In the female group, the initial mean TSR was only slightly lower than previously reported. However, the rate of decrease in TSR was somewhat slower. On the 120th day the mean TSR was 1.12 μg/100 g bw, compared to 0.88 μg in the previous study. Only by the 240th day had the present group reduced to 0.87 μg or 41% below the initial estimation of TSR.

While these two studies are similar in respect to the decline in TSR with age, they indicate that the rate of decline with age may vary in individuals and in groups of rats of the same strain.

Summary. The thyroid hormone secretion rate (TSR) of the same group of 36 male rats was estimated at 30 days and at 30-day intervals to 120 days. They declined from a mean of 1.40 μg/100 g bw to 1.03 μg at 120 days or 26%. A group of 118 male rats at 30 days of age had a mean TSR of 1.34 μg/100 g bw with a range from 0.75 μg to 2 μg/100 g bw. The TSR of a group of 29 females at 30 days of age was 1.47 μg/100 g bw and gradually declined to 0.87 μg at 240 days of age or 41%. It was shown that the TSR of both male and female rats decline with age but the rate of decline may vary in different groups of animals.

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Role of 2-Amino-4-Hydroxypteridine-6-Carboxaldehyde in Folic Acid Biosynthesis.* (31846)

CHARLES M. BAUGH† (Introduced by C. E. Butterworth, Jr.)
Department of Medicine, Division of Dermatology, Washington University, St. Louis, Mo.

Recent studies on folic acid biosynthesis have focused attention on the role of P-6-CH_2OH‡ as an immediate pteridine precursor of the vitamin. This report describes studies which demonstrate the inhibition of growth of *E. coli* ATCC 9723 (Roepke Strain) by P-6-CH_2OH. This inhibition is reversed by dihydrofolic acid and even more effectively by dihydro P-6-CHO.‡ The implications of these results for folic acid biosynthesis are discussed.

The current state of information regarding the biochemical sequence leading to folic acid

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† Present address: Division of Nutrition, Dept. of Medicine, Univ. of Alabama Med. Center, Birmingham.

‡ Abbreviations: 2 - amino-4-hydroxy-6-hydroxymethylpteridine, P-6-CH_2OH; 2-amino-4-hydroxypteridine-6-carboxaldehyde, P-6-CHO; the terms dihydro or DH will in all cases refer to the 7,8-dihydro compounds.

may be summarized as follows: Guanosine-5′-triphosphate is ring-opened in the imidazole portion of the molecule and carbon eight is lost. Subsequent cyclization of the ribosyl moiety forms the pyrazine ring to give 2-amino-4-hydroxy-6-trihydroxypropyl pteridine, probably in a reduced form. This compound is then further metabolized to give 7,8-dihydropteroic acid and ultimately dihydrofolic acid.

The fact that carbons 6,7, and 9 of folic acid originate as carbons 2′, 1′, and 3′, respectively, of the ribosyl moiety of guanylic acid is well established(1). It thus appears clear that a 2-amino-4-hydroxypteridine with a single carbon substituent in position six is an intermediate between the trihydroxypropyl pteridine and folic acid. A number of investigators have indicated this precursor to be a reduced form of P-6-CH_2OH(2,3,4). These earlier findings have now been extended and the proposed intermediate is thought to be dihydro P-6-CH_2OH pyrophosphate(5).

Therefore, although a role for dihydro P-6-CH_2OH pyrophosphate appears to be established, no details of the metabolic sequence from the trihydroxypropyl pteridine to this immediate folate precursor are known. The role of P-6-CH_2OH in this pathway, if any, remains obscure. Like folic acid, P-6-CH_2OH has been indentified as a metabolite of guanine(6). In those studies no attempts were made to prevent oxidation and the conditions of isolation would have hydrolyzed the pyrophosphate ester, if present.

In the cell free studies described by Brown *et al*(4) P-6-CH_2OH was shown to be a potent inhibitor of folate biosynthesis, whereas P-6-CHO was a much more efficient precursor. This inhibition was not seen after reduction. These results were interpreted to indicate the absence of an enzyme system for the reduction of P-6-CH_2OH(4). Koft and Merola(7) reported the coupling of P-6-CH_2OH pyrophosphate with PABA in the absence of enzyme, and Bocchieri and Koft(8) presented evidence to support the proposed role of dihydro P-6-CH_2OH pyrophosphate.

