CCII. THE BIOLOGICAL VALUES OF PROTEINS. II. THE BIOLOGICAL VALUE OF PURIFIED CASEINOGEN AND THE INFLUENCE OF VITAMIN B_2 UPON BIOLOGICAL VALUES, DETERMINED BY THE BALANCE SHEET METHOD.

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In 1909 Thomas introduced the term biological value of protein to express the relative value of different proteins for compensating the daily nitrogenous loss, and defined this as the number of parts of body nitrogen replaceable by 100 parts of the nitrogen of the foodstuff [1909]. He suggested three formulae, differing as regards the treatment of the nitrogen excreted in the faeces, by which to calculate this value from the data of experiments on the nitrogenous exchange. Since that time determinations of the biological values of many proteins have been made by various observers (McCollum [1911, 1914] on the pig; Martin and Robison [1922] on man; Mitchell and his co-workers [1924, 1926, 1927] on the rat; Wagner [1923] on children; and others). The results of these workers show a wide range of variation. For instance, the biological value of the proteins of cow's milk has been variously assessed as 51, 74, 85, 93 and 100 and that of oat protein as 47, 65 and 79.

The provision of adequate amounts of vitamin B in the experimental diet presented serious difficulties to the earlier workers on the subject. Since all the known rich sources of this vitamin were substances rich in nitrogen, as, for example yeast, investigators were faced with the alternatives either of giving a diet deficient in vitamin B, or of adding to the diet appreciable amounts of nitrogen of unknown biological value, and thus decreasing the accuracy of their results. In many experiments the only source of the water-soluble B vitamin in the diet was the protein under investigation. Some proteins would contain considerable amounts of this factor while from others it would be almost entirely absent. In the latter case difficulty would be experienced in maintaining appetite sufficient to prevent the intake of calories falling below the required minimum. A further complication came to light in recent years with the discovery that there are at least two water-soluble B vitamins, having different functions in the body and, in some cases, a different distribution in nature. Mitchell and Carman [1924] attacked the problem of the supply of these vitamins by giving the rats which formed the subject of

their experiments, a daily dose of yeast vitamin-Harris powder (a preparation made from yeast and containing both the B vitamins). The daily ration contained 2·3 mg. of nitrogen.

The work of Kinnersley and Peters [1925, 1927] and of Chick and Roscoe [1927, 1928] has shown that concentrates of these vitamins can be prepared from yeast, with a nitrogen content considerably lower than that of the yeast vitamin-Harris powder. Hence arose the decision to attempt a redetermination of the biological values of proteins by means of metabolism experiments in which the use of such preparations would ensure an adequate supply of both the B vitamins. Rats were selected as the experimental animals. Mitchell has elaborated and tested a technique for use with these animals and has shown that consistent results can be obtained. The method adopted here, however, was that used by Korenchevsky (unpublished results) and Chick and Roscoe [1930].

Purified caseinogen was the first protein selected. There are not many previous determinations of the biological value of this protein. Michaud [1909] fed a dog on a daily ration of caseinogen equivalent to its total daily output of nitrogen on a nitrogen-free diet and obtained a negative balance. Thomas [1909, 1910] gave caseinogen a value of 70 as the result of experiments on an adult human being. McCollum in experiments on pigs in which positive balances of nitrogen were recorded, obtained a value for caseinogen of 67, while the figure given by Mitchell [1924, 2, 4] as the result of experiments on rats was 71 and 75. Martin and Robison [1922], however, gave whole milk proteins a value of only 51.

Since the purified caseinogen (see below) used in this laboratory is devoid of the B vitamins, experiments to determine the biological value of this protein offered a good opportunity to investigate the influence of vitamins B_1 and B_2 on nitrogen metabolism. Previous work on this subject by Karr [1920] was carried out before the dual nature of vitamin B was known. A dog, fed on a diet containing a large amount of caseinogen but no source of vitamin B, maintained a small positive balance of nitrogen for some weeks. When yeast was added to the diet the retention of nitrogen was increased. In similar experiments on two other dogs the ingestion of yeast changed a negative balance of nitrogen into a positive one. Karr attributed these results to the biological value of the yeast protein rather than to the vitamin content of the yeast.

