CLXXVI. THE VITAMIN B_2 COMPLEX. DIFFERENTIATION OF THE ANTIBLACKTONGUE AND THE P.-P. FACTORS FROM LACTOFLAVIN AND VITAMIN B_6

VII. EXPERIMENTS WITH MONKEYS AND OTHER SPECIES

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UNTIL about 1935 "vitamin B_2" was thought to be a single substance. By general usage the term had come to acquire a meaning which in reality covered a number of different properties and phenomena. By "vitamin B_2" was meant the heat-stable component of the accessory factor contained in yeast, which was needed (*a*) by rats as a supplement to vitamin B_1 to support normal growth, and which (*b*) protected against so-called "rat pellagra", as well as (*c*) against blacktongue in dogs, or (*d*) pellagra in human beings. It is now recognized that "vitamin B_2", as thus defined by past usage in the literature, is composite. The first step towards the characterization of the complex was the recognition [György *et al.* 1933] that lactoflavin had growth-promoting activity for rats. For a time it was assumed that lactoflavin was identical with "vitamin B_2". Later it was shown that lactoflavin was not the same as the "rat pellagra" factor, which was thereupon named "vitamin B_6" [György, 1934; 1935, 1; Harris, 1935; Chick *et al.* 1935]. Lastly it has been shown [Birch *et al.* 1935] that the factor which protects against human pellagra (P.-P. factor) is yet a third component of the vitamin B_2 complex. In this same paper it was proved that the antiblacktongue factor for dogs was likewise distinct from lactoflavin or vitamin B_6. No evidence was found to differentiate the antiblacktongue factor from the pellagra-preventing factor. According to the work carried out in this Institute the position to date can therefore be represented as follows:

$$\text{Vitamin } B_2 \text{ complex} = \begin{cases} \text{lactoflavin,} \\ \text{vitamin } B_6, \\ \text{P.-P. (or antiblacktongue) factor.} \end{cases}$$

In the course of the work described in the last mentioned paper, experiments were carried out on a number of different species including rats, dogs, chickens, mice, rabbits and guinea-pigs and it was shown, *inter alia*, that rats differed from dogs or human beings in being relatively insensitive to shortage of P.-P. (or antiblacktongue) factor. The latter conclusion was in agreement with the independent finding of Miller & Rhoads [1935], who incidentally mentioned in the course of their work on swine, that young rats were able to gain weight when restricted to Goldberger's blacktongue diet.

These experiments have been continued and some new observations have been made in which dogs, rats, pigeons and monkeys have been used as experimental animals. The main object of the present paper is to describe the results

of nearly 2 years' tests with monkeys. It is now shown that the monkey resembles the human being or the dog and differs from the rat in requiring the provision of concentrates or supplements carrying the P.-P. factor. For the sake of comparison a few interim observations on other species have also been included.

For a more complete review of the literature the previous paper [Birch *et al.* 1935] may be consulted.

Elvehjem & Koehn [1935] found that lactoflavin was ineffective in preventing "chicken pellagra", a condition which they consider to be identical with human pellagra. Later, Koehn & Elvehjem [1936] independently reached one of the conclusions published by Birch *et al.* [1935], namely that lactoflavin had no curative action on canine blacktongue.[1]

There is no need here to give any detailed account of more recent papers. The work of Dann [1936, 1, 2], Spies & Chinn [1935] and Fouts *et al.* [1936] is in agreement with the conclusion that the human antipellagra factor is different from lactoflavin and/or vitamin B_6. Recently also Sebrell, Hunt & Onstott [1937] have confirmed that lactoflavin is without therapeutic value in acute blacktongue of dogs; an "antiblacktongue fraction" free from lactoflavin exerted a curative action [Sebrell, Onstott & Hunt, 1937]. (These authors suggest that deficiency of lactoflavin in dogs may be the cause of a condition described as "yellow liver".) Finally, several workers [Lepkovsky *et al.* 1936; Elvehjem *et al.* 1936; Gorter, 1936; Richardson & Hogan, 1936; Macrae & Edgar, 1937] have obtained evidence which suggests that for adequate growth rats may need some additional "vitamin B_2" factor, in addition to lactoflavin and vitamin B_6.

