LXXXVI. INVESTIGATIONS ON THE VITAMIN B_2 COMPLEX.

I. THE DIFFERENTIATION OF LACTOFLAVIN AND THE "RAT ANTIPELLAGRA" FACTOR.

By PAUL GYÖRGY.

From the Physiological and Nutritional Laboratories, Cambridge.

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By the end of 1932 three separate components had been distinguished in the vitamin B complex as needed by the rat: (1) vitamin B_1, the antineuritic factor; (2) vitamin B_4, the absence of which was said to be associated also with symptoms of nervous lesions such as disturbances of co-ordination and ataxia; and finally (3) vitamin B_2, the antipellagra factor. In contrast with the first two, the relatively high thermostability of vitamin B_2 had been noted as its special characteristic. All the three factors mentioned display growth-promoting properties under suitable circumstances. Some have held that vitamin B_2 is divisible into two further factors [Sure *et al.*, 1931; Thatcher *et al.*, 1931; Gurin *et al.*, 1931], or at least that this is probably the case [Sherman and Sandels, 1929; 1931]. In this fractionation of vitamin B_2, one factor is alleged to be predominantly growth-promoting, the other being the antipellagra factor. However, none of the supporters of this theory has been able to bring forward convincing proof of his views. Roscoe, on the grounds of her wide experience, recently stated [1933, 2] that "no support was found for the theory postulating the existence of separate dietary factors respectively for preventing and curing dermatitis and for promoting growth".

Chick and Copping [1930] showed that an alkali- and heat-stable, water-soluble, growth-promoting factor, called by them factor Y, probably existed, but no experimental work has yet determined its precise position in the vitamin B complex.

The biological analysis of vitamin B_2 was attended with great difficulties. Almost all workers were agreed that the specific symptoms peculiar to vitamin B_2 deficiency, as first described by Goldberger and Lillie [1926], and regarded by them as analogous with pellagra in man, only appeared irregularly in rats [*cf.* Medical Res. Council, 1932, p. 151]. In another recent paper Roscoe [1933, 1] gives "data concerning the incidence of dermatitis among the vitamin B_2-deficient animals observed in the Nutritional Laboratory of the Lister Institute during the past seven years". Skin changes were noted in only 108 out of a total of 191 rats. The average time taken for symptoms to develop was 10 weeks. Roscoe employed the usual vitamin B-free, synthetic diet, supplemented by vitamin B_1 ($+B_4$) in the form of a concentrate from brewers' yeast prepared according to Peters [*cf.* the new directions of Kinnersley *et al.*, 1933]. Other authors used an alcoholic extract of maize [Goldberger and Lillie, 1926], or of wheat [Bourquin and Sherman, 1931] as the source of vitamin B_1. Dried yeast irradiated with ultra-violet light and liver extracts similarly irradiated [Hogan and Richardson, 1932, 1, 2; 1933] or irradiated rice polishings [Thatcher *et al.*, 1931] are said to be equally suitable forms in which vitamin B_1 may be given

in experiments on vitamin B_2. With these dietetic modifications, and using alcoholic extracts of maize and wheat, the percentage of rats deprived of vitamin B_2 which show pellagra-like dermatitis reaches a satisfactorily high level; but even these experimental methods do not yield uniform results.

The object of our own investigations was the study of the skin changes, and the work of Sherman and Sandels, which appeared about the time we were beginning our experiments, suggested to us that this object could be most rapidly and easily attained by the use of the diet described by Bourquin and Sherman [1931].

These experiments have been reported in several papers in association with Kuhn and Wagner-Jauregg [1933; 1934]. In addition to a number of minor observations, they yielded two new and important pieces of information. (1) Vitamin B_2 is in fact not a single substance but may be subdivided into two subsidiary components; and (2) it was possible chemically to identify one of these subsidiary factors, which was first prepared in crystalline form from milk. This natural dyestuff, called lactoflavin, corresponds to the coloured component of the "yellow oxidation ferment" of Warburg and Christian [1932, 1, 2; 1933], and is present, under natural conditions, in foodstuffs, not only in the free state, but also in the form of Warburg's ferment, *i.e.* in combination with a protein carrier as a non-dialysable "flavoprotein" [György *et al.*, 1934, 3].

When the experimental diet of Bourquin and Sherman is used it is not possible to produce an increase in the weight of animals whose growth has become stationary merely by the addition of pure lactoflavin to the diet. The addition of some other factor (or factors) is found to be necessary. Our first active supplements to the basal diet of Bourquin and Sherman consisted of concentrates from yeast from which the flavins had been removed by adsorption on fullers' earth in acid solution. Such solutions by themselves were however completely inactive, and animals, after a slight initial gain in weight, generally remained at a constant weight for weeks on end. If lactoflavin were administered at this juncture there was a dramatic resumption of growth. The active yeast residue referred to above may be replaced by a concentrate of vitamins B_1+B_4 such as may be obtained by adsorption of purified concentrates from yeast on charcoal, and subsequent elution with alcohol containing hydrochloric acid [Kinnersley *et al.*, 1933; György *et al.*, 1933, 1, 2; 1934]. Since however the results of these experiments were independent of the vitamin B_1, which was present in considerable amounts in the diet of Bourquin and Sherman (extract of wheat), we were inclined at first to correlate the indispensable supplementary substance with vitamin B_4.

In the isolation of lactoflavin, and its identification as one of the components of the vitamin B_2 complex, the growth rate was used exclusively as biological criterion and standard. The unit dose was defined according to the rules laid down by Chick and Roscoe [1927], as that quantity of lactoflavin which would give rise to a gain in weight of 10 g./week for at least 4 weeks. Under the experimental conditions mentioned (diet of Bourquin and Sherman *plus* flavin-free supplementary substance), 7–10 γ of lactoflavin were found to be equivalent to 1 rat day dose [György *et al.*, 1933, 1; 1934, 3]. On the other hand, in order to produce the full effect of lactoflavin, we required as supplementary factor a quantity of yeast concentrate, as prepared by Kinnersley *et al.*, equivalent to 5–15 g. of fresh bakers' yeast.

The growth-promoting action is only one property of vitamin B_2, which owes its peculiar position in the vitamin series to its supposed protective action against pellagra. To what extent lactoflavin also possesses this biological property is an

important question to which the results already referred to give no answer, for, in our numerous original experiments, we failed to observe a single case of genuine, well-marked, pellagra-like dermatitis. Vitamin B_2 deficiency was expressed only as cessation of growth, followed later by a gradually progressive decrease in weight. Thus an important gap arose. The filling of this gap was the task to which the experiments now described were directed.[1]

In the meantime, the scope of the task had widened considerably. The precise nature of the "supplementary substance" which was present in the vitamins B_1+B_4 concentrates of Kinnersley *et al.* was to be submitted to a more exact analysis. Owing to the inconstant chemical composition, and therefore uncertain nature, of the wheat extract which served as the source of vitamin B_1 in the diet of Bourquin and Sherman, I preferred to employ for my other experiments the crystalline preparation of Windaus *et al.* [1931], or a highly purified vitamin B_1 product, of which 8–12 γ were equivalent to 1 pigeon day dose. I was supplied with both these preparations through the generosity of the I. G. Farbenindustrie, Elberfeld, Germany.

