CCIV. THE BIOLOGICAL VALUE OF PROTEINS.

VII. THE INFLUENCE OF VARIATION IN THE LEVEL OF PROTEIN IN THE DIET AND OF HEATING THE PROTEIN ON ITS BIOLOGICAL VALUE.

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MITCHELL [1924, 2], using young growing rats as experimental animals, found that the biological value of proteins was lessened as the proportion of the protein in the diet was increased. He considered that this result might in part be due to the fact that proteins could be used more economically for maintenance than for growth. When supplied in the diet in a low proportion, almost all the protein would be used for maintenance, whilst, as the level was increased, an increasing proportion would be used for growth. Martin and Robison [1922], working on themselves, found that the value of wheat proteins for the maintenance of nitrogenous equilibrium remained steady when these were present in the diet at different levels; the values they obtained for milk proteins, on the other hand, were most irregular. Boas-Fixsen and Jackson [1932], using adult male rats as experimental animals, found that the biological value of maize protein was increased when its level in the diet was lowered, but Boas-Fixsen [1930] found no progressive change in the nutritive value of a sample of dry-heated, purified caseinogen when the amount present in the diet was raised from 5 to 15 %, the values obtained being uniformly low.

The experiments to be described were designed to find out whether the biological value of proteins for maintenance was increased at very low levels in the diet for all proteins, or whether this effect was peculiar to a few. Experiments were therefore planned using diets containing only a low proportion (3-4 %) of the proteins used by Boas-Fixsen and Jackson [1932], *viz.* wheat, white flour, wheat germ, maize endosperm, milk and lactalbumin, for comparison with the results of their previous experiments with diets containing 5-7 % of these proteins. A series of experiments was also performed with caseinogen in various forms, *i.e.* "light white" (commercial), heated, heated and purified, and roasted, to determine the effect of heat and purification on its biological value as well as that of variation in the proportion contained in the diet. The levels of the caseinogen in these diets varied from 3 to 10 %. The effect of heat on lactalbumin was also investigated.

Materials used.

EXPERIMENTAL.

The samples of whole wheat, white flour, wheat germ, maize endosperm and whole milk were the same as those used in the previous investigation [Boas-Fixsen and Jackson, 1932]. The various types of caseinogen used were the following:

(i) "Light white casein" (B.D.H.), a commercial sodium caseinogenate prepared by Hammarsten's method.

(ii) Heated caseinogen. "Light white casein" heated at approximately 120° in an electric oven for 72 hours, being frequently stirred. Two samples were used, sample (1), as ordinarily prepared in the laboratory for basal diets used in vitamin A work and sample (2), of which the maximum temperature reached was checked by the use of maximum thermometers and found to vary from 112° to 125° during the 72 hours.

(iii) Heated extracted caseinogen. "Light white casein" purified by reprecipitation with acetic acid and frequent washing by decantation with 0.05 % acetic acid, followed by extraction with dilute alcohol for 96 hours in a Soxhlet apparatus, drying at a low temperature and finally heating for 3 days at about 120°. This material corresponds to that frequently employed in this laboratory for diets used in work on the B-vitamins [Chick and Roscoe, 1928].

(iv) Roasted caseinogen. "Light white casein" heated for 66 hours in an oven at 143–158° and frequently stirred. This material was definitely browned.

The same sample of lactalbumin was used as was previously investigated by Boas-Fixsen and Jackson [1932]. The heated specimen remained at about 120° for 72 hours in the same manner as the heated caseinogen.

These materials were made up into diets as previously described by Boas-Fixsen and Jackson [1932]; the other constituents of the diet were corn starch, sugar, clarified beef dripping, cod-liver oil, a salt mixture and CaCO_3 . The diets were supplemented with concentrates made from yeast extracts, containing vitamins B_1 and B_2 respectively, given as separate daily doses to each rat. A specially prepared sample of corn starch with a low nitrogen content (0.022 %) was used in the "nitrogen-free" diets and also in those containing 3 % of the various forms of caseinogen, since it was desired to eliminate as far as possible the effect of any "extra" nitrogen, which, at this low level of protein, might be a factor of considerable importance.

METHODS.

