CLVIII. STUDIES ON THE METABOLISM OF ARGININE AND HISTIDINE.

PART II. ARGININE AND HISTIDINE AS PRECURSORS OF PURINES.

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IN the first paper of this series [Stewart, 1925] it was reported that liver perfusion experiments had failed to afford any evidence in support of the suggestion, advanced by Ackroyd and Hopkins [1916], that arginine and histidine are utilised in the animal body as precursors of purines. As was there pointed out, however, the evidence derived from the perfusions, while unable to confirm the hypothesis, was equally unable to invalidate it, and so did nothing to remove the uncertainty as to the function of arginine and histidine which has been produced by the contradictory results of subsequent workers.

It appeared, therefore, that a repetition of the work of Ackroyd and Hopkins might be useful to confirm the experimental basis on which their conclusions were founded. Further, such a series of experiments might throw some light on the cause of the contradictory results which have been published. For it is noticeable that, whereas Ackroyd and Hopkins and also Harding and Young [1919], who obtained similar results, employed young growing animals in their experiments, others, like Lewis and Doisy [1918], who failed to find any connection between purine excretion and histidine and arginine intake, employed adult animals. Indeed, Harding and Young remark, as a possible explanation of the discrepancies, that in the adult animal there may be a cessation or serious impairment of many synthetic processes which are active in the young. There already exists, of course, some evidence for such a state of affairs in the case of tryptophan. Although, as has been abundantly proved by the work of Hopkins and Willcock [1907] and others, young animals are unable to maintain growth on a tryptophan-free diet, Osborne, Mendel and Ferry [1912] have succeeded in keeping adult animals for 178 days on a diet containing no tryptophan without appreciable loss of weight.

The feeding experiments reported in this paper were thus undertaken for a twofold purpose: to confirm the experimental foundation of the suggestion

that histidine and arginine are used as purine precursors and are interchangeable in metabolism, and, by comparing the behaviour of adult and growing animals, to attempt an explanation of the divergent results which have been obtained hitherto.

EXPERIMENTAL.

Four experiments, in each of which two rats were used, are described. The animals were all bred from Wistar Institute stock, and were all females. In Exps. 1 and 2, which were designed to test the effect of arginine and histidine deficiency on adult animals, the four rats were from the same litter, and had passed the menopause. In Exps. 3 and 4, again, all four rats were from the same litter, but were started on the experimental diet when their weight was nearly 50 g. The pairs of rats were kept in cages of the type described by Ackroyd and Hopkins [1916], and the temperature was kept as constant as possible. For the greater part of the time it remained at about 65° F., but occasionally, during the hot weather, it rose to 70° F. The animals, however, did not appear to suffer and continued to eat well.

For the collection of the urine and removal of contaminating faeces and food particles, the apparatus described by Ackroyd and Hopkins was employed. The funnel in which the cage stood was washed down with warm water several times each day, the urine and washings being at once transferred to a stoppered flask containing a little chloroform and sufficient acetic acid to keep the mixture definitely acid. The flask was kept in an ice-chest and the week's collection made up to a convenient volume—usually 500 cc.—for analysis.

The total nitrogen in the urine was determined by a micro-Kjeldahl method. After removal of ammonia, Wiechowski's method [1913] was employed for the determination of allantoin. In the final stage, however, after precipitation of the allantoin by the mercuric acetate reagent, the nitrogen content of the precipitate was determined by the Kjeldahl method, a procedure which preliminary experiments had shown to be reliable.

The diets used were prepared in the manner described by Ackroyd and Hopkins. In the control diet the percentage of nitrogen (2.1) was rather lower than in the particular example given by these authors (3.12), although they state that they used diets containing as little as 2.0 % of nitrogen. Tryptophan was added to the extent of 1.5 %, and cystine to 0.5 % of the weight of the caseinogen originally taken for hydrolysis.

The vitamin supply was drawn partly from the fresh butter used to provide the fat of the diet, and partly from marmite, a commercial yeast extract. The marmite was ground with plaster of Paris and extracted with absolute alcohol. Since the extract contained purines and amino acids, it was evaporated to dryness and the residue dissolved in a little water. Silver sulphate was added in quantity for complete precipitation first in acid, and second in alkaline solution (baryta was used in making the mixture alkaline).

After complete removal of silver, barium, and sulphate ions, the solution, now free from purines, and giving no more than a trace of colour with diazobenzenesulphonate, was evaporated to a syrup, which was mixed with starch and, after drying in a vacuum desiccator, incorporated into the diet in the proportion of 5 g. of original marmite to 100 g. of original caseinogen. No doubt, in the silver precipitations a certain amount of vitamin was carried down, but, as control experiments showed, the supply was adequate for satisfactory growth.

