# Adaptive enzyme changes in liver and muscle of rats during protein depletion and refeeding

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(*Received* 30 *June* 1967—*Accepted* 25 *October* 1967)

1. Young rats were kept on a 6% casein diet for 4–7 weeks and then either killed or returned to a stock diet. Control rats were given a stock diet.

2. Argininosuccinate lyase (EC 4.3.2.1, L-argininosuccinate arginine-lyase) was assayed in liver, and amino acid activating enzymes were assayed in liver and muscle of rats after depletion and while they were refed on the stock diet. Levels of enzymes were compared with those in the control rats.

3. Amino acid activating enzymes were markedly increased in liver at the end of the depletion period, but returned almost to normal levels after 4–6 days of refeeding. In muscle the enzyme levels were not decreased after depletion, but began to rise after 4 days of refeeding.

4. Argininosuccinate lyase activity in depleted female rats was about half that in control rats and rose again slowly on refeeding. Male rats were hardly affected by depletion.

5. It is suggested that these enzyme changes play a part in the adaptation of the rat to a reduced protein intake.

When rats are given diets containing less than the normal content of protein they become adapted to the reduced protein supply by an alteration in the pattern of protein synthesis (Waterlow & Stephen, 1966). It now seems as if in the malnourished animal synthesis of new protein is concentrated in the more vital tissues such as liver at the expense of the less essential ones like muscle (Waterlow, 1959; Bendicenti, Mariani, Paolucci & Spadoni, 1959; Gaetani, Mariani, Spadoni & Tomassi, 1961; Stenram & Hirschman, 1965). This finding was related by the Italian workers to the concentration of the amino acid activating enzymes, the synthetases, in the tissues (Mariani, Spadoni & Tomassi, 1963; Gaetani, Paolucci, Spadoni & Tomassi, 1964). They showed that in adult rats given a protein-free diet for 30–60 days the amino acid activating enzymes in the liver had about twice the activity of those in rats on a stock diet, but were only a little changed in heart or gastrocnemius muscle. On the other hand, Schimke (1962*a*) has shown that, when the diet contains no protein but enough calories to provide energy, the level of the urea cycle enzymes falls, corresponding to the decrease in urea excretion.

These two groups of enzymes therefore seem to promote the adaptation to a low-protein intake by changing their concentrations in opposite directions. The increase in activity of the synthetases means that such amino acids as reach the liver can be used to the best advantage, while the decrease in activity of the urea cycle enzymes acts to prevent the destruction of amino acids to form urea (Waterlow, Alleyne, Chan, Garrow, Hay, James, Picou & Stephen, 1966). Thus, there appears to be an adaptation of enzyme activity to meet the demands of the body in response to changes in diet.

We have studied these adaptive enzyme changes in samples of liver taken by biopsy

from human infants suffering from protein malnutrition and during their recovery in the ward of this Unit. The methods of assay were adjusted to the micro-scale, and results are given in Stephen & Waterlow (1968). It seemed desirable, however, to support these observations by experiments on young rats under conditions more comparable to those of the babies, particularly while they were being refed after a period of depletion lasting several weeks. Mendes & Waterlow (1958) have shown how in refed rats new protein is rapidly formed by the liver and by muscle more slowly. It would be reasonable to expect marked changes in enzyme levels in these two tissues during this stage.

The experiments with rats are reported in this paper. Argininosuccinate lyase (EC 4.3.2.1, L-argininosuccinate arginine-lyase) was the enzyme of the urea cycle selected for measurement in the liver. The study of amino acid activating enzymes, with a mixture of twenty amino acids as substrate, was extended to muscle after the first two experiments.

## EXPERIMENTAL

Male and female albino weanlings or young rats were used. Control rats were given a commercial stock diet (Purina rat chow, containing approximately 20% protein) and the protein-depleted rats a 6% casein diet for 4–7 weeks as described by Waterlow & Stephen (1966). At the end of the depletion period they were either killed for the assay of their enzymes or were refed on the stock diet and weighed daily. They were killed, after anaesthetizing them with ether, by cutting their throats. The livers were removed and placed immediately in ice-cold homogenizing medium (Tris, 0·02 M, containing KCl, 0·05 M; pH 7·4), and a portion of each was homogenized in the cold room at +2°. When muscle enzymes were also measured, the gastrocnemius muscle was dissected from the dead rat in the cold room immediately after removal of the liver. Control rats were killed for comparison at the same time as the depleted ones and again at stages during the refeeding, according to how many rats were available for each experiment.

