CCCXXXV. THE VITAMIN B_2 COMPLEX. DIFFERENTIATION OF THE ANTIBLACK-TONGUE AND THE "P.-P." FACTORS FROM LACTOFLAVIN AND VITAMIN B_6 (SO-CALLED "RAT PELLAGRA" FACTOR). PARTS I–VI.

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IT is now recognised that the vitamin B complex for rats contains at least three components. The position to date may be represented as follows:[1]

Vitamin B { B_1 ; B_2 { Lactoflavin ; B_6 ; ...? ; ...? }

The relation of the human pellagra-preventive factor (P.-P.), as well as of the canine antiblacktongue factor, to these newly differentiated components of the vitamin B_2 complex has remained for investigation and forms the subject of the present paper.

Past work.

"Vitamin B" was first split into vitamin B_1 (heat labile, antineuritic or anti-beriberi factor) and vitamin B_2. The latter by definition is "the more heat-stable, water-soluble dietary factor recently described and named P.-P. ('pellagra-preventive') factor by Goldberger, Wheeler, Lillie and Rogers [1926] and found necessary for maintenance of growth and health and prevention of characteristic skin lesions in rats, and considered by the latter workers to be concerned in the prevention of human pellagra" (adopted by the Accessory Food Factors Committee at a meeting on Nov. 14th, 1927). This distinction between the heat-labile and heat-stable fractions of the vitamin B complex is due mainly to the work of Goldberger.

It was assumed by Goldberger and his associates [*e.g.* Goldberger and Lillie, 1926; Goldberger *et al.*, 1928, 1, 2] that the factor which prevents the "pellagra-like dermatitis" in the rat is identical with the factor which prevents pellagra in human beings (P.-P. factor) and also with the factor which prevents "black-tongue" in dogs. Goldberger's evidence, essentially, was of a similarity in the distribution and in the heat-stability of these factors, and also of the resemblance (superficially at least) of the lesions produced by their absence.

[1] The "vitamin B_4" of Reader is not included in this scheme. It appears that "vitamin B_4 deficiency" can be produced only when the animal is first deprived of vitamin B_1 and that it is cured whenever crystalline vitamin B_1 is given in sufficiently large dose. In other words "vitamin B_4 deficiency" has the appearance of chronic hypovitaminosis B_1. As also it is described as a "heat-labile factor" it does not affect our present discussion. [For a discussion of "vitamin B_4" see further Kinnersley *et al.*, 1935.]

Later work however has made the position somewhat less clear-cut. Other investigators [Chick and Roscoe, 1928; Aykroyd and Roscoe, 1929] found unexpected difficulty in producing the so-called "pellagra-like" dermatitis in the rat with any degree of regularity. They therefore used a method for estimating "vitamin B_2" which depends on the resumption of growth in young rats. For this test the basal vitamin B-free diet was supplemented with "Peters's eluate" as the source of vitamin B_1. Aykroyd [1930] used this method to compare the vitamin B_2 activities of various cereals. He reached the surprising conclusion that while all cereals were relatively poor in "vitamin B_2" as so measured, maize was not in fact so deficient as were certain other cereals, namely millet and rice. Now the clinical literature indicates that pellagra is confined very largely to maize-eating communities, relatively few instances, and those in isolated cases only, having been observed where millet or rice is eaten. Aykroyd was therefore sceptical of the theory that vitamin B_2 deficiency—or at any rate "vitamin B_2" as measured by the rat-growth test—is the cause of pellagra.

However, this objection of Aykroyd's has not been generally regarded as conclusive evidence against the theory that pellagra is a vitamin B_2 deficiency. As he himself indicated, other alternative explanations might be brought forward to account for the known facts. For instance, the striking difference in the incidence of pellagra on maize and rice diets might be explained by the circumstance that milled maize contains vitamin B_1, whilst polished rice is completely devoid of this vitamin: hence on a rice diet—so it might be argued—the subject would develop beriberi instead of pellagra. Hence the maize eater would remain free from beriberi and so liable to fall a victim to pellagra. Secondly, the use of maize as a staple crop is generally confined to areas where there is severe economic depression: therefore individuals in a maize area are less able to supplement their diet with other foodstuffs, which may contain larger amounts of the P.-P. factor, but must rely almost exclusively on this cereal as their principal source of food. Finally it must also be remembered that no yeast is used in the baking of maize bread, and this, at first sight, might account for the difference between maize and other cereals in relation to the incidence of pellagra.

For these and other reasons it has still been generally accepted, even after Aykroyd's work, that vitamin B_2 deficiency is the primary cause of pellagra. It is important at this point to emphasise that carefully controlled clinical tests on human beings have shown that pellagra does clear up with dramatic rapidity and certainty when such sources of vitamin B_2 as liver extract or yeast are exhibited, as small supplements to a pellagra-producing diet [see *e.g.* Ruffin and Smith, 1934].

The position became more complicated when it was shown by György *et al.* [1933; 1934] that vitamin B_2 consists of two components, the water-soluble, yellow pigment lactoflavin and a "complementary factor". It has been later established [György, 1934; 1935, 1; Chick *et al.*, 1935; Harris, 1935] that it is not lactoflavin but the "complementary factor" (vitamin B_6) which protects rats against the so-called "pellagra-like" dermatitis. Apparently the reason why the earlier workers had failed to produce symptoms of "pellagra-like" dermatitis regularly in the rat was because they used "Peters's eluate" as a source of vitamin B_1: this is itself contaminated with the complementary factor.

By general agreement the complementary factor is now known in this country as vitamin B_6, and the term vitamin B_2 is retained for the whole of the more heat-stable part of the vitamin B complex—*i.e.* excluding vitamin B_1, but including lactoflavin and vitamin B_6 [György, 1935, 1; Chick *et al.*, 1935; Harris, 1935].

Objects of present experiments.

Our first object was to obtain evidence as to the identity or non-identity of vitamin B_6, and of lactoflavin, with the pellagra-preventive factor (P.-P.) of Goldberger. With this aim in view we have undertaken a survey of the distribution of vitamin B_6 and of lactoflavin in various foodstuffs and compared the results with the known data concerning the distribution of the pellagra-preventive factor.

The second main object of the present work was to compare the behaviour of different species when fed on the various "pellagra-producing", "blacktongue-producing", or vitamin B_6-deficient diets. The purpose here was to determine whether one and the same factor was concerned in the production of blacktongue, of "rat pellagra" and of various other symptoms which have been described.

We have reached the conclusion that the P.-P. factor and the antiblacktongue factor are distinct both from vitamin B_6 (the "rat pellagra" factor) and also from lactoflavin (see also footnote, p. 2844). Whether the P.-P. factor is identical with the antiblacktongue factor still remains for consideration. So far there seems no cogent evidence against their identity.

Very recently Elvehjem and Koehn [1934; 1935] have stated that lactoflavin does not possess "antipellagra" activity for chickens. We have investigated also this aspect of the problem; but whilst confirming some of the observations of Elvehjem and Koehn, we have been driven to the rather disturbing conclusion that the "chick pellagra" factor again is distinct from the "rat pellagra" factor, vitamin B_6.

Finally the anti-anaemia "extrinsic factor" of Castle and Strauss should be alluded to. It resembles "vitamin B_2" in being relatively heat-stable, and there is some similarity also in distribution [Strauss and Castle, 1932]. It has therefore sometimes been claimed that it is identical with vitamin B_2 (or one of its major components). Until recently it seemed that conclusive evidence was lacking either one way or the other, but we believe it may now be stated with some degree of likelihood that the extrinsic factor is also different both from lactoflavin and from vitamin B_6.

I. Differentiation of the P.-P. factor from vitamin B_6 and lactoflavin.[1]

As already hinted, our conclusion that the human P.-P. factor of Goldberger differs from vitamin B_6 and lactoflavin is based largely on a comparison of their distribution in different foodstuffs, particularly in cereals, fish and liver extract.