Experimental

The monkeys used were young *Macacus rhesus*, of both sexes. The basal diet was modified from the blacktongue diets of Goldberger *et al.* [1928] and Rhoads & Miller [1935], and consisted of:

Maize meal	600 parts by weight
Pea meal	75 ,,
Casein ("extracted casein E", Glaxo Laboratories)	90 ,,
Cotton-seed oil	45 ,,
$CaCO_3$	4·5 ,,
NaCl	15 ,,
The above mixture is made moist and "steamed" for 1½ hr. in a double pan	
Orange juice	12 ml. per monkey per day
"Radistoleum" (= vitamins A and D)	8 drops ,,

This is virtually the same diet as that used for dogs in Part III, except that the allowance of $CaCO_3$ (which as pointed out by Dr Birch seemed to be excessive) has been reduced, and orange juice is provided as a source of vitamin C (which is needed by monkeys but not by dogs or rats). This basal diet is rich in vitamin B_6. It contains only small amounts of lactoflavin. In some of the experiments the effect of addition of futher lactoflavin was investigated, either alone or in combination with the various other supplements tested, which included dried yeast, Eli Lilly "343" liver-extract powder, wheat and cooked herring muscle.

Results

The effects of the diet and the various additions can be summarized as follows.

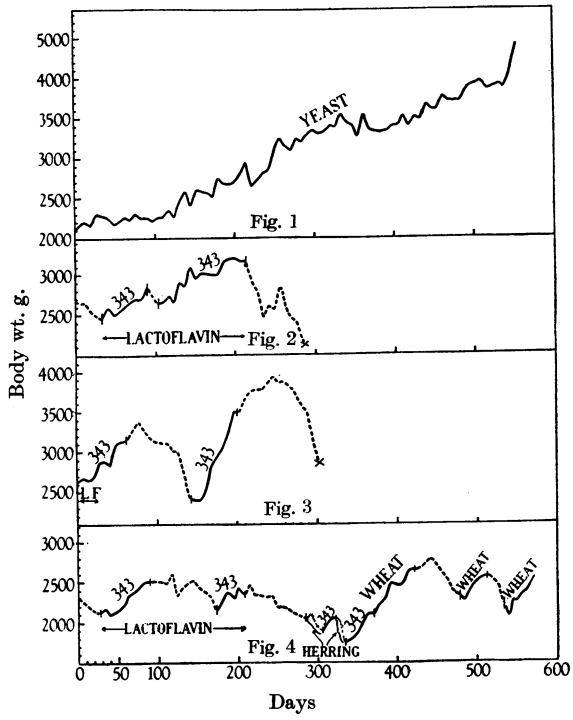
Basal diet. On the basal diet alone the monkeys became noticeably ill and apathetic within the course of a few days to a few weeks (depending on their "reserves"), appetite dropped markedly, there was profuse diarrhoea often

[1] In their most recent paper Koehn & Elvehjem [1937] have given the date of their 1936 paper as "1935", which was the date of presentation.

accompanied by vomiting and, with progressive weakness and emaciation, a fatal termination rapidly followed unless a cure was brought about by the addition of the supplements to be described. The disease seemed to have its most marked effect on the alimentary canal, but there was also a denudation of the fur, and the skin appeared somewhat dry and rough, the backs of the hands and feet becoming "scaly". A description of the pathology will, however, be deferred to a later paper.

Dried yeast. "Positive controls" given the same diet with the addition of 7% of dried yeast continued to thrive indefinitely, e.g. for 21 months in the monkey whose weight curve is shown in Fig. 1.

"343" liver powder. Repeated tests were made with Eli Lilly "343" liver-extract powder,[1] which is one of the most potent agents known for the cure of human pellagra or blacktongue in dogs. The dose given was 1·3 g. per day



Figs. 1 to 4. Experiments with monkeys on "blacktongue" diets. Fig. 1. Protection afforded by basal diet (which contains maize) after addition of yeast. Figs. 2, 3 and 4. Protection with addition of "343" liver powder (thick line), but not without it (broken line). Fig. 4. Protection with substitution of wheat for maize. (Lactoflavin, added for varying periods of time as shown by horizontal arrows in Figs. 2, 3 and 4, did not obviate the need for provision of the "P.-P." supplement.)

(equivalent to 33 g. of fresh liver). In every instance a dramatic cure was brought about (e.g. two examples in Fig. 2, two in Fig. 3, and four in Fig. 4), and when the liver powder was withdrawn the animal quickly became ill again (10 examples are shown in Figs. 2–4).

[1] The "powder" is to be distinguished from Eli Lilly "343" liquid liver extract, which is less potent as source of the antiblacktongue factor; and also from various other liver extracts.

