CCXXIX. TRYPTOPHAN DEFICIENCY.

By ROBERT SAXELBY ALCOCK.

From the Biochemical Laboratory, Cambridge.

(Received August 10th, 1934.)

THE position of tryptophan is very much that of a vitamin; a substance—in this case of known constitution—essential to life, for the supply of which the animal is dependent upon outside sources. As with the vitamins, it is important that the reason for this indispensability should be understood.

The work on tryptophan function has followed three different lines. In the first place, the quantitative and qualitative aspects of the deficiency syndrome have been studied, and as no one has succeeded in measuring any effect other than the failure to grow, this work has resolved itself into following the influence of various factors on the weight curves of animals on diets low in tryptophan. An impressively large number of tryptophan derivatives has been given to such animals, and the only ones able to replace tryptophan itself would seem to be indolepyruvic acid [Jackson, 1929; Berg *et al.*, 1929, 1], *d*-indolelactic acid, but not the *l*-isomeride [Bauguess and Berg, 1934, 1], *d*-tryptophan [Berg and Potgieter, 1931], acetyl-*l*-tryptophan and its ethyl ester hydrochloride [Berg *et al.*, 1929, 2].

As indolepyruvic acid may be converted into tryptophan in the liver, and acetyltryptophan and the ester hydrochloride readily hydrolysed, it cannot be said that the above work has contributed anything of a really positive nature to the problem. An interesting observation is that of Berg and Rose [1929], who showed that feeding 20 mg. of tryptophan per rat at 24 hourly intervals gives submaximum growth, while 10 mg. doses at 12 hourly intervals give maximum growth. It would seem that the vital function is associated with one of the more fluid aspects of the animal's metabolism.

Du Vigneaud *et al.* [1932] report experiments in which tryptophan was injected into deficient animals with a consequent resumption of growth. A similar result was claimed by Schichiri and Sakata [1933], though it would seem from their figures that no very definite effect was obtained; the amounts of the amino-acid injected were never more than 20 % of the daily requirement, and in no case was a resumption of growth observed. The abnormally rapid fall in weight of the control animals would suggest that they were otherwise deficient.

Jackson [1927] reported negative results from tryptophan injections.

A second chapter in the tryptophan story deals with kynurenic acid formation. This has recently been reinvestigated by Kotake and his co-workers, who showed [Kotake and Iwao, 1931] that an intermediary substance, kynurenin, could be detected, and that the reaction is probably located in the liver [Ichihari *et al.*, 1931]. Kotake and Sakata [1931] would associate kynurenin with urochrome, although they ignore the obvious complexity of this group of substances, and their results manifestly refer to one fraction only.

Despite this and more recent work [Kotake, 1933], it can still be said of kynurenic acid that it appears to represent a shunt mechanism which is

available for dealing with an excess of tryptophan. Possibly it should be described as a detoxication mechanism.

Berg [1931] and Bauguess and Berg [1934, 2] have used this system as an index of tryptophan metabolism and have tested a number of substances for their convertibility into kynurenic acid. Among the substances replacing tryptophan in growth experiments they find that tryptophan ethyl ester hydrochloride, indolepyruvic and *dl*-indolelactic acids will, while acetyltryptophan and *d*-tryptophan will not, pass over into kynurenic acid. It seems most probable, however, that the only significance which can be attached to these results is that they may give a measure of the rate at which the substance in question may be transformed into indolepyruvic acid. From the work of Kotake and others, it seems likely that kynurenin and kynurenic acid are produced from tryptophan *via* the pyruvic acid, and any substance following this line of metabolism can be expected to be side-tracked to kynurenic acid only if it is likely, at any stage, to give rise to a large concentration of indolepyruvic acid or tryptophan itself. Thus the failure of acetyltryptophan might be due to the low rate of hydrolysis, and that of *d*-tryptophan to the fact that it is more slowly converted into indolepyruvic acid than the naturally occurring *l*-isomeride. Neither kynurenic acid [Assayama, 1916] nor kynurenin [Jackson and Jackson, 1932] will replace tryptophan in the diet.