### *Homogenization*

*Liver.* In the first two experiments, the rats were being used as controls for the human biopsies, and homogenates were prepared on a micro-scale by gently grinding about 10 mg liver in a tube with a pestle by hand in about 100 $\mu$l homogenizing medium at 0°. It was difficult to obtain good duplicates on such small samples of liver, especially in the protein-depleted animals where the distribution of fat tended to be uneven. In later experiments therefore about 1 g liver was homogenized mechanically in 10 ml medium and the whole homogenate was centrifuged, although incubations and enzyme assays were still done on the micro-scale given below. Comparisons of the macro- and micro-scale showed little difference in concentrations of the amino acid activating enzymes.

Argininosuccinate lyase was measured in samples of liver homogenates before centrifugation.

*Muscle.* About 200 mg gastrocnemius muscle were ground with a little sand in a tube with a pestle by hand in 1 ml ice-cold medium. The suspension was centrifuged

quickly in a bench centrifuge, and the sediment was discarded. For depleted and refed rats, samples were taken from each leg and treated separately, but usually a sample from only one leg was taken from the control rats.

### *Centrifugation*

Homogenates were centrifuged in an MSE Highspeed 18 centrifuge at 0° at about 35000*g* for 1 h. Muscle homogenates and micro-homogenates of liver were centrifuged in small glass tubes, 50 mm long × 5 mm internal diameter, which were encased in a jacket of expanded polystyrene cut with a corkborer to fit in a polypropylene tube inside the stainless steel adapters for the 8 × 50 ml angle rotor. With these precautions very few tubes broke.

### *Enzyme assays*

*Amino acid activating enzymes.* When this work was planned its main object was to measure these enzymes in biopsy samples of liver, weighing 5–10 mg, from human infants. It was not considered practicable to treat the small amount of supernatant liquid available after centrifuging these micro-homogenates in any of the ways recommended by Pennington (1960) or Gaetani *et al.* (1964) to remove the endogenous amino acids which contribute to the blank. Blank values were obtained by incubating without the substrate and subtracting the value so obtained from the sample value. This practice was continued in later experiments. The supernatant liquid was taken for incubation with as little delay as possible. It was, however, found advisable after centrifugation to remove the supernatant liquid with a micro-pipette into a fresh tube in order to distribute the protein content evenly before sampling.

The method was essentially the same as that of Gaetani *et al.* (1964). An incubation mixture was made up for each run as follows: KF, 0·125 M; Tris buffer, pH 7·4, 0·25 M; $MgCl_2.6H_2O$, 0·025 M; ATP (disodium salt, neutralized to pH 7·4), 0·025 M; $^{32}$P-labelled sodium pyrophosphate solution containing approximately $1{\cdot}5 \times 10^6$ counts/min per ml, 0·025 M. All incubations of liver were done on the microscale, even if a macro-homogenate had been made, as follows: 25 $\mu$l of incubation mixture, 25 $\mu$l of an amino acid mixture, containing 0·01 M of each of twenty amino acids in Tris 0·02 M, pH 7·4, and 25 $\mu$l of supernatant liquid were heated in a stirred water bath at 37° for 15 min. The reaction was stopped with approximately 1 ml 5 % (w/v) trichloroacetic acid (TCA). Blanks were incubated at the same time with 25 $\mu$l of incubation mixture and 25 $\mu$l of supernatant liquid, but with 25 $\mu$l of Tris 0·02 M, pH 7·4, in place of the amino acid mixture. Muscle incubations were done with 100 $\mu$l of each of the reactants and stopped with 1 ml of 10 % TCA.

The procedure following the incubations was the same as that used by De Moss & Novelli (1956), except that 0·7 ml 1·2 N-HCl was used to hydrolyse the ATP. From this hydrolysate, 200 $\mu$l were taken for counting as in the method of Waterlow & Stephen (1966) and 200 $\mu$l portions were taken in duplicate for measurement of inorganic phosphate by the method of Taussky & Shorr (1953).

