Crystalline Dihydropteroylglutamic Acid

THE preparation of dihydropteroylglutamic acid has previously been described by O'Dell *et al.*, who used catalytic hydrogenation of pteroylglutamate[1], and by Futterman, who carried out the reduction with sodium dithionite[2]. The acid prepared by catalytic hydrogenation is unsuitable for many biochemical studies, however, because it contains traces of tetrahydropteroylglutamic acid[3]. The material obtained by the method of Futterman is an amorphous discoloured preparation[2], but was found to be uncontaminated by tetrahydropteroylglutamic acid[3]. No analytical figures have been given for this material. By reducing purified pteroylglutamate with sodium dithionite according to the method of Futterman and twice precipitating the product by slow adjustment of the *p*H between strictly defined values, it has now been found possible to obtain dihydropteroylglutamic acid consistently as a white crystalline product giving analytical results agreeing fairly well with the theoretical values.

The procedure adopted was as follows. Ascorbic acid (1 gm.) was dissolved with mechanical stirring in about 5 ml. of water in a 25-ml. beaker. While stirring continued and continuous readings of the *p*H were made with a glass electrode 1 *N* sodium hydroxide was added slowly until the *p*H rose to 6·0. The volume of the solution was brought to 10 ml. by the addition of water and a solution of 38·2 mgm. of pteroylglutamic acid (purified as previously described[4]) in 1·6 ml. of 0·1 *N* sodium hydroxide added. Mechanical stirring was recommenced and 400 mgm. of solid sodium dithionite added. When all the dithionite had dissolved stirring was continued slowly for 5 min. at room temperature (22–25°). An ice-bath was then placed around the beaker and stirring continued until the temperature had fallen below 5°. Addition of 1 *N* hydrochloric acid from a burette at a rate of approx. 0·1 ml./min. was then commenced while the solution was stirred rapidly at 0–5°. Addition of hydrochloric acid was continued until the *p*H fell to 2·8 (approx. 5 ml. of 1 *N* hydrochloric acid required). After stirring the solution for a further 5 min. to allow complete precipitation, the precipitate was recovered by centrifugation (5 min. at 1,000*g*, temperature 0°) and the supernatant discarded. The precipitate, which was mainly amorphous but contained some crystalline material, was re-suspended in 10 ml. of an ice-cold solution of 10 per cent sodium ascorbate, *p*H 6·0, freshly prepared as previously described. The suspension was returned to a 25-ml. beaker cooled in a nice-bath and mechanical stirring and continuous *p*H measurement recommenced. The bulk of the precipitate redissolved at this stage and the solution was between *p*H 5 and 6. If necessary the solution was adjusted to *p*H 6 by cautious addition of 1 *N* sodium hydroxide, and maintained at this *p*H while being stirred at 0° for a further 5 min. Under these conditions only crystalline material remained undissolved, causing marked birefringence. The dissolved dihydropteroylglutamic acid was crystallized from the solution by slow addition of 1 *N* hydrochloric acid performed exactly as previously. When crystallization was complete (5 min. after reaching *p*H 2·8) the heavy, white, birefringent suspension was centrifuged off as before and washed three times with ice-cold 0·001 *N* hydrochloric acid by suspending and centrifuging. After pipetting off the final supernatant the precipitate was quickly dispersed over the walls of the centrifuge tube, placed in a desiccator over phosphorus pentoxide and potassium hydroxide and the pressure quickly reduced to 0·02 mm. mercury. The solid, which was initially white, was stored in the desiccator in the dark until required. It became discoloured while stored in this way over a period of days, and discoloured even more rapidly when removed from the desiccator. Under the microscope the crystals appeared as fine, very small needles (Fig. 1). After preparation, a sample for analysis was dried for 16 hr. over phosphorus pentoxide (pressure 0·02 mm. mercury) at 25°, rather than at higher temperatures, in order to avoid decomposition. Calculated for $C_{19}H_{21}O_6N_7.2\frac{1}{2}H_2O$: C, 46·72; H, 5·37; N, 20·08 per cent. Found: C, 47·57; H, 5·62; N, 19·61