It has already been reported [György, 1935, 2] that fish, although it is known to be an active source of the human P.-P. factor (*e.g.* protective dose of canned salmon = 168 g. [Goldberger and Wheeler, 1929] or flaked canned haddock = 340 g. [Wheeler, 1933]), is poor in lactoflavin. It still had to be shown how vitamin B_6 and lactoflavin are distributed in other natural foodstuffs, more especially in maize (since this cereal is known to be deficient in the P.-P. factor), and, for the sake of comparison, in other cereals (which appear to contain more of the P.-P. factor).

1. *Vitamin B_6 and lactoflavin content of cereals.*

The methods for the estimation of vitamin B_6 and lactoflavin were those previously described by György [1934; 1935, 2]. For the estimation of lactoflavin, the basal vitamin B-free diet was supplemented with crystalline (or highly purified) vitamin B_1 (3 I.U. daily) *plus* vitamin B_6 (in the form of Peters's eluate from baker's yeast: equivalent to 10 g. of fresh baker's yeast daily). For the estimation of vitamin B_6, vitamin B_1 was similarly provided but supplemented in this instance with pure lactoflavin (10γ daily).

[1] A preliminary account of the main results in Part I has already been communicated to the Biochemical Society [Birch and György, 1935, 1].

The basal diet was as follows:

Caseinogen AB "Glaxo"	18
Rice starch	68
Butter fat	9
Salt mixture (B.D.H.)	4
Cod-liver oil	1

The values for vitamin B_6 are based chiefly on the curative effect against dermatitis. The values for lactoflavin were determined by means of the growth test, the skin symptoms being too irregular and frequently insufficiently obvious. The "rat day-dose", taken as provisional unit, was defined as the minimum quantity of the substance which would give rise to a gain in weight of about 10 g. per week for at least 4 weeks.

For each product graded doses were tested, generally on three rats at each level. The values given represent averages.

Table I. *Vitamin B_6 and lactoflavin in cereals. Summary.*

(For animal responses see Table II.)

	Lactoflavin. 1 "rat day-dose" is contained in the following weight of foodstuff g.		Vitamin B_6. 1 "rat day-dose" is contained in the following weight of foodstuff g.
Wheat germ	1·0	Rice polishings	0·1
Wheat bran	1·0	Wheat germ	0·2
Pea meal	2·0	Wheat bran	0·3
Oats (ground)	3·0	White maize	0·5
Yellow maize	5·0	Yellow maize	0·75 (or less)
Rice polishings	>1·0	Pea meal	0·75 (or less)
Whole wheat	>5·0	Oats (ground)	1·0
Polished rice	>5·0	Whole wheat	1·5
		Polished rice	3·0
		"Hominy grits"	3·0

As will be seen (Tables I and II), all cereals are relatively rich in vitamin B_6; and maize in particular is one of the richest. The minimum curative dose of whole maize is no more than 0·5–0·75 g. per rat per day. The vitamin is not uniformly distributed throughout the grain, but the bran and the germ are the richest in it, the endosperm poorer. But even in the endosperm of white maize (in "hominy grits") there appears to be an appreciable amount of vitamin B_6, the minimum curative dose being approximately 3 g. daily.

In contrast with vitamin B_6 the lactoflavin content of cereals is found to be very low (Table I). Indeed, in all the cereals examined the lactoflavin and not vitamin B_6 seems to be the limiting factor of the vitamin B_2 complex. Whilst the cereals are all very poor in lactoflavin, wheat contains even less lactoflavin than does maize. As with vitamin B_6 the germ is richer in it than is the endosperm. Rice polishings were found to be surprisingly poor in lactoflavin.

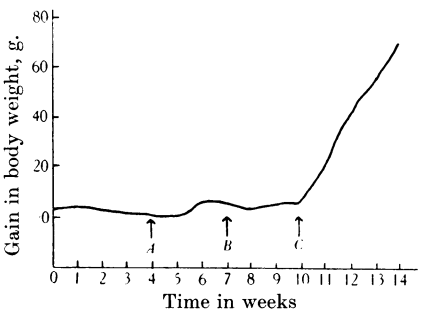

Fig. 1. The following supplements were given daily to a diet containing adequate vitamin B_1 *plus* "Peters's eluate" (for vitamin B_6). At *A*, 3 g. whole wheat. At *B*, 5 g. whole wheat. At *C*, 5 g. whole wheat + 10γ lactoflavin.

An illustration of the poverty of cereals in lactoflavin is recorded in Fig. 1.

Table II. *Tests on cereals* etc., *for lactoflavin and vitamin B_6.*

(For summary see Table I.)

Material fed	Daily amount given g.	Tests for lactoflavin (vitamins B_1 and B_6 provided). Av. weekly increase in weight g.	Tests for vitamin B_6 (vitamin B_1 and lactoflavin provided) Av. weekly increase in weight g.	Healing of specific dermatitis
Yellow maize (ground)	5·0	12	17	Yes
	3·0	8	14	Yes
	2·0	5	9	Yes
	1·5	4	6	Yes
	1·0	—	7	Yes
	0·75	—	6	Yes
White maize (ground)	2·0	—	8	Yes
	1·0	—	7	Yes
	0·5	—	2	Yes
"Hominy grits"	5·0	—	10	Yes
	3·0	—	4	Yes
	2·0	—	1	No
	1·0	—	2	No
Whole wheat (ground)	5·0	6	—	—
	3·0	5	12	Yes
	2·0	2	12	Yes
	1·5	3	8	Yes
	1·0	—	4	Inconstant
Wheat bran	1·0	9	—	—
	0·5	2	9	Yes
	0·3	—	7	Yes
Wheat germ	1·0	9	—	—
	0·5	7	13	Yes
	0·3	—	6	Yes
	0·2	—	6	Yes
	0·1	—	0	No
Oats (ground)	3·0	10	—	—
	2·0	5	11	Yes
	1·0	—	5	Yes
	0·5	—	−2	No
Polished rice	5·0	1	—	—
	3·0	1	3	Yes
	2·0	—	2	Inconstant
	1·0	—	−3	No
Rice polishings	1·0	4	19	Yes
	0·75	4	—	—
	0·5	3	15	Yes
	0·25	2	6	Yes
	0·2	—	8	Yes
	0·1	—	3	Yes
Pea meal	2·0	11	—	—
	1·5	—	8	Yes
	1·0	5	8	Yes
	0·75	—	7	Yes
Black treacle molasses	1·0	—	7	Yes
	0·5	—	5	Yes
	0·25	—	3	Yes
Ruffin and Smith's pellagra-producing diet for humans	3·0	1	11	Yes
	2·0	—	9	Yes
	1·0	—	8	Yes
Elvehjem-Koehn diet for "chick pellagra"	3·0	1	7	Yes
	2·0	0	6	Yes
	1·5	—	4	Yes
	1·0		3	Inconstant

Here not even 5 g. of whole wheat were able to exert a significant growth-promoting action when supplemented with vitamin B_1 and vitamin B_6. The addition of 10γ lactoflavin then gave rise to a marked increase in weight which continued for many weeks.

Comment. The above results furnish a further illustration of the erroneous conclusions which may be reached if the complex nature of vitamin B_2 is disregarded. Since the experiments of Aykroyd and Roscoe [1929] and of Aykroyd [1930] cereals have been commonly regarded as poor in vitamin B_2. As will now be seen this finding holds good only for lactoflavin, and not for the vitamin B_6 component of the vitamin B_2 complex.