Studies on the distribution of vitamin B_2 have shown it to be usually associated with proteins of good biological value such as meat, milk and eggs, rather than with those of low biological value, such as cereals and pulses [Aykroyd and Roscoe, 1929]. This suggested the possibility that the presence of vitamin B_2 might be essential to secure an economical use of ingested nitrogen. Discrepancies between the results obtained by different investigators might be due to differences in the vitamin B_2 content of their diets, and it was conceivable that differences in the biological values of proteins might ulti-

mately prove to be related, in many cases, to variations in vitamin B_2 content rather than to essential differences in protein constitution.

An investigation was therefore planned in which the biological value of purified caseinogen at different levels of intake would be determined in the presence and absence of vitamin B_2.

Experimental.

General plan of experiments.

Two series of experiments were carried out on adult male rats of 350–450 g. wt. In Series 1 rats 1, 2, 3 and 4 were used, and in Series 2 rats 2, 3, 5, 6, 8 and 9. Each experiment involved one pair of rats treated as a single unit in one metabolism cage. The basal nitrogen expenditure was first determined using the "nitrogen-free" diets described below. In the succeeding experiments the same diets were used with added caseinogen in proportions varying from 4 to 16 % of the total dry diet. In half the experiments of each series a daily ration of a watery yeast extract (fraction C [Chick and Roscoe, 1929]), which contains both members of the water-soluble B complex, was administered; in half, Peters's antineuritic concentrate prepared from yeast [Kinnersley and Peters, 1925, 1927], containing vitamin B_1 only, was given. The experiments lasted from 4 to 5 days except in the case of those on the nitrogen-free diet, where, owing to the greater tendency to lose weight, it was thought wiser to terminate them on the third day. In all cases there was a pre-experimental period of 2 days in which the experimental diet and dose were ingested but no analyses were made. Between each experiment in Series 2 the rats were allowed to "rest" for at least 7 days during which time they received a diet containing 16 % of "light white" caseinogen. On each day except the last they also received 1 g. of dried yeast. In Series 1 the length of the "rest" depended on the nature of the preceding experiment.

Materials used.

The caseinogen was prepared from "light white casein" (British Drug Houses), a sodium caseinogenate manufactured by the method of Hammarsten. It was purified by re-precipitation with acetic acid and frequent washing by decantation with 0·05 % acetic acid. It was then extracted with dilute acidified alcohol for 96 hours in a Soxhlet apparatus, dried at a low temperature and finally roasted for 3 days at 120°. Caseinogen prepared by this method contains no B vitamins [Chick and Roscoe, 1928]. The nitrogen content on the dry weight was 14·81 %.

The fraction C, prepared from yeast extract [Chick and Roscoe, 1929] had a sp. gr. of 1·158 and a nitrogen content of 0·278 % by volume. The daily ration of 0·5 cc. per rat contained 1·4 mg. of nitrogen.

The sample of Peters's vitamin B_1 concentrate from yeast had a sp. gr.

of 1·049 and a nitrogen content of 0·633 % by volume. Each rat received 4 drops daily containing 0·7 mg. of nitrogen.

I am indebted to Dr Chick and Miss Roscoe for supplying me with these materials.

Diets. The "nitrogen-free" diets used in Series 1 and 2 respectively were constituted as follows.

Nitrogen-free diet. Series 1:

Corn starch	735 g.
Sugar	60
"Hardened" arachis oil	130
Cod-liver oil	20
Salt mixture	50
Calcium carbonate	8

This diet contained 0·0297 g. nitrogen and 500 kg. Calories per 100 g. of dry weight. As this diet was not well taken in all cases, certain modifications were introduced to make it more palatable. These consisted of an increase in the proportion of sugar and of the substitution of beef dripping for the arachis oil. The modified diet had the following constitution:

Nitrogen-free diet. Series 2:

Corn starch	735 g.
Sugar	90
Clarified beef dripping	100
Cod-liver oil	20
Salt mixture	50
Calcium carbonate	8

This diet contained 0·0279 g. of nitrogen and 380 kg. Calories per 100 g. of dry weight.