Similarly, conflicting reports have appeared concerning the role of P-6-CHO in the biosynthesis of folic acid. In growing cells of *S. faecalis R*, *S. aureus KiAu*(9), and *E. coli* (10) P-6-CHO was found to inhibit folate biosynthesis. With *S. aureus*(10), *E. stei*(11), and *L. arabinosus*(2) P-6-CHO enhanced folate biosynthesis. In adapted strains of *S. faecalis* P-6-CHO was shown to replace the requirement for folic acid(12). This report summarized studies on the role of certain 2-amino-4-hydroxy-6-substituted pteridines in folic acid biosynthesis.

Methods. E. coli was grown in calibrated 18 × 150 mm test tubes in the following media: Solution A: 50 g NaCl, 47.2 g $(NH_4)_2SO_4$, 27.2 g KH_2PO_4, and 20.0 g asparagine dissolved in 1 liter distilled water. The pH was adjusted to 7.0 with 10 N NaOH and the volume taken to 2 liters. Solution B: 100 g glucose in 200 ml H_2O, autoclaved 10 minutes at 15 PSI. Solution C: 1.57 g $FeCl_2 \cdot 4H_2O$, 2.125 g $MgCl_2 \cdot 6H_2O$, 1.97 g $CaCl_2 \cdot 6H_2O$, and 600 mg cysteine to retard oxidation were dissolved in 600 ml H_2O and sterilized by filtration. Solution D: 1 M phosphate buffer, pH 7.0. Solution E: Sodium ascorbate pH 7.0, 100 mg/ml, sterilized by filtration. To make 500 ml double strength media, 200 ml solution A, 260 ml distilled H_2O, and 20 ml solution D were autoclaved 10 minutes at 15 PSI. After cooling, 10 ml solution B, 1 ml solution C, and 10 ml solution E were added aseptically. Double strength medium (5 ml) was added aseptically to sterile test tubes containing calculated amounts of distilled water, so that final volume, when test compounds were added, was 10 ml. All tubes were set up in triplicate, 2 tubes being inoculated with one drop from a 22-hour culture; the third tube served as a blank for each set. The tubes were incubated at 30°. Growth was monitored by determining percent transmission at 650 mμ with a Bausch and Lomb Spectronic 20 colorimeter. Readings were taken between 18 and 24 hours.

The hydroxymethyl pteridines were prepared by the methods of Baugh and Shaw (13). P-6-CHO was prepared by the methods of Waller *et al*(14). Folic acid was purchased from Sigma Chemical Co. All compounds were chromatographically homogeneous and spectrally pure.