The removal of histidine and arginine from the hydrolysed caseinogen was accomplished by the silver precipitation method of Kossel and Kutscher, using silver sulphate and making alkaline with baryta. After quantitative removal of silver, barium, and sulphate ions the solution was evaporated *in vacuo*. When care was taken to add a liberal excess of silver, the resulting syrup was free from all but the merest traces of arginine and histidine. Samples of the syrup corresponding to 1 g. caseinogen, dissolved in 5 cc. water, gave neither the diacetyl test for arginine nor the diazobenzenesulphonate test for histidine.

In those diets from which only one of the diamino acids was to be absent, the other was restored to the silver-precipitated mixture. For every 100 g. original caseinogen, 2 g. amino acid were added, *i.e.* approximately the amount present in the protein. No attempt was made to discover the minimum amount of either histidine or arginine requisite for maintenance or growth, and the amount actually added supplied the animals with a very large excess over the quantity which would be needed to produce the allantoin excreted.

Throughout the experiment the animals received a diet containing the same proportion of fat, carbohydrate and nitrogen. The diets at the different stages of the experiment differed only in the presence or absence of arginine and histidine. The consumption of food was good throughout, and though the daily consumption per pair of rats varied by a gram or two, the weekly averages were fairly uniform and showed no tendency either to fall or rise during the critical periods of the experiments. Thus in Exp. 4 (rats VII and VIII) the daily food consumption per rat varied only from 5.8 to 6.7 g.

RESULTS AND DISCUSSION.

1. *The effect of histidine and arginine deficiency on young rats.*

Fig. 1 shows the rate of growth of the young animals during the experimental period. At first, while the control diet containing a complete supply of amino acids was being given, growth proceeded at a rate which, though rather less than normal, may be regarded as satisfactory. Since each curve gives the combined weight of two rats, the average daily increase per rat was about 0.75 g. On a diet of bread and milk, similar rats were growing at the rate of about 1 g. per day. The withdrawal of histidine and arginine brought about a complete change. At once the animals ceased to grow in spite of an

undiminished food consumption, and during the period in which this deficient diet was being given, they lost weight at an average rate of 0.4 g. per day. When histidine was restored to the diet of rats VII and VIII, growth was resumed and proceeded at a rate which was unaltered when arginine was restored as well, so that the animals were once more receiving the complete amino acid mixture.

Fig. 1.

- Arginine and histidine removed from diet.
- Arginine and histidine restored to diet.
- ◆ Arginine alone restored to diet.
- ◇ Histidine alone restored to diet.

The case of arginine is not so clear. During the period when this amino acid was present in the diet while histidine was absent (rats V and VI), there was a preliminary fall in weight—or rather continuation of the fall—followed by a definite rise over a week and a second slight fall. The net result of a fortnight's feeding on this diet was a very slight increase in weight, very much less than the increase during the similar period in the case of rats VII and VIII. The restoration of the complete amino acid mixture was again accompanied by satisfactory growth. This result does not support that of Ackroyd and Hopkins who suggested that arginine and histidine are essentially equivalent—*i.e.* interchangeable—in metabolism. Certainly the method of carrying out the experiments differs in one respect from that employed by Ackroyd and Hopkins, although the difference does not appear to be significant. Their animals, after a period on the control diet, received a diet which contained arginine but not histidine (or *vice versa*); they never, as in the experiments reported in this paper, had one or other of the amino acids restored to a diet deficient in both.

The fact that on the restoration of arginine to the diet of rats V and VI the fall in weight ceased and the animals succeeded in maintaining themselves at the lower level (if not in growing, for, if one neglects the fall immediately after the resumption of arginine feeding—a fall which occurs also in the case of rats VII and VIII when histidine was first restored—there is a definite increase of weight) may perhaps be significant. It is not in agreement with the observation of Rose and Cox [1924] who found that arginine, as carbonate or nitrate, entirely failed to prevent the loss in weight. That statement is absolutely definite, and quite justified by their experimental observations, but so is the exactly opposite one of Ackroyd and Hopkins.

The three results are not explicable on the assumption that varying amounts of arginine had escaped precipitation in the preparation of the "deficient" diets. There is no test for arginine comparable in delicacy with the diazobenzenesulphonate test for histidine, and, as Rose and Cox point out, the silver salt of arginine is more soluble than that of histidine, so that it is probable that traces, at any rate, of arginine remained in the deficient diets. Nevertheless, if lack of arginine be partly responsible for the fall in weight which does actually occur when it and histidine are withdrawn from the diet (or if it and histidine are interchangeable), it follows that the residual minute amount cannot be sufficient for the animal's needs, and that, consequently, there should be some resumption of growth when arginine is restored; in no case should the fall in weight continue. Such fall in weight can only mean that arginine is not concerned in the nutritional failure which is being manifested, in which case the completeness or otherwise of its removal is unimportant.

