence of the concentration of the extract.

The first experiment was designed to find whether,

Fig. 1. Comparison of growth when two concentrations of beriberi brain extract were used. Concentration of extract in A was ten times that of B.

when vitamin B was deficient, growth-promoting factors were still present in tissue extracts. It was

argued that if growth-promoting factors were present in beriberi brain extracts, their influence would depend on their concentration, as occurs with embryo extract; if growth-promoting factors were absent then no difference in growth would occur when two concentrations were compared. To one group was added 200%, to the other 20% extract produced from dried extract. Measurements were made every 48 hr. Re-feeding at those times was carried out, using freshly dried material in the same dilutions as were used initially. A total of 39 cultures was used.

The group fed with 200% beriberi brain extract grew much better than that fed with 20%. The average relative increases are shown quantitatively in Fig. 1. The difference in the relative increase was found to be highly significant. ($t=3.57$ at 48 hr.; $t=4.41$ at 96 hr.; degrees of freedom (d.f.)=37.) This experiment showed that extracts from brains of pigeons suffering from beriberi were still growth-promoting to fibroblasts, and that the stimulation of growth increased with the concentration of such extracts.

(2) *A comparison of the growth obtained with normal and beriberi brain extracts.* Having found that extracts from pigeons suffering from acute beriberi retained growth-promoting properties, we compared the activity of such extracts with that of extracts of normal tissue. The experimental cultures were fed with 10% fresh beriberi brain extract; for the controls 10% fresh normal brain extract was used. In Exp. 2 there was a total of 35 cultures, and in Exp. 3 a total of 56 cultures. In both experiments the growth of the cultures fed with beriberi brain extract was less than that in the groups fed with normal brain extract, values of $t=1.78$ (Exp. 2; d.f.=33) and $t=1.5$ (Exp. 3; d.f.=54) were obtained. The difference for each experiment taken singly was insignificant, since on the 5% level of significance, $t=2.03$ for d.f. 33 and $t=2.00$ for d.f. 54. A formula for extending the 't' test for multiple samples was used to combine the figures for the two experiments. This test showed the difference in growth to be above the 5% level of significance.

In three further experiments the same comparison was made (Exps. 4-6), the results all tending to show that beriberi brain extract was less growth-promoting than normal brain extract. In Exps. 5 and 6 this difference was significant—'t' for Exp. 5 at 48 hr. being 5.15; at 96 hr.=5.65 (d.f.=70), and 't' for Exp. 6 at 48 hr. being 2.87; at 96 hr.=3.7 (d.f.=83). In Exp. 4 the difference was less. This may have depended on a wartime dietary deficiency in the food of the control pigeons, a defect that was remedied in the two subsequent experiments. The results indicate that extracts from the brains of pigeons suffering from beriberi

are significantly less growth-promoting than are extracts from normal brains. The differences in relative increase of growth are illustrated in Figs. 2 and 3 based on the data of Exps. 5 and 6.

(3) *The influence of the addition of yeast or of aneurin to beriberi brain extracts.* In both experiments, three groups of cultures were used. Each experiment had control cultures fed on 5% normal brain extract, cultures fed on 5% beriberi brain extract, and cultures fed on 5% beriberi brain extract to which yeast extract was added in the concentrations mentioned previously. In Exp. 4 a total of 78 cultures was used, in Exp. 5, 108 cultures.

A comparison of the growth of cultures fed on beriberi extract with those fed on beriberi extract with added yeast extract, showed an increased growth in the latter in both experiments. The difference in growth was significant at 96 hr. for each experiment taken separately. In Exp. 4 the value of t was 2.35 ('t' for significance at 5% level=2.00; d.f.=54). In Exp. 5, $t=2.04$ ('t' for significance at 5% level=1.99; d.f.=70). It can therefore be stated that some component of the B complex of vitamins is growth-promoting *in vitro*. It is unlikely to be biotin, which was lacking in the extract used. The growth curves of Exp. 5 are shown in Fig. 2.

Fig. 2. Comparison of growth when yeast extract was added to media deficient in vitamin B complex. A=normal brain extract. B=beriberi brain extract. C=beriberi brain extract to which yeast extract was added.

In the next experiment aneurin chloride hydrochloride was added in the concentrations described earlier, instead of the yeast extract. A total of 129 cultures was used, divided into three groups. Group A was fed with a 5% normal brain extract, group B with a 5% beriberi brain extract and group C with a 5% beriberi brain extract to which aneurin was added. Mean results showed no significant difference between the growth of cultures fed with beriberi extracts (15-72) and that obtained with beriberi extracts to which aneurin was added

(14.84). The growth obtained with normal brain extract was significantly greater than that in either of the experimental groups.

The values of '*t*' at the two periods of observation in comparisons of the three groups are given in Table 1.

Table 1. Values of '*t*' obtained when the growth data for groups A, B and C (Exp. 6) are compared in pairs

Comparison	Value of ' <i>t</i> ' at 48 hr. (Value of ' <i>t</i> ' for significance at 5% level = 1.98)	Value of ' <i>t</i> ' at 96 hr.
A compared with B	2.87 d.f. = 84	3.7
A compared with C	2.6 d.f. = 84	4.34
B compared with C	0.36 d.f. = 87	0.607

The relative increase in outgrowth of the three groups is shown in Fig. 3.

Fig. 3. Comparison of growth when additional vitamin B₁ was added to media deficient in vitamin B complex. A = normal brain extract. B = beriberi brain extract. C = beriberi brain extract to which vitamin B₁ was added.