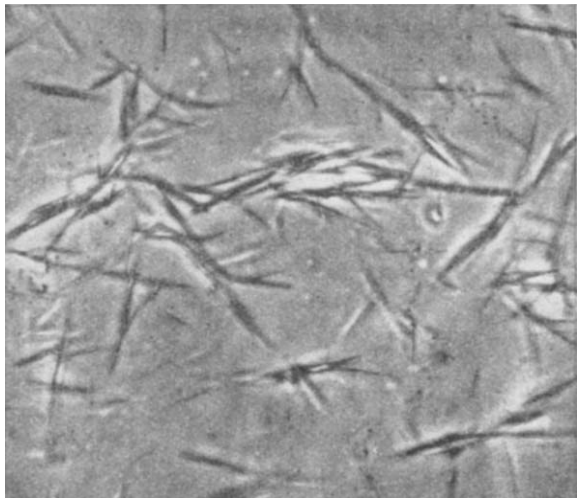

Fig. 1. Crystals of dihydropteroylglutamic acid (× *c.* 1,330)

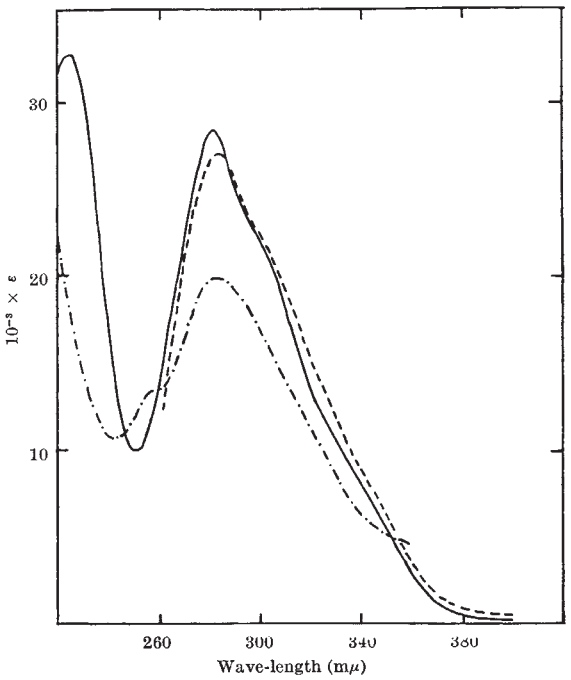

Fig. 2. Absorption spectra of solutions of crystalline dihydropteroylglutamic acid (0·03 m*M*) in 0·1 *M* potassium phosphate buffer (——); in 0·01 *N* sodium hydroxide (– –); and in 0·1 *N* hydrochloric acid (–.–.–)

per cent. Absorption spectra for the crystalline material are shown in Fig. 2.

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Identification of 19-Norandrostenedione in Follicular Fluid

During the course of a recent investigation of the steroids present in the follicular fluid of the mare[1], an unidentified $\alpha\beta$-unsaturated 17-ketosteroid was detected, and it seemed possible that this might represent one of the intermediates in the conversion of androst-4-ene-3,17-dione (androstenedione) to œstrone.

Further work on this compound has now established its identity as 19-norandrost-4-ene-3,17-dione (19-norandrostenedione), the evidence being as follows: (1) R_F value of the unknown compound identical with that of authentic 19-norandrostenedione in ligroin/80 per cent methanol and hexane: benzene/formamide paper chromatographic systems; (2) ultra-violet, sulphuric acid and sulphuric acid–ethanol chromogen spectra of the unknown identical with those of authentic 19-norandrostenedione; (3) R_F value of the sodium borohydride reduction product of the unknown identical with the R_F value of 19-nortestosterone in a ligroin/80 per cent methanol chromatographic system; (4) R_F value of the acetate of this reduction product identical with that of 19-nortestosterone acetate in ligroin/80 per cent methanol and hexane/formamide systems; (5) failure of the unknown to be oxidized by chromium trioxide in pyridine, or acetylated by acetic anhydride in pyridine; (6) Zimmermann colour reaction of the unknown identical with that of authentic 19-norandrostenedione; (7) infra-red spectrum of the unknown identical in every respect with that of authentic 19-norandrostenedione.