Wheat. It was thought of interest to carry out tests with wheat, because of the well-known fact that pellagra is but rarely seen among wheat-eating as opposed to maize-eating populations (wheat is believed to contain significant amounts of the antiblacktongue factor). In these tests the maize meal of the basal diet was replaced by an equal amount of whole wheat meal. Recovery always took place on the wheat, and the monkey quickly declined once more whenever it was restored to the maize diet (see three examples in Fig. 4).[1]

Herring. In Part III [Birch *et al.* 1937] it was found that herring was an effective source of antiblacktongue factor for dogs, and a few attempts have been made in the course of the present work to cure "monkey pellagra" (as the deficiency disease under discussion may conveniently be called) by the same means. It was found difficult, however, to get the animal to eat the herring, and inconclusive results were obtained (e.g. two periods in Fig. 4). Herring may be considered a rather unnatural food for a monkey, however, and it was decided to discontinue the experiment.

Lactoflavin. In various experiments additional lactoflavin was given (60γ per monkey per day), for shorter or longer periods (see Figs. 2, 3 and 4). Alone such a supplement was ineffective in preventing symptoms of the deficiency; while materials containing lactoflavin, in larger or smaller amounts, were only effective when they were also known to be sources of the P.-P. factor (e.g. "343" liver powder, yeast, wheat).

Effect of irradiation. As the skin lesions in human pellagra are intensified by light, the influence of irradiation was tested. The monkeys were exposed three times a week, for from 10 to 45 min., to a carbon arc at a distance of about 2–3 ft. The irradiation did not appear, however, to aggravate their condition.

Conclusions

It is clear from these observations that monkeys resemble dogs and human beings in being unable to survive on a diet known to be deficient in P.-P. factor. Like dogs and human beings also, they are cured or protected by materials rich in the P.-P. factor, "343" liver powder, or yeast, or by the substitution of wheat for maize. In this respect they stand in sharp contrast with rats, which can subsist for long periods on pellagra-producing (or blacktongue-producing) diets (Part II [Birch *et al.* 1935]). Superficially at least the deficiency disease now described in monkeys resembles that of dogs and human beings in that a prominent feature is a disorder of the alimentary system, as evinced particularly by the diarrhoea. Further evidence as to its analogy with the human disease must await a detailed pathological study. But in the meantime there is no need to "multiply hypotheses unnecessarily" by postulating a new vitamin distinct from the already recognized P.-P. (antiblacktongue) factor. Final proof of identity must depend on eventual isolation and identification.

It is obvious that the factor which cures the monkeys is not vitamin B_6, for the basal diet already contains much vitamin B_6 while the active supplements add but little to the amount. Nor is it lactoflavin, since, as already pointed out, the disease still appears and the supplements continue to be effective whether lactoflavin is incorporated in the basal diet or not.[2]

[1] I am informed by Sir Charles Martin that in work still in progress [Birch *et al.* 1937] it has been found that pigs similarly develop symptoms of deficiency disease on a modified Goldberger blacktongue diet and are protected by the substitution of a mixture of wheat and barley for the maize.

[2] It is doubtful whether it would be profitable to discuss, in connexion with the present paper, several attempts which have been made in the past to produce pellagra in monkeys by means other than vitamin deficiency. While it was still generally believed that pellagra was a disease of low

The present results do not enable a decision to be reached whether lactoflavin is needed by monkeys (see p. 1417): to settle this point it will be necessary to carry out further feeding tests using supplements containing the P.-P. factor free from lactoflavin.

Comparison of different species

Further observations with rats. As pointed out in the previous paper in the series, the reason why rats do not develop any obvious symptoms of deficiency disease on a blacktongue-producing diet is still uncertain. The various possible alternative explanations are (1) that the needs of the rat are (relatively) extremely small, or (2) that the rat normally possesses very large stores, or (3) that it is able to dispense with this vitamin, or (4), and most likely, that it makes the vitamin in its own body (cf. vitamin C)—either through symbiotic activity or otherwise. Further work is needed; but it may be mentioned here that later tests have confirmed the finding given in the previous paper [Birch *et al.* 1935] that rats kept for long periods on Goldberger blacktongue diet or on Elvehjem "chicken pellagra" diet, although they show no signs of active disease have submaximum growth rates with a premature flattening of curves. It has been found since that the further addition of "343" liver powder, but not of egg white, produces a slight but appreciable improvement in the growth curve. Thus, rats have been kept on Goldberger diet, supplemented with vitamin B_1 (3 I.U. per day) plus lactoflavin (10γ per day), for periods of many months without apparent illness; if then "343" liver powder were administered a stimulating action could still be detected (see Fig. 8).

