CCXCVIII. THE SUGGESTED RELATION BETWEEN CYSTINE AND VITAMIN B₂.

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It has been suspected that some of the diets used in vitamin work, which contain protein in the form of purified caseinogen, may be deficient in cystine. This is particularly the case with diets used in experiments with the B-vitamins, since those used in the study of the fat-soluble vitamins usually contain the B-vitamins in the form of yeast or yeast extracts, which are rich in cystine.

The realisation of this fact has led some workers to guard against the possibility of a deficiency by adding cystine to the diet [Jansen, personal communication; Block and Farquar, 1933] and has led others to consider whether the syndrome of growth failure and dermatitis, hitherto considered to be due to a deficiency of vitamin B₂, may not in reality be due, in part, to a deficiency of cystine. This view has recently been put forward by Itter *et al.* [1935].

In 1932, with the idea of investigating this question, we studied the effect of variations in the cystine content of the diet on the growth of rats receiving different amounts of vitamin B₂ and on the dermatitis developing in rats deprived of this vitamin. Although it was found that one of the samples of purified caseinogen used had a low content of cystine, the deficiency of cystine in the diet was found to be slight, and no interrelation between cystine and vitamin B₂ could be found. The work was therefore not published. The results we obtained, however, partly confirm and partly extend the recently published work of Itter *et al.*, and as they lead us to different conclusions, it is now thought desirable that they should be published.

METHODS.

Rats weighing 35–50 g., immediately after weaning, were fed on the experimental diets, which all contained caseinogen 20 %, rice starch 60 %, cotton-seed oil 15 % and salt mixture (McCollum's No. 185) 5 % and were cooked in a steamer for 3–5 hours with water. They were supplemented with daily doses of cod-liver oil and concentrates of vitamins B₁ and B₂.

Three preparations of caseinogen were used and two of these were supplemented with extra cystine, making in all five different diets:

(1) Diet K, containing 0.019 % cystine, made with purified caseinogen, "Glaxo physiological caseinogen, AB" of cystine content 0.11 %.

(2) Diet P2L, containing 0.046 % cystine, made with Lister Institute purified caseinogen [v. Chick and Roscoe, 1928] of cystine content 0.22 %.

(3) Diet FL, containing 0.062 % cystine, made with unpurified "Light white casein" of cystine content 0.32 %.

(4) Diet CK, 0.269 % total cystine, made with "Glaxo purified caseinogen" and 0.25 % additional cystine.

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(5) Diet CFL, 0.312 % total cystine, made with unpurified "Light white casein" and 0.25 % additional cystine.

The cystine used was *l*-cystine obtained from Hoffman-La Roche.

Vitamin B₁ was given in the form of Peters's concentrate from yeast [Chick and Roscoe, 1929]; this was found to contain 20 mg./100 g. of cystine, the daily dose of 0.1 ml. ($\equiv$ 0.6 g. dry yeast) thus providing 0.02 mg., a negligible amount.

Vitamin B₂ was given as various watery extracts from yeast, autoclaved either in an acid medium for 5 hours, or in an alkaline medium for 1 hour, in order to destroy vitamin B₁ [Roscoe, 1933, 2]. Both these preparations were found to contain 30 mg./100 g. of cystine, the daily doses of 0.5–1.0 ml. ($\equiv$ 0.25–0.5 g. dry yeast) containing 0.15–0.30 mg. of cystine. The presence of cystine in these autoclaved extracts is interesting, since it has been generally thought that such treatment would completely destroy this amino-acid.

The cystine estimations were carried out by the modified Sullivan method described by Prunty [1933]. This involves reduction of the cystine to cysteine, so that both amino-acids, if present, are estimated together.

The glutathione estimations were made according to the method of Tunncliffe [1925]. The animals were anaesthetised with ether, the thorax was cut open and blood removed from the heart with a syringe, about 4 ml. being the amount obtained from a 100 g. rat. About 10 g. each of liver and muscle were removed as quickly as possible, weighed and ground with sand, the muscle having been first roughly chopped with scissors.

It was not found possible to make duplicate estimations so that it did not seem justifiable to calculate the results to smaller amounts than 10 mg./100 g.

EXPERIMENTAL.

