The work of Stefano & Diermeier (1956) on the effect of growth hormone upon the development of polyploidy in the liver is in keeping with the hypothesis that growth hormone is important in the growth of the liver. Proof of the hypothesis requires work on liver regeneration in hypophysectomized rats. Some preliminary experiments which we have performed gave no positive result, but investigations are being continued.

It is also of interest to enquire whether growth hormone exerts its effect (*a*) in the events which precede DNA synthesis, (*b*) during DNA synthesis, or (*c*) during the mitotic act. In our experiments growth hormone was used in effective concentration during all of these significant periods. The results would suggest that the effect is upon events in stage (*a*) or (*b*), or possibly all three. This problem is capable of experimental investigation and further work is in progress.

Another problem for solution is whether growth hormone acts directly upon regenerating liver, or whether its effect is exerted through some other hormone or combination of hormones. For example, the close co-operation between growth hormone and insulin has been reviewed by Young (1953).

SUMMARY

1. Rats after partial hepatectomy were treated with subcutaneous injections of growth hormone, 2 mg. at 0 hr., 4 mg. at about 8 hr. and 4 mg. at 4–6 hr. before killing. These were compared with control rats in regard to rates of mitosis, deoxynucleic acid synthesis and ribonucleic acid synthesis from 22 to 30 hr. after partial hepatectomy.

2. The wave of mitosis starts slightly earlier (by about 1 hr.), and is two to three times higher in the growth-hormone-treated rats.

3. The rate of deoxynucleic acid synthesis shows a higher and more prolonged peak in the growth-hormone-treated rats.

4. The rate of ^{32}P incorporation into the Schmidt–Thannhauser ribonucleic acid fraction is higher during the peak of synthesis in the growth-hormone-treated rats.

The authors wish to express their thanks to Professor J. S. Mitchell, F.R.S., for his constant advice and encouragement, and to Professor F. G. Young, F.R.S., for his generous gift of growth hormone and for his interest in the project. Mr Basil Slater very kindly prepared the growth hormone and Miss N. C. Burr prepared all the sections.

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L-Cystathionine in the Urine of Pyridoxine-deficient Rats

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Some time ago, when a study was made of the excretion of taurine in rats reared on a diet deficient in pyridoxine, a new ninhydrin-positive spot was seen on two-dimensional paper chromatograms which was absent from normal rat urine (Blaschko, Datta & Harris, 1953) and which was provisionally identified as phosphorylethanolamine. When in the present series of experiments this spot was again noticed it was decided to characterize the compound more fully.

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METHODS

Animals. Two different strains of rats were used: Wistar albinos obtained from the Anatomy Department, Birmingham University, and hooded rats of the Lister strain, which

were obtained at first from the M.R.C. Department for Research in Industrial Medicine, Hampstead, and later from the British Ratin Co. Ltd., East Grinstead, Sussex. The albino rats were freshly weaned and weighed 40 g. on arrival; those of the hooded Lister strain usually had the same initial weights; however, the animals in litter F, which was used for the isolation and characterization of the unknown compound, each weighed about 80 g. at the beginning of the experiment.

The animals were kept on the pyridoxine-deficient diet previously described (Blaschko *et al.* 1953). A daily dose of 30 μg. of pyridoxine was added to the diet of the control rats. The urine passed through a perforated metal disk and a plug of glass wool in a funnel before being collected daily in a flask containing 0·2 ml. of N-HCl per rat. The urine was then stored at $-10°$.

Reference compounds. A sample of cystathionine containing all four isomers was obtained from the California Foundation for Biochemical Research. Small samples of each of the four isomers of cystathionine were kindly given by Professor V. du Vigneaud; the phosphorylethanolamine I owe to Dr S. P. Datta.

Paper chromatography. Urine was examined by two-dimensional paper chromatography at $24 \pm 1°$ by a procedure similar to that of Grobbelaar, Pollard & Steward (1955) and Coulson (1955). Solvent systems were phenol saturated with water, followed by butanol–acetic acid–water (4:1:5) (Partridge, 1948). Chromatograms were sprayed with the ninhydrin reagent of Woiwod (1949).

