The effect of pterins on cell proliferation in a suspension of cells of neoplastic tissue is opposite to the effect on cells of bone marrow suspension or of normal tissue (*3*). Xanthopterin accelerates proliferation of bone marrow cells and inhibits neoplastic cells. 7MP inhibits bone marrow cell proliferation and accelerates proliferation of cells of neoplastic tissue. Table 2 shows the effect of vitamin $B_{14}$ on a cell suspension of Brown Pearce rabbit tumor.

TABLE 1

EFFECT OF VITAMIN $B_{14}$ ON CELL PROLIFERATION *in vitro* IN A BEEF BONE MARROW SUSPENSION*

| Supplement/ml of suspension | Final concentration (RBC/mm³) | Increase in RBC (%) | Final concentration (NC/mm³) | Increase in NC (%) | Retic. /1,000 RBC |
|---|---|---|---|---|---|
| None | 5,850 | 19 | 4,720 | 64 | 15 |
| 5γ xanthopterin | 10,900 | 120 | 8,170 | 184 | 33 |
| 5γ 7MP | 2,680 | −46 | 1,680 | −42 | 5 |
| $1 \times 10^{-6}$γ vit. $B_{14}$ | 12,800 | 161 | 6,850 | 138 | 39 |
| $1 \times 10^{-5}$γ vit. $B_{14}$ | 24,500 | 400 | 19,800 | 665 | 37 |
| $1 \times 10^{-4}$γ vit. $B_{14}$ | 39,000 | 695 | 33,900 | 1,080 | 50 |
| $1 \times 10^{-2}$γ vit. $B_{14}$ | 40,200 | 720 | 34,200 | 1,090 | 46 |
| 10γ vit. $B_{14}$ | 42,500 | 766 | 33,800 | 1,070 | 63 |
| 100γ vit. $B_{14}$ | 41,500 | 750 | 32,400 | 1,020 | 53 |
| $1 \times 10^{-4}$γ vit. $B_{14}$ plus 5γ 7MP | 28,100 | 472 | 23,800 | 760 | 40 |
| $1 \times 10^{-4}$γ vit. $B_{14}$ plus 10γ 7MP | 15,600 | 238 | 10,800 | 276 | 25 |

* Initial concentration of cells in the suspension: RBC, 4,910/mm³; nucleated cells (NC), 2,880/mm³; reticulocytes, 9/1,000 RBC. Time of incubation, 7.5 hrs at 37° C.

TABLE 2

EFFECT OF VITAMIN $B_{14}$ ON CELL PROLIFERATION *in vitro* IN A SUSPENSION OF BROWN PEARCE TUMOR CELLS*

| Supplement/ml of suspension | Final concentration (cells/mm³) | Increase in cells (%) |
|---|---|---|
| None | 11,200 | 26 |
| 5γ 7MP | 15,000 | 68 |
| 10γ 7MP | 17,500 | 96 |
| 5γ xanthopterin | 6,780 | −24 |
| 10γ xanthopterin | 4,600 | −48 |
| $1 \times 10^{-7}$γ vit. $B_{14}$ | 7,960 | −11 |
| $1 \times 10^{-6}$γ vit. $B_{14}$ | 4,850 | −46 |
| $1 \times 10^{-4}$γ vit. $B_{14}$ | 3,120 | −65 |
| $1 \times 10^{-1}$γ vit. $B_{14}$ | 1,320 | −85 |
| $1 \times 10^{-6}$γ vit. $B_{14}$ plus 5γ 7MP | 9,100 | 2 |
| $1 \times 10^{-6}$γ vit. $B_{14}$ plus 10γ 7MP | 11,200 | 26 |

* Initial concentration of suspension: 8,920 cells/mm³. Time of incubation, 5 hrs at 37° C.

The technique used in culturing the tumor cells was the same as that previously described (*3*). Vitamin $B_{14}$ is about 10,000,000 times as effective as xanthopterin in inhibiting cell proliferation *in vitro* in a suspension of tumor cells, or $1 \times 10^{-6}$ γ/ml of vitamin $B_{14}$ had about the same effect as 10 γ/ml of xanthopterin. The effect of $1 \times 10^{-6}$ γ/ml of vitamin $B_{14}$ was completely counteracted by 10 γ/ml of 7MP, or $1 \times 10^{-6}$ γ/ml of vitamin $B_{14}$ plus 10 γ/ml of 7MP gave the same rate of proliferation as with no supplement.

In rats made anemic with sulfathiazole by the procedure previously described (*2*), a single injection of 0.01 γ of vitamin $B_{14}$ was as effective in alleviating the anemia and leukopenia as a single injection of 1 mg of xanthopterin.

