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Studies in Biochemical Adaptation. The Effect of Variation in Dietary Protein upon the Hepatic Arginase of the Rat

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Much of the recent work on enzyme adaptation has been carried out in micro-organisms. Relatively little has been reported on enzyme adaptation in mammals and further work on this subject is desirable. In particular, a quantitative study would be of interest as a test of the predictions made from the extended mass action theory, which should apply to enzyme adaptation in animals as well as in micro-organisms (Mandelstam, 1952).

The enzyme chosen for study was hepatic arginase in the rat and the effect was investigated of variation in dietary protein.

Previous work in this field is inconclusive. Baldwin (1935) reported that there seemed to be a decrease in arginase in the hepatopancreas of the snail during starvation; Baldwin & Yudkin (1939), however, found such a great variation in the activity of the enzyme in different specimens that it was not possible to draw any definite conclusion concerning the effect of starvation. Seifter, Harkness, Rubin & Muntwyler (1948) reported a decrease in hepatic arginase in rats fed on a protein-free diet for 1-3 weeks. Lightbody & Kleinman (1939) found that in rats fed on diets containing 6, 25, 60 and 75 % protein, the amount of enzyme in unit weight of liver was higher with higher amounts of dietary protein. It is possible that the rise in enzyme was due, at least in part, to a general increase in hepatic protein which was not estimated. Folley & Greenbaum (1946) found, with rats, that a diet containing 50 % protein resulted in a higher concentration of hepatic arginase than one containing 20 % protein though, with the small number of animals studied, the difference was not statistically significant. Kochakian, Bartlett & Moe (1948) estimated hepatic arginase in rats fed on a diet containing either no protein or 80 % protein. After 7 days, there was a fall in the arginase in the former, partly due to a general decrease in hepatic protein, and a slight

rise in the animals fed on the high protein diet, which the authors do not consider noteworthy.

Hepatic arginase is of interest in relation to the theory of Krebs & Henseleit (1932) that urea is formed in the liver through a series of reactions involving this enzyme. Although this theory has been the subject of some criticism (e.g. Bach, 1939; Trowell, 1942), it is now accepted by the majority of workers. An increase in dietary protein, and so an increase in production of urea, might well, therefore, cause an increase in the arginase involved in the process. The general problem of the use of enzyme adaptation in studies of metabolic pathways is dealt with elsewhere (see, for example, Yudkin, 1952; Davies & Yudkin, 1951).

EXPERIMENTAL

Animals. The rats were bred in this laboratory and were of an albino strain which we have elsewhere designated as KC1 (Wiesner & Yudkin, 1951). Preliminary tests showed that male animals have some 15 % more hepatic arginase than female animals. In the detailed experiments to be reported, only male animals were used.

Diet. From weaning to the beginning of the experiment, the animals were fed a mixed diet of cubes with additional milk and green vegetables (see Wiesner & Yudkin, 1951). This diet contains approx. 20% protein. The experimental diets containing varying amounts of protein were made according to Table 1. Animals were distributed so that one from each litter was given each of these purified diets. Food and water were given *ad lib*. The animals were weighed twice weekly.

Estimation of arginase. The animals were killed by a blow on the head and the whole liver removed and weighed. Two samples, each of about 20 mg., were accurately weighed on a glass cover-slip. Nitrogen was estimated in these samples by the Kjeldahl method. For the estimation of arginase, about 0.5 g. of liver was accurately weighed and homogenized in a Waring Blendor with 300 ml. distilled water for 90 sec. Into a Warburg cup were placed 1 ml. liver homogenate,

1 ml. water and 0.5 ml. 0.5M-phenolsulphonate-phosphate buffer, pH 8.4 (Hunter & Downs, 1944). A solution of 10 mg. arginine in 0.5 ml. water was placed in the side arm of the cup and the contents mixed after 10 min. at 38°. After a further 30 min., the contents of the cup were washed into 1 ml. 40% trichloroacetic acid, made up to 15 ml. with water and filtered. A portion of 5 ml. was taken for estimation of urea by the urease-aeration method. Urea production in these conditions was directly proportional to the amount of arginase present up to values appreciably higher than those encountered in our experiments.