The distribution of vitamin B_2 in animal products has also been re-investigated [see György, 1935, 1], since, owing to the state of our knowledge at the time when our previous investigations were undertaken, the complex nature of vitamin B_2 could not then be taken into account [György *et al.*, 1934, 1]. Finally I have made a renewed attack on the problem of the photosensitivity of vitamin B_2 [György *et al.*, 1933, 2] or of its components, having regard, this time, to the pellagra-like skin changes [see György, 1935, 2].

Experimental.

A. *Use of the diet of Bourquin and Sherman for the production of pellagra-like dermatitis in rats.*

There were several possibilities which might account for the failure to produce pellagra-like skin changes in the experiments performed with Kuhn and Wagner-Jauregg. The relatively short duration of the preliminary depletion period, during which the animals received no supplements in addition to the diet of Bourquin and Sherman, has already been referred to in previous papers. The animals used for the experiments were very young, generally weighing 20–30 g. The weight frequently became stationary after 1 week only. After 4, or at the most 6 weeks, the animals became so weak that treatment with active B_2 concentrates could usually be no longer postponed. In order to demonstrate the essentially supplementary action of flavin, feeding with flavin-free solutions was begun early, usually in the 3rd to 6th week of the experiment. This was done on the assumption that flavin was the actual pellagra-preventing substance. If this assumption were incorrect, and if the pellagra-preventing substance were contained instead in the supplementary factor, then the absence of pellagra-like dermatitis would be the logical result of this experiment. Finally the possibility has to be discussed whether alcoholic wheat extracts vary in pellagra-preventing activity, so that some of them may be able alone to prevent the development of pellagra.

The experiments performed to clear up this possibility gave positive answers to all the questions which have been considered.

Fifteen rats, ranging in weight from 30 to 40 g., were put on a Bourquin-Sherman diet; five of these received no further supplements, five received 10 γ

[1] A preliminary communication appeared in *Nature*, 133, 498, March 31st, 1934.

each of lactoflavin daily, and the remaining five animals each received 1 ml. of a solution of vitamins B_1+B_4 [Kinnersley *et al.*, 1933] equivalent to 10 g. of fresh bakers' yeast. After 5–8 weeks, two animals of the first and three of the second group showed definite pellagra-like manifestations of the skin, while the third group remained free from symptoms, their weight however remaining stationary in common with the rats of the other two groups. It chanced that the supply of maize-starch impregnated with alcoholic extract of wheat ran out at this stage of the experiment and a new sample had to be used. The result was a gradual disappearance of the pellagra-like skin changes. In addition, the group of animals treated with supplements of lactoflavin displayed an increase in weight side by side with the improvement in the skin symptoms (Fig. 1). The

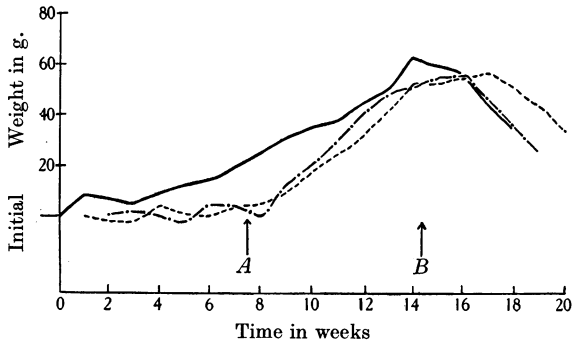

Fig. 1. The weight curves of three rats which received 10γ lactoflavin daily in addition to the diet of Bourquin and Sherman. At *A* the alcoholic extract of whole wheat was replaced by a new sample, and this in its turn was replaced at *B* by a crystalline preparation of B_1. All the animals showed symptoms of pellagra at the end of the experiment.

weight of the animals receiving no lactoflavin remained stationary. It must therefore be concluded that the alcoholic wheat extract used later in the experiment, in contrast to the extract used previously, contained a amount of pellagra-preventing substance which was sufficient to bring about the disappearance of the pellagra-like dermatitis. At the same time another unexpected conclusion probably follows from this observation, namely that lactoflavin is not the pellagra-preventing substance, but that under certain circumstances, large amounts of the pellagra-preventive factor may be present in an alcoholic wheat extract, and that it is also present apparently in the alcohol eluate from charcoal prepared from bakers' yeast [Kinnersley *et al.*, 1933]. Pellagra was cured in all these animals in 7 weeks. The use of untreated rice-starch in place of the maize-starch loaded with alcoholic wheat extract, and the use of a solution prepared from crystalline vitamin B_1 (from the picrolonate) as a source of vitamin B_1 (3 pigeon day doses daily) brought about another immediate cessation of growth of the animals, followed by progressive loss of weight, and after 3–4 weeks, an acute exacerbation of "rat pellagra". This subsequent period gave a further setback to the conclusions which had previously been drawn.

The results of the experiments just described show that, with a suitable wheat extract, the diet of Bourquin and Sherman may be employed with advantage in experiments on "pellagra". The uncontrollable variations in the contents of "pellagra-preventive" factor (probably depending on the type of wheat used and on the composition of the alcoholic wheat extract) constitute a very disturbing element of uncertainty which may under certain circumstances

completely prevent the carrying out of the desired experiments. Accordingly it is recommended that if a crystalline, or similarly well-characterised and highly purified preparation of vitamin B_1, can be obtained the diet of Bourquin and Sherman should be dispensed with in experiments of which the purpose is the study of pellagra-like dermatitis. In addition to this, it must be borne in mind that yet other unknown components of the vitamin B complex may be present in the alcoholic wheat-extract. Thus work on the vitamin B complex, and on the B_2-group in particular, may be made to give quite illusory results.

B. *Use of highly purified or crystalline vitamin B_1 in the analysis of the vitamin B_2 complex.*

By the use of crystalline vitamin B_1 in a diet otherwise free from representatives of the vitamin B group, the analysis of the vitamin B_2 complex became more exact. This advance possesses a certain disadvantage from the biological point of view. For the study of the symptoms of deficiency of any particular component of the vitamin B_2 complex, all the other members of this group must be present in the experiment. It is known from numerous experiences in the study of vitamins that the appearance of the uncomplicated pure, specific, deficiency disease can only be assured if this condition is fulfilled. It is highly probable that the diet of Bourquin and Sherman comes nearer to satisfying this condition than a pure, or highly purified preparation of vitamin B_1. Nevertheless this disadvantage should not be over-estimated, and the attempt at analysing the vitamin B_2 complex should be undertaken by a clear, comprehensive method. The isolation of lactoflavin, with the help of the diet of Bourquin and Sherman, represents an important achievement for the new biological analysis. We now have at our disposal, in the form of crystalline preparations, both vitamin B_1 and lactoflavin. It remained to be shown in separate experiments whether the charcoal eluate obtained from bakers' yeast by the method of Peters was also capable of fulfilling the rôle of supplementary factor in these modified experiments.

