CCIII. THE BIOLOGICAL VALUE OF PROTEINS.

VI. FURTHER INVESTIGATION OF THE BALANCE SHEET METHOD.

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DETERMINATION OF ENDOGENOUS NITROGEN EXCRETION.

IN attempts to compare the nutritive value of different proteins by their relative power to maintain nitrogenous equilibrium, a trustworthy estimate of the daily loss of endogenous nitrogen, for the particular experimental animal employed, is a primary requirement. The usual method is to determine the nitrogen excreted in the urine and faeces, when the diet consumed is as far as possible free of nitrogen and consists only of carbohydrate and fat, with the addition of the necessary minerals and vitamins.

In the course of a long series of experiments, in which the biological value of different proteins has been studied by the "balance sheet method", the variability in the endogenous nitrogen excretion shown by the same animal at different times has been a disturbing factor, and attempts to achieve a greater degree of constancy have not been altogether successful. The method used, employing the adult male rat as experimental animal and the formula adopted for calculating the biological value of the protein under examination have been previously described [Chick and Roscoe, 1930; Boas-Fixsen, 1930; Boas-Fixsen and Jackson, 1932].

The formula used is derived from the original expression of Thomas [1909; 1910] as follows:

The relative biological value of a protein X is expressed as:

$$\begin{aligned} & 100 \times \frac{\text{Body nitrogen saved}}{\text{Food nitrogen absorbed}} \\ &= 100 \times \left\{ 1 - \frac{(\text{N excreted in experiment with } X) - (\text{N excreted on N-free diet})}{\text{True nitrogen intake in experiment with } X} \right\} \\ &= 100 \times \left\{ 1 - \frac{U_x + F_x - (F_x - F_e) - (U_e + F_e)}{I_x - (F_x - F_e)} \right\} \\ &= 100 \times \left\{ 1 - \frac{U_x - U_e}{I_x - (F_x - F_e)} \right\} \quad \dots\dots(1) \end{aligned}$$

where I_x , U_x and F_x are the daily N intake, urinary N excretion and faecal N excretion, respectively, in the experiment with X ,

and U_e and F_e are the daily (endogenous) N excreted in urine and faeces, respectively, as determined in experiments on a nitrogen-free diet.

$F_x - F_e$ will represent the nitrogen in the faeces derived from undigested food and $I_x - (F_x - F_e)$ the true N intake, in the experiment with protein X .

Any irregularity in the values obtained for U_e will affect the value of the above expression (1) more seriously when U_x and I_x are relatively small, i.e. when the protein investigated is present at a low level in the diet. In our experience a greater divergence has been found in the results of experiments with diets containing 3 % protein, than when 10 % was present.

Duration and arrangement of experiments.

In the past the experiments have been so arranged that each observation has lasted 4 days, with daily collection of excreta and washing of the metabolism cage. When a "nitrogen-free" diet was fed, there also was a preparatory period of one day on a low nitrogen diet, followed by 2 days on a "nitrogen-free" diet; with protein-containing diets there were two preliminary days with the diet in question before analyses were made. The rats were used for experiment in alternate weeks and during the intervening "rest" weeks they received a synthetic "complete" diet (CB) which contained 18 % protein in the form of caseinogen; this was sometimes supplemented with liver or other constituents of the ordinary stock diet during the earlier part of the rest period.

One difficulty in securing satisfactory performance during the experimental periods, especially when nitrogen-free or low protein diets were fed, is the maintenance of appetite, so that the amount eaten daily may remain constant during the 4 days of the experiment at a level sufficient to supply all energy needs from the carbohydrate and fat of the diet. We have found it a good plan to limit the consumption both of the "CB" diet in the period immediately before the experiment, and of the experimental diet during the preparatory period, to the amount that will just, and only just, maintain weight, *i.e.* about 11 to 14 g. dry food daily (all diets being arranged to contain 4 Cals. per g. from the fat and carbohydrate constituents). If any surfeit is avoided in these preparatory periods, the appetite is more likely to be maintained during the 4 days of the experiment. It has also been found advantageous to place the rat in the metabolism cage during the preliminary 2-3 day period on the experimental diet, as the change of cage may also affect the appetite. Since these precautions have been adopted, satisfactory experiments have been found easier to achieve.