Urease with no argininolytic activity was prepared from soya flour as follows: 50 g. soya flour was shaken with 200 ml. distilled water for 10 min., centrifuged and the supernatant fluid, containing largely inactive material, discarded. The flour was then extracted three times for an hour each with distilled water, the supernatant fluid from centrifugation being preserved each time. A final centrifugation was made to remove further inactive suspended material and the supernatant fluid then added to twice its volume of 96% ethanol. The precipitate was washed twice with ethanol and finally with ether. The paste was dried on trays at room temperature and finally in a vacuum desiccator. It was kept in a refrigerator and retained its activity for at least a year. For use, 1 g. of the dried preparation was finely ground and 40 ml. water slowly added with further grinding. To this was added 60 ml. glycerol and the mixture stored on ice. This preparation was stable for several weeks and 1 ml. was capable of converting about 3.5 mg. urea in 1 hr. Since the urea produced by arginase in our experiments was never more than one-tenth of this, this preparation of urease was considered satisfactory.

Enzyme activity is expressed as mg. urea N produced in 30 min. at 38° and pH 8.4.

RESULTS

Effect of amount of dietary protein on hepatic arginase

Four rats from each of eight litters were taken at the age of 5 weeks, when they weighed 60–70 g. One animal from each litter was placed on each of four experimental diets, containing 8, 17, 33 and 67% protein (Table 1). The animals on the diet with 8% protein did not thrive and although the protein was increased to 10% after 4 weeks, six of them died in the next few weeks. All the remaining animals were sacrificed after 22–24 weeks of experimental feeding.

Growth. The growth of the animals on the higher protein levels is given in Fig. 1. The most rapid

growth was shown by animals on 33% protein and the slowest on 67% protein. The group consuming 17% protein grew slower initially, but by 19 weeks their weight equalled that of the intermediate group.

Table 1. *Composition of purified diets*

(To 100 g. diet was added 10 ml. vitamin solution, 1 l. of which contained: aneurin 50 mg.; riboflavin 300 mg.; choline chloride 10 g.; inositol 2.2 g.; nicotinic acid 1.0 g.; calcium (+)-pantothenate 1.0 g.; pyridoxin 30 mg.; biotin 2 mg.; 5% acetic acid 300 ml.; ethanol 100 ml. Twice weekly, each animal received three drops of cod liver oil.)

Diet	...	...	...	...	1	2	3	4
						Wt. (g.)		
Casein (Glaxo light white)					10	20	40	80
Sucrose					90	80	60	20
Arachis oil					15	15	15	15
Salt mixture					5	5	5	5

Fig. 1. Growth of rats on purified diets containing varying amounts of protein.

Enzyme activity. Table 2 gives the data which include the total amount of enzyme and the relative amount in relation to the weight of the liver, hepatic nitrogen and weight of the animal. The results for the animals on the lowest amount of protein are not included since only two animals survived, though it might be mentioned that the amount of enzyme was

Table 2. *Effect of variation in dietary protein upon hepatic arginase*

	Diet 2 (17% protein)	Diet 3 (33% protein)	Diet 4 (67% protein)
Body weight (g.)	299 ± 7.4	279 ± 7.1	213 ± 3.8
Weight of liver (g.)	10.3 ± 0.24	10.3 ± 0.36	7.6 ± 0.17
Hepatic N (% of wet wt.)	2.9 ± 0.068	3.0 ± 0.080	3.3 ± 0.098
Hepatic arginase/mg. tissue	34.7 ± 1.03	41.6 ± 1.24	59.2 ± 0.86
Hepatic arginase/mg. N	12.0 ± 0.43	13.9 ± 0.34	19.0 ± 1.36
Total arginase × 10 ⁻⁴	35.8 ± 2.22	43.2 ± 2.54	45.1 ± 1.16
Total arginase × 10 ⁻⁴ /g. body wt.	0.119 ± 0.0048	0.150 ± 0.0059	0.212 ± 0.0014

lower than in any other groups (e.g. 29 units/mg. liver compared with 34.7 units in animals on 17% protein).

