THE RELATIONSHIP OF FOLIC ACID TO FORMATE METABOLISM IN THE RAT: FORMATE INCORPORATION INTO PURINES*

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The considerations which prompted an investigation of the rôle of folic acid in formate metabolism were discussed in an earlier paper (1) in which it was reported that rats deficient in this vitamin incorporated less of a standard dose of C14-formate into certain amino acids and heme than did rats fed folic acid. There is abundant microbiological evidence to indicate an interrelationship between folic acid and the purines (2-5). The accumulation of 4-aminoimidazole-5-carboxamide in cultures of Escherichia coli inhibited with sulfanilamide (6) suggests very strongly that folic acid controls the incorporation of formate at least into carbon atom 2 of the purines. In addition, Skipper et al. (7) have demonstrated that the incorporation of labeled formate into the total nucleoprotein of mice is decreased by the administration of folic acid antagonists.

This study was undertaken to demonstrate whether folic acid affects the incorporation and relative location of labeled formate in nucleic acid purines in the rat.

EXPERIMENTAL

These experiments were performed on nucleic acid fractions from the same animals used in this laboratory for the studies on the incorporation of the carbon of formate into amino acids. The previous paper (1) may be consulted for detailed description of the preparation of the animals. The rats were made deficient in folic acid by feeding a diet containing 2 per cent succinylsulfathiazole. Three deficient animals and three animals in which a remission of leucopenia had been obtained by the administration of 1.1 mg. of folic acid daily for 4 days were injected intraperitoneally with 2 mg. of formic acid (160,000 c.p.s.) per 100 gm. of body weight, and sacrificed 3 hours later.

The livers and viscera (kidneys, spleen, pancreas, heart, testes, and

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intestines) of these animals were extracted with cold trichloroacetic acid, and the nucleic acid fraction (both deoxyribose nucleic acid and pentose nucleic acid) was obtained by treatment of the residue with hot 5 per cent trichloroacetic acid (TCA) (8).

The 5 per cent TCA extracts were deproteinized with chloroform and amyl alcohol (9); the TCA was removed by ether extraction, and the aqueous solution was concentrated in vacuo to a volume of 5 to 10 ml. The purines were released by hydrolysis in 1.0 N HCl at 100° for 1 hour (10). The filtered hydrolysate was made 2 N in HCl, and chromatographed on a column of Dowex-50 cation exchange resin according to the directions of Cohn (11). The eluted guanine and adenine fractions were reduced in volume by reabsorption on short columns of Dowex-50 and elution with 4 per cent NH₄OH. After removal of the ammonia in vacuo, the purines were precipitated as their Cu₂O complexes and washed with water (12). The free purines were regenerated with H₂S. As a precautionary measure, each of the purines was then rechromatographed on Dowex-50. The purine concentration of the eluates was determined spectrophotometrically by measurement of the optical densities at the absorption peaks of the individual purines. The purity of each compound was established by comparison of the ultraviolet absorption spectra in acid and alkaline solutions with values reported in the literature (13, 14).

Guanine was isolated as the free base and adenine as the hydrochloride after the addition of chromatographically pure carrier. All samples were recrystallized at least once; none increased in specific activity on recrystallization. The dilution of the purines with carrier was calculated from the amount of purine originally present, as estimated spectrophotometrically, and from the weighed amount of "carrier" added.

Degradation of Adenine and Guanine—The purines were converted into uric acid by deamination to the corresponding hydroxypurines and oxidation of the hydroxypurines with xanthine oxidase. The uric acid was degraded to obtain carbons 2 and 8 separately by the method of Sonne, Buchanan, and Delluva (15). In some samples a part of the uric acid was treated with MnO₂ in O₂ to obtain carbon 6 (16).

Guanine was deaminated by the method of Fischer (17), and adenine by the method of Kossel (18). The hydroxypurines were isolated in some cases, but it was necessary only to aerate the solutions to remove residual nitrite before carrying out the enzymatic oxidation to uric acid. The xanthine and hypoxanthine solutions were made up to a volume of 150 to 200 ml. and adjusted to pH 8 to 9. 5 ml. of a preparation of xanthine oxidase and 1 ml. of catalase solution were added, and the solutions were

1 Xanthine oxidase was prepared from raw cream by a modified method of Corran et al. (25). The preparation oxidized approximately 350 γ of xanthine per mg.
aerated with a current of O$_2$ in a water bath at 37°C until the reaction, which was followed spectrophotometrically (19), was completed.

The samples were made alkaline (pH 12 to 14), and the protein was removed by treatment with chloroform and amyl alcohol. The chloroform-amyl alcohol gels were then extracted with 0.1 N alkali to recover that part of the uric acid which had been retained in the gel.

In most experiments, it was necessary to add uric acid carrier at this stage, since the yield of uric acid in the oxidation was not high and the material obtained was impure. The solutions were neutralized and concentrated \textit{in vacuo} to about 40 ml., and the uric acid was precipitated by addition of hydrochloric acid. The precipitated uric acid was dissolved in dilute sodium hydroxide, treated with Nuchar C190N, and reprecipitated with acid several times before degradation.