The experiments with the cereal products, milk proteins and lactalbumin, at 3–4 % level in the diet, were performed as described in the previous papers of this series [Chick and Roscoe, 1930; Boas-Fixsen, 1930; Boas-Fixsen and Jackson, 1932], the same group of rats and the same samples of the materials being used as were employed in the previous experiments with these proteins at 5–7 % levels. Certain modifications recently introduced and discussed in the preceding paper [Chick *et al.*, 1935] were adopted in the later work with caseinogen and lactalbumin. It was arranged that, as far as possible, the same rats were used in experiments designed to make comparison between the biological values of the caseinogen after various methods of treatment or when present at different levels in the diet.

In two series of experiments (with rats 77 and 87), the technique adopted was similar to that used by Mitchell [1924, 1; Mitchell and Carman, 1924] and Morgan [1931]. These formed part of the investigation of the effect of differing treatment on the biological value of caseinogen (5 % in the diet). For these special trials the rats were kept on the experimental diets for nearly 3 months without the usual intervening periods of rest on the high protein synthetic "CB" diet (see the preceding paper, p. 1703). Each separate experiment lasted 9 days, during the last 6 days of which analyses of nitrogen intake and output were made. In each series, at the beginning and also 10 weeks later, the rats received the "nitrogen-free" diet in order to estimate the endogenous nitrogen excretion; during the rest of the time they received diets containing 5 % of the various types of caseinogen investigated. The food intake was maintained very nearly constant during the whole period and the body weights fell slowly. For both rats the urinary endogenous nitrogen output was found to be slightly lower in the second "nitrogen-free" period than in the first; with rat 77 it fell from 81.4 to 66.6 mg. N daily and with rat 87, from 63.6 to 51.1 mg. N daily. The endogenous nitrogen excretion corresponding to any particular experiment was estimated from these tests, assuming

a constant fall in endogenous nitrogen excretion to have taken place during the 3 months period. These results give a good example of the gradual fall in endogenous nitrogen expenditure obtained when rats are kept on low nitrogen diets for long periods (see the previous paper, p. 1706). It was thought possible that more consistent results might be obtained with this technique than with our own usual method. The results obtained by both methods were, however, in general agreement (see Table II).

RESULTS.

(1) *Influence of variation of protein level in the diet on its biological value for maintenance of nitrogenous equilibrium.*

(a) *Proteins of wheat, white flour, wheat germ, maize endosperm, whole milk and lactalbumin.* Table I shows the biological values obtained for the proteins from these sources, when 3-4 % was contained in the diet. The mean values obtained by Boas-Fixsen and Jackson [1932], working at 5-7 % levels, are included in the table for comparison. The average figures obtained for B.V. at the lower protein level with whole wheat (100), white flour (84), wheat germ (90), maize endosperm

Table I. *Biological value of various proteins at different levels of protein intake.*

Source of protein used	Low level of protein in the diet			Higher level of protein in the diet*	
	% protein in dry diet	B.V. obtained in individual exps.	Mean B.V.	% protein in dry diet	Mean B.V.
Whole wheat	3.6	100 100 100 100 98 100	100	5.6	68
White flour	3.1	100 69 80 100 92 65	84	7.0	61
Wheat germ	3.6	100 100 70 100 79	90	6.8	69
Maize endosperm	3.4	100 83 94 75	88	6.7	70
Milk, whole, dried	3.4	95 92 92 100 70	89	6.9	86
Lactalbumin	3.1	100 100 89 95 70 100	92	6.7	65

* Results published previously [Boas-Fixsen and Jackson, 1932].

(88) and lactalbumin (92) are consistently greater than those obtained at the higher levels (B.V.=68, 61, 69, 70 and 65, respectively). Whole milk proteins were, however, so well utilised when present as 7 % of the diet (B.V.=86) that lowering the level to 3 % did not apparently increase the biological value appreciably (B.V. at 3 %=89).