The basal diet was constituted as follows:

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

Caseinogen AB "Glaxo" extracted with alcohol was used in the later experiments, but no definite biological differences could be detected, in these experiments, between the two specimens of caseinogen.

The experimental animals employed were albino and piebald rats of various weights. In the earliest experiments the weights ranged chiefly from 35 to 50 g. The stock diet for one group of rats consisted of milk, bread and wheat kernels, with meat and cabbage once weekly. The majority of the rats were descended from a single stock reared on the following regulation stock diet in use at the Nutritional Laboratory:

Monday.	Bread, milk, cabbage and mixed corn.
Tuesday.	Bread, milk, liver.
Wednesday.	Boiled wheat, milk, yeast and cabbage.
Thursday.	Bread, milk, meat and mixed corn.
Friday.	Bread, milk and carrots.
Saturday.	Bread, milk, cabbage and mixed corn.
Sunday.	Bread, milk and carrots.

Lettuce twice a week when obtainable.

The change in the stock diet was without detectable influence on the time of appearance of pellagra-like skin changes, on their intensity or on the absence of growth which occurs with an experimental diet free from vitamin B_2 complex. Differences in sex or in pigmentation (albino or black-and-white) were also found to be of no significance in these investigations.

In addition to the experimental diet the rats received vitamin B_1, at first in the form of a crystalline preparation (from vitamin B_1 picrolonate) in doses of 3–4 pigeon day doses dissolved in 2 ml. of water daily (Sundays excepted). A lengthy series of experiments showed that a highly purified vitamin B_1 concentrate, 1 pigeon day dose of which was equal to about 8–12 γ of the dry residue, performed the same function as the pure picrolonate. The latter substance, which is more easily prepared, was therefore used in preference. Henceforth every new experimental result was invariably controlled with the crystalline preparation serving as the source of vitamin B_1.

The first task was the repetition of the results previously obtained with the diet of Bourquin and Sherman. The most urgent question was whether the character of lactoflavin, as a component of the vitamin B_2 complex, could be brought out by the modified experimental method also.

To this end the animals were given, together with the preparation of vitamin B_1, Peters's eluate [Kinnersley *et al.*, 1933] from bakers' yeast, such as had already proved of value as a supplementary factor when the diet of Bourquin and Sherman was being used [György *et al.*, 1934, 3]. 1 ml. of a solution, equivalent to 10 g. of fresh bakers' yeast, was given daily (again Sundays excepted). Despite these supplements however the growth of the animals remained almost completely stationary, apart from a slight increase in the first weeks of the experiment. If, after a longer or shorter preliminary period, the animals are given lactoflavin, a striking increase in weight at once sets in which persists with practically no diminution for 4–6 weeks. If the order of the experiment be now reversed and the animals be fed first with vitamin B_1 *plus* lactoflavin, growth will remain stationary in precisely the same way. In fact the inhibition of growth in this case is generally most impressive and is as a rule absolute. In this experiment the subsequent administration of Peters's eluate causes as sudden a resumption of growth as did that of lactoflavin with the animals which were given vitamin B_1 *plus* Peters's eluate in the first place and lactoflavin subsequently. The change in the order of the experiment does not affect the result, at all events so far as the resumption of growth is concerned (Fig. 2).

Thus the information obtained in previous researches [György, Kuhn and Wagner-Jauregg, 1933; 1934] was confirmed once more with the aid of this clear and simplified experimental technique. Vitamin B_2 therefore may be subdivided into two subsidiary factors: (*a*) the crystalline, well-defined lactoflavin and (*b*) the "supplementary factor" (or perhaps supplementary factors) contained in Peters's "charcoal eluate" from bakers' yeast. Growth can only be brought about by the combined actions of both these components, independently of the order in which they are exhibited.

This result is of critical importance, the key as it were to all further attempts at analysing the vitamin B_2 complex. It was confirmed by numerous subsequent experiments. Of these eleven were devoted exclusively to this purpose. In seven of these the animals received Peters's eluate in addition to vitamin B_1 in the preliminary stage. In four others they received lactoflavin only in addition to vitamin B_1. Lactoflavin was given in doses of 7–10 γ daily, and Peters's eluate in amounts equivalent to 10 g. fresh bakers' yeast. The average weekly gain in weight for the eleven experiments was 11 g. in an experimental period

of 4 weeks, and 10 g. in a period lasting 6 weeks. It should be emphasised that the variations in the values obtained for individual animals were very slight. Increasing the quantity of lactoflavin to 20 γ or of yeast eluate to the equivalent of 20 g. fresh bakers' yeast did not increase the rate of growth to any notable

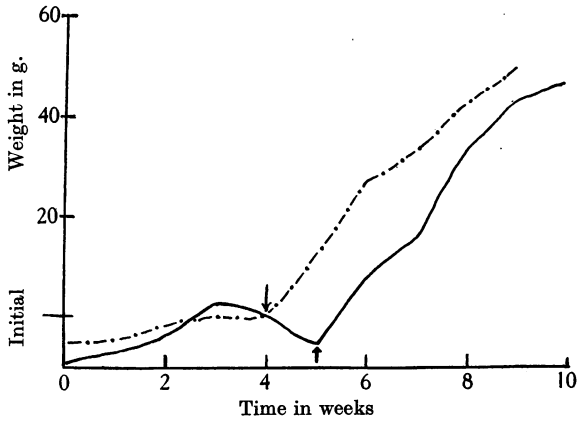

Fig. 2. Apart from the basal diet both animals received 3 pigeon doses daily of vitamin B_1. In addition to this the animal —— received 10γ lactoflavin, and the animal —·— 1 ml. Peters's eluate daily (equivalent to 10 g. of fresh bakers' yeast). At ↑ the animal —— was given 1 ml. of Peters's eluate also, and at ↓ the animal —·— 10γ lactoflavin. An increase in weight was only produced by the combined administration of vit. B_1 + lactoflavin + Peters's eluate.

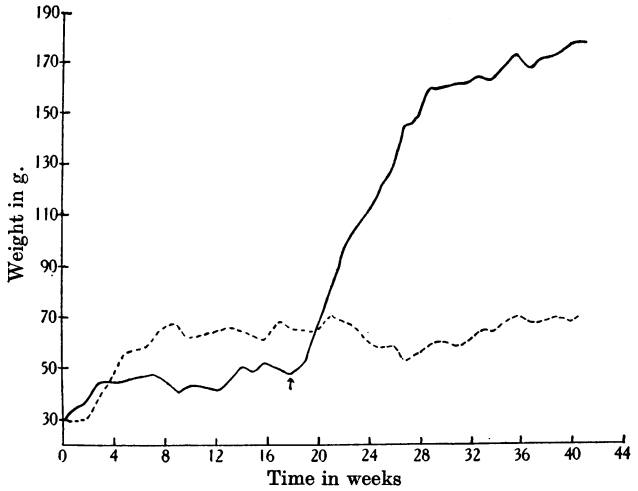

Fig. 3. The weight-curves of two animals which, apart from the basal diet, received at first only vitamin B_1 + Peters's eluate. At ↑ the animal —— was given 10γ lactoflavin daily in addition. The doses of Peters's eluate and lactoflavin were doubled in the 32nd week.

extent. On the other hand, reduction of the doses, whether of lactoflavin or of yeast eluate, led to an insufficient acceleration of growth.