The "nitrogen-free" diet used in these experiments was constituted as follows: corn starch 735, clarified beef dripping 130, salts (McCollum mixture No. 185) 50, cod-liver oil 20, and CaCO_3 8. Its nitrogen content varied from 0.02 to 0.04 %. Each rat in addition received concentrates from yeast extract containing the B vitamins, the nitrogen in these amounting to 2.4 mg. per rat per day. Thus the total daily intake of nitrogen from these sources for a rat eating 12-16 g. "nitrogen-free" diet was 6-7 mg.

Endogenous urinary nitrogen.

There appeared to be some doubt whether the 2-3 day preparatory period was long enough to eliminate the effects of the preceding diet, especially when the change was made from a high protein to a "nitrogen-free" diet, as in experiments made to determine the endogenous nitrogen excretion. Ashworth and Brody [1933] found that the urinary nitrogen per kg. body weight excreted by rats living on a nitrogen-free diet did not reach a minimum value until 10-15 days after the beginning of the diet and that thereafter the daily variations were large. Roche [1933] obtained similar results; in some cases the endogenous urinary nitrogen per unit body weight fell continuously until a sudden rise occurred, immediately preceding the death of the animal from nitrogen starvation. Mitchell *et al.* [1922] found a rapid fall in endogenous urinary nitrogen during the first day on a "nitrogen-free" diet and thereafter a rough constancy with 2 out of 4 rats, but a slow decrease with the other two.

In order to ascertain whether in our experiments the urinary nitrogen output did further decrease after the preliminary period, a series of experiments was performed in which collections of excreta and analyses were made during

Table I.

Average daily excretion of N of rats maintained on "N-free" diet for 10-14 days, no analyses being made during first 3 days (usual preparatory period).

Rat No.	Date	Average daily N excretion (mg. N)					
		Days 4-7		Days 8-10		Days 11-14	
		Urine	Faeces	Urine	Faeces	Urine	Faeces
58	12. vi. 33	63.2	15.7	62.3	18.6	—	—
59	10. vii. 33	92.8	15.8	81.2	21.0	—	—
62	*19. vi. 33	101.1	20.6	83.4	21.9	—	—
	10. viii. 33	82.0	26.7	73.5	21.7	—	—
63	12. vi. 33	84.0	18.4	69.7	19.0	—	—
64	*19. vi. 33	85.3	18.0	72.7	21.3	—	—
	8. viii. 33	73.6	21.7	91.1	16.7	—	—
66	*12. vi. 33	88.2	14.6	82.0	16.9	—	—
72	1. v. 34	86.7	19.6	No analyses made		70.7	16.2
78	1. v. 34	104.5	19.7			85.9	21.0
77	11. v. 34	†69.1	†17.7	63.3	17.0	—	—
87	11. v. 34	†51.7	†21.2	50.2	20.1	—	—

* Average from experiment in Table II.

† Average of 3 days only.

Fig. 1. Urinary and faecal nitrogen excretion, food intake and body weight of rats 72 and 78 during 14 days of "N-free" diet. Average daily nitrogen intake 5.2 mg. (approx.).

Table II.

Nitrogen excretion of rats on "N-free" diet; results of daily analyses of excreta.

Rat No.	Day of experiment ...	1	2	3	4	5	6	7	8	9	10	11	12	Body weight	
														At beginning of exp. period g.	Average daily loss during exp. period g.
62	mg. N per day in {	155.2	109.6	94.8	92.5	84.0	78.5	73.2	81.8	69.6	69.1	—	—	468	3.3
	Urine	33.5	30.7	20.1	30.4	—	28.1	26.7	18.1	21.9	25.2	—	—		
	Dry food eaten (approx.) (g.)	14.0	15.7	15.2	15.8	16.4	16.2	16.4	15.6	15.2	15.9	—	—		
64	mg. N per day in {	*	104.3	70.4	72.7	60.6	78.1	82.9	86.4	91.2	84.7	68.6	78.2	481	5.1
	Urine		33.4	28.0	26.1	21.1	21.0	18.5	13.5	20.2	16.5	21.9	17.7		
	Dry food eaten (approx.) (g.)	15.3	16.2	17.1	17.7	15.2	13.8	10.6	12.7	11.2	12.6	13.9	12.2		
66	mg. N per day in {	177.8	117.0	91.4	99.4	87.4	91.6	86.1	72.7	—	—	—	—	449	5.4
	Urine														
	Dry food eaten (approx.) (g.)	15.9	16.5	13.7	11.7	10.2	9.4	10.1	15.7	—	—	—	—		
91	mg. N per day in {	*	—	80.1	88.4	81.4	111.9	94.0	—	—	—	—	—	431	4.5
	Urine			24.3	24.2	20.8	17.1	26.8	—	—	—	—	—		
	Dry food eaten (approx.) (g.)	12.1	12.5	13.0	13.0	14.4	9.3	14.0	—	—	—	—	—		
93	mg. N per day in {	*	105.3	64.0	67.5	85.9	65.8	76.9	—	—	—	—	—	336	2.5
	Urine		22.4	21.2	20.5	18.8	20.5	20.1	—	—	—	—	—		
	Dry food eaten (approx.) (g.)	14.0	14.0	14.0	14.9	14.9	14.9	14.4	—	—	—	—	—		