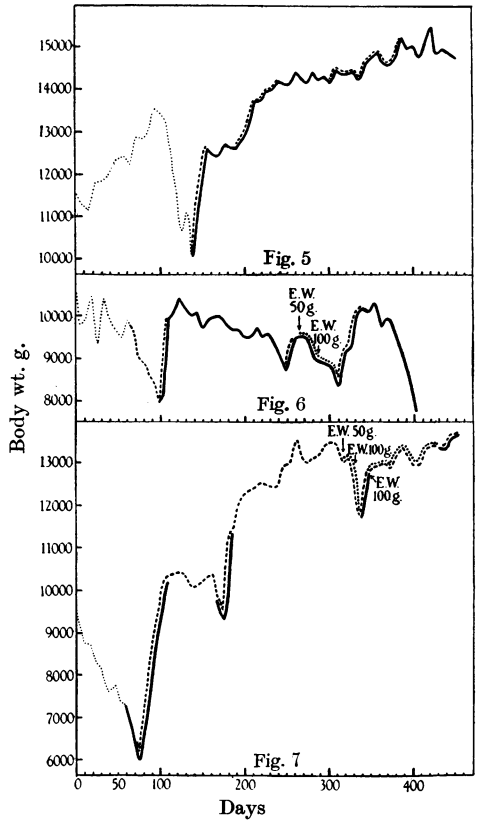
A full discussion of the implications of this finding seems outside the scope of the present paper; but it is thought worthy of mention since it may be of interest to other workers in the field as a further confirmation of the existence of an apparent "third" vitamin B_2 factor for rats (see p. 1415).

Further experiments on dogs. The experiments previously described [Birch *et al.* 1935] have been continued during a further experimental period of 18 months (Figs. 5–7), and the earlier results have been repeatedly confirmed. That is to say, on the synthetic basal diet, plus lactoflavin (30γ per day per kg. body weight) and vitamin B_1 (10 I.U. per day per kg. body weight) it has been consistently found that recovery only takes place when two factors are present simultaneously, i.e. vitamin B_6 (as provided by maize) and the antiblacktongue factor (as provided by "343" liver powder). A new result is that boiled egg white (50–100 g. per dog per day) was found to be ineffective either as a substitute for the maize or for the "343" liver powder (Figs. 6 and 7).

The fact that egg white cannot replace maize fits in with the observation of György [1935, 2] that it is deficient in vitamin B_6. It serves also to confirm the view that the factor provided by the maize and needed as a dietary essential by dogs is in fact vitamin B_6 (or a substance with a somewhat parallel distribution).

quality protein, "probably a tryptophane deficiency", Chick & Hume [1920] obtained symptoms in monkeys, which were thought to resemble closely those of human beings. The "low-quality protein" diets used contained varying amounts of sugar, maize starch, salts and gluten, with vitamins A, B, C (and D) added. Administration of tryptophan to one of the monkeys staved off death for a long time, but lysine, arginine and histidine were without effect.

More recently Stockman & Johnson [1933] stated that maize and all cereal grains examined were poisonous to monkeys, rabbits and guinea-pigs when they constituted the bulk of the diet, even when large amounts of vitamin carriers were consumed along with them. Acids isolated from the various grains produced similar symptoms, which were thought to bear a resemblance to human pellagra.



Figs. 5 to 7. Requirements of dogs for two separate "vitamin-B_2" supplements. Fig. 5. Failure on basal diet (containing vitamin B_1 and lactoflavin); recovery with simultaneous addition of vitamin B_6 fraction (maize) plus antiblacktongue factor ("343" liver powder), but not on either alone. Fig. 6. Inability of egg white to replace maize (vitamin B_6). Fig. 7. Inability of egg white to replace "343" liver powder (P.-P. or antiblacktongue factor). Similar results were obtained with two further dogs.

..... Basal diet.
——— Source of antiblacktongue (P.-P.) factor added.
- - - - Source of vitamin B_6 (maize) added.
▬ ▬ Maize and "343" liver powder added simultaneously.
ooooo Egg white added.