(b) *Caseinogen*. The results obtained with the various forms of caseinogen, when present in the diet at 10 %, 5 % and 3 % levels, are summarised in Table II. In every case the average biological value was increased as the percentage of protein in the diet was decreased. Thus, as the percentage of protein in the diet was lowered from 10 % to 5 % and 3 %, the average biological value was raised from 62 to 64 to 78 for "light white casein", from 55 to 67 to 84 for "heated" caseinogen (sample 1), from 54 to 68 to 80 for "heated" caseinogen (sample 2) and from 53 to 67 to 71 for the "heated extracted" caseinogen. Since the roasted caseinogen was only tested at one level (5.5 %) in the diet there is no evidence as to the effect of variation in this case. The values obtained with different rats on the same diets varied considerably, but with one exception (rat 78, Table II) there was always an increase in biological value when the proportion of protein in the diet was lowered. It should be noted, however, that, for caseinogen, this variation in biological value was not as great as was found with the other proteins investigated. It appears that, even at a very low level (3 %) in the diet, the caseinogen was not as completely utilised as were the cereal proteins investigated or those of whole milk.

Table II. *Biological values of caseinogen, before and after various types of treatment, when present as 3-10 % of the diet.*

Kind of caseinogen % in diet Rat No.	"Light white"			"Heated" sample 1			"Heated" sample 2			"Heated, extracted"			"Roasted"
	9.6-9.9	5.5	3	10	5	2.6	9.4	5.3	2.9	10	5.6	2.8	
59	64	—	—	48	74	100	—	—	—	—	—	—	—
60	81	—	—	—	—	—	—	—	—	—	—	—	—
61	—	—	—	58	—	—	—	—	—	—	—	—	—
62	59	—	—	{67 57	—	89	—	—	—	—	—	—	—
63	—	—	—	—	56	76	—	—	—	—	—	—	—
64	—	—	—	45	—	63	—	—	—	—	—	—	—
66	—	—	—	—	71	94	—	—	—	—	—	—	—
72	55	68	—	—	—	—	64	{69 68	78	53	67	62	—
73	—	—	56	—	—	—	45	64	67	55	—	51	—
74	52	59	94	—	—	—	42	—	85	{47 44	58	57	22
77	63	69*	89	—	—	—	63	61*	84	64	67*	89	45*
78	60	{52 67	64	—	—	—	58	—	(34)	—	75	—	48
79	—	—	—	—	—	—	—	71	—	—	—	—	—
87	—	69*	—	—	—	—	—	73*	—	—	—	—	43*
91	—	—	102	—	—	—	—	—	76	—	—	93	—
93	—	—	—	—	—	—	—	—	64	—	—	57	—
94	—	—	61	—	—	—	—	—	107	—	—	89	—
Average	62	64	78	55	67	84	54	68	80†	53	67	71	40

* Results obtained using a modified technique similar to that used by Mitchell [1924, 1; Mitchell and Carman, 1924] and Morgan [1931].

† Omitting the one very low value obtained with rat 78.

The relatively slight improvement in the utilisation of caseinogen at the 3 % level might be partly due to a supplementary effect of the "extra" nitrogen in the diet (made up of the small amounts given in non-protein constituents and in the B-vitamin supplements), if this contained cystine, an amino-acid important for maintenance, in which caseinogen is somewhat deficient. The amount of "extra" nitrogen the rats were receiving in these supplements was from 5.7 to 8.4 mg. per day, as compared with a total nitrogen intake of 50-70 mg. on diets containing 3 % protein, 80-140 mg.

on those containing 5 % and 150–220 mg. on those containing 10 %. Mitchell [1924, 1] found that, to raise the biological value of caseinogen at an 8 % level from 75 to 86, an addition of cystine nitrogen equivalent to 1/60 of the total nitrogen in the diet was necessary. In Greaves and Morgan's experiments [1934], the addition of 0.02 % of cystine to a diet containing 9 % caseinogen (*i.e.* the addition of 1.6 mg. cystine nitrogen to 100 mg. caseinogen-nitrogen) raised the biological value from 68 to 80. The possibility that the diets used by us might contain traces of extra cystine is not excluded, and the effect of such traces might be greater at a low level of protein intake, but it is unlikely that enough could have been present to be a factor of importance in influencing the results.

With all the proteins investigated, therefore, (except whole milk proteins) significantly lower biological values were obtained as the level of the protein in the diet was raised. The differences obtained by Mitchell [1924, 2] in similar experiments, but with young growing rats, were not greater. Therefore no support is given to his theory that these differences might be due to the fact that the biological values of these proteins are greater for growth than for maintenance, since in our experiments no growth occurred. They can be more readily explained on the assumption, also suggested by Mitchell, that when present at higher levels in diet the proteins are less economically used, owing to the presence in the blood stream of a greater proportion of amino-acids to other food constituents.