Technique of the metabolism experiments.

The main features of the technique are described in the preceding paper [Chick and Roscoe, 1930]. In Series 2 an improvement was introduced into the method of measuring the nitrogen intake. The procedure of estimating the nitrogen content in the basal diet after mixing the caseinogen with the other constituents of the diet was abandoned owing to the difficulty of ensuring a sufficiently even mixture for correct sampling. The following procedure was substituted. A large quantity of the nitrogen-free diet was prepared without the addition of water, and samples were taken for analysis. This air-dry diet was kept in a tin in the refrigerator room, the diet bottle for each experiment being filled from this store. The purified caseinogen was dried at 110° for 24 hours and transferred to a heated weighing bottle. After cooling in a desiccator this was weighed. When the daily ration of the nitrogen-free food was weighed out from the diet bottle into the daily ration pots, the approximate amount of caseinogen needed to give the required protein content

to the diet was added from the weighing bottle. The diet and the caseinogen were well mixed in the daily ration pots with enough water to give a liquid paste. At the end of the experiment the weighing bottle, containing the unused caseinogen, was heated, cooled and weighed as before, the difference between the two weighings giving an accurate measure of the amount of caseinogen removed. The diet bottle was also weighed before and after the experiment to arrive at the amount of basal diet consumed. Any residue remaining in the daily ration pots was collected and analysed for nitrogen, in order to make allowance for the unconsumed nitrogen. After experience of the animal's appetite it could be usually arranged that this amount was small and insignificant.

Calculation of the biological value of a protein.

Martin and Robison [1922] and Mitchell [1924, 1, 3] have discussed at length the various problems involved in the calculation of the biological value of a protein from the data provided by experiments in which the nitrogen balance is determined. The method of calculation used here is based on the work of these observers, their methods being in turn based on the formulae of Thomas [1909, 1910].

Martin and Robison showed that the situation could be expressed on a graph (Fig. 1) in which the abscissae represent real nitrogen intake (x) and

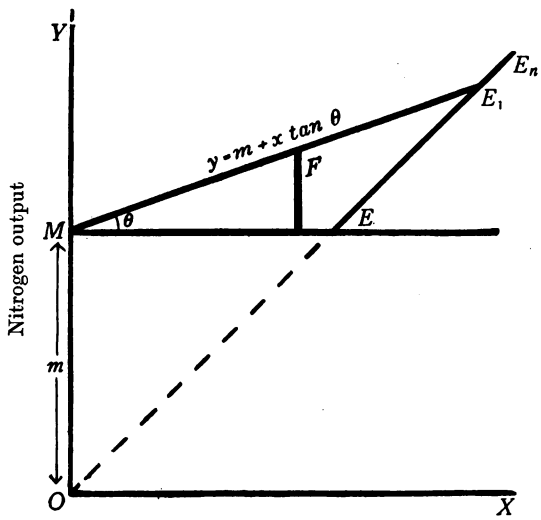

Fig. 1.

the ordinates real nitrogen output (y); m ($= OM$) is the output on a nitrogen-free diet. The formula used here for calculation of biological values is based on that of Mitchell but has two modifications. To express output, the "true" output (*i.e.* urine nitrogen + endogenous faecal nitrogen) is used instead of urine nitrogen alone, as it is thought that the endogenous faecal nitrogen should be regarded as forming part of the basal nitrogen expenditure. Secondly no

correction is made in the estimation of the endogenous urine nitrogen to allow for the variations in body weight of the rats, as such a correction is not applicable to experiments with adult rats where variations in body weight will be related to alterations in the amount of body fat and will not therefore be associated with variations in basal nitrogen expenditure.