2. *Vitamin B_6 and lactoflavin in various foods.*

Further assays on a variety of foodstuffs illustrate once again that their vitamin B_6 and lactoflavin contents bear no apparent relation to their reputed values as pellagra preventives (Tables III and IIIA). For example Eli Lilly

Table III. *Comparison of P.-P. value with vitamin B_6 and lactoflavin in various foods.*

Food	Reputed P.-P. value, after Sebrell [1934] (dose fed, g. shown in brackets)	Lactoflavin dose. 1 "rat day-dose" is contained in following weight of foodstuffs g.	Vitamin B_6 dose. 1 "rat day-dose" is contained in following weight of foodstuffs g.
Dried yeast	Good (15–30)	0·1–0·2	0·1–0·2
Liver, dried pig	,, (64)	—	—
Liver, ox, fresh	,, —	0·2	0·3
Liver extract, Eli Lilly 343	,, (100)*	—	>5·0*
Wheat germ	,, (150)	1·0	0·2
Salmon	,, (168)	>3·0	0·5
Beef, fresh	,, (200)	3·0	1·0
Chicken	,, (325)	3·0	1·0
Milk, skim	Fair (*a*)	10 ml.	10 ml.
Haddock	,, (340)	>3·0	0·5
Caseinogen	Slight (85)	>2·0	>2·0
Butter	,, (135)	>2·0	>2·0
Whole wheat	,, (400)	>5·0	1·5
Oats, rolled	None (400)	3·0	1·0
Maize meal, whole white	,, (450)	5·0	0·5

* Expressed as weight of fresh liver.
(*a*) = 30 ml. per kg. of body weight.

Table IIIA. *Vitamin B_6 and lactoflavin values of pellagra- and blacktongue-producing diets.*

	Rat dose, g. per day	
	For vitamin B_6	For lactoflavin
Human pellagra-producing diet (Ruffin and Smith)	0·5	>6·0
Canine blacktongue-producing diet (Rhoads and Miller)	0·7	6·0
"Chick pellagra" diet (Elvehjem and Koehn)	1·5	>6·0

liver extract (powder) No. 343, known to be one of the most highly potent of all antipellagra remedies (see below), contains relatively little vitamin B_6 whilst cereals and molasses which have little or no antipellagra activity are rich in

vitamin B_6. Or again, there is no more lactoflavin in fish, which is an active source of P.-P., than in cereals, which are poor in it.

These differences are perhaps made more convincing when expressed in figures. For example, it may be seen from Table III that with maize containing 900 doses of vitamin B_6 there is no antipellagra action, whilst with liver extract containing less than 20 doses of vitamin B_6 rapid cure results (see below).

3. *Direct clinical trials with lactoflavin.*

We are allowed here to refer also to direct clinical trials carried out through the co-operation of our colleague Dr W. J. Dann at Duke University, North Carolina, which have confirmed the foregoing conclusions that the P.-P. factor is different from both lactoflavin and vitamin B_6. Patients kept on the "pellagra-producing basal diet", under strictly controlled conditions (including regular exposure to light *etc.*), failed to improve when lactoflavin was administered, but they recovered rapidly and dramatically when liver extract, or other concentrated source of the P.-P. factor, was administered [Dann, *et al.* 1935]. As the basal diet itself contains maize and molasses, both rich in vitamin B_6, these results indicate that neither this factor nor lactoflavin has antipellagra action for human beings.[1]

II. Maize in relation to the vitamin B_2 complex and human pellagra.

It is apparent from the results described in Part I that the rat antidermatitis factor, vitamin B_6, is present in large amount in all the cereals examined, and particularly so in maize—*i.e.* in the "pellagra-producer" *par excellence*. Moreover the different dietaries used for the production of blacktongue in dogs, or pellagra in human beings (such as the diet of Ruffin and Smith), are also very rich in vitamin B_6 (curative dose for rats = 1 g. per day or less) (Tables II and III A).

It remains to be explained, therefore, how the theory came to be so generally adopted that the antidermatitis factor for the rat is identical with the P.-P. factor for human beings. We believe the explanation to be as follows. The theory had its origin in Goldberger's experiments in which dermatitis in rats was produced by means of a synthetic diet which was supplemented not with whole maize but with an extract of maize made with cold alcohol. The latter appears to be deficient in vitamin B_6. It has been found [Birch and György, 1935, 2] that quantitative extraction of the vitamin from natural foodstuffs is only accomplished with difficulty. Even with extraction with hot alcohol containing HCl low yields are obtained. It may be safely concluded therefore that when cold alcohol is used very little of the vitamin B_6 is extracted. Unfortunately Goldberger does not appear to have attempted to use a diet containing whole maize (or milled maize), and this, as our experience indicates, is actually effective in preventing or curing rat dermatitis.

Later, when maize was examined by Aykroyd and Roscoe [1929] it was found to be a poor source of "vitamin B_2"—*i.e.* of the whole "vitamin B_2" complex as determined by growth tests. The explanation here, no doubt, is that there is little lactoflavin in maize: poor growth would therefore have resulted. As already indicated such experiments were merely measuring lactoflavin as the "limiting factor".

[1] Simpson [1935] has recently reported that a case of secondary pellagra following gastrectomy was partly improved by a "vitamin B_2 concentrate" from egg-white: since egg-white is deficient in vitamin B_6 [György, 1935, 2] this result might seem to furnish still further evidence of the differentiation of the P.-P. factor from vitamin B_6.

1. *Experiments with rats fed on maize diets.*

Further experiments were also undertaken to try and ascertain whether rats need the true antipellagra factor (human P.-P. factor) or the canine antiblacktongue factor, *i.e.* in addition to vitamin B_6. In one test a group of 10 young rats was placed on the following diet, the components of which, judged by clinical experience, are deficient in the P.-P. factor:

White maize	...	...	85 %
Arachis oil	...	...	15 %
Cod-liver oil	...	...	6 drops per rat per week

This diet was supplemented with additional pure crystalline vitamin B_1 and lactoflavin. These additions were made to be sure that any symptoms due to possible shortage of either of these factors should not obscure the hypothetical lesions due to the lack of the P.-P. (or antiblacktongue) factor. Since exposure to light appears to play an important part in the production of the dermatitis amongst pellagrins, half of the group of rats was exposed under a quartz mercury-vapour lamp for half an hour each day.[1]

The growth curves of this group of animals are shown in Fig. 2. It is seen that both the irradiated and the non-irradiated animals had subnormal growth rates. At the point on the curve marked ↓ (102 days from the beginning of the experiment) an addition of 10 % of caseinogen was made to the diet and the maize

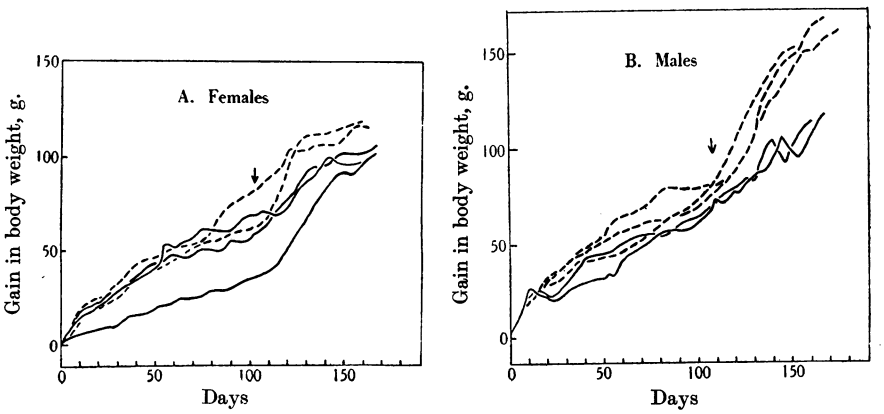

Fig. 2. Growth curves of rats fed on diet deficient in P.-P. factor. *A*, females; *B*, males. —— non-irradiated animals. ----- irradiated animals. Caseinogen given at ↓.

reduced to 75 %. At this time some of the animals had begun to show bald patches and loss of fur together with a slight scurfy condition of the skin. With this addition of caseinogen however the growth rate increased and the fur became almost normal again. It is thought therefore that these symptoms were probably due to the lack of an essential amino-acid, perhaps cystine or tryptophan. Subsequently the irradiated animals developed small brown scales on their

[1] Aykroyd [1930] had previously attempted without success to produce the pellagra-like dermatitis in rats by feeding a maize diet and submitting the animals to ultraviolet irradiation. His experiment differed from ours in that he fed Peters's eluate as a source of vitamin B_1: this might possibly contain the P.-P. factor. Also the period of irradiation was limited to 3 min. per day. The possibility therefore remained that with supplements of pure vitamin B_1 *plus* lactoflavin, and with the amount of irradiation increased some 10 times, symptoms due to the lack of the true P.-P. factor might appear.

backs, and a whitish opacity was seen on the eyeballs, due apparently to the excessive irradiation with ultraviolet light. Nevertheless, characteristic skin lesions analogous to pellagra were not seen and diarrhoea, a characteristic symptom in pellagra, was absent.[1]

It appears therefore that rats do not need the P.-P. (or antiblacktongue) factor (or their needs are remarkably small), or, as with vitamin C, are able to synthesise it for themselves. Experiments are at present in progress on this last point.

