LXXXVIII. INVESTIGATIONS ON THE VITAMIN B₂ COMPLEX.

III. THE INACTIVATION OF LACTOFLAVIN AND VITAMIN B₆ BY VISIBLE LIGHT.

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THE inactivation of preparations containing vitamin B₂ by irradiation with visible light [György *et al.*, 1933, 1, 2] necessitated an extension of our experiments, for whilst the destruction of the activities of vitamins by ultra-violet light has been the subject of close study for some time, and has been successfully applied also to the study of vitamin B₂ [Hogan and Hunter, 1928], investigations by the present author in association with Kuhn and Wagner-Jauregg were the first to show that visible light can also exercise the same effect. The inactivation of lactoflavin by visible light is the logical consequence of what has later been shown to be its nature as a dyestuff.

Our first observations were concerned mainly with relatively unpurified concentrates of lactoflavin, which for the most part still contained considerable amounts of "vitamin B₆". In order to demonstrate the effect of light, we mostly employed an artificial source of light (500 k.w. bulb). The complete loss of the vitamin B₂ activity of liver concentrate, not only after direct irradiation but also after it had been exposed in a room to diffuse daylight for 18 months, has previously been reported [György *et al.*, 1934, 1]. It naturally seemed important to correlate this photosensitivity of lactoflavin with the conditions of occurrence of pellagra in man; but after it was seen that lactoflavin was not identical with the pellagra-preventing substance, and that in consequence deficiency of the former could play no part in the development of pellagra, any such correlation lost a great deal of its practical significance. Evidence was still lacking on the photosensitivity of the newly discovered factor, the actual "pellagra-preventing factor", vitamin B₆. This gap can be filled now that the production of pellagra-like dermatitis no longer presents any technical difficulties.

The first experiments of this nature served to confirm our earlier findings regarding the inactivation of lactoflavin by visible light. A concentrate from cow's liver prepared in the usual way [*cf.* György *et al.*, 1934, 2] was placed in a thick-walled, rectangular glass bottle at a distance of about 30 cm. from a 500 k.w. electric bulb, for 24 hours; cooling was carried out by a fan. Irradiation was performed not only at a neutral reaction, but in two further specimens at acid (p_H 2-3) and alkaline (p_H 9-10) reactions. At the termination of the irradiation the two latter solutions were neutralised, as was also another control solution which had not been irradiated but kept for 24 hours at the same alkaline reaction. This control was necessary because, as we have already shown [György *et al.*, 1934, 1] when confirming the finding of Chick and Roscoe [1929], the activity of a vitamin B₂ concentrate may diminish in intensity at alkaline reactions, even at room temperatures. Rats, which for the few weeks preceding

had received the basal diet and supplements of vitamin B₁ (3 pigeon units daily), were treated with irradiated and non-irradiated specimens of the liver concentrate (L₂; 4 ml. daily, equivalent to 2 g. fresh liver). The animals were already stationary in growth but showed no signs as yet of pellagra-like skin changes owing to the short time the experiment had been in progress. After the administration of specimens of the liver concentrate, we obtained the following figures for growth (in g. weekly) in the first group:

Non-irradiated		Irradiated		
Neutral	Alkaline	Neutral	Acid	Alkaline
g.	g.	g.	g.	g.
9	9	1	3	2

A second group of animals reacted similarly. Growth, which failed to increase in the animals treated with the irradiated specimens of liver concentrate, could be restored once more by the addition of lactoflavin (10 γ daily). The exact data in Fig. 1 give an impressive proof of the inactivation of lactoflavin by visible light.

Fig. 1.

Fig. 2.

Fig. 1. The weight curves of five rats which were given vitamin B₁ + liver concentrate irradiated at neutral (o—o), at alkaline (— · —) and at acid (— —) reactions; and non-irradiated liver concentrate at neutral (—) and at alkaline (— · —) reactions. Addition of lactoflavin at the end of the 4th week.

Fig. 2. Weight curves of rats which, apart from vitamin B₁ + lactoflavin, received Peters's eluate daily, the latter irradiated at neutral (— · —) or at alkaline (o—o) reactions, and non-irradiated at neutral (—) or at alkaline (— —) reactions.