The daily requirements established in this case hold good only for the experiment described. It is quite possible, for example, as Euler *et al.* [1934] have recently emphasised, that the daily requirement for lactoflavin may be lowered if

the animals are given all the other components of the vitamin B_2 complex at the same time, or at all events if they are given them in a more favourable combination than in the above experiment. In this respect Peters's yeast eluate does not fulfil the highest requirements as a supplement to lactoflavin. This may clearly be seen from the fact that animals receiving vitamin B_1, lactoflavin and Peters's eluate maintain the normal rate of growth only up to a body weight of 150–170 g. Growth cannot be pushed beyond these limits even by increasing the doses of lactoflavin and yeast eluate. The curve reproduced (Fig. 3) is particularly instructive in this respect.

Crystalline lactoflavin may also be replaced by egg-white.

Animals receiving 3–5 ml. daily of raw egg-white in addition to a basal diet *plus* purified vitamin B_1 react in the same manner as in the experiments with vitamin B_1 and crystalline lactoflavin. Growth remains at a complete standstill and shows no change for weeks on end. Feeding with Peters's preparation at this point promptly brings about the expected increase in weight (Fig. 4). Up to the

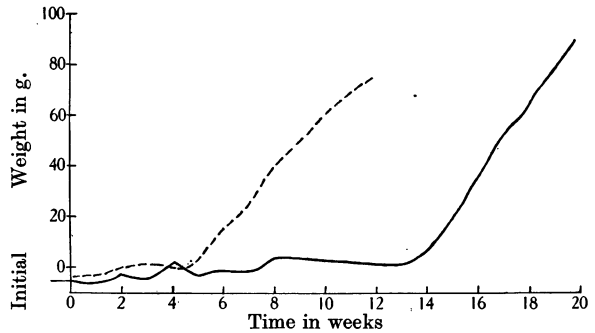

Fig. 4. Vitamin B_1 + egg-white (3 ml. daily) do not produce growth. This is stimulated immediately Peters's eluate is added. With a longer preparatory period the animal —— displayed typical "pellagra" while being treated with egg-white + vitamin B_1; this rapidly healed coincidently with the increase in weight after addition of Peters's eluate.

present we have been unable to find any definite differences between the reactions of animals receiving crystalline lactoflavin and those in receipt of raw egg-white as the source of lactoflavin. The amount of Peters's eluate required in this experiment was again equivalent to 10 g. of fresh bakers' yeast. A dose of 3 ml. of egg-white as the source of lactoflavin is sufficient to maintain the growth at the standard rate. An increase in the dose of egg-white to 5–10 ml. produced no additional increase in growth, nor did an increase in the dose of lactoflavin from 7–10 γ to 20 γ.

The experiments which have just been described possess special significance with regard to the question of the existence of vitamin B_4 [Reader, 1929; 1930, 1, 2]. It is worthy of note that rats receiving crystalline vitamin B_1 (3–4 pigeon doses daily) and no further addition of vitamins (with the possible exception of crystalline lactoflavin only) do not exhibit any symptoms which can be taken as specific of vitamin B_4 deficiency (Reader), even in experiments extending over as long as 10–15 weeks and more. Nervous manifestations typical of this condition in particular were absent, such as ataxia, loss of co-ordination and spastic gait.[1]

[1] The nature of the symptoms held to be due to vitamin B_4 deficiency is discussed in a separate paper by Birch and György in press.

C. *Pellagra-like dermatitis*[1] *and the vitamin* B_2 *complex.*

The incidental observations made in the experiments with the diet of Bourquin and Sherman, that pellagra-like dermatitis was dependent not on the absence of lactoflavin but on that of the "supplementary factor", was convincingly confirmed by a further extensive series of experiments with the modified technique. In a group of over fifty rats which received purified vitamin B_1 and Peters's eluate in quantities equivalent to 10 g. fresh bakers' yeast, not a single case of skin changes of a pellagra-like character was observed. This negative result cannot be explained by the shortness of the period during which the animals were under observation, for a large number (24) of the experimental animals were treated with vitamin B_1 and Peters's eluate for at least 12 weeks. At the same time the growth curve, after an initial but definite rise, took a horizontal, or a moderately rising course (*cf.* as example Fig. 3). The relevant figures are given in the following table:

No.	Weeks	Increase in wt. (g.)	No.	Weeks	Increase in wt. (g.)
1	19	21	13	16	18
2+	15	16*	14+	14	7*
3	20	40	15+	27	79
4	19	28†	16	23	63
5	20	13	17+	26	44
6	20	35	18+	13	10*
7	19	37	19	21	38
8	24	46	20	41	39
9+	23	17	21+	21	34
10+	29	50	22+	12	6
11+	18	35*	23+	20	39
12+	26	27*	24+	22	38

* Died. † 33 g. in first 5 weeks and − 5 g. in the remaining 14.

In about one half of the rats submitted to over 12 weeks' experimentation (*i.e.* in 13 marked + out of 24 in the above table) the deficiency of lactoflavin expressed itself in these experiments not only as stationary weight but in certain well-marked associated symptoms, the most important of which were changes in the skin and fur. These latter, however, were fundamentally distinct from the specific "pellagra-like" changes. They differ chiefly in being much less striking. In deficiency of lactoflavin (at least under the experimental conditions selected by us) a gradually progressive loss of hair occupies the foreground of the picture. It is localised to certain areas, such as the back between the shoulders, the lines joining the eyes and ears leaving a median comb of hair remaining in between and finally the chest. In many cases there may be observed on the uninflamed skin, in the midst of the scanty patches of fur, minute, almost punctate whitish-yellow scales. In advanced cases the eyes are sometimes surrounded by a spectacle-like loss of hair, while the eyelids are gummed together. The extremities and the ears invariably escape all changes whatsoever. If treatment, *i.e.* addition of lactoflavin, is not begun in time, the animals succumb to general exhaustion. The end is usually ushered in by meteorism (which is presumably an expression of intestinal atony), by dyspnoea (which is probably due to the last-named symptom and is reminiscent of the laboured breathing seen in pneumonia) and by blue discoloration of the extremities (circulatory failure?). The administration of lactoflavin at the appropriate time will avert this end with

[1] Throughout this series of papers the term "pellagra", as applied to rats, is used without prejudice as to its identity or non-identity with human pellagra.

absolute certainty. With the resumption of growth all the other associated symptoms of lactoflavin deficiency begin to resolve.