The influence of normal chick-embryo extract containing added aneurin on the growth of fibroblasts in vitro

It has been shown in the previous experiments that vitamin B₁ acting by itself does not restore the growth-promoting capacity of beriberi brain, while the vitamin B complex does so, at least partially. The latter action may be due either to the presence of vitamin B₁ acting in concert with other members of the vitamin B complex, or it may be due to some factor in the vitamin B complex other than vitamin B₁. If the former explanation were true, it would be likely that vitamin B₁, in the presence of the normal embryonic extract and plasma, would improve the growth. Exp. 7 was designed to test this. A total of 54 cultures divided into two groups of 27 was used. Both groups were fed with equal volumes of 10% extract from chick embryos, and plasma from normal birds. In addition,

aneurin was added to one group in the same concentration as before.

In this experiment the growth obtained was almost equal in the two groups. The relative increase for the group without added vitamin B₁ was 4.86, that for the group with added vitamin B₁ was 4.49. The difference was insignificant since '*t*' = 0.41. At 5% levels, *t* = 2.008 for d.f. = 53.

Fig. 4. Comparison of growth when additional vitamin B₁ was added to 10% extract of normal chick embryos. A = normal embryo extract. B = normal embryo extract to which vitamin B₁ was added.

The graphs of the relative increases of growth (Fig. 4) compared with those in Fig. 3 showed that the types of growth curve obtained are similar in form whether aneurin is added to a normal medium, or to a medium obtained from beriberi birds. Within the technical limits of the experiment the members of each pair of curves can be regarded as identical.

DISCUSSION

Until now the effect of the B complex of vitamins on growth has largely been examined on animals *in vivo*. Experiments *in vitro* have the advantage of affording a more direct picture of the effect on cells grown in a standard substrate and they exclude to some extent influences which are indirect. The disadvantage of tissue culture experiments is the difficulty of defining and of measuring growth.

In the above experiments growth has been assessed as increase in area of the culture. It is appreciated that this 'growth' is a combination of cell division and outwandering. In order to ensure as far as possible that the ratio of division and outwandering for any experiment was a constant, the plasma coagulum was kept as constant as possible [Willmer, 1933]. All the plasma used for an entire experiment was obtained from one bird and the quantity used for each culture and the size of the spread drop were constant. The extracts used differed in their action, but except in Exp. 1 the dilution used in all sets of cultures in any one experiment was the same. It is therefore felt that in any experiment the mechanical properties of the medium and the accessibility of oxygen to the cells

were very similar. In Exp. 1 the dilutions of extract were of necessity different, but if the greater dilution induced more outwandering this would operate to lessen the significance of the results obtained.

In all the experiments in which extracts from normal brains were compared with extracts of brains from pigeons suffering from beriberi, it was shown that while growth occurred in the latter, it was less than that produced by brain extracts from normal birds. In beriberi it is very probable that there is a deficiency not only of aneurin, but of at least one other component of the B group of vitamins [Williams & Spies, 1939]. The reduced growth of cultures fed with beriberi brain extract might therefore be regarded as due to a deficiency of components of the vitamin B complex other than aneurin.

In examining the effect of the addition of vitamin B₁ or of the vitamin B complex, it had to be kept in mind that these substances may not necessarily act in the free state on cells. The results of those experiments, in which an extract of the vitamin B complex was added to media deficient in vitamin B, showed that increased growth could be produced in the presence of the media supplied. It is therefore presumed that any other factor necessary for the effect was present in the media. Since the extract of the vitamin B complex used was deficient in vitamin B₁ and in biotin, it is assumed that the growth-promoting substance was neither of these two.

In those experiments in which vitamin B₁ was added, no effect on growth could be shown. This was true whether or not the medium was obtained from animals judged to be lacking not only in those substances necessary for the formation of co-carboxylase, but also in other components of the vitamin B complex. No correlation could therefore be made between growth and the carbohydrate metabolism of the cell, and no effect could be shown whether vitamin B₁ was used alone or in the presence of the vitamin B of normal embryonic extract. The findings suggest that vitamin B₁ has no specific

effect on cell growth as measured in these experiments. It would follow that the use of growth rate in animals as a measure of vitamin B₁ must be dependent on an indirect influence on the animal as a whole, as for example an effect on appetite.

It is felt that the methods described are satisfactory for further direct testing of the growth-promoting properties of constituents of the B complex of vitamins.

SUMMARY

1. An investigation was carried out to demonstrate the influence of the vitamin B complex and of vitamin B₁ (aneurin) on the growth of fibroblasts *in vitro*.

2. Extracts of the brains of pigeons suffering from beriberi were found to contain substances which promoted a satisfactory growth of fibroblasts. The growth obtained was, however, significantly less than that obtained when extracts of brains of normal pigeons were used.

3. The addition of yeast extract, which was deficient in vitamin B₁ and biotin, improved significantly the growth of fibroblasts which were grown in a medium deficient in vitamin B.

4. The addition of vitamin B₁ (aneurin) could not be shown to increase growth whether the medium was or was not deficient in its content of vitamin B complex.

5. From the data obtained, it is concluded that the vitamin B complex, or some component of it, has a specific effect on growth *in vitro*. This growth-promoting factor is probably not aneurin.

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REFERENCES

- Carrel, A. & Ebeling, A. H. [1921]. *J. exp. Med.* **34**, 317.
Ebeling, A. H. [1921]. *J. exp. Med.* **34**, 231.
Flosdorf, E. W. & Mudd, S. [1935]. *J. Immunol.* **29**, 389.
Kinnerley, H. W., Peters, R. A. & Reader, V. [1928]. *Biochem. J.* **22**, 276.
Peters, R. A. [1936]. *Lancet*, **1**, 1161.
Williams, R. R. & Spies, T. D. [1939]. Vitamin B₁ and its use in Medicine. New York: The Macmillan Co.
Willmer, E. N. [1933]. *J. exp. Biol.* **10**, 317.