The inactivation of a concentrate of vitamin B₂ by irradiation with visible light is however not limited solely to the lactoflavin. Experiments carried out for a similar length of time with suitable doses of a second liver concentrate (L₃; 1.5 ml. = 1 g. of fresh liver) submitted to the same treatment, showed conclusively that vitamin B₆ was destroyed by visible light. In this experiment the specimens of liver concentrate were fed to animals in receipt of a basal diet supplemented by vitamin B₁ plus lactoflavin. In the following table are given the mean values of the weekly increases in weight over a period of 10 weeks and indications of the appearance or otherwise of symptoms of pellagra.

It will be seen from this table that, despite the addition of lactoflavin, the growth-promoting activity has been markedly diminished by irradiation with visible light. The differences in the weight curves do not appear so much in the

Liver concentrate daily ml.	Non-irradiated		Irradiated		
	Neutral g.	Alkaline g.	Neutral g.	Acid g.	Alkaline g.
3	12	9	13	10	7
	No pellagra	No pellagra	No pellagra	No pellagra	No pellagra
2	12	7	7	9	8
	No pellagra	No pellagra	Pellagra	Pellagra	Pellagra
1.5	8	6	3	6	3
	No pellagra	No pellagra	Pellagra	Pellagra	Pellagra

first weeks of the experiment as later on, when a definite flattening of the curve makes its appearance in the case of the irradiated specimens. With the largest doses employed (3 ml. daily) this diminution in the growth-promoting property is manifested only by the specimen irradiated at alkaline reaction, and to some extent also by the non-irradiated specimen kept at alkaline reaction for 24 hours. Symptoms of pellagra did not develop. With doses of 2 and 1.5 ml. daily all the animals in receipt of the irradiated specimens developed definite symptoms of pellagra, whilst these were completely absent from the animals which were given corresponding non-irradiated control solutions. The slighter increases in weight of the animals treated with the non-irradiated alkaline liver concentrate as compared with those of the animals treated with the non-irradiated neutral liver concentrate are again very striking.

Very similar results were obtained in further experiments with heart concentrate (1 ml.=0.5 g. cow's heart). Observations extended over 10 weeks in this case also. The administration of heart concentrate to animals which again had been receiving vitamin B₁ *plus* lactoflavin from the commencement of the experiment, was begun quite early, before the appearance of pellagra-like symptoms. The latter then developed, if at all, during the period in which the heart concentrate was administered.

Heart concentrate daily ml.	Non-irradiated		Irradiated		
	Neutral g.	Alkaline g.	Neutral g.	Acid g.	Alkaline g.
5	9	7.5	4	6	6
	No pellagra	No pellagra	Pellagra	Pellagra	Incipient pellagra

These and similar experiments, which, to avoid repetition, will not be given here, appear to prove that the pellagra-preventing substance is destroyed by visible light. The objection that the inactivation is an indirect effect of the sensitising action of the fluorescent lactoflavin, which is also present in concentrates of heart and liver, is disposed of, or if not completely disposed of at least rendered extremely improbable, by the fact that the fall in vitamin B₆ activity also occurs in the specimens irradiated at strongly acid or alkaline reactions. As is well known, lactoflavin no longer shows any fluorescence at these extreme reactions [Kuhn *et al.*, 1933]. Experiments which were performed with lactoflavin-free solutions of vitamin B₆, with Peters's eluate, and with concentrates or alcoholic extracts of salmon, also oppose the theory of the indirect nature of the photo-inactivation of vitamin B₆, at all events so far as lactoflavin as the photosensitive agent is concerned.

Fig. 2 gives the weight curves of four animals treated with Peters's eluate

before and after irradiation (at neutral and alkaline reactions). Here again it will be seen that even irradiation at neutral reaction lowers the rate of growth in experiments extending over corresponding lengths of time. This effect is less marked with the specimen irradiated at alkaline reaction than with a non-irradiated control specimen which was kept at alkaline reaction. Both specimens gave rise to a much smaller increase in growth than did the original concentrate. There was however a marked difference in the development of rat-pellagra. Pellagra-like symptoms were only seen in animals which received the irradiated specimens of Peters's eluate, independently of whether irradiation were carried out at alkaline or neutral reaction.

