The reactive intermediate formed by formaldehyde and tetrahydropteroyl glutamate

Both tetrahydropteroylglutamic acid (tetrahydroPGA) and dihydropteroylglutamic acid (dihydroPGA) act as coenzyme for an enzyme present in avian and mammalian liver which synthesises serine from glycine and formaldehyde[1]. The actual mechanism of this synthesis is unknown but it is generally assumed that the hydrogenated derivatives of PGA combine with formaldehyde to form a "reactive" compound. It would seem possible that this latter reaction is an enzymic process.

It has been shown that after treatment with HCHO, tetrahydroPGA and dihydroPGA readily undergo aerobic degradation (as shown by loss of coenzyme activity)[1]. Hence no *stable* compound is formed between formaldehyde and either tetrahydroPGA or dihydroPGA. On the other hand, the rate of aerobic degradation of the hydrogenated PGA derivatives is a little slower in the presence of formaldehyde than in its absence, suggesting the non-enzymic formation of readily *dissociating* compounds made up of tetrahydroPGA or dihydroPGA and formaldehyde. It is likely that these dissociating compounds are N^5- and possibly N^8-hydroxymethyl derivatives. No evidence could be found for enzymic formation of a stable compound of formaldehyde and tetrahydro- or dihydroPGA.

With the aid of $H^{14}CHO$ it has now been found that tetrahydroPGA and dihydroPGA combine with formaldehyde. Similar results have been briefly reported by KISLIUK[2], who claimed that the compounds are non-dissociating. In the present work, solutions of dihydroPGA, tetrahydroPGA and related compounds (approximately 0.01 *M*) were treated under H_2 with 1–3 molecular proportions of $H^{14}CHO$. After standing 0.5–20 h at room temperature, the pteridine derivative was precipitated, usually as the barium salt, by the addition of ethanol. The precipitate was redissolved and reprecipitated one or more times and finally dissolved in the minimum volume of water before being freeze dried. The radioactivity of the dry residue was determined under a conventional end-window Geiger counter and the molecular ratio of formaldehyde to pteridine calculated. The results are shown in Table I.

TABLE I

PROPORTION OF $H^{14}CHO$ COMBINED WITH VARIOUS PTERIDINE DERIVATIVES

Pteridine	*Moles $H^{14}CHO$/mole pteridine*
5,6,7,8-TetrahydroPGA	1.08, 0.93, 1.02, 0.59, 0.89, 0.83
DihydroPGA	1.39, 0.75, 0.21
Leucovorin	0.02
5,6,7,8-Tetrahydro-2-amino-4-hydroxy-6-methyl pteridine	0.87
5,6,7,8-Tetrahydro-N^{10}-formylPGA	0.60, 0.56, 0.32
5,6,7,8-Tetrahydro-4-amino-4-deoxyPGA	0.78

The following points are noteworthy:

(1) The amount of formaldehyde combined varied within wide limits, suggesting that freely dissociating compounds were formed.

(2) With one exception, the amount of formaldehyde combined was equal to or less than one mole/mole of pteridine derivative, indicating the combination of one molecule of formaldehyde with one site of the pteridine molecule.

(3) Leucovorin combined a negligible amount of formaldehyde, although tetrahydroPGA combined with almost one molecule of formaldehyde. This indicates that formaldehyde combined with N^5 of tetrahydroPGA, the position blocked by formylation in the case of leucovorin.

(4) Since 5,6,7,8-tetrahydro-2-amino-4-hydroxy-6-methylpteridine and 5,6,7,8-tetrahydro-N^{10}-formylPGA combined as readily with formaldehyde as did tetrahydroPGA, N^{10} of tetrahydroPGA does not combine with formaldehyde.

To confirm that the compound of tetrahydroPGA and formaldehyde readily dissociates, a preparation was dissolved in O_2-free 0.1 *M* formaldehyde (unlabelled) and reprecipitated as Ba salt by the addition of ethanol. The formaldehyde was crystallized as the dimedone compound and its radioactivity determined together with that of the Ba tetrahydropteroylglutamate. The process was repeated. The results shown in Table II clearly indicate a small but definite dissociation.