*Argininosuccinate lyase.* This enzyme was assayed in liver by the method of Schimke (1962*b*). A portion (5 $\mu$l) of homogenate was incubated for 30 min at 37° in 150 $\mu$l of

medium containing approximately: argininosuccinate, 0·02 M (barium salt, Sigma Chemical Co., adjusted to pH 7 with 0·1 N-$H_2SO_4$ and the $BaSO_4$ centrifuged down); sodium phosphate, 0·033 M, pH 7·4; arginase, 0·25 mg. The reaction was stopped by the addition of 1 ml 5 % TCA. Blanks contained all the reagents and the homogenate but had the TCA added at zero time. After centrifuging down the protein precipitate, urea was measured in two portions of 400 $\mu$l of the supernatant liquid by the method of Ceriotti & Spandrio (1963).

### *Metabolic experiments to measure urea excretion*

It was thought possible, in the light of the results for argininosuccinate lyase activity, that male and female rats kept on low-protein diets might excrete different amounts of urea. In order to test this, two male and two female rats were kept in individual metabolism cages on the 6 % casein diet for 13 days (period 1), and then refed on a stock diet for a further 13 days (period 2). One male and one female rat were also kept in metabolism cages on the stock diet throughout as controls. The rats were weighed daily, the amount of food eaten was measured as far as possible, and urine was collected over alternate 24 h periods. Total nitrogen was determined on the urines by nesslerization, urea plus free ammonia by the action of urease followed by nesslerization, and urea alone by the method of Ceriotti & Spandrio (1963). All the six rats were killed at the end of this experiment and argininosuccinate lyase activity was measured in their livers. In addition to the six rats in the metabolism cages, one male and one female rat on the stock diet and one male and one female on the low-protein diet were killed at the end of period 1 to provide an intermediate value for argininosuccinate lyase activity.

### *Measurement of protein*

The protein content of the supernatant liquids used for the amino acid activating enzyme assays was measured on solutions in 0·1 N-NaOH of the TCA precipitates in each of the incubation tubes by the method of Lowry, Rosebrough, Farr & Randall (1951). The protein content of the homogenates was also measured on solutions of TCA precipitates.

## RESULTS

### *Growth of rats*

On the 6 % casein diet the young rats did not usually grow, but they maintained their initial weight; they remained lively, although sometimes their coats deteriorated. At the time of killing, their liver weight as a percentage of body-weight was high, being about 50 % above that of controls. The livers were moderately fatty, containing 10–15 % of the wet weight as fat. When the low-protein diet was replaced by the stock food, the rats immediately began to put on weight, gaining as much as 15–18 % of their body-weight in the first 24 h; some of this increase must have been due to retention of water. After the 1st day they continued to gain weight steadily at the rate of about 5 g/day, and within 4 days the liver fat content had dropped to 5 %. Liver weight as a percentage of body-weight fell, and after 12 days of refeeding it was nearly the same as in the controls.

Table 1. *Enzyme activity in liver and muscle of rats given a 6 % casein diet for 4–7 weeks followed by a stock diet and of rats given a stock diet throughout*

| | Tissue | After depletion | After refeeding for: 3 days | 4 days | 6 days | 8 days | 12–13 days | Controls |
|---|---|---|---|---|---|---|---|---|
| Amino acid activating enzymes (μmoles P/mg protein per h) | Liver | 3·81 | 2·84 | 2·44 | 2·14 | 2·45 | 2·27 | 1·78 |
| No. of rats | | 12 | 4 | 3 | 6 | 4 | 6 | 15 |
| Difference from controls | | +2·03** | +1·06** | +0·66* | +0·36 | +0·67* | +0·49 | |
| SE of difference | | ±0·20 | ±0·30 | ±0·33 | ±0·25 | ±0·29 | ±0·25 | |
| Amino acid activating enzymes (μmoles P/mg protein per h) | Muscle | 0·40 | 0·46 | 0·52 | 0·61 | 0·53 | 0·55 | 0·41 |
| No. of rats | | 7 | 2 | 4 | 5 | 4 | 6 | 13 |
| Difference from controls | | −0·01 | +0·05 | +0·11 | +0·20** | +0·12* | +0·14* | |
| SE of difference | | ±0·041 | ±0·071 | ±0·058 | ±0·046 | ±0·052 | ±0·045 | |
| Argininosuccinate lyase (μmoles urea/mg protein per h) | Liver | 1·13 | 1·24 | 1·36 | 1·47 | 1·47 | 1·71 | 2·33 |
| No. of rats (♀) | | 8 | 5 | 5 | 8 | | 4 | 16 |
| Difference from controls | | −1·20** | −1·09** | −0·97** | −0·86** | | −0·62** | |
| SE of difference | | ±0·17 | ±0·21 | ±0·20 | ±0·17 | | ±0·23 | |

Significance levels: * $P < 0.05$; ** $P < 0.01$.