Specimens of Peters's eluate (equivalent to 10 g. fresh baker's yeast) irradiated at alkaline reaction were compared with non-irradiated controls in two further experiments. The corresponding figures for growth (average per week) were

	Non-irradiated	Irradiated at alkaline reaction
	g.	g.
1.	10	5
2.	12	2

Here again symptoms of pellagra appeared only in those animals which were given irradiated Peters's eluate. By doubling the dose in the second experiment we were able to bring about disappearance of the pellagra together with simultaneous increase in growth (9 g. weekly).

In all the experiments mentioned so far a powerful electric light (500 k.w.) was used for the destruction of the pellagra-preventing substance and lactoflavin by irradiation for 24 hours. If the results obtained by this technique were in all respects uniform, the photo-inactivation produced by exposure to diffuse daylight for correspondingly longer periods was still more impressive.

A liver concentrate (L_4), in a thick-walled transparent glass bottle, was kept in a room with diffuse daylight behind a window of opal glass. When this specimen was compared with a control specimen kept in the same room, but in a dark bottle wrapped in black paper, it showed a very marked fall in its activity in lactoflavin and vitamin B_6 . Our earlier work, which, as has already been mentioned, was concerned with the total vitamin B_2 content of a liver concentrate which had been exposed to diffuse daylight for 18 months, is thus not only supported by these new findings but supplemented in another way. It was now shown that under the influence of diffuse daylight alone the vitamin B_6 as well as the lactoflavin titre of this liver concentrate had fallen to a very marked extent. In Fig. 3 are reproduced the weight curves of two animals, one of which received in addition to the basal diet 4 ml. daily of a liver concentrate kept in the dark and the other 4 ml. of the specimen exposed to light; apart from this both animals were given 10 γ lactoflavin *plus* vitamin B_1 . The animal treated with the specimen exposed to light died in 5 weeks of pellagra, with weight completely stationary, whilst in the control animal the pellagra healed completely and the body weight increased by 97 g. Two animals which had also been submitted to the same treatment (basal diet *plus* vitamin B_1 *plus* lactoflavin), and were also already suffering from pellagra, were given 2 ml. of the specimen not exposed to light, and as a result showed an average weekly increase in weight of 17 and 16 g. respectively (68 and 65 g. respectively in 4 weeks), associated with rapid cure of the pellagra symptoms. The "exposed" specimen given in the same dose was again unable to supplement the lactoflavin. The body weights of two other animals, otherwise submitted to the same conditions, showed an average weekly fall of 2 and 1 g.

respectively, whilst the pellagra symptoms were aggravated. The irradiated liver concentrate also failed as a source of lactoflavin, *i.e.* as a supplement to Peters's eluate (vitamin B₆), not only in doses of 2 ml. but even 4 ml. daily. Fig. 4 shows the weight curve of one of these animals, which received 2 ml. of irradiated liver concentrate *plus* Peters's eluate (equivalent to 10 g. fresh baker's yeast) in addition to the basal diet. The sudden effect of the addition of lactoflavin supplement in this case affords the best proof of the assumption that lactoflavin was, in fact, absent from the "exposed" liver concentrate, at least in the doses given.

Fig. 3.

Fig. 4.

Fig. 3. Liver concentrate unexposed to light + vitamin B₁ + lactoflavin ——. " " " " exposed " " " " — — —.

Fig. 4. "Exposed" liver concentrate + vitamin B₁ + Peters's eluate. Addition of lactoflavin at ↑.

Experiments with the liver extract "campolon"¹ yielded results just as impressive as those already recorded. Specimens were exposed to diffuse daylight in thick-walled transparent bottles for 7½ weeks (Aug. 18th–Oct. 10th, 1934), (a) at a neutral reaction, (b) at a highly acid reaction with HCl (p_H 1–2), and (c) at a highly alkaline reaction with NaOH (p_H 11.0). Three corresponding controls were kept under similar conditions, except that they were protected from light of any kind. After 7½ weeks the specimens, neutralised and diluted 1:10, were tested biologically on animals which, apart from the basal diet, had received vitamin B₁ *plus* lactoflavin and already displayed symptoms of pellagra. The daily doses given were 5 ml. in one group, and 3 ml. in the second group, of each of the six different solutions of "campolon". In both series of experiments the non-irradiated specimens produced pronounced increase in weight with rapid healing of the pellagra. The non-irradiated specimens kept at alkaline reaction again gave rise to the slightest acceleration of growth only. Under the influence of diffuse daylight all the "campolon" solutions irrespective of their reactions

¹ Kindly supplied by the I.G. Farbendindustrie, Wuppertal-Elberfeld, Germany.

had completely lost their vitamin B₆ activity, at least in the doses in which they were given, 3–5 ml. daily. In spite of treatment, the symptoms of pellagra were aggravated without exception. A few animals died (Fig. 5). Substitution of the

Fig. 5.