Corresponds to "preparatory" period

Corresponds to usual 4-day "experimental" period

* On low nitrogen diet, see p. 1703.

successive periods of 4 and 3 days after the preliminary period had elapsed. The results are collected in Table I and, in 9 cases out of 12, show a significant lowering of the urinary nitrogen excretion during the second period. The results of two of these experiments, with rats 72 and 78, are illustrated graphically in Fig. 1. These two observations show great similarity in the amount and constancy of the daily food intake, in the rate of fall in urinary nitrogen and in body weight over the two periods investigated and in the constancy of the amount of the faecal nitrogen.

In a further series of tests the daily collections of excreta were analysed separately over periods the duration of which was extended to 7 or 12 days. The results of five such observations on a nitrogen-free diet are given in Table II. In these experiments the daily food intake was maintained fairly constant throughout the observations. It will be seen that the rapid fall in urinary nitrogen which took place during the first 2-3 days (corresponding to the preparatory period of the ordinary test) was afterwards checked; the fall was then continued more slowly in 2 cases (rats 62 and 66), whilst a rough constancy was achieved in 3 cases (rats 64, 91 and 93). The daily variations were large, as was also found by Ashworth and Brody, and, in some cases, this probably masked the gradual fall in urinary nitrogen excretion usually shown in the longer experiments. On the whole the results of the experiments set out in Tables I and II support the view held by Ashworth and Brody [1933] and others, that the excretion of endogenous nitrogen in the urine does tend to diminish with the length of time that the rat is fed on a diet devoid of nitrogen. They also show that the preliminary period of 2-3 days, which elapsed before the experiment proper was made, was long enough to include the initial rapid fall in nitrogen excretion.

A further series of similar experiments was made with diets containing various percentages of protein, to determine whether a gradual decrease in urinary nitrogen excretion also occurred when protein was present in the diet. Table III shows the results of experiments with diets containing 5 and 7 %

Table III.

Average daily excretion of N of rats maintained on a protein-containing diet for 9-10 days, no analyses being made during first 2 days (usual preparatory period).

Protein in diet	Rat	Date	Average daily N excretion (mg. N)					
			Days 3-6		Days 7-9		Days 7-10	
			Urine	Faeces	Urine	Faeces	Urine	Faeces
5 % heated caseinogen	58	17. vii. 33	123.8	24.0	—	—	99.4	24.0
	63	12. vii. 33	113.0	23.1	99.7	22.7	—	—
	66	17. vii. 33	121.5	23.7	—	—	102.6	23.7
7 % heated lactalbumin	58	1. vii. 33	105.6	58.8	102.4	61.3	—	—
	66	1. vii. 33	102.0	60.9	84.3	56.3	—	—

caseinogen and lactalbumin, respectively, which were continued for 7-8 days after the preliminary period. The excreta collected during the two divisions of the experimental period were analysed separately though this decrease was sometimes only small. In all cases there was a lowered excretion of urinary nitrogen during the second period, though the decrease was sometimes only small. A few experiments were performed with diets containing 3 and 10 % caseinogen, in which daily analyses of excreta were made for 4 days only, following the preliminary period of 2 days (see Table IV). They show that the 2-day period was long enough to include the first rapid fall in urinary nitrogen,

Table IV.

Daily excretion of nitrogen in urine of rats receiving diets containing 3 and 10 % caseinogen.