*Amino acid activating enzymes*

*In liver.* No difference between males and females was found. The enzyme activity tended to be high in young rats on the stock diet although there was considerable variation. The average for twelve weanling rats was 2·45 μmoles phosphorus exchanged/mg protein per h. In forty-two rats weighing between 100 and 200 g, some of which were not included in the enzyme experiments, the average activity was 1·69 μmoles P exchanged/mg protein per h (range 0·93–2·71); in rats weighing more than 200 g, the activity decreased slightly.

Table 1 shows that the enzyme activity per unit of protein rose markedly on a low-protein diet and began to fall immediately the rats were returned to the stock diet. At 6 days the value was not significantly different from that of the controls, although in the period 8–13 days the value appeared not to return completely to that in the controls.

*In muscle.* In muscle, as in liver, the activity of the amino acid activating enzymes was high in young rats; the average value for six weanling rats was 0·72 μmoles P exchanged/mg protein per h, which was significantly higher than the control value of 0·41 μmoles P exchanged/mg protein per h ($P < 0·01$).

Table 2. *Argininosuccinate lyase activity in the livers of male rats after a period on a 6% casein diet and at stages during refeeding on a stock diet, expressed as a percentage of the values for control rats given a stock diet throughout*

| | Argininosuccinate lyase activity | | | | |
|---|---|---|---|---|---|
| Period of depletion (weeks) | After depletion | After refeeding for: 3 days | 6–8 days | 12–13 days | Controls* |
| 4–7 | 111 (3) | 96 (1) | 116 (4) | 101 (4) | 100 (4) |

* Control rats were killed at the end of the depletion period and at intervals afterwards. Number of rats for each value shown in parentheses.

The effects of depletion and refeeding are shown in Table 1. In depleted rats the activity did not fall appreciably below control levels. However, when the rats were refed, the activity began to rise after 4 days and on average reached a peak at 6 days. At this time the activity was significantly higher than that of the controls and it was still significantly raised after 12–13 days refeeding.

*Argininosuccinate lyase activity in the liver*

In our rats, this enzyme showed different activity in the two sexes. In twenty male rats given a stock diet the average was 1·52 μmoles urea/mg protein per h and in forty-nine females 2·61 μmoles urea/mg protein per h. This difference, which was not at first noticed, means that the results for males and females have to be treated separately.

The results for depleted female rats are shown in Table 1. Enzyme levels were reduced to about half the control values by depletion. Even after 12–13 days of refeeding the activity was still significantly lower than that of the controls. Table 2 shows that male rats were scarcely affected by depletion.

### *Urea excretion*

Table 3 shows that liver argininosuccinate lyase activity in the male rat hardly changed after 13 days on the low-protein diet, whereas in the female rat it dropped to 63 % of the value for the control, a result which fits in with the findings in the main experiment. There was, however, no difference in urea excretion between the sexes. It was difficult to measure exactly the amount of the 6 % casein diet eaten because some of it was scattered, but the intake was between 6 and 8 g per day for each rat. This would contain approximately 0·45 g protein or 70 mg N. These rats excreted in the urine between 53 and 66 mg N daily/100 g body-weight, compared with about 200 mg N for the two controls. There appeared to be no difference between the male and female rats in the growth curves or total N excretion. When the depleted rats were refed, their total N excretion rose within the first 2 days to normal levels.

Table 3. *Urea excretion and argininosuccinate lyase activity in liver of male and female rats given a stock diet or a 6 % casein diet followed by a stock diet for two periods of 13 days each. Urine was collected over alternate 24 h periods*

| | | | Urea N/total N (mean for period) (%) | | Argininosuccinate lyase activity at end of period (μmoles urea/mg protein per h) | |
|---|---|---|---|---|---|---|
| Period | Days | Diet | ♂ | ♀ | ♂ | ♀ |
| 1 | 0–13 | Stock | 86·7 | 87·5 | 1·58 | 2·28 |
| 2 | 14–27 | Stock | 82·8 | 81·3 | 1·33 | 2·12 |
| 1 | 0–13 | 6 % casein | 74* | 69* | 1·37 | 1·44 |
| | | | 70 | 73 | | |
| 2 | 14–27 | Stock | 79·5 | 80 | 1·34 | 1·95 |
| | | | 78 | 78 | 1·28 | 2·45 |

* Urea excretion of rats on the 6 % casein diet during the first 24 h period has not been included in this mean value, since the rats were becoming adjusted to the reduced protein intake.