Other researches into the function of tryptophan have been concerned with its haematopoietic effect. Hirazawa [1922] showed that anaemic animals recover the faster for tryptophan injections. Okagawa and Tatsui [1931] would associate this with the spleen, but their evidence does not seem adequate to support their conclusions. It has been shown here (unpublished work) that tryptophan is oxidised by peroxidases in the spleen, but the nature of the products has not yet been established, and the observation has in no way been correlated with the above findings.

Matsuoka and Nakao [1931], in demonstrating that 2-methyltryptophan, which will not replace dietary tryptophan, is efficacious in relieving anaemia, indicated that the haematopoietic effect is of a stimulatory nature, rather than the provision of a building-stone. In an earlier paper [Alcock, 1933] it is shown that tryptophan deficiency is not accompanied by anaemia, and that blood-formation can go on in the absence of dietary tryptophan. This would appear to dismiss the idea that the tryptophan is providing an essential constituent of the blood-protein, such as haematin.

Kynurenin has no haematopoietic effect [Iwakura *et al.*, 1933]. Fontés and Thivolle [1930] reported the relief of anaemias in human subjects by subcutaneous injection of a mixture of tryptophan and histidine, but this, together with the other findings reported in the same paper, has not been substantiated elsewhere.

From this mass of experimentation few positive conclusions can be drawn. Tryptophan is essential for growth and maintenance, and the need is immediate. Excess of tryptophan causes production of kynurenin and kynurenic acid, though no physiological significance has been attached to these substances. Injections of tryptophan will accelerate the recovery from anaemia, though such recovery is possible in the absence of either injected or dietary tryptophan. A further possible association is with the spleen, the reticulo-endothelial system generally and urochrome, but information on this point is meagre.

The really positive finding, and the one which is linked with tryptophan's essential nature, is the original one of Hopkins and Willcock [1906], that without this amino-acid animals decline and die. The other two series of observations would appear to be dealing with side-lines.

There are two possible causes for this decline on a tryptophan-deficient diet. It may be, and indeed it is usually assumed, that the animal is wholly unable to make tryptophan, and that therefore the fall in weight measures the rate of utilisation of the body proteins and of the tissue involved; the non-cyclopoietic animal being powerless to make good the loss. The other possibility is that tryptophan, or a derivative of it, exerts some specific effect upon the tissues or upon some one tissue—a “hormone” effect—entirely independent of the protein “Baustein” function mentioned above. The distinction between these two is important, not only in connection with tryptophan metabolism and significance, but also because it has a bearing upon the problem of the mode of protein synthesis *in vivo* and on the question of limiting factors to growth generally.

An attempt has therefore been made to decide to which of these the indispensable nature of tryptophan is to be ascribed. The preliminary work deals with the quantitative aspects of loss of weight in tryptophan deficiency. There follow experiments on the injection of (*a*) tryptophan and (*b*) the anterior pituitary growth hormone into tryptophan-deficient animals.

EXPERIMENTAL.

Most of the work on tryptophan deficiency has been carried out on rats, and the diet described by Berg and Rose [1929] has been almost universally adopted. The various workers have introduced slight variations into the composition of the diet, but nothing which would account for the wide variations in the results. Table I gives some idea of the falls in weight obtained by different workers using approximately the same conditions.

Table I.

Duration in days	Loss in g.	Reference
12	17	Matsuoka and Nakao [1931]
20	20	Berg <i>et al.</i> [1929, 2]
40	18	Jackson and Jackson [1932]
40	52	Schichiri and Sakata [1933]
40	35.5	See Table III

This wide variation is manifestly undesirable, as it becomes impossible to compare the results of one worker with those of another on a quantitative basis. In all cases there should be record of a control experiment where the diet itself has been supplemented with tryptophan; the “control” applied by some workers, a replacement of the hydrolysed caseinogen by unhydrolysed, is no control in effect.