While in lactoflavin deficiency the skin changes just described appear irregularly and are not very striking, in rats which received in addition to the basic diet vitamin B_1, or vitamin B_1 *plus* lactoflavin or egg-white, but not the "supplementary factor" contained in Peters's eluate, the skin disorders completely fill the picture. It is here that we encounter the specific symptom-complex of pellagra-like dermatitis first described by Goldberger and Lillie [1926]. This peculiar skin disorder appeared in every case provided the experiments were prolonged for a corresponding length of time. We already possess observations on 211 cases of this nature which we collected during 1934. Of these 55 have had the disease twice, *i.e.* having been successfully treated on the first occasion they again fell ill when submitted to the original conditions once more. The animals may be placed in six groups according to the supplements they received.

Group	Supplement	Total no. of animals	First symptom of "pellagra" after av. no. of weeks	Animals ill twice	First symptom of "pellagra" of second illness after av. no. of weeks
1	Crystalline vitamin B_1 (3–4 pigeon units daily)	10	6	1	7
2	Purified vitamin B_1 preparation (3–4 pigeon units daily)	12	7	3	8
3	Crystalline vitamin B_1 + lactoflavin (10γ daily)	31	6	8	6
4	Purified vitamin B_1 preparation + lactoflavin (10γ daily)	65	7	18	7
5	Purified vitamin B_1 preparation + 3 ml. egg-white daily	59	7	18	8
6	Purified vitamin B_1 preparation + 5 ml. egg-white daily	34	8	7	11
	Total	211	7	55	8

Thus with this experimental technique it has been possible to overcome the element of uncertainty which has hitherto prevailed in the experimental production of pellagra-like dermatitis. Differences in the supplements used did not affect the incidence of the clinical picture of pellagra-like dermatitis. Within narrow limits it was a matter of indifference whether the animals received only vitamin B_1 in addition to the basal diet, or vitamin B_1 *plus* lactoflavin (the latter in the form of crystalline lactoflavin, or as egg-white in doses of 3–5 ml. daily). It was found that the only factor of importance in this respect was the daily quantity of vitamin B_1 administered. If less than 3 pigeon day doses were given daily the appearance of pellagra-like dermatitis was often delayed. In a few cases, examples of which are shown in the accompanying curves (Fig. 5), spontaneous healing of the "pellagra" was observed while these subliminal doses of vitamin B_1 were being given, either with or without the simultaneous appearance of polyneuritic symptoms. But a subsequent increase in the dose of vitamin B_1 to 3–4 pigeon day doses daily brought out the pellagra-like dermatitis once more, at the same time suppressing the nervous symptoms. Thus it is an indispensable condition for the production of pellagra-like dermatitis that the animals be adequately supplied with vitamin B_1. Excessive doses of vitamin B_1, 6–8–10 pigeon day doses daily instead of 3–4, did not accelerate the development of the dermatitis, nor did they predispose towards its appearance. We are therefore unable to agree with Kellogg and Eddy [1933], who see a relationship between the

development of pellagra-like dermatitis and a hypothetical toxic action on the part of vitamin B_1. It is also worthy of mention in this respect, that in our experiments excessive dosage with vitamin B_1 does not affect the weight curves of the animals. This must be taken as evidence on the one hand that the vitamin

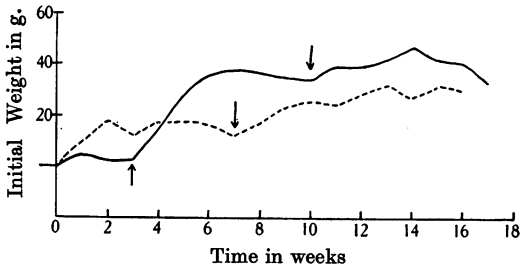

Fig. 5. Both animals received at first vitamin B_1 only (1·5 pigeon day doses daily); the animal —— from ↑ onwards, and the other rat ----- from the very commencement. One week before ↓ both rats showed definite signs of healing of the pellagra symptoms which had just recently appeared. Doubling of the dose of vitamin B_1 at ↓ produced an exacerbation of the pellagra symptoms.

B_1 requirements of the animals were amply satisfied by 3–4 pigeon day doses, and on the other hand that the vitamin B_1 preparation employed was uniform in the biological sense.

We were unable to find any correlation between the season of the year when the experiments were performed, or between the initial weight of the experimental animals on the one hand and the development of "rat pellagra" on the other. The relation between the initial weight of the animals and the time taken to produce symptoms of "pellagra" is shown in the table below.

Initial body-weight (g.)	No. of exp. animals	First symptom of "pellagra" after av. no. of weeks	Animals ill twice	First symptom of "pellagra" of second illness after av. no. of weeks
26–35	21	7	—	—
36–45	51	7	1	13
46–55	35	7	—	—
56–65	17	6	2	8
66–75	21	7	5	7
76–85	17	9	7	10
86–95	18	9	14	9
96–105	16	6	13	6
106–115	7	7	6	7
116–125	4	7	3	8
126–135	4	6	4	6

The onset of the second illness in animals which have been successfully cured of a first attack of "pellagra" and are then re-submitted to the disease-producing conditions is determined by the intensity of the original therapeutic treatment. The reappearance of the disease may be considerably postponed by intensive treatment, which must be taken as evidence that the "pellagra-preventing factor" is capable of being stored in the body.

The skin manifestations met with when vitamin B_1 or B_1 *plus* lactoflavin (egg-white) are given may be distinguished readily and with certainty from those which appear when vitamin B_1 *plus* Peters's eluate are fed. The former symptoms are identical with the "pellagra-like" dermatitis of Goldberger and Lillie [1926],

while the irregular skin manifestations of lactoflavin deficiency as seen with our experimental technique are chiefly of an unspecific nature.

The leading feature of "pellagra-like" dermatitis is the involvement of the most peripheral parts of the body. The disease begins with swelling and hyperaemia of the ears which is soon followed by the involvement of the fore and hind paws and the nasal region. The distribution is for the most part perfectly symmetrical but exceptions to this rule are met with occasionally. In the latter the symmetry is frequently, though not invariably, restored during the course of the illness. The second stage, which follows the swelling and hyperaemia, consists in the formation of crusts on the pinnae, which becoming thickened and indurated may develop into flower-like excrescences. Scabs form in the vicinity of the raw and inflamed nostrils, corresponding in their distribution to the follicular areas of the vibrissae, and also on the eyelids, which are gummed together in these cases. The vibrissae do not fall out. The paws undergo either a desquamation in small flakes or larger scales, or else their dorsal surfaces become markedly oedematous with a serous discharge, associated in many cases with gangrene of individual toes and secondary auto-amputation. Among the hairs on other parts of the body seborrhoeic scales or often only inspissated secretion are not infrequently found. The hair shows very little external alteration. In a few animals bleaching may be observed, hairs formerly black becoming grey. Ulceration of the buccal mucuous membrane, particularly the lower surface of the tongue, is a very common associated symptom. The pulse-rate per minute is normal (450–530); diarrhoea is a rare occurrence. If no specific treatment be undertaken the animals succumb, without exception, with progressive loss of weight which towards the end falls steeply.