The formula of Mitchell,

$$\text{B.V.} = 100 \times \frac{\text{nitrogen retained}}{\text{nitrogen absorbed}},$$

is therefore modified thus:

$$\text{B.V.} = 100 \times \frac{\text{true intake} - (\text{true output} - \text{output in nitrogen-free experiment})}{\text{true intake}}.$$

From the graph of Martin and Robison (Fig. 1) this becomes:

$$\text{B.V.} = 100\frac{x-(y-m)}{x}.$$

Since from the graph $y = m + x \tan \theta$,

$$\text{B.V.} = 100\frac{x-(m+x\tan\theta-m)}{x} = 100\,(1 - \tan\theta).$$

This is the same equation as that deduced by Martin and Robison from Thomas's formula B.

The method of calculating the biological values of proteins used in these experiments was, therefore, as follows.

From the faecal nitrogen of the nitrogen-free experiments, the endogenous faecal nitrogen per 100 g. of dry food ingested was calculated for each set of rats [see Mitchell, 1924, 1]. This figure was used in each experiment to determine the endogenous and exogenous fractions of the total faecal output. The endogenous faecal nitrogen was added to the total urinary nitrogen to obtain the figure for the true output, while the exogenous portion, representing unabsorbed food nitrogen was subtracted from the total nitrogen ingested to obtain the true intake. The nitrogen of the yeast concentrates was, contrary to the procedure of Mitchell, included in the total nitrogen intake. The figures for true intake and true output were used to plot the point F on the graph, m having been already plotted from the intake and output in the nitrogen-free experiment. Tan θ was measured and the biological value calculated from the equation B.V. $= 100\,(1 - \tan\theta)$.

Discussion of experimental results.

In those experiments in which vitamin B_1 only was supplied considerable difficulty was experienced in maintaining the intake at a sufficiently high level, for the rats showed a marked loss of appetite within 48 hours of deprivation of vitamin B_2. The rapidity with which this loss of appetite appeared is consistent with the inability of the rat to store large reserves of this vitamin [Chick and Roscoe, 1927]. When the total calorie intake falls below a certain figure, the animal will use some of the protein of the diet to supply energy rather than to replace body nitrogen. Under these circumstances the bio-

Table I. *Details of the experiments in which an adequate intake of calories from fat and carbohydrate combined was secured. The figures given represent average daily values for one rat.*

Exp. No.	Rats No.	Date	B vitamins given	Amount of protein in diet % of dry weight	Non-protein intake of calories per kg. of body weight Cals.	Average body weight g.	Change in body weight, as % of initial body weight	Intake of nitrogen		Faecal nitrogen			Nitrogen in urine mg.	Output of nitrogen		Balance of nitrogen mg.	Biological value $100\times(1-\tan\theta)$ using true output and true intake	Biological value $100\times(1-\tan\theta)$ using urine nitrogen and true intake
								Total mg.	Total mg.	Total mg.	Endogenous mg.	Exogenous mg.		Total mg.	True mg.			
Series 1:		1929																
1_3	2 and 4	May 22–25	B_1 and B_2	0·2 "N-free"	150	310	−1·3	4	4	17	17	—	65	82	82	−78	—	—
9_3	1 and 3	Aug. 20–24	B_1 and B_2	5·6	125	294	−0·8	75	74	19	18	1	101	120	119	−45	**49**	49
9_5	2 and 4	,, 20–24	B_1	5·6	130	320	−1·5	84	78	23	17	6	106	129	123	−44	**45**	45
7_3	2 and 4	,, 13–17	B_1 and B_2	4·4	170	331	−0·4	90	87	26	23	3	108	134	131	−44	**41**	48
4_3	1 and 3	July 1–5	B_1 and B_2	7·7	140	294	−0·5	122	116	27	21	6	124	151	145	−29	**44**	43
6_5	2 and 4	July 29–Aug. 2	B_1 and B_2	15·4	142	299	+5·0	278	260	38	20	18	204	242	224	+36	**45**	45
5_3	1 and 3	July 23–27	B_1 and B_2	15·4	145	294	+6·0	282	274	32	24	8	196	228	220	+54	**49**	51
Series 2:																		
10	2, 3, 5, 6, 8 and 9	Nov. 18–21	B_1 and B_2	0·2 "N-free"	185	402	−4·0	7	7	21	21	—	71	92	92	−85	—	—
11_2	5 and 8	Dec. 1–7	B_1	5·6	130	417	−4·0	155	113	65	23	42	128	193	151	−38	**45**	47
11_3	3 and 6	,, 1–7	B_1	5·6	130	415	−2·0	160	126	57	23	34	133	190	156	−30	**47**	48
11_7	2 and 9	,, 1–6	B_1 and B_2	5·6	150	425	−1·0	190	153	62	25	37	145	207	170	−17	**46**	49
12_3	3 and 6	,, 15–20	B_1 and B_2	7·2	135	413	−3·0	206	160	70	24	46	144	214	168	− 8	**51**	52
8_{3a}	5 and 8	Aug. 26–29	B_1 and B_2	12·9	130	413	+2·0	278	255	41	17	24	214	255	231	+24	**43**	44
8_3	5 and 8	,, 19–23	B_1 and B_2	12·9	145	410	−1·0	304	284	38	18	20	240	278	258	+26	**40**	39
																Mean biological value	**45**	47