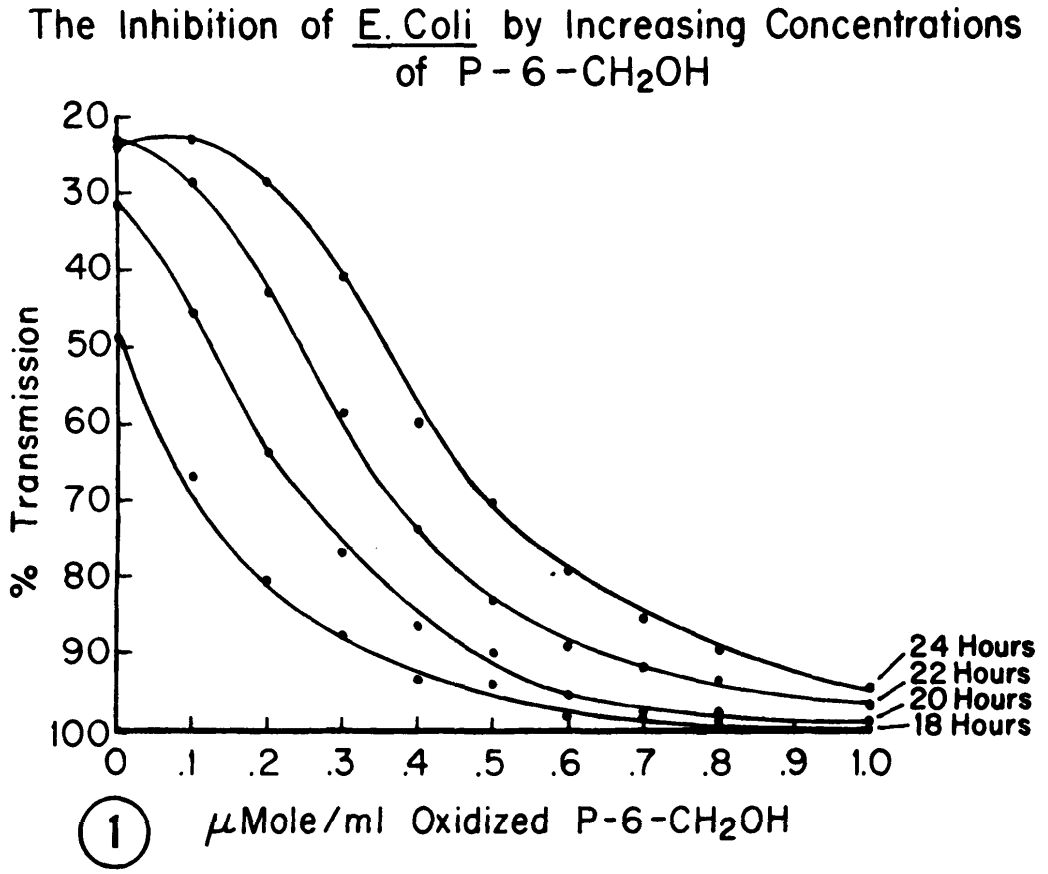

FIG. 1. Inhibition of *E. coli* by increasing concentrations of P-6-CH_2OH. Readings are shown between 18 - 24 hr.

The various pteridines and folic acid were reduced by dithionite using the method of Blakley(15). Dihydrofolic acid and dihydro P-6-CH_2OH were quantitated spectrophotometrically. Dihydrofolic acid was quantitated at 283 mμ in 0.1 N KOH, A_m, 28,000 (16). Dihydro P-6-CH_2OH was quantitated at 254 mμ in 0.1 N HCl, A_m, 8,700(3). Dihydro P-6-CHO was obtained as a crystalline compound, and was quantitated by weighing. As a check on the gravimetric quantitation of dihydro P-6-CHO a weighed aliquot was oxidized in 0.1 N NaOH with excess 2% $KMnO_4$, 30 minutes at room temperature, and 15 minutes at 100°. Excess $KMnO_4$ was then destroyed with a few drops of ethanol. Manganese dioxide was removed by filtration and washed with water. The washings were combined with the filtrate and brought to 100 ml with water. The 2-amino-4-hydroxypteridine-6-carboxylic acid so obtained was quantitated spectrally at 262 mμ in 0.1 NaOH, A_m, 20,800(17). Agreement between the two methods was in all cases above 90%.

After reduction and quantitation all reduced compounds were parcelled out in 100 μmole batches, stabilized with 50 mg/ml sodium ascorbate and stored at —20°. New batches of all reduced pteridines and folic acid were prepared at two-week intervals. Prior to use all reduced compounds were dissolved in a minimum volume of 0.1 N NaOH and adjusted to the desired volume with distilled water and the necessary amount of sodium ascorbate added to give a final concentration in 5 mg/ml. In all cases oxidized and reduced compounds were sterilized by filtration, and added to the assay tubes just prior to inoculation.