2. *Absence of pellagra from rats fed on blacktongue or human pellagra-producing diets.*

A further group of rats was fed on the diet given below (Goldberger diet), which is more closely based on that used by Goldberger *et al.* [1928, 1, 2] for the production of blacktongue in dogs, and which we ourselves have confirmed (see Part III) to be satisfactory for this purpose. All the rats throve on this diet (see Fig. 3), and remained free from skin lesions, although eventually their

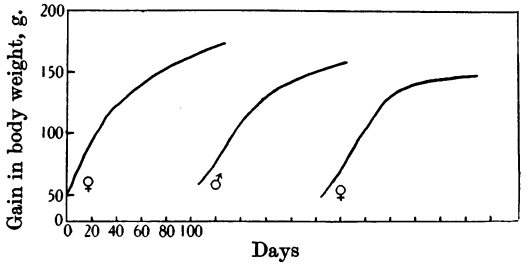

Fig. 3. Growth curves of rats fed on Goldberger blacktongue diet *plus* vitamin B_1 and lactoflavin.

growth rates became subnormal. Further tests were also made with diets similar to those used for experimental work on pellagra in human beings (see below) and with the same result.

Composition of pellagra-producing diets (after Ruffin and Smith [1934]).

Goldberger diet.

	g.		g.
Corn meal	229	Cane sugar	58
Grits	29	Sweet potatoes	92
Corn starch	35	Turnip greens	21
Wheat flour	137	Cabbage	9
Rice	23	Collards	13
Cane syrup	34	Pork fat	107

Ruffin and Smith diet.

	g.		ml.
Corn meal	92	Cod-liver oil	90
Cane sugar	105	Tomato juice	45
Flour	111	Iron ammonium citrate	6
Lard	81	Calcium gluconate	6
Rice	25	Cheese	60
Field peas	90		
Hominy grits	51		
Fat salt pork	60		

[1] It appears that the irradiation had a beneficial effect on the animals, for in all cases the irradiated ones grew better than the non-irradiated. After 166 days the irradiation was stopped and the rats were mated. Three out of the five does produced litters, the two not having litters being both from the non-irradiated group. Such a result suggests that the diet is not grossly deficient in any factor needed by the rat, although the young rats died shortly after birth.

III. Differentiation of the "antiblacktongue" factor from vitamin B_6 and lactoflavin.

The purpose of the experiments to be described in this section was to produce symptoms of blacktongue in dogs and test the curative and preventive actions of the known components of the vitamin B_2 complex.

1. *Experiments with Goldberger's blacktongue-producing diets.*

Diet. In the first experiment 3 young bitches of about 6–7 kg. in weight were placed on a basal diet slightly modified from that of Goldberger *et al.* [1928, 1] and Rhoads and Miller [1935, 1].

White maize meal	600
Dried pea meal	75
"Glaxo" extracted caseinogen ("free from vitamin B_2") ...	90
Cod-liver oil	28
Cottonseed oil	45
$CaCO_3$	45
NaCl	15

As will be seen from the results given in Part I this diet is rich in vitamin B_6 but contains only small quantities of lactoflavin.

To prepare the diet, the maize meal, the pea meal and the caseinogen were cooked in a double saucepan for 2 hours and the other ingredients added subsequently. The diet was offered *ad lib.* About 100–200 g. were consumed by each dog per day. All dogs received a daily injection subcutaneously of 80 I.U. of vitamin B_1. This was given in the form of a highly active concentrate, free from lactoflavin and vitamin B_6. We are indebted to Messrs I. G. Farbenindustrie of Germany for their generosity in supplying this preparation.

Experimental observations. One dog, kept as a positive control, was given autoclaved yeast, 10 % by weight of the diet. In contrast with the other animals, this one grew well, remained in excellent health and had none of the characteristic symptoms. During the latter part of the experiment an additional supplement of lactoflavin was provided with no apparent influence on the progress of the animal, showing that the amount of lactoflavin in the added autoclaved yeast was already adequate.

The two dogs on the unsupplemented blacktongue-producing diet lost weight rapidly, and became emaciated and increasingly weak (see Fig. 4). After 76 days, incipient lesions were just distinguishable on their tongues, in the form of transverse ridges on the upper surface. Lactoflavin was then administered, 30γ per kg. body weight per day, intraperitoneally. It failed to protect the animals, for the loss in weight continued, diarrhoea developed, and the condition of the tongue quickly deteriorated. In another fortnight the tongues had become markedly pale, the ridges across the surface were more pronounced and there was some atrophy at the edges. Excessive salivation was prominent in one dog.[1]

Cures with liver extract and fish. After the dogs had been 102 days on the experimental diet, one was treated with a supplement of 4 g. per day of Eli Lilly powdered liver extract No. 343 (=100 g. fresh liver), and the other with 35 g. per day of fresh herring. In both dogs, the improvement was dramatic. They

[1] Although the lactoflavin clearly had no specific action against "blacktongue" it seems likely that it is a necessary component of the diet of the dog, as of the rat. Following its administration the dogs seemed more lively and alert and the loss in weight less steep.

immediately started gaining weight again, and already after 5 or 6 days the tongue showed some improvement; the general behaviour and appearance of the dogs changed from one of dejected apathy to normal liveliness.

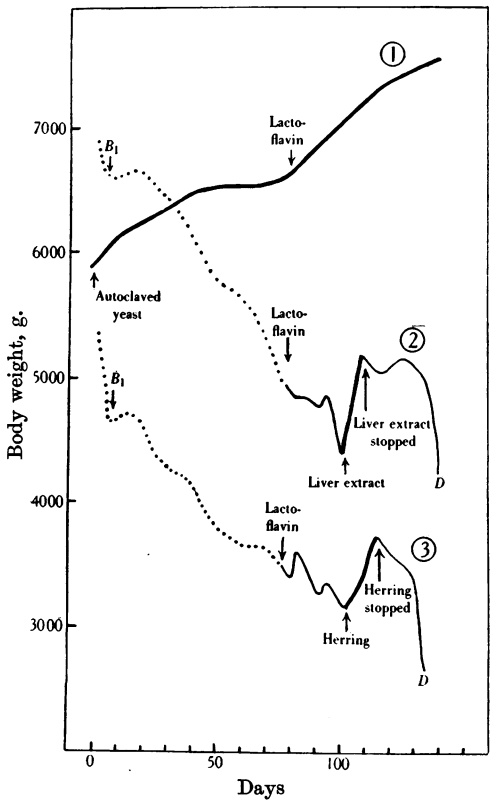

Fig. 4. Experiments with dogs on Goldberger blacktongue diet. No. 1, positive control, received autoclaved yeast, 10% of diet daily. Starting from arrows marked "B_1", 80 I.U. of vitamin B_1 were given daily; from "Lactoflavin" 30γ of lactoflavin per kg. body weight were injected daily; at "Liver extract" (dog No. 2) 4 g. of Eli Lilly liver powder 343 (=100 g. fresh liver) daily; and at "Herring" (dog No. 3) 35 g. fresh herring daily.