## DISCUSSION

The emphasis in these experiments was on young growing rats, because the work was designed as a background to the studies on malnourished infants, who grow very rapidly once they begin to recover on a good diet. Srinivasan & Patwardhan (1955) have pointed out that the effects of protein-deficient diets are much quicker and more severe in young animals because of the demands of growth as well as those of maintenance.

The values of argininosuccinate lyase reported in this paper for young rats agree very well with those which Schimke (1962*a*) found for adult male rats. In his experiments, the decrease in argininosuccinate lyase to a third of its initial value took place within 4 days of changing from a diet containing 15 % casein to a protein-free diet. We found it more difficult to make rats eat a protein-free diet and our depleted rats therefore received a diet containing 6 % casein over a longer period with less dramatic

effects. On refeeding, the return of the argininosuccinate lyase to normal levels was slow in young female rats, presumably because of the continuing need for conservation of N for the purpose of protein synthesis. The finding that male rats did not respond in the same way as females is so far unexplained. In both sexes the proportion of total urinary N found as urea N was higher than would be expected from the work of Kiriyama & Ashida (1964); they found that, in rats of a similar age given a protein-free diet, the urea N in total urinary N was 54 % and with a 14 % casein diet it was 62·5 %. Even with a 6 % casein diet, our rats excreted 70 % of their total N as urea. It does not seem, therefore, that the reduction in activity of this one enzyme of the urea cycle found in female rats results in any serious impairment of urea production. However, it could be said that with respect to argininosuccinate lyase activity male rats do not become adapted as well as females to a low-protein diet. Radhakrishnan (1966) has noted that, after a period of protein depletion, the growth of young male rats was more retarded than that of young females. There is also evidence that malnutrition in early life has a more lasting effect on the learning behaviour of male rats than on that of female rats (Barnes, Cunnold, Zimmermann, Simmons, MacLeod & Krook, 1966).

The original experiments of Mariani *et al.* (1963) and Gaetani *et al.* (1964), like those of Schimke (1962*a*, *b*; 1963), were done with adult rats. Although the activity of the amino acid activating enzymes was increased in the livers of the protein-depleted rats, no difference in the activities of these enzymes was found in the muscle unless the results were expressed in terms of DNA-P. In later work, Mariani, Migliaccio, Spadoni & Ticca (1966) measured these enzymes in the livers of growing rats and related them to the number of nuclei and the DNA-P content. In the young rats on a normal diet, the total amino acid activating enzyme activity and the total DNA content both increased with increase in weight of the liver. The enzyme levels per unit DNA were therefore unaffected by growth. When growth was prevented by protein deprivation, the total liver DNA content remained constant but the enzyme levels per unit DNA were almost doubled 30 days after weaning. We also found changes of this order of magnitude in the enzyme levels in protein-depleted young rats when expressed in terms of protein.

The results presented here for amino acid activating enzymes therefore agree with those of the Italian workers in that protein-depleted rats show high levels of activity in the liver but little change in muscle when expressed in terms of protein; the values found in our experiments are very similar to theirs. We found that, on returning the rats to a stock diet, the liver enzymes immediately began to decrease, and when they were almost normal the muscle enzymes began to increase (Fig. 1). Mendes & Waterlow (1958) have shown that on refeeding young rats after a low-protein diet recoveries of liver protein and liver DNA follow very similar patterns. Therefore it might be expected that the activity of these enzymes at this stage would change in the same way whether they are expressed in terms of protein or of DNA. If the levels of amino acid activating enzymes in the tissues reflect the amount of protein synthesis taking place, as Gaetani *et al.* (1964) suggested, then it would seem that 4–6 days after the return to the stock diet the emphasis of protein synthesis shifts from the liver

to the muscle. This immediate response of liver to refeeding and the slight time lag before muscle takes over have also been observed by Mendes & Waterlow (1958).