EXPERIMENTAL

When urine from deficient rats of the hooded Lister strain was examined, the spot noticed by Blaschko *et al.* (1953) was readily observed. This spot was absent from the urine of rats from the same strain fed on the pyridoxine-supplemented diet. The spot was also absent from the urine from pyridoxine-deficient animals of the albino Wistar strain.

A chromatogram of rat urine from a deficient animal is shown in Fig. 1. The most conspicuous spot is that of the unknown compound (*A*). Two less conspicuous spots (*E* and *F*) were absent from the urines of normal rats but present in the urine of all deficient animals.

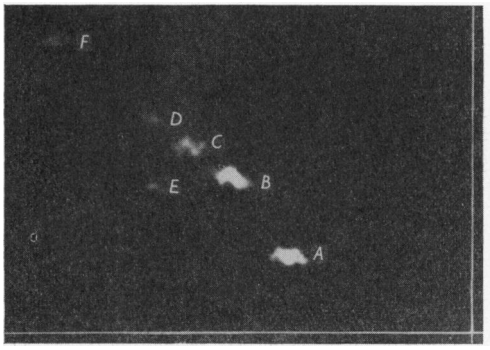

Fig. 1. Two-dimensional paper chromatogram of urine from a hooded rat deficient in pyridoxine for 6 weeks. *A*, unknown compound; *B*, glycine and serine; *C*, threonine; *D*, alanine; *E* and *F*, not identified.

In litter F the time course of the appearance of the unknown spot was followed more closely. Detectable amounts of the compound appeared in the urine of the three males at the end of the second week. Four females were excreting it by the end of the fourth week and urine from the remaining three females contained the compound at the end of the fifth week. It thus appears that the compound was excreted at an earlier stage in the males than in the females. At the end of the fifteenth week nine of the ten animals had survived: all excreted the unknown compound.

Two closely adjacent spots were found on chromatograms of deficient-rat urine to which phosphorylethanolamine had been added. This showed that the unknown compound could not be phosphorylethanolamine. From a comparison with a sample of urine run in the absence of added phosphorylethanolamine it appeared that the unknown spot ran faster in phenol. A comparison with the chromatographic map of amino acids given by Dent (1948) showed that cystathionine behaved in a similar way. When a sample of the commercial cystathionine was added to the urine one spot only was seen; this showed that in the two solvent systems used the R_F values of the unknown were the same as those of cystathionine.

Cystathionine gives the test for S compounds with the platinic iodide reagent described by Winegard, Toennies & Block (1948); this test was applied to the paper chromatogram from the urine and was positive: the paper sprayed with the reagent was pink, but the region occupied by the unknown substance remained white.

Isolation of unknown compound

In the first experiment 500 ml. of urine was collected between the 32nd and 48th day of deficiency from the three male rats of litter F. The filtered urine was passed down a column (3·5 cm. × 20 cm.) of Dowex-50 × 8 (200–400 mesh) in its acid form. The column was then washed with water until the eluate was neutral (800 ml.). The amino acids were then eluted with 2 N-ammonia (250 ml.) and the column was washed with water (250 ml.). The eluate and washings were combined and evaporated on a steam bath to about 10 ml. This material was applied in closely adjacent spots to two sheets of Whatman no. 3 MM paper. The chromatogram was run in the butanol–acetic acid–water system for 48 hr. The unknown compound was detected about 8 cm. from the origin and a strip about 3 cm. wide was cut out, so as to include this. The strip obtained from one sheet was extracted with water in a Soxhlet apparatus. The extract, 20 ml., was passed down a small column (0·9 cm. × 6 cm.) of Dowex-50 in its acid form. The column was washed with water (50 ml.); the eluate, with 10 ml. of 2 N-ammonia and the washings, with 10 ml. of water, were combined and concentrated to a syrup. Some crystalline material separated after several days, and

a larger amount was obtained by the addition of a few drops of ethanol. The product was collected and washed first with a mixture of ethanol and water and then with ethanol alone. It was dried over H_2SO_4 *in vacuo* (yield, 11·6 mg.). It gave a positive Lassaigne test; the test with the cyanide–nitroprusside reagent for S-S-groups was negative.