Figures constructed from these results show the increase in enzyme with increase in dietary protein (Fig. 2). This increase is linear for enzyme concentration related to weight of liver and to hepatic

Rate of adaptation and reversibility of adaptation in young and adult rats

Groups of twenty-four young and thirty-eight adult male rats were available. They had been weaned at 3 weeks on the stock diet described above. Sixteen of the younger animals and fifteen of the

Fig. 2. Effect of variation in dietary protein on hepatic arginase. Individual graphs show enzyme activity related to weight of tissue, nitrogen of tissue, total liver and weight of animal. Arginase activity expressed as mg. urea N liberated in 30 min. at 38° and pH 8.4.

nitrogen. There was also a linear increase in enzyme related to body weight, but since the absolute weight of the body, and of the liver, fell with high dietary protein, it follows that the total amount of enzyme in each animal did not rise linearly. From the functional point of view, it is of more interest to relate the amount of enzyme to unit weight of animal or of liver tissue, since the liver is the main site of urea formation.

older animals were placed on a purified diet containing 67% protein when they were 8 weeks and 26–28 weeks old respectively, and weighed an average of 130 and 270 g.; the remaining animals were kept on the stock diet and served as controls. At intervals over 2 weeks for the younger animals, and 3 weeks for the older animals, hepatic arginase was estimated in both the control and the experimental animals. The results are reported as if they

were obtained at weekly intervals, although the actual analyses were carried out at 5-9 and 12-16 days and on the nineteenth day.

The remaining animals on the high protein diet were then placed back on the stock diet—four young rats after 2 weeks and eight adult rats after 3 weeks. After a further 4 and 3 weeks respectively, these animals were also used for determination of hepatic arginase.

Growth. In both groups of animals, there was a loss of weight after transfer to the high protein diet (Fig. 3). Recovery began at the end of 1 week in the younger rats and at the end of a fortnight in the older rats.

Fig. 3. Growth of rats on purified diet and on subsequent return to stock diet. ●—●, adult animals on purified diet for first 3 weeks; x---x, young animals on purified diet for first 2 weeks.

Enzyme activity. Results were calculated in the same way as in the previous experiment. For simplicity, they are shown only in the form of graphs (Fig. 4).

The concentration of enzyme in unit weight of liver was initially lower in the young rats than in the older rats but rose higher during adaptation. Reversion to the stock diet resulted in a fall in both, but the final value, as expected, did not fall to the original low value in the younger rats, which by now had grown considerably and weighed over 200 g.

The curve relating arginase to hepatic nitrogen shows that the initial gain in enzyme in adult rats was only due to a relative increase in hepatic nitrogen. In the younger rats, however, there was a real gain in enzyme in this period, and the total gain in the second week was greater than that of the adult rats. A similar result is seen in the amount of enzyme related to body weight, and here it is observed that reversion on the stock diet is complete in both groups. The total amount of enzyme shows a regular rise and fall with young animals put on the

high protein diet and then back on the stock diet. With the older animals, there was a fall in the first week which was no doubt due to the fact that the animals were in a state of semi-starvation as shown by the considerable loss in weight.

During the same period of 6 weeks, the adult control animals showed no change, whilst the young control animals showed only the increase in enzyme expected during that period of growth. For example, the arginase per mg. liver for the adult rats was 34 and 33 units at the beginning and end of the experiments, whilst for young rats it was 28 and 34 units.

DISCUSSION

The results show that an increase in dietary protein is followed by an increase in arginase, which seems to be more or less complete within 3 weeks; reversion to the previous diet results in a decrease of the enzyme to its normal values. A possible explanation of adaptation to an increase in dietary protein is that the larger amounts of protein being metabolized involve a greater use of the enzyme in producing urea. According to the mass action theory of enzyme adaptation, more arginase would be produced if more of the enzyme is in a combined form. This might occur with a high protein intake because of a greater formation of ammonia, so that more is passing through the arginine cycle, more arginine is present and hence more of the enzyme is combined. It is also possible that the substances combining with the enzyme are derived directly from the protein; these would include arginine itself and also ornithine and lysine which are known to act as competitive inhibitors for arginase (Hunter & Downs, 1945). Other explanations may be advanced for the increase of arginase with increase in dietary protein, for example, a general increase in hepatic enzymes. Although this seems unlikely, since there was no appreciable increase in hepatic nitrogen, such a possibility cannot at present be excluded.