Fig. 5. Campolon solution "unexposed" at neutral reaction ——. " " " " at acid " ——. " " " " at alkaline " ——. " " " " "exposed" at neutral " ——. " " " " at acid " o—o. " " " " at alkaline " Δ—Δ.

All the animals received vitamin B₁ and lactoflavin.

Fig. 6.

Fig. 6. (a) Campolon solution "exposed" at neutral reaction ——. Exchanged at A for corresponding "unexposed" specimen. (b) Campolon solution "exposed" at acid reaction o—o. Exchanged at B for corresponding "unexposed" specimen. (c) Campolon solution "exposed" at alkaline reaction Δ—Δ. Exchanged at C for corresponding "unexposed" specimen.

All the animals received vitamin B₁ and lactoflavin.

corresponding non-irradiated controls for the irradiated specimens resulted in the improvement anticipated, with increase in weight and resolution of the manifestations of pellagra (Fig. 6). As the animals were in receipt of vitamin B₁ plus lactoflavin, in addition to the "campolon" solutions, the difference in action between the "unexposed" and "exposed" solutions can only be explained by the differences in their contents of "pellagra-preventing substance" depending on its photo-inactivation.

The prolonged action of diffuse daylight, and especially of its visible portion, upon solutions which are practically free from lactoflavin, such as Peters's eluate, or concentrates or alcoholic extracts of salmon, leads to the destruction of the pellagra-preventing substance. For example, we obtained the following average

figures for growth on using Peters's eluate exposed for 4 weeks to diffuse daylight.

	Non-irradiated			Irradiated		
	2 ml.	1.5 ml.	1 ml.	2 ml.	1.5 ml.	1 ml.
Weekly gain (g.)	10	8	7	4	1	0

All the animals had received lactoflavin from the very beginning and had displayed pellagra-like manifestations. Treatment with the unexposed specimens led, with all doses, to rapid resolution of the symptoms of pellagra. The highest dose of exposed specimen (2 ml. daily) also produced a definite improvement, if not a complete cure of the pellagra; but the pellagra was unaffected by 1.5 and 1 ml. daily.

The results of experiments with an ordinary concentrate and with an alcoholic extract of salmon used in addition to vitamin B₁ and lactoflavin were equally unequivocal. The alcoholic extract was prepared by the same process as that described by Bourquin and Sherman [1931] for the preparation of alcoholic extract of wheat. Both salmon extracts were exposed to the visible portion of the spectrum of daylight for 6 weeks (Aug. 29th–Oct. 11th, 1934). Control solutions were kept in the dark. Biological experiments performed later showed that the irradiated specimen had been completely inactivated (Figs. 7 and 8). This was in solutions which were, again, practically free from lactoflavin.

Fig. 7.

Fig. 8.

Fig. 7. Salmon concentrate, non-irradiated — (3 ml. $\equiv$ 3.5 g. salmon), and "exposed" --- (5 ml.), and likewise "exposed" o—o (3 ml.). All the animals received vitamin B₁ and lactoflavin.

Fig. 8. Alcoholic salmon extract, non-irradiated — (3 ml. $\equiv$ 3 g. salmon), and "exposed" --- (3 ml.), and likewise "exposed" o—o (5 ml.). All the animals received vitamin B₁ and lactoflavin.

All these identical results led to the indisputable conclusion that the pellagra-preventing substance (vitamin B₆) like lactoflavin is destroyed by visible light. This inactivation may also occur in the absence of lactoflavin. Under these circumstances it should be of importance to correlate this phenomenon, using the example of lactoflavin as an analogy, with the nature of the pellagra-preventing

substance as a dyestuff, and not to regard it as a secondary photosensitisation. However the possibility must be considered that there may have been present, in all these solutions of vitamin B₆, yet another fluorescent substance, differing from lactoflavin, which might have exercised a photosensitising action.