It is possible that these results are also due to other factors whose influences are at present obscure. Such factors may be concerned with the extent to which proteins can act as a whole in nitrogenous metabolism and the constituent amino-acids can act separately. Even proteins deficient in essential amino-acids may appear to function to good advantage when the intake is low, yet, however much the intake is increased, nitrogenous equilibrium will never be attained and the nitrogen balance will remain negative. This fact is well shown in the relatively high B.V. obtained by McCollum [1911] for zein, when fed to pigs at a low level to provide an amount of nitrogen equivalent only to the daily endogenous output, and in the experiments of Robison [1922] with gelatin.

(2) *Effect of heat.*

(a) *Caseinogen.* Table II shows that heating at 112–125° and also purification (reprecipitation and extraction with water and alcohol) of caseinogen have no significant detrimental effect on its biological value for the maintenance of nitrogenous equilibrium. The mean figures obtained for the four different types of caseinogen were similar, at whatever levels of protein in the diet they were compared. Thus, the mean figures for the biological values of the untreated caseinogen, "heated" caseinogen (1), "heated" caseinogen (2) and "heated extracted" caseinogen were respectively, 62, 55, 54 and 53 at the 10 % level, 64, 67, 68 and 67 at the 5 % level and 78, 84, 80 and 71 at the 3 % level.

When, however, the caseinogen was more strongly heated, at about 150° for 66 hours, its nutritive value was definitely reduced, the average biological value being 40, as compared with 64 for the untreated caseinogen (5 % level of protein in the diet). This result is in agreement with that obtained by Greaves and Morgan [1934], who found that heating caseinogen at 140° reduced its biological value from 68 to 58. The slight lowering of biological value of caseinogen by heating at 112° previously reported from this laboratory [Boas-Fixsen and Jackson, 1932], has not been confirmed by this further investigation and the previous result was probably based on the results of too few experiments (3 rats only).

These results show that the nutritive value of caseinogen is not significantly lowered by the treatment which it receives before incorporation in basal diets

used for vitamin work, if the temperature at which it is heated does not exceed about 125°. It should be remembered, however, that the above results refer to maintenance tests on adult rats, whereas in vitamin work, young growing rats are usually employed. The usual proportion of caseinogen in these basal diets, on the other hand, is relatively very high (18–20 %).

Table III. *Effect of heat on the percentage digestibility of caseinogen (5 % protein in the diets).*

Rat No.	Unheated caseinogen	"Heated" caseinogen (approx. 120° for 72 hrs.)	"Roasted" caseinogen (approx. 150° for 66 hrs.)
59	—	95	—
63	—	92	—
66	—	95	—
72	90	{ 95 100	—
73	—	98	—
74	92	—	63
77	96	97	84
78	94	—	69
79	—	92	76
87	92	94	—
Average	93	95	73

Table III shows the percentage digestibilities of the raw, "heated" and "roasted" caseinogen, as calculated from the results of experiments with 5 % protein in the diet. They show that prolonged heating at about 120° did not affect the digestibility, but that stronger heating at approximately 150° reduced it from 93 to 73 %.

(b) *Lactalbumin*. To investigate the effect of heat (112–125°) on the nutritive value of lactalbumin, diets containing 5 % lactalbumin and 7 % heated lactalbumin were used (see Table IV). The heating decreased the digestibility of the lactalbumin from 95 to 69 % (average values). Hence the content in each of the two diets was equivalent to about 5 % digestible protein. The heating decreased the biological value by about one-fifth (B.V.=66 for the unheated, and 56 for the heated, specimen).

Table IV. *Effect of heat on the biological value and on the digestibility of lactalbumin (72 hours at approximately 120°).*

Rat No.	Biological value*		% digestibility	
	Unheated	Heated	Unheated	Heated
75	64	59	98	75
76	65	52	92	67
79	72	58	96	73
87	64	47	93	61
89	64	65	94	69
Means	66	56	95	69

* Both diets contained 5 % digestible protein.

Heating the lactalbumin appeared to cause some physical change, so that it could not be so well digested, whilst the biological value of the absorbed portion was also reduced, but to a less degree. It should, however, be noted that these experiments were concerned with the value of the lactalbumin for maintenance, and the effect of this degree of heating might have been much more serious on its value for supporting growth.