The first need was clearly a tryptophan-deficient diet in which the tryptophan lack represented the limiting factor to growth, and which would give reasonably uniform results. It was felt that, if possible, the hydrolysed caseinogen should be replaced by a whole protein—the advantages are obvious; slower absorption, better taste, controlled blood-amino-acid concentration and so on. Gelatin is unsatisfactory and could not be used, even in conjunction with zein. Zein itself contains no lysine, and supplements of lysine would have proved expensive. However, a solution was found in various mixtures of zein and hydrolysed caseinogen.

The zein is prepared as follows. Maize gluten, obtainable from British Corn Products, Ltd., in a calico bag, is immersed in 80 % alcohol and allowed to soak for some hours. The bag is then allowed to drain, and the alcohol actually

soaked up by the gluten is squeezed out in a press. The squeezed gluten is again extracted, drained and pressed. Three or four such extractions can profitably be made. The solution of zein is then poured into a large volume of cold water, with vigorous stirring, and the suspension is allowed to settle overnight in the ice-chest. The zein is then picked out (by hand), placed in a muslin bag and rubbed through into a beaker of ice-cold water, from which it is allowed to settle. The water is decanted, the sludge of zein filtered on a Büchner funnel and spread out on large trays to dry. It may be necessary at this stage to grind it, but usually, if it has been kept cold, it dries as a fine powder. If at any time it sets into a dense rubbery mass, a few days in the ice-chest under water will render it friable and capable of being powdered through muslin. It should never be allowed to get warm. The yield is about 20 %.

The caseinogen is hydrolysed by three hours' treatment in an autoclave with 25 % sulphuric acid. This is removed with baryta, and the filtrates are evaporated in open pans and finally dried in a vacuum-oven.

In all the following experiments the vitamins were given in the form of three drops of cod-liver oil, and an alcoholic extract of autolysed yeast, the equivalent of 0.75 g. fresh yeast per rat daily. This preparation contains vanishingly small quantities of tryptophan, as does also the zein. The food was always given *ad lib.*, and from time to time, throughout the experiments, tests of intake were made. These gave a constant figure—between 6 and 8 g. per rat daily, with a tendency to a higher value on the better-tasting diet 25. This is probably set off by the relative indigestibility of the zein; in any case, the rates of growth on the supplemented diets do not differ markedly.

Three diets have been tested, the main difference being the relative proportions of hydrolysed to unhydrolysed protein. Diet 1 differs from that of Berg and Rose only in the slightly higher caseinogen content and the absence of a cystine supplement. This was found to make no difference.

Table II.

Constituents	Diet 1	Diet 15	Diet 25
Hydrolysed caseinogen	18	9	5
Zein	—	9	20
Rice starch	40	40	40
Sucrose	20	20	20
Palm kernel oil	17	17	10
Salt mixture	5	5	5

Table III summarises the results shown in Figs. 1, 2 and 3. It shows the average losses and gains on the different diets and the corresponding supplemented diets. The supplement consists of 0.25 % of tryptophan.

Table III.

<i>Basal diets.</i>		Average loss in weight, g.		
Duration in days	Diet 1	Diet 15	Diet 25	
20	27.5	8.2	3.7	
40	35.5	14.8	7.8	
60	42.5	23.6	—	
<i>Supplemented diets.</i>		Average gain in weight, g.		
20	20	26.5	21	
40	45	43.5	49.5	

The results given in Table III and Figs. 1, 2, 3 indicate that, although no great improvement in the growth rate on the supplemented diet could be

Fig. 1. Weight curves of rats on diet 1. At Tr ↑, B and E received tryptophan.

Fig. 2. Weight curves of rats on diet 15. Rats J and H received tryptophan.

Fig. 3. Weight curves of rats on diet 25. Rats H and G received tryptophan.

achieved, the replacement of the hydrolysed protein by zein prevented the extensive losses in weight and kept the animals in much better condition. Blood counts for such animals are to be found in a previous paper [Alcock, 1933].

The almost negligible loss of weight on diet 25 would suggest that the tryptophan in proteins lies within the animal's synthetic powers. During the experimental period, the rats have been making some skin and hair, intestinal secretions, haemoglobin, hormones, *etc.*, involving appreciable amounts of tryptophan. To suggest that the amino-acids liberated from autolysed tissue proteins were utilised would imply an unbelievable economy and one which the results of Fürth and Lieben [1922] would make to appear all the more unlikely, as they showed that only 3 to 8% of ingested tryptophan was available for tissue building.