Contamination with histidine of the arginine used by Ackroyd and Hopkins and by the author would, of course, explain the results at once, but seems extremely unlikely. Certainly in the sample of arginine used in these experiments there was no admixture of histidine (as shown by the absence of any coloration with diazobenzenesulphonate) and Hopkins has stated that his supply was similarly pure.

For a full solution of the problem more data appear to be required. The essential question in the author's experiments is whether the administration of arginine was the factor responsible for the resumption of maintenance, or whether, in the absence of arginine, the loss of weight would still have ceased. Experiments on this point are in progress. Ackroyd and Hopkins point out a tendency, in their experiments, to the attaining of an equilibrium after a period of arginine and histidine deficiency, stating that it may possibly be due to slightly incomplete removal of these substances. Nevertheless, in all their experiments the rats continued to lose weight over a period of 4 weeks during which they received only such traces of arginine and histidine as had escaped precipitation. In the experiments of Rose and Cox the loss of weight continued, though later at a diminished rate, for much longer periods. Hence, although a definite conclusion must be withheld until the termination of the

experiments now in progress, there does seem, in the growth curve of rats V and VI, to be an indication that arginine deficiency plays some part in the nutritional failure which follows the removal of it and histidine from the diet.

Fig. 2.

- Arginine and histidine removed from diet.
- Arginine and histidine restored to diet.
- ◆ Arginine alone restored to diet.
- ◇ Histidine alone restored to diet.

Fig. 2 shows the weekly excretion of allantoin by each pair of rats during the experimental period. When the rats were receiving the control diet, and were growing at a satisfactory rate, the allantoin excretion increased slightly with the weight—a result in accordance with that published by Rose and Cook [1925] while this work was in progress. When arginine and histidine were withdrawn, the allantoin excretion rapidly fell, at a rate proportionately much greater than did the weight, until in the third week of the deficient diet it reached a value almost half the original. Restoration of histidine to the diet (rats VII and VIII) was followed by a rise in the allantoin excretion accompanying the resumption of growth. So far the results are in complete agreement both with those of Ackroyd and Hopkins and of Rose and Cook. The results following the restoration of arginine, however, present the same difficulties here as in the question of growth. They are indefinite, confirming neither the positive results of Ackroyd and Hopkins nor the negative ones of Rose and Cook. If anything, they incline towards the latter, for the allantoin excretion appeared to have reached a minimum before the arginine was added to the diet. Still, the protocols of Rose and Cook show that in a long continued deprivation of arginine and histidine the fall in allantoin excretion was often irregular, and that such a halt was often followed by a further fall. Hence the present experiment can neither affirm nor deny that arginine has an effect on the purine excretion; one can only say that if such an effect exists, it is much less than that of histidine.

It must not be forgotten that in any case the conversion of histidine and arginine to purines (if such a conversion takes place) is only one of perhaps

several possible metabolic paths. It may well be that in the case of histidine, which already contains the iminazole ring, this conversion proceeds rapidly in comparison with other competing reactions; whereas the reactions which convert arginine to a form suitable for purine synthesis have a velocity which is small compared with that of reactions which divert it into other metabolic paths (*e.g.* the formation of ornithine).

In considering whether these results justify the conclusion that histidine is a precursor of purines, it is of importance to note not merely the variations in the absolute amount of allantoin excreted when histidine is withdrawn from and restored to the diet, but also the variations of the ratio between the allantoin nitrogen and the total nitrogen of the urine. Should both allantoin and total nitrogen vary in the same sense and to the same degree (*i.e.* should the ratio of allantoin nitrogen to total nitrogen remain constant) it would appear probable that the alterations in the allantoin excretion were signs, not of any special disturbance of purine metabolism, but simply of nutritional failure generally. Now actually, while the nitrogen intake remained roughly constant, the total excretion of nitrogen in the urine by rats VII and VIII was 1.36, 1.40, 1.31, 1.28, 1.26, 1.34, 1.40, 1.39 and 1.43 g. in successive weeks. The slight decrease in the third, fourth, and fifth weeks, when the animals were receiving the diet free from histidine and arginine, is almost equal to the decrease in the allantoin excretion. In this experiment, as in that with rats V and VI, when arginine and histidine were withdrawn from the diet, the ratio of allantoin nitrogen to total urinary nitrogen showed a very considerable drop, corresponding to the fall in the absolute amount of allantoin excreted (Fig. 3). When histidine was restored to the diet the ratio again rose. With restoration of arginine alone, however, the ratio remained low, showing that any effect on purine metabolism possessed by this substance can only be slight. The evidence in favour of the conclusion that histidine bears a very intimate relationship to the purine metabolism is considerably strengthened by the fall in the allantoin excretion relative to the total nitrogen excretion when the two amino-acids are withdrawn from the diet and the subsequent rise when histidine is alone restored.