The balance sheet method.

The balance sheet method, by which the nutritive values of different proteins are compared in regard to their relative values for maintenance of nitrogenous equilibrium in the adult animal, has many drawbacks. The method is difficult and laborious in practice and the results are subject to a margin of error which is large even for biological experiments. Long experience and skill in management of the experimental animals are needed in order to achieve a series of comparative observations, possessing even the somewhat low degree of accuracy of the work reported in this paper and the preceding ones of this series. There is also a limit to the duration of the experiment, which militates against accuracy. This is specially true for the observations on "N-free" diet, made to determine the endogenous nitrogen expenditure, which form an essential part of the method. Even in the experiments in which protein is fed, the nitrogen balance should be negative and animals cannot be maintained in health for more than short periods in negative nitrogen balance. It is also possible that a large nitrogen debt may affect the biological value of a protein for maintenance.

Other methods of studying the value of proteins, in which the criteria adopted are the support of growth and development in young animals [Osborne *et al.*, 1919] or the complete maintenance of the species including reproduction and lactation [Osborne *et al.*, 1919; McCollum *et al.*, 1921, 1, 2, 3] are simpler in conception and appear, at first sight, to yield more useful information. In practice, however, such experiments are often very difficult to carry out in such a way that the proteins compared are observed under truly comparable conditions. The appetite and preferences of the experimental animals often present serious complications under these more "natural" conditions. For these reasons the results of the observations are often difficult of interpretation or of utilisation for conclusions having a quantitative value.

Maintenance of body weight has been used by Osborne and Mendel [1916, 2] as a criterion of the value of different proteins for maintenance, apart from growth. Their experiments were made with rats of weight from 100 to 170 g., *i.e.* rats rather less than half grown, whose bodies would contain relatively little fat. If the method were applied to adult animals, variations in the fat content of the carcass might prove a serious complication in interpreting the results.

It is not easy to appraise the relative merits of the different types of investigation. These are well discussed in Mitchell's [1924, 3] admirable review, and, more recently, by Boas-Fixsen [1935]. Biological values of proteins for maintenance as opposed to growth and other functions, are, however, of interest since their relative values for different functions are probably different, depending on their make-up of amino-acids. In the experiments of Osborne and Mendel [1916, 1, 2] lactalbumin showed a degree of superiority over caseinogen and edestin for supporting growth, which was much greater than the superiority for maintenance. This is doubtless to be explained by the large requirement for growth of lysine and the relatively small need of this amino-acid for maintenance. Conversely, the proteins of raw ox muscle were found by Morgan and Kern [1934] to be inferior to those of milk or cereals for maintenance, whilst superior for the support of growth.

In the one instance in which both the "balance sheet" and the "growth" methods were used by the present group of workers to compare the same proteins, those of wheat and maize, and the more obvious errors of the second method were satisfactorily avoided, the results obtained were concordant, *i.e.* no significant difference was detected by either method in the values of the proteins from the two sources compared [Boas-Fixsen and Jackson, 1932; Boas-Fixsen *et al.*, 1934].

SUMMARY.

1. The biological value for the maintenance of nitrogenous equilibrium in the adult rat has been determined for proteins from the following sources, at levels in the diet varying from 3 to 10 %: whole wheat, white flour, wheat germ, maize endosperm, whole milk, lactalbumin, heated lactalbumin (at approximately 120° for 72 hours), caseinogen, heated caseinogen (at 112–125° for 72 hours), heated caseinogen purified by reprecipitation and extraction with dilute alcohol.

2. With the exception of whole milk proteins, the B.V. of which remained uniformly high at all levels, increasing the proportion of protein in the diet from 3 to 5, 7 or 10 %, lowered the biological value.

3. The biological value of caseinogen was not lowered significantly by heating the caseinogen at 112–125° for 72 hours or by further purification. Heating at about 150° for 66 hours did, however, decrease the biological value from 64 to 44 (5 % protein in the diet), and also reduced the digestibility from 93 to 73 %.

4. The biological value of lactalbumin was only slightly reduced by heating the lactalbumin at about 120° for 72 hours, but the digestibility was lowered from 95 to 69 %.

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