Spadoni and her co-workers (Mariani *et al.* 1963; Gaetani *et al.* 1964; Mariani *et al.* 1966) suggest that the increase in amino acid activating enzymes in the livers of protein-depleted rats is independent of the DNA and is brought about by a reduction in the size of the circulating free amino acid pool. Waterlow & Stephen (1966) have been unable to find any evidence that the free amino acid content of livers in protein-deficient rats is significantly different from that of control rats. Furthermore, in the experiments reported in this paper, the 'blank', which is attributable to the endogenous free amino acids, did not differ appreciably between control and depleted animals.

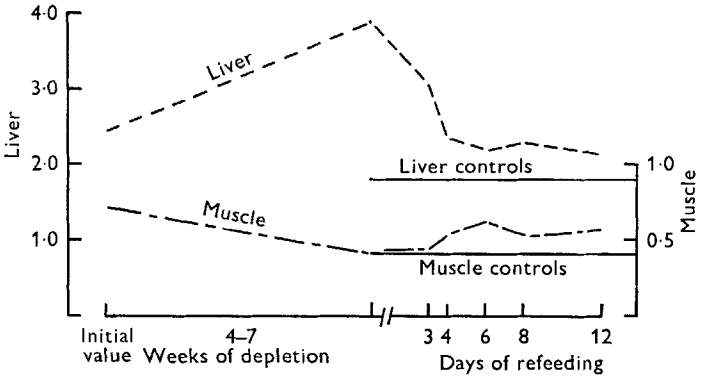


Fig. 1. Amino acid activating enzyme activity (μmoles phosphorus exchanged/mg protein per h) in liver and muscle of rats during a period of protein depletion followed by refeeding on a stock diet.

As a result of the findings of Alleyne & Young (1966, 1967) that blood cortisol levels were high in children with protein malnutrition, we have considered the possibility that the enzyme changes found in these rats could have been affected by hormones. Durbin & Heard (1962) have also found that protein-deficient pigs excrete larger amounts of adrenal steroids than normal pigs. The protein-depleted rats in the experiments reported here had livers which were enlarged in proportion to their body-weight. Some of this increase could have been due to fat accumulation, but in those livers in which the fat content was measured, it only amounted to 15% of the wet weight at the most, and this could not have accounted for the increase in total weight of the depleted livers.

An increase in the ratio of liver weight to body-weight can be caused by administration of cortisone (Weber & Singhal, 1964). The enlargement of the livers which we found, therefore, could be explained by an increased production of adrenocorticoid hormones. Weber & Singhal (1964) also reported that cortisone produced increased glucose-6-phosphatase activity. We also found (Stephen, unpublished) high glucose-6-phosphatase levels in the livers of these protein-deficient rats, which was unexpected in view of the contrary results of Freedland & Harper (1959) and Fletcher (1966).

Cortisone is known to stimulate the incorporation of amino acids into liver (Munro,

1964), so that high levels of amino acid activating enzymes might be expected. Mariani *et al.* (1963) discounted the effect of steroids on these enzymes on the basis of the work by Smith, Koeppe & Franz (1961). However, the experiments of Smith *et al.* (1961), in which tryptophan oxygenase activity in the liver was increased after the injection of cortisone but the amino acid activating enzymes showed no change, were done with adult rats, and conditions may well be different in younger animals with a potential for rapid growth. McCorquodale & Mueller (1958) showed very convincingly that the amino acid activating enzymes in another tissue, rat uterus, were considerably enhanced in activity by the administration of oestradiol.

The effect of adrenal hormones on the enzymes of the urea cycle seems to be much more complex. McLean & Gurney (1963) found that arginine synthetase, argininosuccinate lyase and arginase were all reduced after adrenalectomy and administration of growth hormone in rats. Cortisone given in small doses has little effect on urea excretion and the urea cycle enzymes but, when excessive protein catabolism is induced by larger doses of cortisone or by diabetes, the levels of these enzymes are raised (Freedland & Sodikoff, 1962; Schimke, 1963; McLean & Gurney, 1963), as they are in starvation (Schimke, 1962*a*). If cortisol levels were raised in our rats, therefore, it is difficult to predict how this would have affected the argininosuccinate lyase activity.

The results reported in this paper for these two groups of enzymes connected with protein synthesis demonstrate one way in which the growing animal can become adapted to a reduced protein intake. The high level of amino acid activating enzymes in the liver enables this organ to make use of such amino acids as are available. On the other hand, if argininosuccinate lyase can be taken as representing the enzymes of the urea cycle, reduced levels prevent amino acids from being broken down and excreted. Synthesis of protein in muscle takes second place until the supply of raw materials for other more vital organs is assured. The effect that adrenocorticoid and other hormones have on these various processes is a subject for further study.