Summarising, one may say that the experiments completely confirm those of Ackroyd and Hopkins so far as histidine is concerned; that they do not support the suggestion that histidine and arginine are completely interchangeable in metabolism; and that they leave it doubtful whether arginine has any effect either in growth or in purine metabolism. The experiments of Ackroyd and Hopkins, confirmed here, and by Rose and Cook, afford ample evidence for concluding that histidine is, or may be, utilised by the growing animal for the manufacture of purines.

2. *The effect of arginine and histidine deficiency on adult rats.*

The experiments in which adult rats were used yielded a perfectly definite result. Rats I and II (Fig. 4) received the complete amino acid mixture through-

Fig. 3.

- Arginine and histidine removed from diet.
- Arginine and histidine restored to diet.
- ◆ Arginine alone restored to diet.
- ◇ Histidine alone restored to diet.

Fig. 4.

- Arginine and histidine removed from diet.

out the experimental period. Their weight remained constant, and so did both the amount of allantoin excreted per week and the ratio of allantoin nitrogen to total urinary nitrogen. Rats III and IV, from the same litter, gave similar constant results on a diet from which arginine and histidine had been withdrawn. Although the rats received the deficient diet only for 28 days, it seems quite certain that there is a very marked difference in histidine requirements between adult and growing animals, and the fact that the former are able to maintain themselves without external supplies of the amino acids readily explains the failure of such workers as employed them over similar periods to confirm the results of Ackroyd and Hopkins.

So far as body weight is concerned, Geiling [1917] claimed that addition of either arginine or histidine to a diet otherwise deficient rendered the nitrogenous portion of the diet adequate for maintenance of body weight in adult mice. The sole source of nitrogen in his deficient diet was that portion of a tryptic digest of caseinogen which was not precipitated by phosphotungstic acid, with the addition of cystine and tryptophan. Apart from the fact that, according to the data of Rose and Cox [1924], the protein was probably incompletely digested, the figures published by Geiling seem hardly able to support the interpretation put upon them.

Abderhalden [1922] has published the results of a series of feeding experiments, using adult rats, in which the nitrogen of the diet was derived entirely from a mixture of pure amino acids. He states that both arginine and histidine are indispensable amino acids. In one of his experiments addition of arginine did not remedy a deficiency caused by absence of histidine, so that his work does not support the idea of the interchangeability of the two substances. His curves certainly show that his animals lost weight when histidine and arginine were absent, but the multiplicity of the deficiencies renders his results exceedingly difficult of interpretation.

The experiments reported in the present paper do not, of course, necessarily mean that in the adult animal histidine no longer functions as a purine precursor. It may be that over the comparatively short period of the experiment the adult animal, though not the growing one, may be able to draw the necessary supplies of purine from some source as yet undiscovered; that histidine, whilst an important source of raw material in the young animal with its relatively greater need for purines, becomes a minor factor in the adult animal. In that case, just as readily as if histidine had no part whatever in purine metabolism, the adult, though normally utilising a certain amount of histidine to synthesise purines, would be able to subsist, at any rate for some time, without it. It is certain, however, that some difference exists in the need for histidine during, and after the cessation of growth.

The actual figures for the total nitrogen and the allantoin excretion show very clearly that in the young animal, not only is the nitrogen metabolism as a whole more active than in the adult, but that the purine metabolism is more active to an even greater degree. While all the animals were receiving

the complete supply of amino acids, the young ones, in a week when their average weight was 61.5 g., excreted an average of 0.78 g. nitrogen and 0.209 g. allantoin per rat, while the allantoin nitrogen formed 9.55 % of the total. In the same week, the adults, with an average weight of 217.5 g. excreted 1.29 g. nitrogen and 0.243 g. allantoin per rat, the allantoin nitrogen being 6.7 % of the total. Thus the adults, more than three times the weight of the young ones, excreted only about 60 % more nitrogen, and only 16% more allantoin. In their case, too, the allantoin nitrogen formed a much smaller percentage of the total than in the case of the young ones.

SUMMARY.

On a diet deficient in histidine and arginine young rats lose weight, and their allantoin excretion, but not the total nitrogen excretion, falls almost to half the normal value. Restoration of histidine to the diet is followed by resumption of growth, and by increased allantoin excretion. It is concluded that, as suggested by Ackroyd and Hopkins, histidine functions as a precursor of purines in the animal body.

Restoration of arginine to the deficient diet is not followed either by appreciable growth or by increased allantoin excretion. The experiments, therefore, do not support the idea that arginine and histidine are completely interchangeable. Since, however, the restoration of arginine was followed by cessation both of the fall in weight and in allantoin excretion, it remains doubtful whether arginine plays any part at all in the purine metabolism.

Adult rats were able to live for 28 days without loss of weight or decrease in the allantoin excretion on a diet containing no more than traces of histidine and arginine.

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