Protein in dry diet %	Protein used	Rat No.	Daily excretion of N in urine (mg.)			
			3rd day	4th day	5th day	6th day
3	"Light white casein"	77	113.7	109.3	90.3	91.5
"	Heated caseinogen No. 2	94	67.0	72.6	78.4	69.4
"	Heated extracted caseinogen	93	106.3	96.7	89.9	102.0
"	"	91	85.9	96.2	96.7	86.7
"	"	94	85.7	81.6	77.4	85.1
10	"Light white casein"	74	161.6	139.5	137.8	158.6
"	"	77	149.9	176.9	158.0	137.4
"	"	78	159.7	173.7	161.1	150.7
"	Heated caseinogen No. 2	72	178.0	109.0	215.8	127.1

but thereafter the daily fluctuations were rather great and the experiments were of too short a duration to show whether the urinary nitrogen might not have continued to fall, if they had been continued longer. The results given in Table III with the 5 and 7 % protein diets do suggest, however, that with low proportions of protein in the diet there is a continued gradual drop in the urinary nitrogen excretion, similar to that observed with a nitrogen-free diet. This may be due to a lag in attaining equilibrium or to a diminution in the rate of nitrogenous metabolism induced by the experimental conditions. At any rate, since this drop occurs on both the nitrogen-free and low protein diets, it cannot greatly affect the calculation of the "biological value".

The estimated endogenous urinary nitrogen (as required for the calculation of biological value of proteins by the balance sheet method) must be regarded as a somewhat arbitrary quantity. It seems fair to conclude, however, that the data obtained from the analysis of the excreta collected during 4 days, after a preliminary period of 2-3 days, does give a fairly good estimate of the endogenous urinary N output when a nitrogen-free diet is fed, and of the total urinary N output when a low protein diet is fed.

Endogenous faecal excretion. Relation to food intake.

In the observations set out in Tables I and II the relative constancy shown in the endogenous faecal N excretion during the experimental periods is in contrast to the gradual fall in that excreted in the urine.

Mitchell [1924] found that for any given animal the amount of endogenous faecal nitrogen was proportional to the food intake. This relation did not appear to hold in the observations of Boas-Fixsen and Jackson [1932]. Schneider's [1934] explanation of this discrepancy points to a difference in size of the animals employed in the two investigations; young growing rats were used by Mitchell and large adult rats by Boas-Fixsen and Jackson. Schneider considers that the faecal endogenous nitrogen consists of two parts, the first being related to the food intake and the nitrogen lost in the intestinal secretions concerned in the digestive processes, and the second related to the body weight, representing an expenditure connected with the general metabolism. In small rats the amount of the first would be much greater than that of the second and there would be an apparently simple relation between the food intake and faecal nitrogen of endogenous origin.

The results of 78 experiments made recently with 21 rats of weights ranging from 250 to 500 g., fed on a "nitrogen-free" diet, are plotted in Fig. 2, and show

only a rough relationship between food intake and faecal nitrogen. The line drawn through the points is in about the same relative position as that drawn by Schneider [1934, Fig. 1]. According to Schneider's reasoning, the points corresponding to experiments made with lighter rats should show a grouping

Fig. 2. Relation between faecal nitrogen and food intake of rats receiving a "N-free" diet. +, rats weighing <350 g.; x, rats weighing 350–405 g.; •, rats weighing 405–450 g.; ○, rats weighing >450 g.

below the line, while the results of those with heavier rats should tend to lie above it. Of 39 experiments with rats of body weight 250–405 g., 15 gave points above and 24 points below the line; of 39 experiments with rats of weights 405–500 g., 22 gave points above and 17 points below the line. These differences, though pointing in the direction of Schneider's reasoning, must be considered too small to be significant. It would seem that the differences in weight among our rats were not great enough to demonstrate any variations that might occur in the distribution of endogenous faecal excretion between the two constituents postulated by Schneider. When, however, the same results are plotted with the faecal nitrogen per g. food ingested against the food intake, as in Fig. 3, the shape of the curve closely resembles that given by Schneider [1934, Fig. 2], and suggests a rough general proportionality between faecal nitrogen and food intake even for rats of 250–500 g. weight, provided the food intake is greater than 9–10 g. daily (approx. 100–120 Cal./kg. body weight). In our experiments, therefore, the direct proportion between food intake and faecal nitrogen is only very approximate (see Fig. 2). Seeing that in any case the amount of the faecal endogenous nitrogen has relatively little influence on the value of the expression used for calculating the biological value (see p. 1702), we have taken the mean value of the actual faecal nitrogen excreted in all the nitrogen-free experiments

performed on any rat, as a reasonably good estimate of the endogenous faecal nitrogen for that rat, following the previous usage in this laboratory.