Second experiment. On the 114th day the curative supplements of liver extract and herring were discontinued. At once the animals began to lose weight again. They died on the 140th and 134th day respectively from the beginning of the tests.

Blood counts. A few days from the end of the experiment determinations of haemoglobin and red blood cells were carried out by Dr M. A. Abbasy. His fuller results will form the subject of a later communication; but it may be noted that pronounced anaemia was present in the animals suffering from blacktongue.

Table IV.

	Haemoglobin value %	Red blood cells per μl.
Blacktongue	40	3,320,000
Blacktongue	35	2,500,000
Positive control	88	5,200,000

Post mortem *findings*. The two dogs which died under the influence of the deficient diet were examined by Dr J. R. M. Innes of the Institute of Animal Pathology, who stated that their condition conformed with that described for "blacktongue" by Goldberger, and by Rhoads and Miller and others.

Conclusions. It is apparent that the dogs lost weight and developed "blacktongue", with symptoms of diarrhoea, anaemia *etc.*, on a diet containing relatively large amounts of vitamin B_6. The addition of large quantities of lactoflavin was also unable to protect against the disease, whereas small amounts of autoclaved yeast, fresh fish or liver extract, containing relatively little additional vitamin B_6 or lactoflavin, prevented or cured the symptoms. (The calculated amounts of vitamin B_6 or lactoflavin in this basal diet and in the various supplements are shown in Table V.) It may be concluded that the "blacktongue" factor present in the autoclaved yeast, in the liver extract and in the herring is distinct from lactoflavin or vitamin B_6.

Table V.

	No. of rat units		Result (protection against blacktongue)
	Vitamin B_6	Lactoflavin	
Basal diet alone (approximately 150 g. per day)	230	22	No protection
,, + lactoflavin	230	50	,,
,, + lactoflavin + 35 g. herring per day	300	50	Protection
,, + lactoflavin + 4 g. liver extract per day	240	100	,,
,, + lactoflavin + 10% autoclaved yeast	275	95	,,

2. *Experiments on synthetic diets.*

So far as we can tell from a study of the literature, past workers who have used dogs for the study of blacktongue have usually restricted their attention to the use of "natural" diets, and have apparently not succeeded in maintaining dogs satisfactorily on "synthetic" diets. However, it seemed desirable, for scientific accuracy, to attempt to work with the "synthetic" type of diet, in order that the various dietary components might be more accurately controlled and varied. Our experiments indicate that this is in fact possible. Control dogs fed on the "synthetic" diet *plus* 7% of yeast throve normally (Figs. 5 and 6). The composition of the basal diet used throughout these experiments was as follows:

Cane sugar	67
Extracted caseinogen	20
Salt mixture	3
Arachis oil	10
Cod-liver oil	20 ml. per day

From the earlier results it might be anticipated that this basal diet, even when supplemented with vitamin B_1 *plus* lactoflavin, is still deficient in not one but in two factors—namely the "rat pellagra factor" (vitamin B_6) and the antiblacktongue factor. Former workers have wrongly assumed that these two are identical. Our supposition seems confirmed by the observations recorded in Fig. 7. Here a dog receiving vitamin B_1 and lactoflavin was not cured when vitamin B_6 was added in the form of maize (50 g. per day of cooked white maize meal), but was cured with the further addition of the antiblacktongue factor, given in the form of liver extract 343. Further tests on other dogs (Figs. 8, 9 and 10) uphold this view, namely that the dog is unable to thrive when given either of these supplements separately, but does so when the two are combined. That this result is quantitatively significant—and is not due to the second supplement augmenting the amount of vitamin B_6 in the first supplement—is

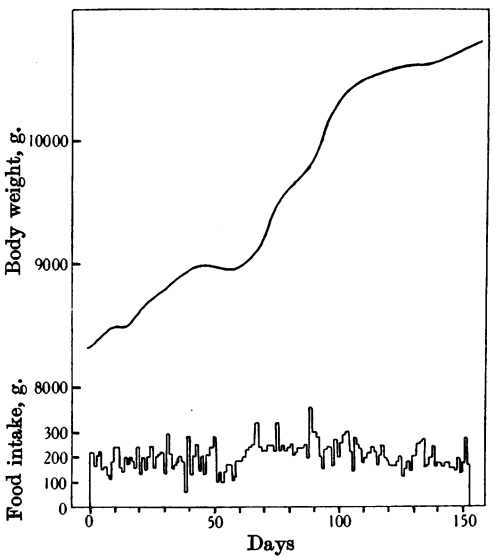

Fig. 5. Dog on "synthetic" vitamin B-deficient diet *plus* 7% dried yeast (positive control).

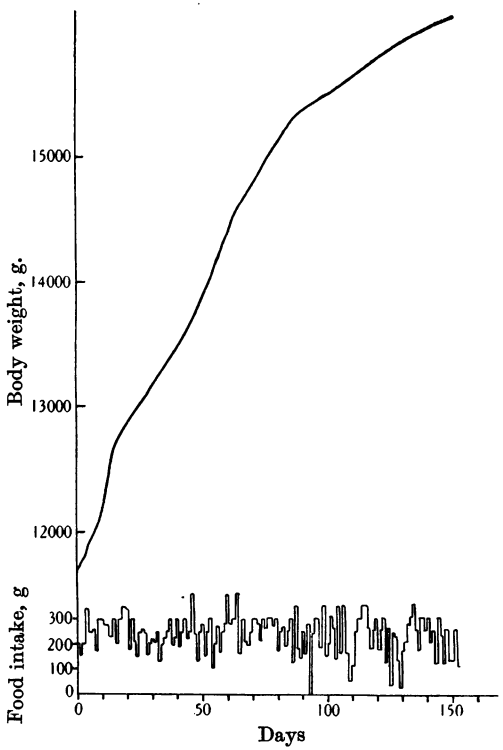

Fig. 6. Dog on "synthetic" vitamin B-deficient diet *plus* 7% dried yeast (positive control).

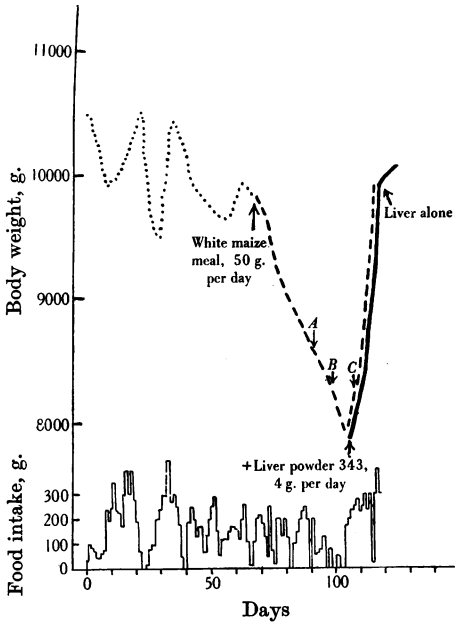

Fig. 7. Dog on "synthetic" vitamin B-deficient diet + vitamin B_1 + lactoflavin. *A*, tongue pale, mouth inflamed, charac. diarrhoea; *B*, very ill, "blacktongue" pronounced; *C*, improved.

..... = basal diet.
– – – – = + maize alone.
——— = + liver extract alone.
– – – – = + maize + liver extract.