The very striking differences in the characters of the skin manifestations afford the best proof that the disturbances of growth seen in lactoflavin deficiency on the one hand, and those met with during the administration of vitamin B_1, or B_1 *plus* lactoflavin (egg-white) on the other hand, do not depend on the same disturbances of metabolism. As we have already shown, administration of lactoflavin and of the "supplementary factor" in Peters's eluate both cause resumption of growth under suitable conditions. In the same way both lactoflavin and Peters's eluate bring about the disappearance of the skin manifestations in a short time.

The effect of lactoflavin in this respect runs parallel with the increase in growth and consists in disappearance of the fine whitish yellow scales and in shedding of the old hairs and their replacement by new ones of the normal density and gloss. This result is attained by 7–10 γ lactoflavin daily, whereas a manifold increase in the dose of Peters's eluate has no more effect on the skin changes than on growth, if the simultaneous administration of lactoflavin is withheld.

The healing of "pellagra-like" dermatitis under the influence of adequate doses of Peters's eluate begins with shedding of the vegetation on the ears, paws, nasal regions and eyelids. The ears when first freed from scabs are thickened and hyperaemic with ragged, irregular margins. In the vicinity of the eyes and nose, and in other parts of the body, the hair frequently comes out in tufts at the same time as the scabs and scales are shed, exposing bald areas until new hairs have reappeared. The transitory spectacle-like borders surrounding the eyes arise in this way. The last sign to disappear by which a case of healed "rat pellagra" may be recognised is the reddened, and later somewhat shiny, folded ears; finally, with sufficient treatment, even this residual symptom disappears.

In pellagra-like dermatitis developing during the administration of vitamin B_1 *plus* lactoflavin (10 γ daily), or vitamin B_1 *plus* egg-white (3–5 g. daily), ad-

ministration of Peters's eluate in doses equivalent to 10 g. of fresh bakers' yeast will produce rapid healing of the skin changes with simultaneous increase in weight, equal to, or even exceeding, that demanded by the criterion (10 g. per week) (*cf. e.g.* Fig. 4). In very advanced cases the acceleration of growth in the first week of treatment may fall short of that anticipated, only to compensate for this delay in the 2–3 weeks following. The skin is generally restored to its normal condition after 3–4 weeks of treatment. Bleaching of the hairs, which may have arisen during the disease, generally persists after all the other symptoms have been successfully cured. With a dose of Peters's eluate equivalent to 5 g. of fresh bakers' yeast, the skin manifestations resolve in the same way, if somewhat more slowly, as with double this amount; but the increase in growth falls considerably short of the criterion. Still smaller doses leave even the skin manifestations unaffected. These subliminal doses may be of no avail even prophylactically. The symptoms however do not as a rule become intensified but exhibit considerable variations which in some instances may approach spontaneous healing. If the Peters's eluate be prepared from brewers' instead of from bakers' yeast, then doses equivalent to double the quantity of the original yeast will be necessary.

From the experiments described thus far, the fact emerges that the pellagra-preventing substance cannot be identical with lactoflavin but should be looked for in Peters's eluate. The correctness of this assumption is strongly confirmed by the following observations.

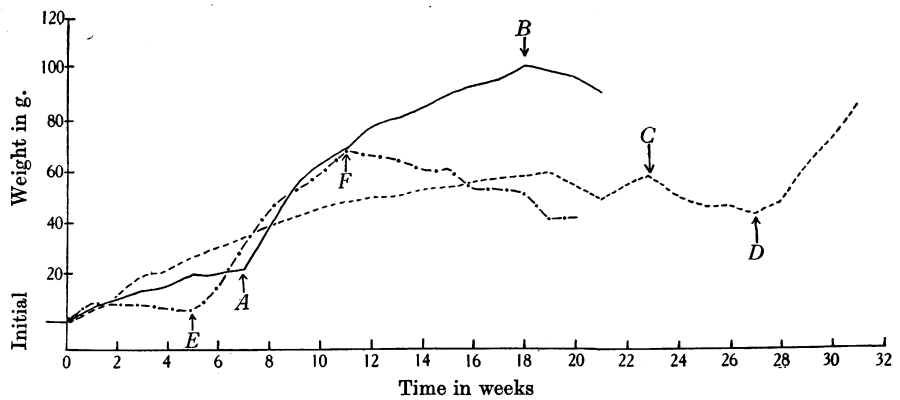

Fig. 6. The rat —— at first received vitamin B_1 + Peters's eluate only; at *A* lactoflavin was added, and at *B* Peters's eluate was omitted, *i.e.* administration of vitamin B_1 + lactoflavin only. Result: "pellagra". The rat ----- was treated at first with vitamin B_1 + lactoflavin and with doses of Peters's eluate insufficient to produce growth. At *C* this rat was given vitamin B_1 + lactoflavin only. Result: "pellagra" which was cured by Peters's eluate (*D*), this time in large doses. The rat —·— received at first vitamin B_1 + lactoflavin. Addition of Peters's eluate at *E* and again omitted at *F*. Result: "pellagra".

In animals which have suffered either from deficiency of lactoflavin or from "pellagra" and are being treated with vitamin B_1 *plus* lactoflavin *plus* "supplementary factor" (Peters's eluate), and no longer show any pathological skin changes, if then Peters's eluate be suddenly omitted, the specific skin manifestations of pellagra-like dermatitis will infallibly reappear sooner or later. A few examples from this extensive series of experiments are given in Fig. 6. On the other hand elimination of lactoflavin with continued administration of Peters's eluate does not lead to the development of "pellagra".

The conclusion is therefore irresistible that the "pellagra-preventing substance" is contained in the flavin-free, charcoal eluate of Peters, and that it is

not identical with lactoflavin. When engaged in proving the growth-promoting properties of lactoflavin in our earlier experiments [György, Kuhn and Wagner-Jauregg, 1933; 1934] we made use of Peters's eluate as a "supplementary factor". We then expressed the opinion that the active principle to which the properties of Peters's eluate were due was vitamin B_4. In the specific vitamin B_4 deficiency disease, as described by Reader, nervous symptoms resembling polyneuritis are prominent, whereas in our experiment these symptoms were completely lacking, the picture being dominated by the pellagra-like dermatitis. Furthermore we succeeded in curing the pellagra-like dermatitis by using, instead of Peters's eluate, a yeast preparation (marmite) autoclaved for 4 hours at p_H 10 [György, 1934]. This finding cannot be reconciled with the fact that vitamin B_4 is highly sensitive to alkalis (Reader). In this respect we may refer to our earlier remarks (p. 748). In our preliminary communication, to avoid misunderstandings, we chose for the component of the vitamin B_2 complex, which was to be regarded as the pellagra-preventing substance, the provisional designation of vitamin B_6, leaving open the question of its identity with the Y-factor of Chick and Copping [1930], and with the pigeon-factor B_5. We propose to adhere to this designation for the time being and shall return to the problem of nomenclature later.