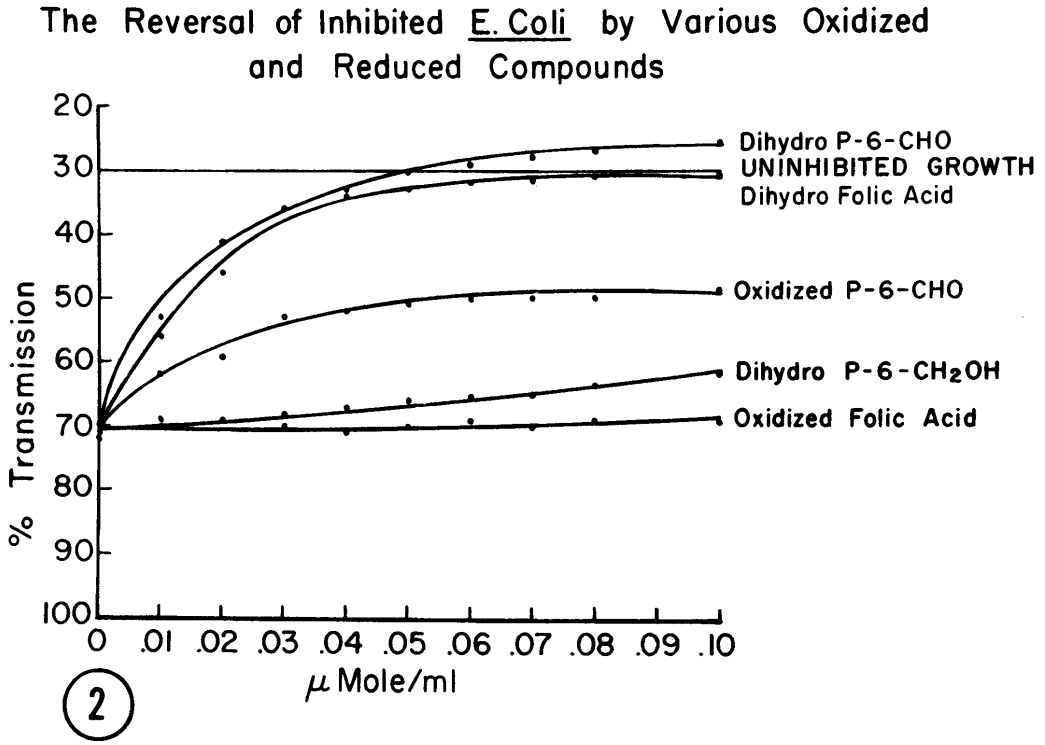

Fig. 2. Reversal of 50% inhibited *E.coli* (0.3 μmole/ml P-6-CH_2OH/tube) by various oxidized and reduced pteridines and folic acid. All readings were taken 20 hr after inoculation.

Experimental results. When increasing concentrations of P-6-CH_2OH were tested in growing cultures of *E. coli,* a dramatic inhibition of growth was seen. The concentration required for 50% inhibition was usually found between 0.2-0.3 μmole/ml at 20 hours. Under the growth conditions described in the methods section, maximum growth was obtained after 21 hours. Inhibition curves from readings taken at two-hour intervals between 18 and 24 hours may be seen in Fig. 1. It was clear that some reversal of inhibition was occurring with increasing incubation after maximum growth was reached; however complete growth was never seen at higher inhibitor concentrations.

A study of various compounds for their ability to reverse the inhibition by P-6-CH_2OH was next carried out. In all cases a concentration of 0.3 μmole/ml of P-6-CH_2OH was used as inhibitor. Inspection of Fig. 2 will indicate that in all cases the reduced compounds were more effective reversal agents than were the corresponding oxidized compounds. It is interesting to note that dihydro P-6-CH_2OH was not inhibitory and was unable, except in high concentrations, to overcome even slightly the inhibition produced by P-6-CH_2OH. Although not shown here, none of the reversal agents tested have inhibitory effects on the growth of *E. coli* when tested alone over a concentration range of 0-0.8 μmole/ml. Dihydro P-6-CHO was the only compound among those tested which gave stimulation of growth above controls. It should also be observed that the most effective compound tested as a reversing agent was dihydro P-6-CHO.