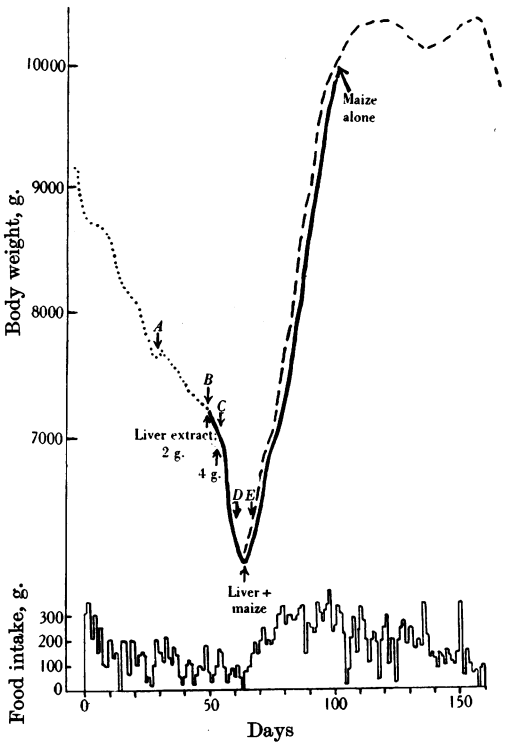

Fig. 8. Dog on "synthetic" vitamin B-deficient diet + vitamin B_1 + lactoflavin. *A*, losing hair, dermatitis; *B*, blacktongue; *C*, very weak; *D*, excessive salivation, diarrhoea, blood in faeces; *E*, much improved.

..... = basal diet.
——— = + liver extract alone.
– – – = + maize alone.
═══ = + maize + liver extract.

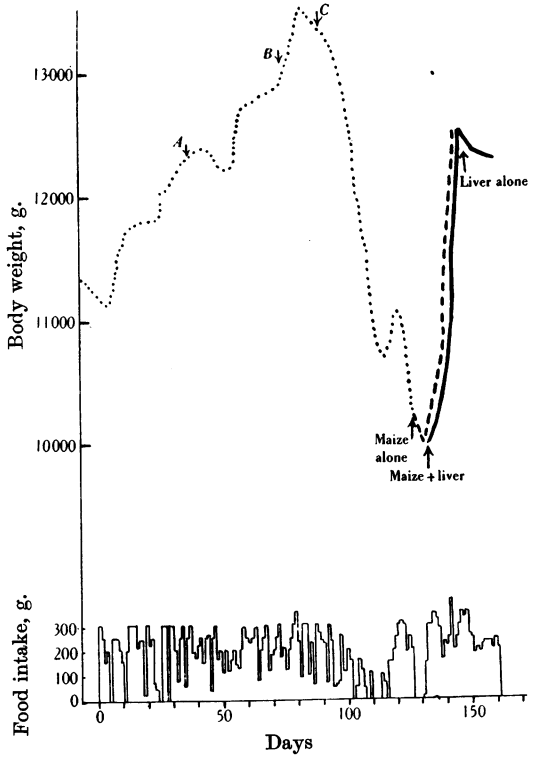

Fig. 9. Dog on "synthetic" vitamin B-deficient diet + vitamin B_1 + lactoflavin. *A*, tongue ridged and pale; *B*, loss of hair, skin inflamed; *C*, diarrhoea, tongue inflamed, severe dermatitis and loss of hair.

..... = basal diet.
——— = + liver extract alone.
– – – = + maize alone.
═══ = + maize + liver extract.

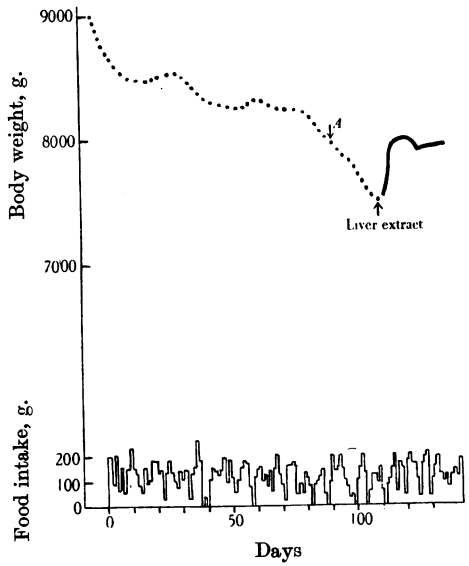

Fig. 10. Dog on "synthetic" vitamin B-deficient diet + vitamin B_1 + lactoflavin. *A*, tongue pale, mouth inflamed.

..... = basal diet.
——— = + liver extract.

seen from the contrasting vitamin values given in Table VI. The individual effects of deficiency of vitamin B_6, lactoflavin and the antiblacktongue factor in the dog are still under investigation.

Table VI.

	Vitamin B_6	Antiblacktongue factor
White maize meal, 50 g. per day	100 rat units	0
Liver extract, No. 343, 4 g. per day (=100 g. fresh liver)	< 10 rat units	+ + +

Comments. Observations by earlier workers have tended to suggest that the presence of maize is a necessary condition for the production of symptoms of blacktongue in dogs. (Many have supposed that maize contains a pellagra-producing, or blacktongue-producing, toxin [*cf.* Chick, 1933].) Recently Cowgill *et al.* [1934] have reported that on artificial vitamin B_2-deficient diets containing no maize the symptoms which develop are different from those of blacktongue, being marked by stomatitis and glossitis: Zimmerman and Burack [1934] indeed suggest that "blacktongue", as ordinarily observed, may be a multiple deficiency. The experimental results recorded in this section point to the following as a possible explanation of such observations: namely, that the addition of maize to the diet helps in the production of regular symptoms of blacktongue, not so much because of a toxin present in it, as because in its absence the dog may sometimes develop earlier vitamin B_6 deficiency instead of blacktongue.[1]

IV. Observations with chickens and other species.

The observations made in Parts I–III indicate that there are considerable differences between species in their need for the components of the vitamin B_2 complex, the rat appearing to differ markedly from the dog or the human being. It was thought advisable to pursue this aspect of the problem by observations on a variety of other species. We wish here to record our observations on chickens, mice, rabbits and guinea-pigs.

1. *"Chick pellagra" of Elvehjem and Koehn.*

Elvehjem and Koehn [1934; 1935] recorded that chicks fed on the following diet:

Maize meal	580
Middlings	250
Extracted caseinogen	120
Common salt	10
Calcium carbonate	20
Cod-liver oil	2% daily

(the maize meal, middlings and extracted caseinogen are heated in shallow trays at 100° for 144 hours)

developed skin lesions (considered by them to be the analogue of human pellagra). The lesions were not cured by lactoflavin. We have confirmed the

[1] A recently published preliminary note by Rhoads and Miller [1935, 2] states that rats do not develop vitamin B_2 deficiency when fed on Goldberger diet. These authors postulate therefore that blacktongue is not due to a deficiency of "vitamin B_2". This experimental observation with rats is independently confirmed in Part I, but our own results with dogs lead to a rather different summing up of the position: that is, that the antiblacktongue factor is found to be distinct from two known constituents of the vitamin B_2 complex, namely vitamin B_6 and lactoflavin, but is to be regarded as an additional constituent of the vitamin B_2 complex, possibly identical with the human P.-P. factor. So far we have been able to find no cogent evidence to distinguish the canine blacktongue factor from the human P.-P. factor.

findings of Elvehjem and Koehn that chicks fail to thrive on this diet and may develop skin lesions, especially around the beak (which however in our experiments were not very well marked). Loss in weight and skin symptoms are prevented when yeast is added to the diet, or if the diet is fed in an unheated condition instead of after the heating process (see Fig. 11). In accordance with the

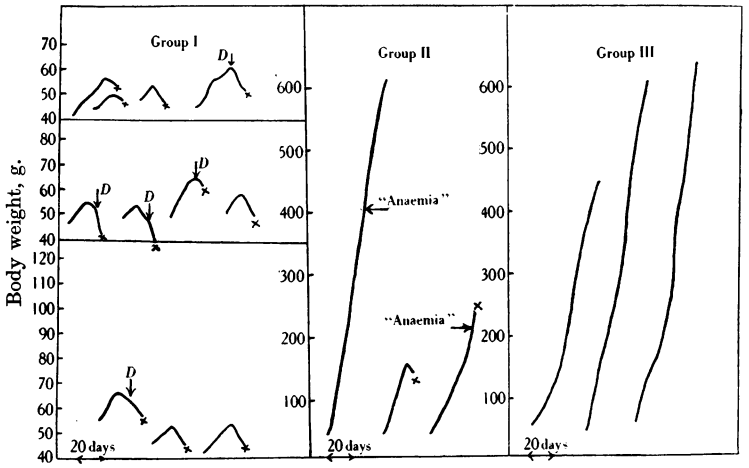

Fig. 11. Chicks on various modifications of Elvehjem-Koehn diet. In group I (heated E.K. diet), 10 I.U. of vitamin B_1 and 30γ of lactoflavin were injected twice weekly. "*D*" indicates incidence of dermatitis. Group II. Heated E.K. diet + 7% dried yeast. Group III. Unheated E.K. diet.