logical value will be underestimated. The experiments described here indicate that the use of protein for fuel is not significant if the calorie intake from fat and carbohydrate combined is greater than about 125 kg. Calories per day per kg. of rat. (The calculations of calorie intake are only approximate.) All experiments in which it falls below this figure have, therefore, been rejected as unreliable for calculating the biological value of the protein.

Table I gives details of six experiments in Series 1 and six experiments in Series 2 in which the calorie intake was regarded as satisfactory. The figures for the experiments with nitrogen-free diets are given at the head of each Series. In Series 1 the experiment with a nitrogen-free diet on rats 2 and 4 is not used in the calculation, as vitamin B_1 only was given. As a result of loss of appetite, the calorie intake became too low and the loss of body weight too high for the figures to be trustworthy. As the two sets of rats behaved similarly in other experiments it was thought best to calculate the biological value in the experiments on rats 2 and 4 from the values obtained in the experiment with a nitrogen-free diet on rats 1 and 3.

In Series 2 biological values are calculated using the average figures derived from the nitrogen-free experiments on the three pairs of rats, as the individual figures obtained from the experiment with nitrogen-free food on rats 2 and 9 and that on rats 3, 5, 6 and 8, were almost identical.

In the last column but one of Table I are given the biological values of purified caseinogen as calculated from the results of each experiment. These range from 40 to 51 with a mean value of 45. Four experiments are included in which a positive nitrogen balance was obtained.

In Table II are collected all the experiments rejected on account of low

Table II. *Some details of the experiments rejected on account of insufficient calorie intake.*

Exp. No.	Rats No.	Date	B vitamins given	Amount of protein in diet % of dry wt.	Non-protein intake of calories per kg. of body weight Cals. per day	Average body weight g.	Change in body weight as % of initial body weight per day	Biological value 100× (1 − tan θ) using true output and true intake
Series 1:		1929						
1_3	2 and 4	May 22–25	B_1 and B_2	0·2 "N-free"	150	310	−1·3	—
7_5	1 and 3	Aug. 13–17	B_1	4·4	100	302	−1·2	29
4_5	2 and 4	July 1–5	B_1	7·7	120	309	−1·1	29
5_5	2 and 4	,, 23–27	B_1	15·4	80	310	−1·3	27
6_3	1 and 3	July 29–Aug. 2	B_1 and B_2	15·4	105	293	−0·5	42
Series 2:								
10	2, 3, 5, 6, 8 and 9	Nov. 18–21	B_1 and B_2	0·2 "N-free"	185	402	−4·0	—
12_2	5 and 8	Dec. 15–20	B_1	6·5	105	407	−1·3	40
12_7	2 and 9	,, 15–20	B_1 and B_2	6·7	115	419	−0·9	37
		1930						
13_3	3 and 6	Jan. 12–17	B_1	11·3	75	426	−0·8	31
13_2	5 and 8	,, 12–17	B_1 and B_2	12·4	100	408	−0·4	36

calorie intake. It will be seen that, as was anticipated, these have, with two exceptions, 12_2 and 6_3, biological values of less than 40.