It follows from the experiments described above that no causal significance can be ascribed to lactoflavin, either in respect of the development or the cure of "pellagra-like" dermatitis. The appearance of "pellagra" was observed in 189 cases in which vitamin B_1 *plus* lactoflavin was given, but not a single case was seen when B_1 *plus* B_6 (Peters's eluate) only were given. Pellagra-like dermatitis developed in 22 animals which were given vitamin B_1 only, with no simultaneous administration of lactoflavin. In these cases the administration of vitamin B_6 alone, in the form of Peters's eluate (*cf.* Fig. 7), eventually brought

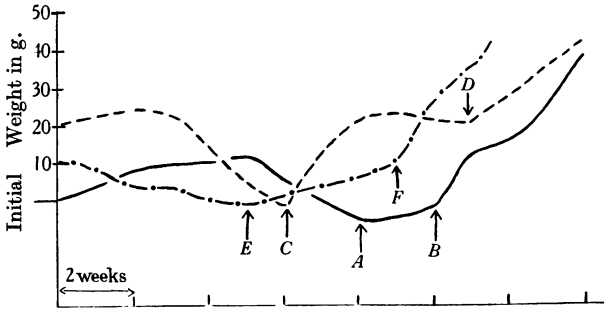

Fig. 7. The weight-curves of three rats which had developed "pellagra" with vitamin B_1 as the only supplement. At *A*, *C* and *E* addition of Peters's eluate, and at *B*, *D* and *F* of lactoflavin also.

about the cure of the pellagra-like skin manifestation, just as it did also in the case of those animals which developed "pellagra" when given vitamin B_1 *plus* lactoflavin. Nevertheless the two cases differ in two respects. There is, as might be expected, no resumption of growth in the absence of lactoflavin on the one hand, in spite of the administration of vitamin B_6, and on the other hand the skin changes take much longer to clear up than when B_6 is given in combination with lactoflavin; although the "pellagra" shows considerable improvement after 4 weeks, it is by no means healed. The symptoms of lactoflavin-deficiency, where this exists, remain in all their intensity. It should however be pointed out that with more prolonged treatment all the manifestations of pellagra will eventually disappear under the influence of vitamin B_6 (Peters's eluate) alone. In many cases

where marked loss of weight has occurred before the beginning of treatment, administration of vitamin B_6 even without simultaneous administration of lactoflavin may cause a transitory stimulation of growth, but this increase comes to a stop after 1–2 weeks and can only be renewed by giving lactoflavin (Fig. 7). If feeding with lactoflavin is begun during a stage when the administration of vitamin B_6 has not yet completely cured the pellagra, the rapid improvement which sets in may be erroneously ascribed to the action of lactoflavin. The falsity of this conclusion has been finally proved by our experiments.

Discussion.

Analysis of the vitamin B_2 complex into lactoflavin and a residual factor (or factors) constitutes the first step towards the successful analysis of this group of water-soluble vitamins. The growth-promoting action of lactoflavin and the presence of a lactoflavin-free "supplementary factor" which was necessary in order to demonstrate this growth-promoting action (György, Kuhn and Wagner-Jauregg) have been confirmed once more by the experiments described in this paper, in which a simplified and easily comprehensible technique was employed. There can be no doubt of the correctness of these findings, as they have since been confirmed by other workers, such as Euler *et al.* [1933; 1934], Chick and Copping [1934] and others.

The biological activity of lactoflavin was also recognised by Booher [1933; 1934], who claims, in a recently published paper, that a lactoflavin concentrate prepared by her stimulated growth in rats maintained on the diet of Bourquin and Sherman without any supplementary factor being present. This claim however rests upon the assumption that the lactoflavin prepared by Booher is comparable with the pure lactoflavin which was employed by us. A glance at the doses of the two preparations required is sufficient to invalidate this assumption. The daily requirement of Booher's preparation is 600γ, that of pure lactoflavin $7–10\gamma$. It seems certain that the lactoflavin concentrate of Booher represents a heterogeneous mixture, which contains among other things the supplementary factor which we found it necessary to add to lactoflavin in our experiments.

The recent synthesis by Kuhn and Weygand [1934] of a lactoflavin which was biologically active under the usual conditions (*i.e.* in the presence of Peters's eluate as a supplementary factor) represents the climax of the series of researches which had as their object the isolation of lactoflavin as one of the components of the vitamin B_2 group.

The vitamin character of lactoflavin was first established by the growth test exclusively, while the relationship of lactoflavin to pellagra-like dermatitis, which according to the original definition, represented the real specific symptom of vitamin B_2 deficiency, was at first obscure. In the first place the technique originally employed failed to produce "pellagra". This essential condition was only satisfied after the experimental technique was modified. The utilisation of a crystalline or highly purified preparation of vitamin B_1 instead of an alcoholic extract of wheat as the source of this vitamin together with a diet otherwise free from vitamin B_1 led invariably to the development of "pellagra". In our experience with over 200 animals, the average time elapsing before the appearance of the specific skin manifestations was 7 weeks. Using this experimental technique it was found that lactoflavin had nothing to do with either the prevention or the cure of "pellagra-like dermatitis". The actual pellagra-preventing substance is the supplementary factor, or at all events a part of it, together with

which lactoflavin is able to reveal its growth-promoting powers. A deficiency of this factor, which we had provisionally termed vitamin B_6, leads invariably to the appearance of "pellagra", even in the presence of lactoflavin.

The identification of the pellagra-preventing substance as one of the components of Peters's eluate also makes it possible to understand certain apparent discrepancies between our experimental results and the views held by Chick and her collaborators. Roscoe [1933, 1] observed pellagra-like dermatitis in about 50 % only of their experimental animals, and after an average experimental period of 10 weeks. In a personal communication Dr Chick states that the skin and manifestations were not generally severe in degree. Chick and Copping [1934] found that the action of lactoflavin upon the skin manifestations was a variable one. In many cases the "skin lesions" improved during the course of treatment with lactoflavin, while proving refractory in others. These results, which at first sight appear contradictory and irreconcilable with our own, are readily explicable in the light of our present knowldege. Chick and her collaborators employed Peters's eluate as the source of vitamin B_1 and thus continually supplied their rats with small quantities of pellagra-preventing substance in addition. Very often these quantities fell short of the threshold dose, as they were equivalent to only 0·6 g. of dried yeast, instead of 10 g. fresh yeast (equivalent to about 2 g. dried yeast). Further, brewers' yeast used by Chick is poorer in pellagra-preventing substance than bakers' yeast. At the same time, doses smaller than the therapeutic unit are effective prophylactically. It can therefore be understood why under these circumstances the appearance of pellagra in the experiments of Chick and her collaborators was inconstant, and that the symptoms of pellagra never reached the greatest intensity or developed in a short space of time. The slight therapeutic affect of lactoflavin occasionally observed by Chick and Copping [1934] also becomes easy to understand, since in our own experiments we have seen that lactoflavin occasionally accelerates the action of the "pellagra-preventing substance" and is thus apparently concerned in the healing process. On the other hand, the actual healing power is the property of the pellagra-preventing substance which was administered to the animals with the Peters's eluate.

Chick and Copping also suggest the identity of the supplementary factor (pellagra-preventing substance or vitamin B_6) with the factor Y previously described by them [1930]. This possibility was discussed also in a preliminary communication [György, 1934]. In view of the fact that Chick and Copping [1930] did not postulate any connection between the factor Y and the skin affections developed in absence of vitamin B_2, and that they did not take into account its character as supplementary substance within the vitamin B_2 complex, we thought it best, in order to avoid misunderstanding, to give the pellagra-preventing substance an entirely new designation.