findings of Elvehjem and Koehn, lactoflavin had no effect on the symptoms. On the other hand we believe that the "chick pellagra" factor of Elvehjem and Koehn is different from vitamin B_6, for we have found that rats fed on this diet remain free from any symptoms of vitamin B_6 deficiency, and in fact the diet cures rats suffering from vitamin B_6 deficiency in doses of 1·5 g. per day (see Table II).[1]

The nature of the deficiency (or deficiencies) in the diet of Elvehjem and Koehn seems for the moment uncertain. We have noted that rats fed on it show a subnormal growth rate and a premature flattening of the growth curve (Fig. 12) after a time, although, as remarked above, they remain free from the skin lesions of vitamin B_6 deficiency. This might appear to indicate a deficiency in the Elvehjem-Koehn diet of some additional factor which is needed by the rat and hence is presumably different from the P.-P. or antiblacktongue factor (which the rat appears able to dispense with or at any rate to require in only very minute amounts (Parts I–III)).

A further observation we have made is that chicks fed on Elvehjem-Koehn diet supplemented with yeast show a condition suggestive of anaemia, as judged by the strikingly pale condition of their combs. The anaemia-preventing factor concerned, therefore, as it is not contained in yeast, should not perhaps be regarded as a component of the vitamin B_2 complex. When unheated Elvehjem-Koehn diet was fed the anaemia was found to be entirely prevented.

[1] Since this was prepared for press Lepkovsky and Jukes [1935] have also published the conclusion (reached by a quite different line of evidence from our own) that the Elvehjem-Koehn chick factor is distinct from the rat "vitamin G".

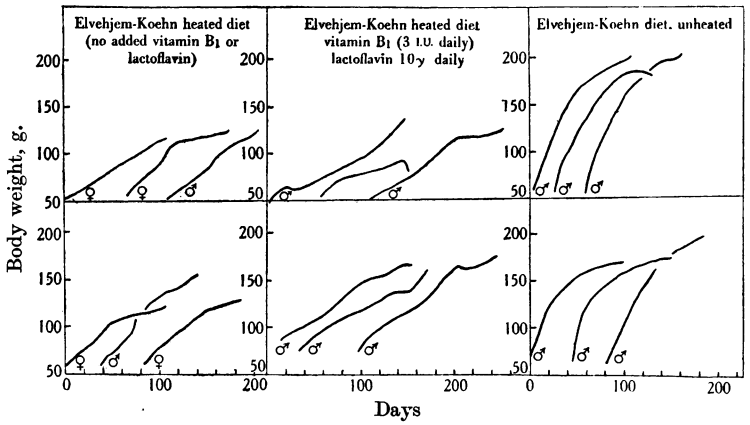

Fig. 12. Rats fed on Elvehjem-Koehn "chicken pellagra" diet with various modifications.

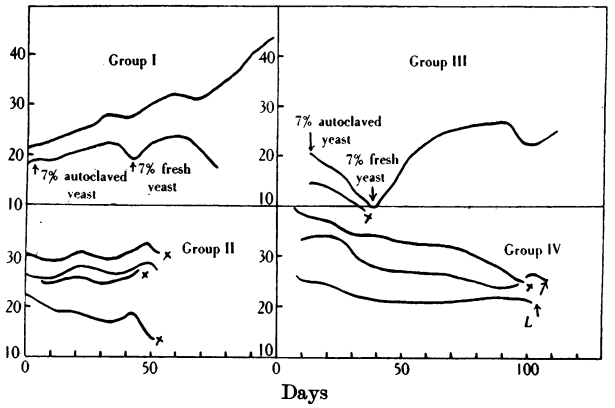

Fig. 13. Mice fed on various vitamin B-deficient diets. (In groups II and IV vitamin B_1 was given as 5 I.U. injected twice weekly and lactoflavin as 15γ injected twice weekly.) Group I. Goldberger blacktongue diet *plus* 7 % fresh dried yeast. Group II. Goldberger blacktongue diet *plus* vitamin B_1 and lactoflavin. Group III. Synthetic vitamin B-deficient diet *plus* 7 % fresh dried yeast. Group IV. Synthetic vitamin B-deficient diet + vitamin B_1 + lactoflavin.

2. *Mice.*

Preliminary observations with mice show that they fail to grow and develop loss of hair with slight skin lesions when kept on a synthetic diet, supplemented with vitamin B_1 *plus* lactoflavin (5 I.U. and 15γ respectively, injected twice weekly). Addition of yeast improves their condition (Fig. 13). It is possible therefore that mice may prove a useful and convenient experimental species for the investigation of the vitamin B_2 complex and further investigations are in progress. So far all mice fed on Goldberger's blacktongue diet, on which rats are able to thrive, have lost weight and succumbed.

3. *Guinea-pigs and rabbits.*

Attempts to produce "vitamin B_2" deficiency in rabbits or guinea-pigs, by the feeding of modified Goldberger-blacktongue diets, or synthetic diets, were unsuccessful, as the animals failed to eat sufficient quantities of food.

V. Vitamin B_6 as the rat anti-acrodynia factor.

Previous workers have referred to the dermatitis seen in rats on vitamin B_2-deficient diets as being "pellagra-like". But, as has been pointed out above, this specific lesion is due to deficiency of vitamin B_6—a factor which is certainly different from the antipellagra (P.-P.) factor of Goldberger. It would therefore be misleading to refer to the results of vitamin B_6 deficiency, certainly in their dermatological aspects, as "pellagra-like". Furthermore the "dermatitis" of vitamin B_6 deficiency differs in its clinical picture from pellagra as seen in human beings. For instance it seems to have no relation to light sensitisation; there is no association with diarrhoea, and there are other marked differences. On the other hand, it has certain very characteristic features of its own, involving chiefly the most peripheral parts of the body, such as the paws, the nose and the ears. The condition resembles a peculiar disease of infancy, known as "pink disease", Swift's disease, or acrodynia. This latter term is appropriate as giving an accurate description of an outstanding feature of the disease, namely the localisation of the most affected parts.

This is not the first time that a possible connection has been discussed between pink disease and skin alterations produced experimentally in rats. Findlay and Stern [1929] emphasised the similarity between acrodynia in man and certain pathological changes occurring in young rats fed on a diet rich in egg-white. On the other hand, Parsons [1931] and others have stressed the "pellagra-like" appearance of the condition produced by the "egg-white injury" [Boas, 1927].

Our own observations lead to the conclusion that the "egg-white injury" is distinct from vitamin B_6 deficiency. The most characteristic feature of vitamin B_6 deficiency is that the earliest and most distinctive lesions appear on the peripheral parts of the body (ears and paws), whereas in the "egg-white injury" the extremities are not necessarily affected. The factor, the deficiency of which is responsible for the "egg-white injury", was termed "factor X" by Boas, and appears to correspond with "vitamin H" of György. The latter has been differentiated from vitamin B_6 and lactoflavin and has widely dissimilar chemical properties. For instance, vitamin B_6 is basic, whilst vitamin H is an acid ampholyte.

The characteristic acrodynia type of abnormalities in rats has been seen only in vitamin B_6 deficiency. We propose therefore to name this specific dermatitis *rat acrodynia*—without prejudice as to its identity or otherwise with human acrodynia.