A. *The effect of the proportion of cystine in the diet on the occurrence of dermatitis in rats deprived of vitamin B₂.*

Itter *et al.* [1935], discussing the possible rôle of the sulphydryl group in vitamin B₂ deficiency, suggested that "the variability of the cystine content of different caseins may account for the inconstant results frequently observed in producing the deficiency". In support of this they quote the conclusion of Chick and Roscoe [1928], that in order to obtain dermatitis it was necessary rigidly to purify the caseinogen of the basal diet, which process would be liable to reduce the cystine content.

Roscoe [1933, 1] however, as the result of a more prolonged study, found that the purification of the caseinogen had no significant effect on the incidence of dermatitis. These results are given again in Table I, Exp. 1. Neither the number of rats developing dermatitis nor the time during which they received the deficient diet before symptoms developed was affected by the amount of cystine in the diet.

Table I. *Incidence of dermatitis and weight increase among rats deprived of vitamin B₂ and receiving diets containing varying amounts of cystine.*

Exp. No.	Diet	Cystine content of diet %	No. of rats observed	No. of rats developing dermatitis	% of rats developing dermatitis	Average time for development of dermatitis weeks	Average weight increase in 5 weeks g.
1	K	0.019	22	13	64	8	4
	P2L	0.046	107	61	57	11	8
	FL	0.062	54	36	67	9	20
2	FL	0.062	17	13	76	9	21
	CFL	0.312	11	8	73	9	19

In Table I, Exp. 2, are shown the results of a further small experiment, in which the diet FL and the same diet with added cystine (CFL) were fed to rats from the same litters. The incidence of dermatitis was in no wise affected by this addition to the diet.

B. *The effect of the proportion of cystine in the diet on the growth of rats.*

In the last column of Table I are shown the increases in weight of rats deprived of vitamin B₂ during the first 5 weeks of deficiency. It will be seen that, although the cystine content of the diet did not affect the incidence of dermatitis, it appears to have influenced the growth of the animals. Thus, in Exp. 1, the average weight increases observed during the 5 weeks were 4, 8 and 20 g. for rats receiving the diets containing 0.019, 0.046 and 0.062 % cystine respectively. Increasing the cystine content beyond this point (Exp. 2) from 0.062 to 0.312 % did not improve the growth and it may therefore be supposed that the 0.062 % level was adequate.

These results are striking, but it should be borne in mind that the different methods employed for purifying the caseinogen may have affected it in other ways than by altering the cystine content, and that the different rats included in Exp. 1 were not observed concurrently, but over a number of years, so that variations in their reserves and in other experimental conditions may possibly have had an effect on growth [*v. Chick et al.*, 1935].

Three more comparable experiments were carried out, in which rats received diets containing varying amounts of cystine together with sub-optimum amounts of vitamin B₂. These results are given in Table II. In Exp. 3 the

Table II. *Weight increase of rats receiving diets containing varying amounts of cystine and varying sub-optimum amounts of vitamin B₂.*

Diet	Cystine content %	Weight increase observed during 5 weeks, g.		
		Exp. 3*	Exp. 4	Exp. 5
K	0.019	—	♂ 54	♂ 89
			♀ 52	♀ 66
			—	♀ 69
			Average	75
CK	0.269	—	♂ 64	♂ 92
			♀ 60	♀ 62
			—	♀ 79
			Average	78
FL	0.062	♂ 57, 56	♂ 63	♂ 86
		♀ 60, 37	♀ 54	♀ 77
		Average	58	80
		52		
CFL	0.312	♂ 63, 63		
		♀ 54, 42		
		Average	55	
		55		

* These rats were observed for two periods of 5 weeks each.

addition of 0.25 % cystine to the diet FL containing unpurified caseinogen was again found to have no effect on weight increase. In Exps. 4 and 5, 0.25 % cystine was added to the diet K, containing the purified Glaxo caseinogen, whilst rats receiving the diet FL, containing unpurified caseinogen, were observed simultaneously as controls. In Exp. 4 a low level of vitamin B₂ was fed and there was a slight improvement in growth, 62 g. against 53 g. in 5 weeks, when the

additional cystine was given. In Exp. 5 where the vitamin intake was higher, there was no significant difference, the increase in weight with the extra cystine being 78 g. in 5 weeks, as against 75 g. In both Exps. 4 and 5 the growth of the rats receiving the Glaxo caseinogen diet supplemented with cystine was as good as that of the rats with the diet containing unpurified caseinogen.

It thus appeared that the purified Glaxo caseinogen diet and the Lister purified caseinogen diet did not contain enough cystine for the growth of young rats, but that this deficiency did not affect the occurrence of dermatitis due to vitamin B₂ deficiency.