In the last column of Table I are shown the biological values obtained when the calculations are made using urine nitrogen in place of true output, as is done by Mitchell. It will be seen that the values obtained by this method do not show significant differences from those obtained by the other method, the mean values being respectively 47 and 45.

The influence of vitamin B_2 on the biological values of proteins.

An examination of these results shows that vitamin B_2 does not itself exert an influence on the economical use of ingested nitrogen. In Table I there are three experiments in which the animals received no vitamin B_2, but the estimates of biological value (45, 47 and 46) are not lower than those of the experiments in which both vitamins were supplied. In Table II, on the other hand, there are three experiments in which, in spite of the ingestion of both B vitamins, the calorie intake was low, and in these the biological values were also low.

It was originally intended to investigate the influence of vitamin B_1 on nitrogen metabolism as well as that of vitamin B_2, but so rapid is the decline in appetite when the first factor is removed from the diet that it has not so far been possible to secure a sufficiently high intake to give trustworthy results.

Biological values at different levels of intake.

Martin and Robison [1922] questioned on theoretical grounds the assumption of Thomas that the biological value of a protein would be a constant for different levels of protein intake. If it were not constant, ME_1 in their graph (Fig. 1) would be curved. They found, however, that in the case of wheat protein ME_1 did indeed appear to be a straight line. The results obtained in this paper give no evidence of a variation of biological value with variations in the level of protein intake when caseinogen is used. The validity of the equation $\text{B.V.} = 100\,(1 - \tan\theta)$ is based on the assumption that ME_1 is a straight line. Confirmation of this is given by the fact that the biological values calculated by means of this equation for levels of caseinogen intake varying from 4 to 16 % of the diet show no wide range of variation.

Mitchell [1924, 2] determined the biological values of a number of proteins when the intake represented 5 % and 10 % of the diet, respectively. With the higher percentage of protein in the diet, though there might not be increased intake of nitrogen, the values obtained were consistently lower. He attributed this in part only to variations in biological value at different levels of intake. Since in his first set of experiments the animals were stationary in weight, while in the second they were growing, he also suggested that the biological value of a protein for growth was different from the value when maintenance only was required. The experiments described in this paper do

not support this theory. In those experiments in which the rats were increasing in weight the average biological value was 46, while the average figure for the entire series was 45. It is to be noted, however, that the rats used in these experiments were slowly-growing adults, whereas those used by Mitchell were young and growing rapidly.

The biological value of 46 for caseinogen is much lower than those determined by McCollum and Mitchell, 67 and 71 respectively. This may be due to differences in technique, or possibly to a higher purity in the sample of caseinogen used. Contamination with lactalbumin would, of course, increase the apparent biological value of the caseinogen.

Summary.

1. The biological value of purified caseinogen has been redetermined by means of 12 metabolism experiments on adult male rats. In these experiments the B vitamins were supplied by means of concentrates containing only small amounts of nitrogen—an improvement on the technique previously available.

2. The formula used here to calculate the biological value of a protein, B.V. $= 100\,(1 - \tan\theta)$, is based on the work of Thomas, Martin and Robison, and Mitchell.

3. The biological value of purified caseinogen was found to be 45, a figure considerably lower than that found by previous workers.

4. In experiments, in which the intake of calories from fat and carbohydrate combined fell below a certain value, the use of ingested protein for fuel is at once indicated by the underestimation of the biological value.

5. The absence of either vitamin B_1 or B_2 from the diet caused a decline in appetite within 48 hours. So great was the decline in appetite in the absence of vitamin B_1 that the intake of calories was too low to allow reliable figures to be obtained for the calculation of biological values.

6. The absence of vitamin B_2 from the diet does not appear to prevent the economical use of ingested nitrogen, provided the calorie intake from fat and carbohydrate is adequate.

7. There is no evidence in these experiments either of variations in biological value at different levels of intake or of the existence of different biological values for growth and maintenance.

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