The results are also interesting from the quantitative point of view. We have seen that the concentration of enzyme produced is directly proportional to the amount of dietary protein and that this proportionality is linear. The mass action theory of enzyme production originally suggested in respect to adaptation in micro-organisms (Yudkin, 1938) should apply equally to enzyme adaptation in multicellular organisms (Mandelstam, 1952). The linear relationship between dietary protein and hepatic arginase is in conformity with the quantitative deductions from that theory for enzyme adaptation in higher organisms and, in certain conditions, in micro-organisms. This matter is dealt with in the accompanying papers (Mandelstam, 1952; Mandelstam & Yudkin, 1952).

SUMMARY

1. A study has been made in rats of the effects of variation in dietary protein upon hepatic arginase.

4. The rate and extent of increase of enzyme is greater in young animals than in older animals.

5. There is a linear relationship between dietary protein and amount of enzyme produced. This is in

Fig. 4. Reversible adaptation of hepatic arginase. Arrows indicate return from purified diet (67% protein) to stock diet (20% protein). ●—●, adult animals; ×---×, young animals. Individual graphs show enzyme activity related to weight of tissue, nitrogen of tissue, total liver and weight of animal. Arginase activity expressed as mg. urea N liberated in 30 min. at 38° and pH 8.4.

2. The amount of the enzyme, both absolute and in relation to unit weight of liver, hepatic nitrogen and body weight, increases with increase in dietary protein and reverts to normal with reversion to a normal diet.

3. The adaptation to the increase or decrease in dietary protein is almost complete within 2 or 3 weeks of changing the diet.

conformity with the mass action theory of enzyme adaptation.

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Studies in Biochemical Adaptation. Some Aspects of Galactozymase Production by Yeast in Relation to the 'Mass Action' Theory of Enzyme Adaptation

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The experiments to be described were designed to test some of the predictions of the extended 'mass action' theory (Mandelstam, 1952). The enzyme system chosen for study was that involved in the fermentation of galactose by yeast cells. Several enzymes are involved in this reaction, at least two of which are adaptive, so that it is not perhaps the ideal enzyme system for such a study (Stanier, 1951). It has, however, been the subject of so much work by such investigators as Stephenson & Yudkin (1936) and Spiegelman (1946), that it was nevertheless chosen for this work. For simplicity, we shall speak as if one enzyme, galactozymase, is involved.

The predictions selected to be tested were:

(1) The rate of adaptation in the presence of a given amount of galactose.

(2) The effect of substrate concentration on the rate of adaptation.

(3) The effect of substrate concentration on the amount of enzyme formed.

(4) The relative amounts of two adaptive enzymes (galactozymase and maltozymase) formed in the presence of varying amounts of both substrates.

The detailed theoretical treatment for each of these situations is given by Mandelstam (1952). A simpler diagram is given here (Fig. 1) to facilitate the necessary reference to the theory.

A general description will first be given of the techniques used. A summary of the expectations

from the theory for each of the selected situations, the experiments designed to test them, and the results obtained will then be presented in order.

Fig. 1. Simplified diagram illustrating mass action theory of enzyme adaptation. *B*, pool of building blocks; *P*, enzyme precursor; *E*, enzyme; *S*, substrate. Concentrations are denoted by small letters. k_1 , k_2 , etc., are velocity constants. Broken arrows indicate that a number of reactions may be involved.

METHODS

The organism used was a pure strain of *Saccharomyces cerevisiae* (N.C.T.C. No. 815). It was maintained on slopes, the medium containing bacto-peptone 2%, yeastrel 3%, glucose 4%, agar 1.5%. The pH was adjusted to 6.0 and the organisms grown at 29°. Cultures for the experiments were made in a synthetic medium containing 50 g. glucose, 4.7 g. $(\text{NH}_4)_2\text{SO}_4$, 750 mg. KH_2PO_4 , 100 mg. MgSO_4 , 6.6 mg. inositol, 3 mg. nicotinic acid, 3 mg. calcium (+)-pantothenate, 150 μg . aneurin, 900 μg . riboflavin, 240 μg . pyridoxin, 6 μg . biotin and water to 1 l. The pH was adjusted to 6.0 and the organisms were grown at 29° for 40–48 hr. The cultures were centrifuged and the cells washed twice in saline and suspended in galactose containing phosphate buffer ($1.67 \times 10^{-2}\text{M}$, pH 6.0). The suspensions were made up so that 1 ml. contained about 30 mg. dry weight of cells.