The ultimate nomenclature of the vitamin B_2 complex will be a matter for the competent authorities to decide. We would urge that the generic term vitamin B_2 for the whole group be retained, and the individual components distinguished by special names. This question can be considered as already settled in the case of lactoflavin in the same way as the term ascorbic acid has been justified for vitamin C. For the time being the "pellagra-preventing substance" may be referred to as such, or as vitamin B_6, until its isolation has been accomplished, when it may receive its final designation. Elvehjem and Koehn [1934] have recently brought forward the proposal that the pellagra-preventing substance be termed vitamin B_2, and that lactoflavin be renamed, as they find that it has no connection with pellagra in chickens. In our opinion this proposal,

if only from the point of view of the antecedent literature, would increase the confusion.

The systematic investigation of the vitamin B_2 complex does not form an end in itself. The clinician will view this analysis as only the first step essential for the better understanding of the relationship between the individual representatives of the vitamin B_2 complex and the corresponding specific diseases in man. Now that the separation of the pellagra-preventing substance (vitamin B_6) from lactoflavin has been accomplished, the problem of the prevention of human pellagra will have to be subjected to a new experimental investigation. In this respect nutritive products will be considered according to their content of vitamin B_6 and not as hitherto, according to their total vitamin B_2 content. Our experiments [György, 1935, 1] have already led to the noteworthy discovery that fish, the great value of which for the treatment of pellagra in man was expressly pointed out by Goldberger, is among the natural products richest in vitamin B_6. Thus the value of fish, *e.g.* herring, salmon *etc.* as a foodstuff is supported by this experimental finding.

Further research must be devoted to a close examination of the vitamin B_6 content of cereals, especially of maize, if only in view of the criticism of Aykroyd [1930] which was fully justified in the previous state of our knowledge. However, our ultimate object is the testing of concentrates rich in vitamin B_6 on cases of human pellagra; and experiments in this direction are already being contemplated. It will be the results of these experiments which will finally confirm or disprove Goldberger's theory, which regards human pellagra and rat pellagra (as well as "black-tongue" in dogs) as identical in nature.

In the absence of personal clinical experience of human pellagra I must refrain from taking up any definite point of view with regard to the identity of the clinical pictures in man and in the rat. On the other hand, I am struck by the resemblance of pellagra in rats to a peculiar disease of infancy, known as "pink disease" or acrodynia, which has only been recognised in the last 20 years. It would be of great value if the results of animal experimentation were applied to human pathology in this instance, especially as successes with the feeding of liver have recently been claimed in many quarters. One important clinical difference between pink disease and rat-pellagra is the absence of an increased pulse-rate in the animals, whereas in the disease of infants it is one of the most constant symptoms.

Although there are indications as to the part which the "pellagra-preventing factor" may play in human pathology, we are completely in the dark in the case of lactoflavin. The symptoms of lactoflavin deficiency in animals are not sufficiently striking or specific. Important information in this respect will probably only be obtained by empirical means.

The relation between the vitamin B_2 complex and Castle's so-called extrinsic factor (which, in combination with the intrinsic factor of the stomach, is responsible for the production of an antipernicious anaemia substance) must be decided by experiments specially devoted to this problem. All we know from the literature at the moment is, that this extrinsic factor is present in meat and in yeast autolysate (marmite), that it is very thermostable and soluble in water and alcohol [Castle and Rhoads, 1932; Wills, 1931; 1933; 1934]. According to Miller and Rhoads [1934] the extrinsic factor is also present in egg-white. Since egg-white is known to be rich in lactoflavin, it might appear that the extrinsic factor will be more closely related to lactoflavin than to the other known components of vitamin B_2. It is interesting to compare foodstuffs according to their lactoflavin contents on the one hand and their anti-anaemic powers on the other. The latter was studied by Whipple and Robscheit-Robbins [1927; 1928; 1929; 1930], working with nutritional anaemia, sustained and regulated by repeated

bleedings in dogs, who found that fish flesh and fish liver were inactive, and our experiments have shown these to be the animal products which are poorest in lactoflavin [György, 1935, 1]. On the other hand, ox liver possesses the greatest anti-anaemic powers and the greatest lactoflavin content. Veal has a more potent anti-anaemic action than beef and is also richer in lactoflavin. Even allowing for the fact that the salts (Fe, Cu) and the haemoglobin contents of the nutritive products tested by Whipple and Robscheit-Robbins were also concerned in the therapeutic effects on anaemia investigated by them, the parallelism which is revealed between the anti-anaemic values and the lactoflavin contents is still a striking one. On the other hand, it must be pointed out that no sign of anaemia could be seen in our rats fed on a lactoflavin-free diet. True, this negative result might also be explained by the fact that rats are not suitable for the study of anaemia in this way, and further, the continued effect of repeated haemorrhages was also absent in the case of our rats in contradistinction to Whipple and Robscheit-Robbins's dogs. This question too will only be finally decided by direct clinical experiments.

In recent years the development of cataract in rats fed on a diet free from vitamin B_2 has been frequently described [Day *et al.*, 1931; Langston *et al.*, 1933]. In an extensive series of experiments in which over 500 rats were involved, we never observed this phenomenon either with complete or partial deficiency of the vitamin B_2 complex or with separate deficiencies of lactoflavin or vitamin B_6.

SUMMARY.

1. "Rat pellagra" can be produced, almost without exception, by the use of a crystalline or highly purified preparation of vitamin B_1 (3–4 pigeon day doses daily) added to a vitamin B-free basal diet.

2. The addition of lactoflavin, whether in the form of the pure substance (7–10 γ daily) or as egg-white (3–5 ml. daily), does not affect this result. Lactoflavin therefore can have no connection with "pellagra-like dermatitis". These observations extend over 211 animals.

3. The "pellagra-preventing" substance corresponds to the "supplementary substance" which is needed to demonstrate the growth-promoting action of lactoflavin. The combined administration of lactoflavin and the supplementary substance leads to a prolonged and marked increase in growth in rats in receipt of vitamin B_1 as the only member of the total vitamin B complex. Neither lactoflavin nor the "supplementary substance" by itself possesses any growth-promoting action.

4. Peters's "charcoal eluate" from yeast represents a suitable source of "supplementary substance" free from lactoflavin.

5. In view of its causal rôle in the development of "rat pellagra" the supplementary substance is termed the "rat pellagra-preventing factor" or, without making any further assumption, vitamin B_6.

6. The relations between the vitamin B_2 complex and its individual components and disease in man are discussed.

7. It is proposed that the designation vitamin B_2 be retained for the total complex. Lactoflavin might be known as such, and the pellagra-preventing substance as such, or provisionally as vitamin B_6.

It is with great pleasure that I express my deep gratitude to Prof. J. Barcroft whose hospitality has made possible the continuation of the experiments recorded in this and the accompanying papers.

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