I thank Professor J. C. Waterlow for his constant interest and advice during these experiments; and Mr A. V. Swann and Mr M. J. R. Healy of the Division of Computing and Statistics, Clinical Research Centre, for their statistical analysis of the results.

## REFERENCES

Alleyne, G. A. O. & Young, V. H. (1966). *Lancet* i, 911.
Alleyne, G. A. O. & Young, V. H. (1967). *Clin. Sci.* **33**, 189.
Barnes, R. H., Cunnold, S. R., Zimmermann, R. R., Simmons, H., MacLeod, R. B. & Krook, L. (1966). *J. Nutr.* **89**, 399.
Bendicenti, A., Mariani, A., Paolucci, A. M., & Spadoni M. A. (1959). *Boll. Soc. Biol. sper.* **35**, 1997.
Ceriotti, G. & Spandrio, L. (1963). *Clinica chim. Acta* **8**, 295.
De Moss, J. A. & Novelli, G. D. (1956). *Biochim. biophys. Acta* **22**, 49.
Durbin, P. A. J. & Heard, C. R. C. (1962). *Proc. Nutr. Soc.* **21**, xxviii.
Fletcher, K. (1966). *Am. J. clin. Nutr.* **19**, 170.
Freedland, R. A. & Harper, A. E. (1959). *J. biol. Chem.* **234**, 1350.
Freedland, R. A. & Sodikoff, C. H. (1962). *Proc. Soc. exp. Biol. Med.* **109**, 394.
Gaetani, S., Mariani, A., Spadoni, M. A. & Tomassi, G. (1961). *Boll. Soc. Biol. sper.* **37**, 1685.
Gaetani, S., Paolucci, A. M., Spadoni, M. A. & Tomassi, G. (1964). *J. Nutr.* **84**, 173.
Kiriyama, S. & Ashida, K. (1964). *J. Nutr.* **82**, 127.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
McCorquodale, D. J. & Mueller, G. C. (1958). *J. biol. Chem.* **232**, 31.
McLean, P. & Gurney, M. W. (1963). *Biochem. J.* **87**, 96.
Mariani, A., Migliaccio, P. A., Spadoni, M. A. & Ticca, M. (1966). *J. Nutr.* **90**, 25.
Mariani, A., Spadoni, M. A. & Tomassi, G. (1963). *Nature, Lond.* **199**, 378.
Mendes, C. B. & Waterlow, J. C. (1958). *Br. J. Nutr.* **12**, 74.
Munro, H. N. (1964). In *Mammalian Protein Metabolism.* Vol. 1, chapter 10. [H. N. Munro and J. B. Allison, editors.] New York and London: Academic Press Inc.
Pennington, R. J. (1960). *Biochem. J.* **77**, 205.
Radhakrishnan, M. R. (1966). *Indian J. med. Res.* **54**, 486.
Schimke, R. T. (1962*a*). *J. biol. Chem.* **237**, 1921.
Schimke, R. T. (1962*b*). *J. biol. Chem.* **237**, 459.
Schimke, R. T. (1963). *J. biol. Chem.* **238**, 1012.
Smith, A. L., Koeppe, O. J. & Franz, J. M. (1961). *Endocrinology* **69**, 872.
Srinivasan, P. R. & Patwardhan, V. N. (1955). *Indian J. med. Res.* **43**, 1.
Stenram, U. & Hirschman, R. (1965). *Acta anat.* **61**, 445.
Stephen, J. & Waterlow, J. C. (1968). *Lancet* i, 118.
Taussky, H. H. & Shorr, E. (1953). *J. biol. Chem.* **202**, 675.
Waterlow, J. (1959). *Nature, Lond.* **184**, 1875.
Waterlow, J. C., Alleyne, G. A. O., Chan, H., Garrow, J. S., Hay, A., James, P., Picou, D. & Stephen, J. M. L. (1966). *Archos lat.-am. Nutr.* **16**, 175.
Waterlow, J. C. & Stephen, J. M. L. (1966). *Br. J. Nutr.* **20**, 461.
Weber, G. & Singhal, R. L. (1964). *J. biol. Chem.* **239**, 521.

*Printed in Great Britain*