Schneider [1934] considers that estimations of endogenous faecal nitrogen should be made with nitrogen-free diets containing the same amount of roughage

Fig. 3. Relation between daily food intake and faecal nitrogen per g. dry food eaten for rats receiving a "N-free" diet.

as the protein-containing diet under examination, since this secures more regular elimination of faeces and more comparable amounts of food to be digested. We have made a number of comparable observations with nitrogen-free diets and those containing caseinogen as protein, in which filter-paper was present in the proportion of 4 % of the food, but in these experiments the effect of the paper appeared to be unimportant.

*Urinary endogenous nitrogen in relation to food intake
and body weight.*

Mitchell [1924] found the urinary endogenous nitrogen excreted by rats to be proportional to the body weight. In our experiments, we have found no greater constancy in the ratio U_e per kg. body weight than in the value U_e itself, which, for the adult male rats used, was usually between 80 and 100 mg. per day. This constancy may be due to the fact that our rats were all large and adult and that the difference in body weight (250–500 g.) largely consisted in differences in body fat. There was, however, a tendency for the urinary excretion to be high when the loss in body weight during an experiment was great, corresponding to a low intake of the "N-free" diet. When the results of 65 experiments on 21 rats are considered together, the mean urinary nitrogen for the

20 experiments in which the daily intake of dry food was less than 10 g. (approximately 100–120 Cal./kg.), was found to be greater (average 96.5 mg. daily) than for the 45 experiments in which more than this amount was eaten (average 80.2 mg.). This difference, on analysis, was found to be statistically significant. If, however, the results of experiments in which less than 6 g. of the “N-free” diet were consumed daily are omitted, the difference of these averages was no longer found to be significant (average daily urinary excretion, 89.5 mg. for intake of 6–10 g., and 80.2 mg. for intake of over 10 g.). In any case, the experiments in which less than 10 g. daily of dry food were eaten formed only a small proportion of the total number of “N-free” experiments. It was thought wise, therefore, in our estimate of the endogenous nitrogen excretion for use in the calculation of the biological value of proteins (see the following paper), to exclude only the results of “N-free” experiments in which less than 6 g. dry food were eaten and the urinary N output was abnormally high. With this exception in our calculation of biological values, we have continued to use, as estimate of the endogenous urinary nitrogen excretion, the average of all values obtained with the rat in question, during a 3-month period before, and a 3-month period after, the date of the experiment with the protein under investigation.

The results and conclusions set forth in this paper are in accord with some published quite recently by Mason and Palmer[1935] in an investigation of the biological value of gelatin, caseinogen and zein. In special experiments made to determine the length of time on a N-free diet required to bring the urinary nitrogen to the endogenous level, only a very slight decrease was found after the third day; a 3-day preparatory period was therefore adopted. The authors also found that a “fall in calorie intake somewhat below the theoretical requirements during the ‘nitrogen-free’ feeding period was of no apparent significance” and that, with large adult male rats, loss of weight during a short experiment was without effect on the urinary nitrogen excretion.

SUMMARY.

1. Adult rats, when transferred from a high protein (18 %), to a “nitrogen-free”, diet and maintained thus for periods extending to 10–14 days showed a rapid fall in urinary nitrogen during the first 2–3 days, followed by a very gradual decrease subsequently. Similar changes in urinary nitrogen excretion were observed when the change was made from a high to a low protein diet.
2. The faecal endogenous nitrogen excretion showed a greater degree of constancy in experiments of similar duration and the amount was roughly proportional to the food intake when this was equivalent to not less than 100–120 Cal./kg.
3. The “balance sheet” method, as used in this laboratory, in which the endogenous nitrogen output on a nitrogen-free diet and the nitrogen intake and total output on the protein-containing diet are estimated during a 4-day period following a preparatory 2–3 days on the diet in question, is considered to afford reasonably satisfactory estimates of these values for use in the calculation of the biological value of the protein investigated.

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