C. *Glutathione content of the tissues of rats receiving synthetic diets.*

In most cases in which estimations have been made of the sulphydryl content of the tissues of animals deprived of the B-vitamins, the animals were deprived of the entire vitamin B complex or of vitamin B₁ [Abderhalden and Wertheimer, 1923; Randoïn and Fabre, 1927; 1931; Drummond and Marrian, 1926]. The results have been, in any case, conflicting.

The effect of a diet low in cystine on the sulphydryl content of tissues was also investigated by Abderhalden [1922], who found that tissues from rats on such diets gave a very weak nitroprusside reaction. In this case the animals were supplied with the B-vitamins.

Itter *et al.* [1935] found that rats fed on their experimental diet, containing purified caseinogen, showed variations in the glutathione content of the tissues which bore a relation to the vitamin B₂-containing supplements fed. Thus when no vitamin B₂ was given, the average glutathione content of the blood was 29.4 mg./100 ml. and that of the liver 143 mg./100 g.; when 5% dried yeast was added to the diet, these amounts were increased to 40.0 mg. and 177 mg. respectively. Supplements of autoclaved yeast, of glutathione or of cysteine hydrochloride raised the glutathione level of the liver to the same extent as dried yeast but did not affect that of the blood.

The results which we obtained for the glutathione content of blood, liver and muscle, are given in Table III. They show that the glutathione content of the tissues was not influenced by the variations in the cystine content of the diets. It is possible, however, that had the amounts of cystine fed been still lower

Table III. *Glutathione content of the tissues of rats (ca. 100 g. weight) receiving diets containing varying amounts of cystine, with and without vitamin B₂ (1.0 ml. of autoclaved yeast extract containing enough vitamin to promote 50 g. weight increase in 5 weeks).*

Diet	Cystine content of diet %	Vitamin B ₂	No. of weeks during which diet was fed	Glutathione		
				Blood mg./100 ml.	Liver mg./100 g.	Muscle mg./100 g.
K	0.019	+	6	40, 40, 30	200, 180, 180	40, 40, 30
			13	40, 30	240, 150	30, 30
CK	0.269	+	6	40, 40, 30	190, 190, 160	40, 40, 30
			13	40, 40	170, 170	30, 30
FL	0.062	+	12	40, 40	230, 160	40, 30
			13	40, 30	190, 170	30, 30
			12	40, 40	230, 210	40, 30
Mixed stock diet			—	40, 30	240, 230	50, 30

there might have been a reduction in the glutathione levels, and the fact that Itter *et al.* observed values as low as 20 mg./100 ml. for blood and 100 mg./100 g. for liver, as compared with our lowest values of 30 mg. for blood and 150 mg. for liver, would seem to indicate that their purified caseinogen contained less cystine than did the Glaxo purified caseinogen used by us (see Diet K, Table III).

The two rats totally deprived of vitamin B₂ receiving the adequate cystine diet (FL) showed a glutathione content of the tissues as high as that of the animals receiving the same diet with sub-optimum amounts of the vitamin, or of those receiving an adequate stock diet. Thus, if a diet contained adequate cystine, it did not appear that the vitamin B₂ intake influenced the glutathione content of the tissues. Glutathione determinations could not be made on the tissues of rats receiving a cystine-deficient diet, without any vitamin B₂, owing to the small size of these animals.

DISCUSSION.

The results described above confirm the fact that caseinogen, which in any case is not rich in cystine [Osborne and Mendel, 1915], may be so affected by the extraction and heating processes used during its "purification", that a level of 20% in the diet is no longer adequate for the growth of rats. The glutathione content of the tissues, however, was not found to be affected by a level of cystine intake which was sufficiently low to retard growth.

It may perhaps be noted that Osborne and Mendel found that 15% unpurified caseinogen in the diet was the lowest level which provided sufficient cystine for the normal growth of young rats. It is therefore to be expected that reducing the cystine content of the caseinogen by more than one quarter will produce a cystine deficiency when the caseinogen is fed at a 20% level.

From such results as these however it is impossible to compute accurately the quantitative needs of the rat for this amino-acid. Not only is there no accepted standard method of estimating cystine in biological materials, but as yet there is also no agreement as to the extent to which cystine and cysteine can be replaced by methionine. It has been suggested recently [Brand *et al.*, 1935] that cystine and glutathione are metabolised by a separate path from cysteine and methionine. If this holds true for normal animals as well as for those suffering from cystinuria, on which the experiments were performed, it would seem probable that even cysteine cannot replace cystine in the diet. In our work, which was carried out before the more recent papers on the subject were published, no distinction was made between cystine and cysteine, and methionine was not considered.