30 to 40 mg. of uric acid were suspended in 3 ml. of 6 N HCl at room temperature. Potassium chlorate was then added, a few crystals at a time, until the uric acid had all gone into solution; this required from 5 to 10 minutes. The solution was treated with H$_2$S to reduce the alloxan to alloxantin. The free sulfur was centrifuged and the residue washed with hot water to remove adherent alloxantin.

The alloxantin was isolated by concentrating the solution \textit{in vacuo} and by addition of ethanol. After crystallization had been completed by chilling the solution overnight at 5°C, the alloxantin was collected by filtration and washed with liberal quantities of cold absolute ethanol. The filtrate, which contained the urea from atoms 7, 8, and 9 of the original purine, was decomposed with urease at pH 5, and the CO$_2$ representing carbon 8 was collected in Ba(OH)$_2$. The washed alloxantin was dissolved in 5 ml. of hot water and treated with about 25 mg. of PbO$_2$ at 100°C in a stream of CO$_2$-free air for 20 minutes. The CO$_2$ evolved was collected in Ba(OH)$_2$ solution. The PbO$_2$ was filtered and washed, and the filtrate and washings treated with urease to liberate CO$_2$, representing carbon 2. The alloxantin from the visceral guanine samples was degraded in a different way by treatment with 1.0 ml. of 30 per cent hydrogen peroxide in 10 ml. of 0.1 N NaOH for 1 hour at room temperature, followed by treatment with acid to remove the CO$_2$ formed. The solution was then neutralized, the peroxide removed with catalase, and the remaining urea decomposed with urease.

of protein per hour under Kalckar's (19) assay conditions and contained 12.5 mg. of protein per ml. The catalase solution was a commercial preparation of "catalase Sarett" obtained from the Vita-Zyme Laboratories, Inc., Chicago, Illinois.

* Obtained from the West Virginia Pulp and Paper Company, Tyrone, Pennsylvania.
The crude pyrimidine nucleotide mixtures from the first fractions of the Dowex-50 chromatograms (11) were evaporated to dryness and counted. The total radioactivity was so small that it seemed impractical to attempt to isolate the free pyrimidines.

RESULTS AND DISCUSSION

The specific activities and the distribution of radioactivity between carbons 2 and 8 of the purines are shown in Table I. Folic acid deficiency caused a rather marked decrease in the incorporation of the carbon of formate into the liver purines, but had no effect on the incorporation into the visceral purines. Totter, Volkin, and Carter (20), in a recently reported study with folic acid-deficient chicks, have found no difference, in the extent of radioactive formate incorporation into the purines of the "total viscera," between normal and deficient birds. It is possible that their results diverge from ours because of species difference, employment of a longer period of time for incorporation of the radioactive formate, or combination of the livers of the birds with the other viscera.

The distribution of radioactivity between carbons 2 and 8 is close to 1:1 (C-2:C-8 = 0.7 to 1.1) in all samples except that of the visceral guanine from the folic acid-deficient animals (C-2:C-8 = 0.52). There is a possibility that the observed radioactivities for carbon atoms 2 and 4, 5, and 6 of this guanine sample are not comparable to the others, since the alloxantin was not degraded by Buchanan’s method. Heinrich and Wilson...
(21) have determined the ratio C\textsubscript{2}:C\textsubscript{8} in rat nucleic acid guanine after prolonged formate administration and found it to be 1.31.

From the fact that 4-aminoimidazole-5-carboxamide accumulates in bacteria poisoned with sulfanilamide, it might be inferred (see (22)) that formate is normally incorporated into position 2 of the purine molecule through the agency of a folic acid-containing enzyme, while the fixation into position 8 is less dependent on this vitamin. It is apparent from the results of this investigation, however, that the decreased incorporation into liver purines resulted from decreased labeling of both carbons 2 and 8 and not specifically from failure of formate to be fixed as carbon 2. Therefore, these in vivo experiments, under the conditions employed, have not provided evidence for the above postulate.

Carbon atom 6 of the visceral adenine and the liver adenine and guanine, derived from the uric acid by treatment with MnO\textsubscript{2}, contained relatively insignificant amounts of radioactivity (less than 1 per cent of the total recovered). The CO\textsubscript{2} from carbon atoms 4, 5, and 6 evolved in the oxidation of the alloxantin also contained little C\textsuperscript{14}, except in the case of that obtained from the visceral guanine of the deficient rats. That very little incorporation occurred into either carbons 4 and 5 or 6 is in agreement with the results of Heinrich and Wilson (21). In view of the fact that formate is converted to bicarbonate in the rat (1, 23, 24), it is peculiar that there was no appreciable incorporation of isotope into carbon 6.

**SUMMARY**

Rats deficient in folic acid incorporated less of a standard dose of C\textsuperscript{14}-formate into the purines of their liver nucleic acids than did rats which had been fed this vitamin. No difference in total counts between deficient and repleted animals was noted in the visceral purines.

A method was developed for the conversion of adenine and guanine into uric acid; the latter was degraded to determine the distribution of C\textsuperscript{14} between carbons 2 and 8. Results of the degradations showed that folic acid deficiency did not give rise to marked differential labeling, but decreased the incorporation of formate into both C-2 and C-8 of the liver adenine and guanine.

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