A portion (2 mg.) of the substance was dissolved in 4 ml. of water and 100 mg. of damp Raney nickel (Mozingo, Wolf, Harris & Folkers, 1943) was added. The mixture was refluxed on a water bath for 1 hr. and the solid removed by centrifuging. The supernatant was shaken with an equal volume of 8-hydroxyquinoline in chloroform (1%, w/v), the aqueous layer was evaporated to dryness and the residue dissolved in 0·5 ml. of water. Chromatography of this solution in phenol showed the presence of two conspicuous spots in the positions expected for alanine and α-aminobutyric acid. This is consistent with the behaviour of cystathionine (Berridge, Newton & Abraham, 1952).

The mixture methanol–water–10 N-HCl–pyridine (32:7:1:4, by vol.) was used by Rhuland, Work, Denman & Hoare (1955) for resolving the isomers of αε-diaminopimelic acid. The system was used with the four isomers of cystathionine and *allo*cystathionine on Whatman no. 1 paper. The R_F values for L-, D-, L-*allo*- and D-*allo*-cystathionine were 0·21, 0·17, 0·22 and 0·22 respectively. The unknown material (5 μg.) had an R_F value of 0·21; this excludes D-cystathionine.

In a second experiment larger amounts of the compound were isolated; the amino acids from 500 ml. of urine were separated by the Dowex 50 column as described above. The ammoniacal eluate was evaporated to remove free ammonia. The brown neutral concentrate (about 150 ml.) was passed through a column (2 cm. × 15 cm.) of Dowex 2 × 8 (200–400 mesh) in its chloride form, prepared according to Stein & Moore (1954). The column was washed with water (50 ml.) and the colourless eluate and washings were concentrated on the water bath to 30 ml. Solid material separated from the concentrate at +3° after a few hours; this was filtered off, washed with water and ethanol and dried *in vacuo*. From 500 ml. of urine collected between the 43rd and 58th day of deficiency 0·194 g. of solid material was obtained; the yield from 500 ml. collected during the 59th and 78th day was 0·048 g.; no solid material separated out from the urine collected between the 79th and 108th day. The material collected in the first period, which represents an excretion of 1·3 mg. of cystathionine/rat/day, was chromatographically homogeneous in phenol and it gave a negative test for S-S groups with the cyanide–nitroprusside reagent. It was dissolved in 2 ml. of N-HCl and filtered; filtrate and washings were neutralized with N-NaOH; 146 mg. of the solid material separated. This material, recrystallized from water, had m.p. 294–295° (with slight browning at 270°), $[\alpha]_D^{20\cdot5} + 22\cdot0°$ in N-HCl (*c*, 1·0) (Found: C, 37·6; H, 6·4; N, 12·8; Calc. for $C_7H_{14}O_4N_2S$: C, 37·8; H, 6·4; N, 12·6). For L-cystathionine du Vigneaud, Brown & Chandler (1942) reported browning at 270° with m.p. at 312° and $[\alpha]_D^{20} + 23\cdot7°$. The dibenzoyl derivative was prepared from 50 mg. as described by Anslow, Simmonds & du Vigneaud (1946). The crude product (76 mg.) was recrystallized from ethanol–water (1:1, v/v) and had m.p. 226–227° (uncorr.). Dibenzoyl-L-cystathionine has m.p. 229° (du Vigneaud *et al.* 1942); the dibenzoyl derivatives of the *allo*cystathionines have m.p. 186–189° (Anslow *et al.* 1946).

I am grateful to Dr D. Hodgkin, F.R.S., and Mrs M. Webster for taking the X-ray-diffraction photographs of single crystals of the unknown and of L-cystathionine; the patterns indicated that the two crystals were identical. Powder photographs also gave identical diffraction patterns (Fig. 2).