Although it must now be recognised that, in many cases, animals fed on diets thought to be complete in every factor except vitamin B₂ were in fact also inadequately supplied with cystine, it does not appear that this cystine deficiency materially affected the syndrome of vitamin B₂ deficiency.

Both cystine and vitamin B₂ are necessary for the growth of rats, and since a number of vitamin B₂-containing substances are also rich in cystine, it is possible that quantitative errors have occurred in vitamin B₂ estimations in which the basal diet employed contained purified caseinogen. It is doubtful however whether these errors would have been large enough to be significant.

The only observation in our work which suggested that vitamin B₂ and cystine might have a supplementary action was the fact that the cystine inadequacy of the purified caseinogen diet (K) was less apparent at the higher levels of vitamin B₂ intake (see Table II). This effect was not due to the addition

of more cystine in increasing doses of autoclaved yeast extract, for the vitamin B₂ preparations used contained varying proportions of cystine. Thus the vitamin B₂-containing dose given in Exp. 5, where the largest amount of vitamin B₂ was fed, contained less cystine (0.22 mg. daily) than the dose given in Exp. 4 (0.30 mg. daily), whilst the growth in Exp. 5 was definitely superior. It was however possible that this result might be explained by the fact that the rats receiving little or no vitamin B₂ ate poorly, whilst those receiving more vitamin B₂ ate more freely. In this way the cystine intake might be increased sufficiently to satisfy the physiological requirements.

Growth, being dependent on so many factors, is at best an unsatisfactory criterion for work of this type, and the absence of correlation between the cystine content of the diet and the incidence of vitamin B₂ deficiency dermatitis disproves any relationship between cystine and vitamin B₂ more clearly than can any observations on growth.

Our results do not agree with those of Itter *et al.* [1935], who found that the skin symptoms developed by their rats receiving a vitamin B₂-deficient diet were cured by the addition of glutathione or cysteine hydrochloride to the diet. Our rats however exhibited an acute inflammatory dermatitis [Goldberger and Lillie, 1926; Chick and Roscoe, 1927; Roscoe, 1933, 1], described by György [1934] as the "specific" type of dermatitis, and by Chick *et al.* [1935] as the florid or (a) type. Rats developing the (b) type described by the latter workers, the "non-specific" type of György, were not included in our observations. The rats observed by Itter *et al.*, however, did not show a florid inflammatory dermatitis, and the alopecia they describe is not specific for vitamin B₂ deficiency and may be caused when rats, fed on any insufficient diet, pluck themselves or each other. Such a condition might well be cured more rapidly when cystine is added to the diet, since sulphur-containing amino-acids are needed for the growth of hair.

In our experiments the failure of large amounts of cystine (CFL diet) to prevent the occurrence of the dermatitis characteristic of vitamin B₂ deficiency would seem to indicate clearly that this type of skin lesion is not connected with cystine metabolism.

Since this work was carried out, vitamin B₂ has been resolved into two factors [Kuhn *et al.*, 1933, 1; 2; *etc.*], one factor being a lyochrome (flavin) and the second known as the supplement or vitamin B₆. In our work, the effects stated to be due to absence of vitamin B₂ were due to absence of the whole vitamin B₂ complex, for it has been shown that the yeast concentrate used in this laboratory as source of vitamin B₁ contains no significant amounts of either flavin or supplement in the doses given. Separate experiments might therefore seem to be needed to show whether the failure of cystine to ameliorate symptoms of vitamin B₂ deficiency was due to the simultaneous need for another component of the complex. But it is obvious that cystine cannot take the place of either the flavin or the supplement, for Chick *et al.* [1935] have produced symptoms both of flavin deficiency and of supplement deficiency in rats fed on diets containing the unpurified "Light white casein" shown here to contain adequate amounts of cystine.

SUMMARY.

1. Confirmation has been obtained of the fact that some specimens of purified caseinogen, used in basal diets for vitamin work, are deficient in cystine.

2. When vitamin B₂ deficiency was complicated by a deficiency of cystine, to such an extent that growth was affected, the incidence of dermatitis was not influenced.

3. No relation has been found between cystine and the vitamin B₂ complex in the nutrition of the rat.

4. Within the limits observed, the cystine intake of rats was not found to influence the glutathione content of the tissues.

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