It is clear that if the limit to growth is imposed by the lack of tryptophan in the tissues for protein synthesis, then injections of this amino-acid will have the same positive effect upon the growth rate as that given by the mouth. The blood-concentration of free tryptophan will be restored, and protein building can continue. The work of Jackson [1927] suggests that this is not the case, but du Vigneaud *et al.* [1932] and Schichiri and Sakata [1933] both report the opposite result, that tryptophan injected under the skin is efficacious in relieving the symptoms of tryptophan deficiency.

The experiment has therefore been repeated here. Four rats were kept in the same cage and given tryptophan-deficient diet 25. Two were taken out daily and given a subcutaneous injection of 1 ml. of 2 % tryptophan solution, which had been filtered through a Seitz filter. The injections were continued for 15 days, and from the 20th day onward all four were given diet 25 with a supplement of 0.25 % tryptophan. It will be seen from Fig. 4 that the injections had no effect, although the animals responded normally to oral tryptophan subsequently.

Fig. 4.

Fig. 4. Weight curves of rats on diets 25. C and D received daily injections of tryptophan to D ↓. At ↑ Tr all four given tryptophan in the food.

Fig. 5.

Fig. 5. Rats on diet 25. G, F, and E received pituitary injections from I ↑ to I ↓, and H, tryptophan in the food to Tr ↓.

This suggests that the limit to growth is not imposed by the inability to make the tryptophan necessary for tissue proteins. If this is actually so, it might be possible to raise the growth limitation by other means than oral tryptophan, and thus get tissue production, with its local tryptophan synthesis, on a tryptophan-deficient diet.

This was accidentally arrived at in one experiment, in which a rat which had been placed on diet 25 was found to be pregnant—some 9 days gone, apparently—at the time of starting the experiment. Throughout the gestation, her weight did not alter in the least, and ultimately, she gave birth to a litter of six—small, and stillborn. The tissues of these rats had however been built up without any exogenous tryptophan.

In the course of a research on the question of the significance of growth limitation on deficient diets (Alcock and Mapson, to be published shortly), animals on diet 25 were given daily injections of a preparation of the anterior pituitary growth hormone [Evans and Simpson, 1931]. 0.1 to 0.15 ml. of this material was injected daily. The results are shown in Fig. 5, the three lower lines representing the rats so treated and the uppermost line a rat given 20 mg. of tryptophan by the mouth daily.

The weights of these three animals ran up steadily for the 15 days. After the injections had been discontinued, the weights fell off again, and the weight curves of the three injected animals closely paralleled that of the control. It is important to observe that there was no sudden falling off on the first day, but that the decline was gradual. This is significant, as the weight-increases resulting from such injections, in the normal animal, are due in part to water intake and in part to real growth, but while the growth effect remains, the whole of the excess water is excreted within the first 48 hours of the cessation of the injections. The shape of the curves makes it most probable that a real growth effect is involved, but the experimental period was too short to permit of any definite statement being made. The variations lie within the experimental error zone of nitrogen-balance determinations, and therefore these cannot be used to settle the matter.

An extension of the experiment to a longer period is made difficult by the sensitiveness of the skin of the deficient animals. Instead of becoming accustomed to the injections, as do normal animals, they become increasingly apprehensive as the time goes on, and seem to suffer real pain on the insertion of the needle, even though the injections are made at a different place on each occasion. It was this which also prevented an extension of the experimental period in the tryptophan injection work. The same effect was observed in animals suffering from vitamin deficiencies.

The evidence here presented would seem to justify the conclusion that the limiting factor to growth on a tryptophan-deficient diet does not arise from the demand of the tissues for amino-acids to be used for protein synthesis. There is no positive suggestion as to how the limitation can be accounted for, except that some material which is essential to life appears to be made in the liver (see experiments on tryptophan injection). This is probably not associated with kynurenic acid or kynurenin and is independent of the haematopoietic effects which tryptophan exerts when injected.