Since there still remained the possibility that this compound might have been an artefact produced by the action of alkali on 19-hydroxyandrost-4-ene-3,17-dione, 20 μgm. of the latter substance was added to a sample of follicular fluid prior to extraction; however, this did not increase the amount of 19-norandrostenedione present in the final extract.

With the exception of two œstranediols reported by Marker *et al.*[2] in non-pregnant human urine, this is the first time that a non-aromatic 19-nor steroid has been found as a natural product. The mean concentration in eight samples of equine follicular fluid was 20 μgm./100 ml. fluid (range 4–45 μgm./100 ml.), and since it is present in considerable amounts, it is interesting to consider its possible biological significance.

It is now well established that androstenedione serves as a precursor in the biosynthesis of œstrogens, and as a result of *in vitro* studies with a number of endocrine tissues, it was suggested[3] that 19-hydroxyandrostenedione was an intermediate in this conversion. This has now been amply confirmed[4,5], and it has been shown that human placental microsomes *in vitro* can convert androstenedione to œstrone via 19-hydroxyandrostenedione. Since 19-oxoandrostenedione is also readily converted to œstrone, the following biosynthetic sequence has been proposed[6]:

androstenedione → 19-hydroxyandrostenedione → 19-oxoandrostenedione → œstrone

If the results of these *in vitro* experiments with human placental microsomes are an accurate reflexion of the events taking place in the ovary *in vivo*, one might expect to find 19-hydroxyandrostenedione and 19-oxoandrostenedione in follicular fluid. However, this is not the case; repeated attempts to identify 19-hydroxyandrostenedione in large quantities of equine follicular fluid have met with negative results, although recoveries of the pure steroid are satisfactory. Neither is there as yet any evidence to suggest that 19-oxoandrostenedione is present in equine follicular fluid. These negative results could be ascribed to the fact that both intermediates are so rapidly converted to œstrone that they do not accumulate in the ovary. It would be tempting to conclude that in the mare's ovary 19-norandrostenedione serves as an alternative intermediate in the conversion of androstenedione to œstrone. However, it must be remembered that 19-nortestosterone is a relatively inefficient œstrogen precursor *in vitro*[4].

I wish to express my thanks to Dr. R. K. Callow and Mr. Paul Miners for carrying out the infra-red analysis. Reference samples of 19-hydroxyandrostenedione and 19-norandrostenedione were kindly supplied by Dr. Maximilian Ehrenstein (Philadelphia) and the Medical Research Council Steroid Reference Collection (Dr. W. Klyne).

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Isolation of Purified Trichophytin

Commercial preparations of trichophytin[1] are generally aqueous extracts of the fungal mycelium (for example, *Trichophyton mentagrophytes*) together with a proportion of the culture medium. They thus contain a variety of substances which are not specific to trichophytin sensitivity and which may contribute in a variety of ways to the production of the skin reaction. In order to limit this non-specific response and to define the significance of the trichophytin reaction, an attempt has been made to isolate the antigenic material in a purified form and to obtain preliminary data as to its constitution.

The fungus, *Trichophyton mentagrophytes* (N.T.C. *D*281), was grown in a medium containing glucose (50 gm.), 1 per cent ammonium sulphate (100 ml.), 1 per cent sodium nitrate (100 ml.), 5 per cent acid hydrolysed casein (100 ml.), trace metal ions (1 ml.; ferrous sulphate, 200 mgm.; manganous sulphate, 100 mgm.; zinc sulphate, 100 mgm. in water, 100 ml.) and solutions *A* (540 ml.) and *B* (105 ml.) of