VI. The extrinsic factor for pernicious anaemia.

Before it was recognised that vitamin B_2 was complex in nature Strauss and Castle had reported [1932] that the extrinsic factor for pernicious anaemia bore a resemblance to vitamin B_2 both in its distribution in nature and also in its heat-stability. Strauss and Castle believed that the two substances might be identical. This latter conclusion was disputed by Wills and Naish [1933], but more recently Miller and Rhoads [1934] have given cogent reasons for questioning the validity of Wills's proof. But now that two separate constituents of vitamin B_2 have been characterised, as lactoflavin and vitamin B_6, the whole question demands reconsideration.

A clue to the probable non-identity of the extrinsic factor with vitamin B_6 or lactoflavin may be found in the work of Miller and Rhoads [1934]. These workers have found that two materials in particular serve as highly potent sources of extrinsic factor, namely egg-white and an acetone extract of rice polishings. Now egg-white is known to be deficient in vitamin B_6 [György, 1935, 2], and rice

contains very meagre amounts of lactoflavin, and acetone extracts of it, no doubt, still less. Unless therefore it can be shown that other sources of the extrinsic factor are in fact active in much smaller doses than hitherto considered likely, this finding of Rhoads and Miller seems to rule out the possibility of either vitamin B_6 or lactoflavin being the extrinsic factor.

The liver substance curative of pernicious anaemia (which is held to be the result of the interaction of this extrinsic factor with the intrinsic factor of the gastric juice) clearly cannot be identical with any constituent of vitamin B_2, since yeast, without previous digestion, is inactive [Cohn *et al.*, 1928; Strauss and Castle, 1932].

Discussion.

The principal conclusions to be drawn from the experimental work described in Parts I to VI may now be given as follows. The human P.-P. factor is different both from the rat vitamin B_6 and from lactoflavin. The canine antiblacktongue factor is also different from vitamin B_6 and lactoflavin: it may be identical with the P.-P. factor. Human pellagra is a distinct condition from rat pellagra, and much past evidence based on their supposed identity must accordingly be abandoned. Perhaps the most striking evidence for the differentiation of vitamin B_6 from P.-P. and the antiblacktongue factor is the finding that maize is so rich in vitamin B_6.

The question which will now be raised is, What is the nutritional significance of vitamin B_6 for human beings and for dogs? Further work will have to be undertaken to examine this problem.

An objection which may possibly be advanced to our differentiation of the P.-P. and antiblacktongue factors from vitamin B_6 is to suppose that the difference is merely one of degree—*i.e.* that the factors are identical but that dogs and human beings merely need more than do rats. This criticism is immediately answered by a study of Tables III A and V which show that blacktongue in dogs and pellagra in human beings are produced by diets extremely rich in vitamin B_6 and are cured when supplements are given containing no more than a slight trace of it. The same argument applies also to the differentiation of lactoflavin from the human pellagra and canine blacktongue factor.

It might be suggested that perhaps the vitamin B_6 in maize is readily absorbed by a rat, but, for some reason, inaccessible to a human being or to a dog. There is however no evidence to favour such a supposition; on the contrary it may be pointed out that fish, from which vitamin B_6 can be recovered less readily by treatment with solvents, is a potent source of P.-P. (or antiblacktongue) factor, whilst maize, from which vitamin B_6 is more readily dissolved, is deficient in P.-P. Moreover, treacle, which is rich in vitamin B_6 and contains it in an immediately soluble form, has no P.-P. activity (molasses being indeed a principal constituent of the human pellagra-producing diet—"the three M's"—maize-meal, meat (=salt pork) and molasses).

Finally allusion should be made to the commonly held view that pellagra is caused by a toxin assumed to be present in maize. It should be pointed out that, even if such a toxin be concerned in the production of pellagra, it is none the less apparent from the controlled clinical observations of Ruffin and Smith [1934] and others, that the lesions are readily cured by small amounts of a special substance—which therefore deserves the designation of the antipellagra vitamin or "P.-P factor"—a substance which is present in liver extract and certain other sources, and which as we have shown is distinct both from the so-called "rat pellagra" factor (vitamin B_6) and from lactoflavin—the two hitherto recognised constituents of the vitamin B_2 complex.

SUMMARY.

I. The human pellagra-preventing ("P.-P.") factor is different both from vitamin B_6 (hitherto called the "rat pellagra" factor) and from lactoflavin, two known components of the vitamin B_2 complex.

There are marked differences in distribution. Thus, maize and molasses, which are known to be deficient in P.-P. are rich sources of vitamin B_6, whilst liver powder "343" is rich in P.-P. but deficient in vitamin B_6. Fish which is moderately rich in P.-P. is relatively deficient in lactoflavin. Further data are given of the distribution of vitamin B_6 and lactoflavin in various cereals and other products and are contrasted with the known distribution of the P.-P. factor.

Furthermore lactoflavin fed direct to human pellagrins [Dann *et al.*, 1935] in controlled tests under standardised conditions failed to cure, whereas known sources of P.-P., such as liver extract *etc.*, gave dramatic cures. The basal pellagra-producing diet used in these trials was itself rich in vitamin B_6, which again illustrates the difference between P.-P. and vitamin B_6.

The P.-P. factor should therefore be regarded as a third component of the vitamin B_2 complex.

II. Rats were fed on known pellagra-producing diets identical with those used in clinical experiments, or based on diets actually consumed by pellagrins, and on blacktongue-producing diets. Such rats, even although exposed to intensive irradiation remained free from symptoms of pellagra. On the other hand, rats suffering from vitamin B_6 deficiency (misnamed "rat pellagra") were cured when these pellagra-producing diets were fed.

"Rat pellagra" therefore is not the analogue of human pellagra, but is a separate condition. The mistaken identification of "rat pellagra" with human pellagra had its origin in experiments by Goldberger, in which alcoholic extracts of maize (containing no vitamin B_6) were unfortunately used in the diet, in lieu of maize itself which is a potent source of vitamin B_6.

Rats do not need the human P.-P. (or canine blacktongue) factor in any significant amount, or are able to synthesise it.

III. Dogs lost weight and developed "blacktongue" with symptoms including diarrhoea and anaemia when fed on a Goldberger maize diet containing large amounts of vitamin B_6. The addition of lactoflavin had no curative action. Symptoms were prevented or cured by the addition of supplements of autoclaved yeast, fresh fish (containing negligible lactoflavin), or Eli Lilly liver powder, 343 (containing negligible vitamin B_6).

It is therefore concluded that the antiblacktongue factor is different from lactoflavin or vitamin B_6. It may be identical with the human P.-P. factor, but evidence is so far not conclusive.

Dogs kept on "synthetic" diets containing vitamin B_1 and lactoflavin, appear to need supplements of two further factors, one contained in maize (presumably vitamin B_6) and one in liver extract (P.-P. or antiblacktongue factor). It is supposed that the addition of maize to the diet helps in the production of regular symptoms of blacktongue, not so much because of a toxin present in it, but because in its absence the dog may sometimes develop earlier vitamin B_6 deficiency instead of blacktongue.

IV. So-called "chicken pellagra" of Elvehjem and Koehn appears to be distinct from vitamin B_6 deficiency, and its relation to human pellagra and blacktongue is undecided. An anti-anaemia factor is destroyed in the method of preparation of the Elvehjem-Koehn diet.

Preliminary observations on vitamin B_2-deficient mice are recorded.

Guinea-pigs and rabbits appear unsuitable for work on vitamin B_2.

V. Since vitamin B_6 deficiency is most characteristically a disease of the extremities and the lesions are not truly "pellagra-like", it would be more appropriate to call vitamin B_6 the "rat acrodynia factor".

VI. The extrinsic factor for pernicious anaemia also appears to be different from lactoflavin or vitamin B_6.

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Note added December 12th, 1935. A private report from Drs T. D. Spies and A. B. Chinn (Cincinnati) states that "under controlled conditions two pellagrins failed to respond dramatically if at all to the administration of lactoflavin in fairly large doses". It will be seen that this finding is in conformity with the results of Dann, Ruffin and Smith (Part I).