In the microbiological tests, kindly carried out by Professor D. D. Woods, two strains of *Escherichia coli* were used. *E. coli* 26–18 grows well in the presence of either L-homocysteine or L-methionine, but poorly in the presence of L-cystathionine. *E. coli* 6/5 grows almost equally well in the presence of these three compounds. In a preliminary experiment with the crude material isolated by paper chromatography, strain 28–18 grew poorly in the presence of the

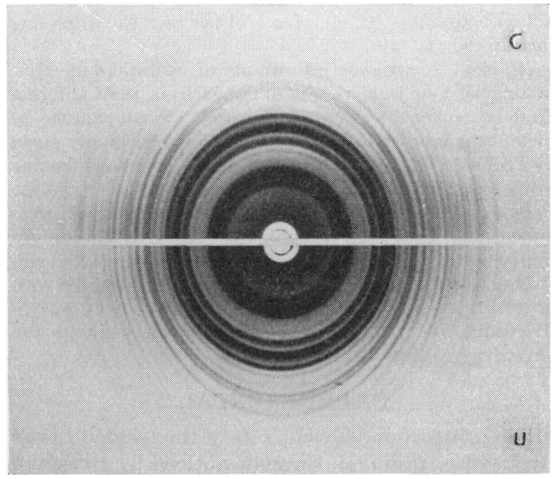

Fig. 2. X-ray diffraction powder photographs of L-cystathionine (*C*) and the unknown compound (*U*).

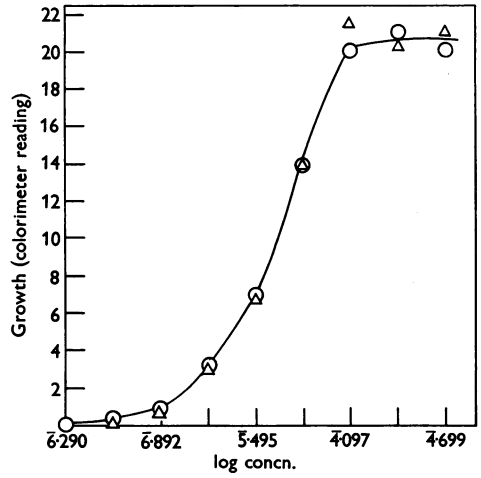

Fig. 3. Comparison of the growth-promoting properties of L-cystathionine and the unknown metabolite from urine of pyridoxine-deficient rats on *Esch. coli* strain 6/5. Growth determined by nephelometric readings with EEL photoelectric colorimeter. △, unknown metabolite; ○, L-cystathionine.

unknown material but strain 6/5 grew well. The recrystallized material was compared quantitatively with L-cystathionine by Professor Woods. The results are shown in Fig. 3.

DISCUSSION

The evidence given in the preceding section shows that the unknown compound seen by Blaschko *et al.* (1953) is L-cystathionine. This confirms a suggestion made by Chatagner, Tabechian & Bergeret (1954), who tentatively identified as cystathionine a spot on chromatograms of urine of vitamine B_6-deficient rats. Dr Chatagner (personal communication) has since found that synthetic cystathionine moves to the same position.

It is known that vitamin B_6 is involved in different stages of the trans-sulphuration reaction (for review see Blaschko & Hope, 1956). Pyridoxal phosphate is essential for the enzymes responsible for both the formation and the breakdown of cystathionine. The observations described can best be understood if it is assumed that in the vitamin B_6-deficient animals the enzyme which cleaves cystathionine is lost at a faster rate than the enzyme that forms it (Binkley, Christensen & Jensen, 1952; Goryachenkova, 1952). The reason why cystathionine had not previously been found in urine from vitamin B_6-deficient rats may be connected with the strain difference noted in this study. The absence of cystathionine from the urine of the deficient albino rats remains to be explained.

The diet used in the present experiments was poor in cystine. Thus a disability to carry out the trans-sulphuration, i.e. the formation of cystine from methionine, may contribute to the decrease in taurine excretion in pyridoxine deficiency (Blaschko *et al.* 1953; Chatagner *et al.* 1954).

SUMMARY

1. The appearance of a ninhydrin-positive spot in chromatograms of urine from hooded rats deficient in pyridoxine has been confirmed.

2. The substance responsible has been isolated and shown to be L-cystathionine.

3. This observation is discussed in the light of what is known about the importance of vitamin B_6 in trans-sulphuration.

I wish to thank Professor J. H. Burn, F.R.S., for the privilege of working in his Laboratory, and Dr H. Blaschko for his help and guidance. The work has been carried out during the tenure of a Medical Research Council Scholarship for which I wish to express my gratitude.

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