TABLE II

	C.p.m./cm² at infinite thickness	
	Ba tetrahydroPGA	HCHO dimedone
Before treatment with 0.1 *M* HCHO	7,168	—
After 1st treatment	6,556	561
After 2nd treatment	6,194	220

As a final proof of dissociation, saturated dimedone solution was added to a solution of Ba tetrahydroPGA (6,194 c.p.m./cm² at infinite thickness) in O_2-free water. White crystals immediately commenced to separate and after standing were dissolved by the addition of ethanol. The Ba tetrahydroPGA was precipitated, washed with ethanol and prepared for radioactivity determination. It had 2,060 c.p.m./cm² at infinite thickness. The ethanolic supernatants from the precipitation of the barium tetrahydroPGA were treated with carrier formaldehyde and the dimedone compound crystallized. It had 1,750 c.p.m./cm² at infinite thickness. The expected radioactivity of the formaldehyde calculated from the loss of activity of the Ba tetrahydroPGA was 1,420 c.p.m./cm².

These results indicate that formaldehyde combines non-enzymically with tetrahydroPGA to form N^5-hydroxymethyltetrahydroPGA, a compound which dissociates appreciably in solution. On incubating enzyme, glycine and N^5-hydroxymethyltetrahydroPGA labelled in the hydroxymethyl group with ^{14}C, approximately 69 % of the ^{14}C appeared in the serine synthesised. A similar result was obtained with N^5-hydroxymethyl-N^{10}-formyltetrahydroPGA. Present evidence therefore suggests that N^5-hydroxymethyltetrahydroPGA reacts on the enzyme surface with the Schiff's base of glycine and pyridoxal phosphate to form the Schiff's base of serine and pyridoxal phosphate with regeneration of tetrahydroPGA.

Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra (Australia) R. L. BLAKLEY

[1] R. L. BLAKLEY, *Biochem. J.*, (1957) (in the press).
[2] R. L. KISLIUK, *Federation Proc.*, 15 (1956) 289.

Received December 19th, 1956

Preliminary Notes

Studies on the nature of steroid 11-β hydroxylation

Previous work in several laboratories has shown that adrenal preparations are able to hydroxylate steroids at the 11-position in the presence of TPNH and molecular oxygen[1,2]. Studies with ^{18}O have indicated that the oxygen atom which is introduced is derived exclusively from the gaseous atmosphere and not from the solvent[3,4,5]. These findings suggest a similarity between the mechanisms for the hydroxylation of steroids and of aromatic compounds, as well as a number of apparently unrelated oxidations[6,7].

The present communication deals with further studies on steroid 11-β hydroxylation carried out with extracts of calf adrenal acetone powder which were prepared by homogenization in 0.1 *M* tris(hydroxymethyl)aminomethane (TRIS) buffer, pH 7.4, containing 0.5 % digitonin and in which enzyme activity was not sedimentable on centrifugation at 100,000 × *g* in 1 h. The reaction was detected by a modification of the method described by SWEAT[8] based on the fact that in acid-ethanol mixtures, 11-hydroxysteroids display an intense fluorescence not shown by their 11-deoxy analogues. The substrates were 4-pregnen-21-ol-3,20-dione (DOC) and 4-pregnene-17,21-diol-3,20-dione (compound *S*), which on 11-hydroxylation yielded 4-pregnene-11,21-diol-3,20-dione (compound *B*) and 4-pregnene-11,17,21-triol-3,20-dione (compound *F*), respectively. These reaction products were further identified by paper chromatography[9].

The data presented in Table I indicate that in the presence of TPNH and O_2, at least two distinct enzymes were required to catalyze the overall reaction. Furthermore, it can be seen that one of these enzymes was present not only in the adrenal, but also in extracts of rabbit liver.