Another point of interest in connection with the photo-inactivation of the pellagra-preventing substance is the fact that all the vitamin B₆ solutions examined so far have been coloured yellow, including the purified Peters's eluate, and that under the influence of diffuse daylight (from which the ultra-violet rays are excluded) they take on a darker, reddish brown colour. This change in colour is especially marked in concentrated solutions (3–5 rat day doses of vitamin B₆ in 1 ml.) of salmon extract, and also in Peters's eluate, *i.e.* again in the absence of lactoflavin. The curves in Fig. 9 give the quantitative data of the spectro-

Fig. 9. Spectrogram of "unexposed" (—) and "exposed" (---) salmon concentrate (cf. Fig. 7) after concentration to 1:10.

photometric examination of a salmon concentrate. (I am very much indebted to Prof. R. Kuhn for carrying out this investigation at his Institute.) The only fact which emerges from these measurements with certainty is the shift in the absorption of light of long wave-length in the irradiated extract, which was also obvious to the naked eye. The curves exhibited no characteristic maxima. Further chemical purification of the vitamin B₆ concentrates is now required; experiments in this direction have already been begun. The results of these experiments will decide whether this change of colour in solutions containing vitamin B₆ after exposure to the visible portions of diffuse daylight depends on the pellagra-preventing substance, or only on an associated substance which differs from it and is biologically inactive.

According to Hogan and Richardson [1932, 1, 2; 1933] extracts of yeast or liver lose their activity as sources of pellagra-preventing substance on irradiation with ultra-violet light, and such loss of activity is much more rapid than is the case with vitamin B₁ activity. These authors have shown that this observation may be utilised in a process for the production of pellagra-like dermatitis in rats. This statement has been confirmed by the present writer in a large series of experiments involving over 80 rats. Daily doses of 0.1 g. of dry yeast which had been exposed in thin layers to ultra-violet radiation, led in fact almost constantly

to the development of "rat pellagra", when given to rats fed on the vitamin B-free diet. However, this procedure possesses several disadvantages, which led us to discard its further use. In the first place numerous spontaneous healings were observed, and secondly there was a long "preparatory period" before the pellagra-like symptoms disappeared. Nevertheless, the use of this method on a large number of animals suffering from pellagra gave convincing evidence of the complete therapeutic inefficacy of lactoflavin, and of the curative effect of the "supplementary factor" (vitamin B₆).

The slight diminution in the growth-promoting action of solutions which have not been irradiated but which have been kept for some time at an alkaline reaction appears from the above experiment to be independent of any action of lactoflavin and probably of the pellagra-preventing substance also. This finding constitutes the sole support which we possess at present for the assumption that, in addition to the pellagra-preventing substance and vitamin B₁, Peters's eluate must contain yet another substance with the character of a vitamin. Further investigations are necessary to elucidate this last problem.

SUMMARY.

Both lactoflavin and the pellagra-preventing substance (vitamin B₆) may be destroyed by visible light. The inactivation of the pellagra-preventing substance also occurs in lactoflavin-free solutions, so that if it is dependent on an indirect photosensitisation it must be through some agency distinct from lactoflavin. The possibility is discussed and left open as to whether the pellagra-preventing substance is, like lactoflavin, a natural dyestuff.

REFERENCES.

- Bourquin and Sherman (1931). *J. Amer. Chem. Soc.* **53**, 3501.
Chick and Roscoe (1929). *Biochem. J.* **23**, 498.
György, Kuhn and Wagner-Jauregg (1933, 1). *Naturwiss.* **21**, 560.
— — — (1933, 2). *Klin. Woch.* **12**, 1241.
— — — (1934, 1). *Z. physiol. Chem.* **223**, 21.
— — — (1934, 2). *Z. physiol. Chem.* **223**, 241.
Hogan and Hunter (1928). *J. Biol. Chem.* **78**, 433.
— and Richardson (1932, 1). *Mo. Agric. Exp. Sta. Research Bull.* **178**, 3.
— — — (1932, 2). *J. Biol. Chem.* **97**, vii.
— — — (1933). *J. Biol. Chem.* **100**, lv.
Kuhn, György and Wagner-Jauregg (1933). *Ber. deutsch. chem. Ges.* **66**, 317.