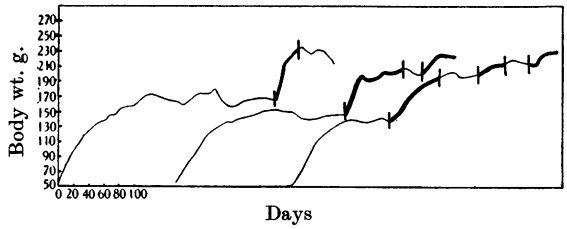


Fig. 8. Protection of rats for prolonged periods on diet devoid of P.-P. factor (Goldberger black-tongue diet supplemented with vitamin B_1 plus lactoflavin): cf. with the behaviour of dogs, monkeys (figs. 1–4) and human beings. Evidence of a state of "partial deficiency" only (sub-optimum growth), which shows some response to administration of "343" liver powder.

——— Basal diet alone.
——— Basal diet supplemented with "343" liver powder.

The inability of egg white when given in moderate amounts to replace the liver powder indicates that it is relatively deficient in antiblacktongue factor and probably therefore it cannot be used, as an observation of Simpson [1935] once seemed to offer hope that it might, as a concentrated source of P.-P. free from vitamin B_6 [see Birch *et al.* 1935, p. 2836, footnote].

Tests on pigeons. Experiments have also been started with the object of determining whether the pigeon needs any of the newly recognized constituents of the vitamin B_2 complex. It was found that pigeons on a basal diet of white rice, vitamin B_1 and radiostoleum lost weight. The addition of lactoflavin alone (20γ per day) had an insignificant effect in staying the loss in weight, the addition of "343" liver powder alone or in combination with lactoflavin was followed by growth at a subnormal rate, while the best growth response in these experiments was seen with the simultaneous administration of lactoflavin, "343" liver powder and 15% of maize meal (a source of vitamin B_6). On the other hand in experiments with Goldberger blacktongue diet (supplemented with vitamin B_1) the addition of "343" liver powder produced surprisingly little improvement in growth. It is evident that these results cannot be adequately interpreted without further studies—in which attention will have to be paid to such additional considerations as the intake of protein and other main constituents of the diet, the supplies of "vitamin B_3" and "vitamin B_5" and the use of a wider range of sources of the factors now under consideration.

Results with mice. In further work [cf. Part IV, Birch *et al.* 1935] it has been found possible to maintain mice for long periods on Goldberger blacktongue diet supplemented with vitamin B_1 (1·5 I.U. per day) and lactoflavin (5γ per day). Growth was improved by the further addition of "343" liver powder (0·06 g. = 1·5 g. fresh liver per day).

Conclusions. The needs of different species for the known constituents of the vitamin B_2 complex may be summarized as in Table I. A plus sign indicates that the constituent is needed by the animal in question; a negative sign that it can be dispensed with, or seems to be needed in relatively small amounts only; and a question mark that the needs are doubtful or as yet unknown.

Table I. *Needs of different species for components of vitamin B_2 complex*

	Lactoflavin	Vitamin B_6	P.-P. factor
Rats	+	+	−
Dogs	+	+	+
Human beings	?	?	+
Monkeys	?	?	+
Pigeons	?	?	?

SUMMARY

Monkeys resembled human beings or dogs, but differed from rats, in being unable to survive on a basal diet deficient in the human antipellagra, or canine antiblacktongue, factor (vitamins B_1 and B_6 and lactoflavin provided). They were protected by concentrates or supplements known to contain the P.-P. (or antiblacktongue) factor, viz. Eli Lilly "343" liver-extract powder, or yeast, or by the substitution of whole wheat for the maize of the basal diet. The more obvious symptoms of the deficiency disease in monkeys included marked loss of appetite, diarrhoea and vomiting, and there was a rapid fatal termination, the lesions of the skin not being prominent.

It is concluded that the third portion of the vitamin B_2 complex, distinct from lactoflavin and vitamin B_6, which is needed to prevent pellagra in human beings and blacktongue in dogs is likewise concerned in protection against the newly described disease of monkeys.

Further experiments confirm the conclusion that dogs need supplements both of vitamin B_6 (e.g. maize) and antiblacktongue factor (e.g. liver extract) to prevent nutritive failure on a synthetic diet containing vitamin B_1 and lactoflavin. Egg white in moderate amounts was ineffective as a source of either vitamin B_6 or antiblacktongue factor for dogs.

The comparative needs of rats, dogs, monkeys and pigeons for the several components of the vitamin B_2 complex are discussed.

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