The effectiveness of these compounds for reversing the inhibition by P-6-CH_2OH is more clearly presented when the data in Fig. 2 are converted to percentage reversal and plotted *versus* concentration. These curves may be seen in Fig. 3. Dihydro P-6-CMO at a ratio of 4.28:1 with P-6-CH_2OH completely overcomes the inhibition. At higher concen-

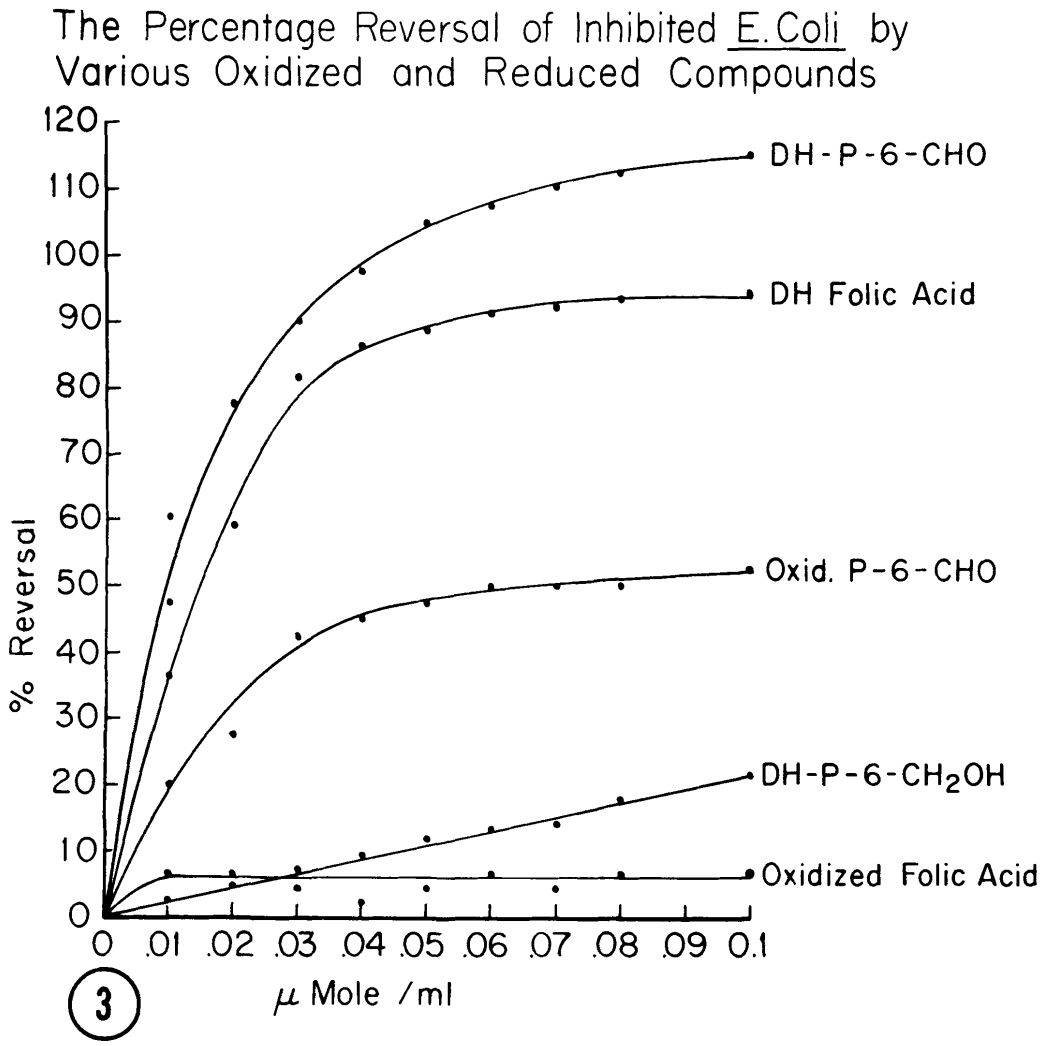

Fig. 3. Data presented are calculated as percent reversal and plotted *vs* concentration of reversal agent.

trations a stimulation of growth was seen. No other compound tested demonstrated the capacity to completely overcome the inhibition produced by P-6-CH_2OH. Although dihydrofolic acid effectively overcomes the inhibition, complete growth was not obtained.