The fact, however, that the animal can synthesise proteins while receiving no tryptophan, means that, for this particular purpose it is able to make its own tryptophan. This carries with it the implication that the synthesis of protein probably starts at a lower stage than the fully formed amino-acid, and that the amino-acid groups actually found in the animal proteins have been formed there, not assimilated from the blood-stream as such. This brings the synthesis of protein in the animal into line with that in other organisms, in particular the plant, where the nitrogen source in protein building is necessarily of a simple nature.

It might be objected that, if the animal can make the tryptophan for its

proteins, it should be able to use that machinery for providing itself with what may be styled "essential," or hormonal tryptophan, when the normal supply of this fails. This, however, can be ruled out on two grounds; the quantitative and the teleological. On the latter, the need for such a transfer would never arise in nature; specific tryptophan deficiency is unknown. And on the former, the rat needs 20 mg. tryptophan daily—this means 1 g. of protein, or say 10 g. of tissue, which would have to be synthesised and autolysed during the course of 24 hours. Not only that, but it has been shown here that tryptophan delivered elsewhere than in the portal vein is not available for the "essential" purpose, and the autolytic tryptophan postulated above would be generally distributed.

So that, while it has not been possible to show why it is that tryptophan is essential to life, a study of the deficiency has thrown some light on protein synthesis, and the essential function has probably been identified as a prerogative of the liver.

SUMMARY.

1. A brief review of the knowledge of tryptophan function is given.
2. An improved tryptophan-deficient diet is described.
3. Rats on the above diet, injected with tryptophan, failed to grow.
4. Rats on the above diet gave a positive response to the anterior pituitary growth hormone and probably grew new tissues.

This work was carried out during the tenure of a Senior 1851 Studentship.

I would like to express my indebtedness to Sir F. G. Hopkins for his constant encouragement and advice.

REFERENCES.

- Alcock (1933). *Biochem. J.* **27**, 754.
 Assayama (1916). *Biochem. J.* **10**, 466.
 Bauguess and Berg (1934, 1). *J. Biol. Chem.* **104**, 675.
 ——— (1934, 2). *J. Biol. Chem.* **105**, viii.
 Berg (1931). *J. Biol. Chem.* **91**, 513.
 ——— and Potgieter (1931). *J. Biol. Chem.* **94**, 661.
 ——— and Rose (1929). *J. Biol. Chem.* **82**, 479.
 ——— and Marvell (1929, 1). *J. Biol. Chem.* **85**, 219.
 ——— (1929, 2). *J. Biol. Chem.* **85**, 207.
 Evans and Simpson (1931). *Amer. J. Physiol.* **98**, 511.
 Fontés and Thivolle (1930). *Le Sang*, **4**, 658.
 Fürth and Lieben (1922). *Biochem. Z.* **132**, 325.
 Hirazawa (1922). *J. Osaka Med. Soc.* **21**, 9.
 Hopkins and Willcock (1906). *J. Physiol.* **35**, 88.
 Ichihari, Otani and Tsujimoto (1931). *Z. physiol. Chem.* **195**, 179.
 Iwakura, Otani and Taniguchi (1933). *Z. physiol. Chem.* **214**, 22.
 Jackson (1927). *J. Biol. Chem.* **73**, 523.
 ——— (1929). *J. Biol. Chem.* **84**, 1.
 ——— and Jackson (1932). *J. Biol. Chem.* **96**, 697.
 Kotake (1933). *Z. physiol. Chem.* **214**, 1 et seq.
 ——— and Iwao (1931). *Z. physiol. Chem.* **195**, 139.
 ——— and Sakata (1931). *Z. physiol. Chem.* **195**, 184.
 Matsuoka and Nakao (1931). *Z. physiol. Chem.* **195**, 208.
 Okagawa and Tatsui (1931). *Z. physiol. Chem.* **195**, 192.
 Schichiri and Sakata (1933). *Z. physiol. Chem.* **214**, 14.
 Du Vigneaud, Sealock and van Etten (1932). *J. Biol. Chem.* **98**, 565.