Discussion. These results are interpreted to indicate that in *E. coli* (a strain which has no folate requirement) there is no role in folic acid biosynthesis for P-6-CH_2OH. No inference can be made on the basis of these data to substantiate or refute the proposed role of dihydro P-6-CH_2OH pyrophosphate in this pathway. However, if P-6-CH_2OH, in the dihydro form and phosphorylated, is the immediate folic acid precursor one must conclude either (1) the enzyme system required for the reduction of P-6-CH_2OH is absent in this organism, or (2) the phosphorylation system is not operative. Since the organism can synthesize folic acid, and since the inhibition of growth produced by P-6-CH_2OH disappears upon reduction to the dihydro form, one must conclude that (1) is the more likely possibility. However, the fact that dihydro P-6-CHO completely overcomes the inhibition produced by P-6-CH_2OH, whereas dihydro P-6-CH_2OH does not, cannot be ignored.

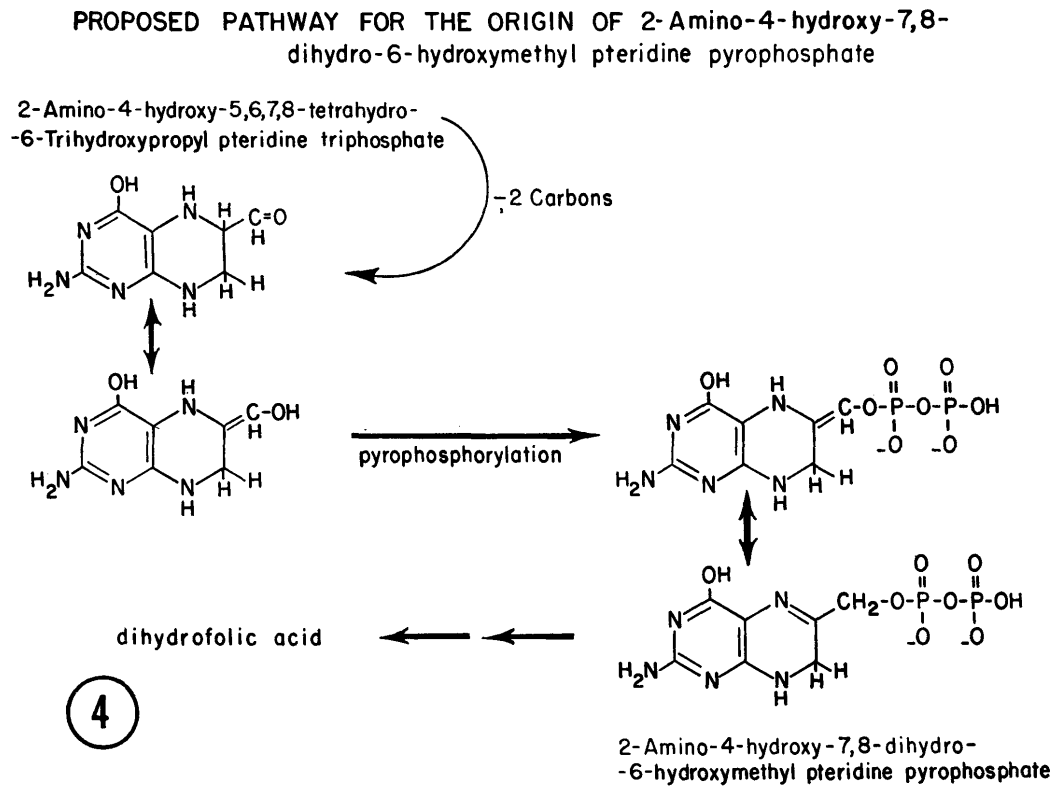

Fig. 4. A hypothetical scheme for the origin of dihydro P-6-CH_2OH pyrophosphate eliminating P-6-CH_2OH, and dihydro P-6-CH_2OH as intermediates in the pathway.

A hypothesis that permits the retention of dihydro P-6-CH_2OH pyrophosphate in the biosynthetic sequence leading to folic acid, and includes the carboxaldehyde, while excluding P-6-CH_2OH is outlined in Fig. 4. This hypothesis is in general agreement with the literature. However, confirmation must await further investigations into the individual enzymes between the guanosine-5′-triphosphate ring closure product and the conjugation step with PABA. These studies are in progress.

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