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## Action of Enzymes on Vitamin $B_{14}$ and Pteridines


**Earl R. Norris and John J. Majnarich**

*Department of Biochemistry*
*University of Washington, Seattle*


The activity of xanthopterin and pteroyl derivatives in accelerating the rate of cell proliferation *in vitro* and in producing hemopoiesis in anemic rats has been found to be greatly increased by the action of certain enzymes.

Xanthine oxidase was prepared from milk and purified by repeated precipitation with ammonium sulfate. The enzyme preparation was very active when measured in a Thunberg tube with methylene blue, using either xanthine or xanthopterin as the substrate. Xanthopterin and folic acid (pteroylglutamic acid) were incubated with the enzyme, under toluene, at 37° C with a phosphate buffer of pH 7.4. Aliquots were removed at intervals for analysis. There was a very marked change in the absorption spectrum, which will be reported later. Kalckar, *et al.* (*1, 2*) have studied some phases of the action of the milk enzyme on xanthopterin.

The effect on cell proliferation of the products resulting from the enzyme action was measured by the technique previously described (*3*). Table 1 gives a summary of typical results obtained.

The activity of both xanthopterin and folic acid was increased by the action of the enzyme. The effect is most noticeable in the case of folic acid. At zero time the number of cells produced in the incubation of the bone marrow culture were the same with folic acid present as with no supplement. After the action of the enzyme the product formed from folic acid very greatly increased the rate of proliferation of both RBC and

nucleated cells (NC). As previously pointed out (*3*), the reticulocyte count is roughly proportional to the rate of erythrocyte formation.

TABLE 1

EFFECT OF THE PRODUCTS FORMED BY THE ACTION OF XANTHINE OXIDASE FROM MILK ON RATE OF CELL PROLIFERATION IN BONE MARROW CULTURE*

| Supplement/ml of suspension | Final concentration (RBC/mm³) | Increase in RBC (%) | Final concentration (NC/mm³) | Increase in NC (%) | Retic. /1,000 RBC |
|---|---|---|---|---|---|
| None | 10,600 | 24 | 6,120 | 44 | 18 |
| 5γ 7MP | 4,520 | −47 | 280 | −93 | 4 |
| 1 × 10⁻⁷γ vit. $B_{14}$ | 17,800 | 108 | 13,640 | 222 | 36 |
| 5γ xanthopterin enzyme—zero time | 16,880 | 98 | 11,700 | 175 | 32 |
| 5γ xanthopterin enzyme—5 days | 25,200 | 195 | 21,900 | 416 | 52 |
| 5γ folic acid enzyme—zero time | 10,640 | 24 | 6,560 | 55 | 18 |
| 5γ folic acid enzyme—5 days | 24,300 | 183 | 18,900 | 345 | 42 |

* Time of bone marrow incubation, 6 hrs; concentration of initial bone marrow suspension: RBC, 8,550/mm³; NC, 4,240/mm³; reticulocytes, 11/1,000 RBC.

TABLE 2

EFFECT OF THE PRODUCTS FORMED BY THE ACTION OF ENZYMES FROM RAT LIVER HOMOGENATE ON THE RATE OF CELL PROLIFERATION IN BONE MARROW CULTURE*

| Supplement/ml of suspension | Final concentration (RBC/mm³) | Increase in RBC (%) | Final concentration (NC/mm³) | Increase in NC (%) | Retic. /1,000 RBC |
|---|---|---|---|---|---|
| Liver homogenate (LH)—zero time | 9,680 | 91 | 5,690 | 119 | 24 |
| Liver homogenate (LH)—5 hrs, 37° C | 11,400 | 124 | 8,400 | 223 | 32 |
| 5γ xanthopterin plus LH—zero time | 18,800 | 270 | 15,100 | 480 | 34 |
| 5γ xanthopterin plus LH—5 hrs | 20,400 | 300 | 19,600 | 650 | 56 |
| 5γ folic acid plus LH—zero time | 9,100 | 79 | 7,100 | 173 | 20 |
| 5γ folic acid plus LH—5 hrs | 16,400 | 224 | 13,400 | 455 | 42 |
| 5γ teropterin plus LH—zero time | 9,360 | 84 | 6,880 | 165 | 24 |
| 5γ teropterin plus LH—5 hrs | 16,200 | 219 | 12,000 | 360 | 36 |

* Time of bone marrow culture incubation, 8 hrs; concentration of initial bone marrow suspension: RBC, 5,080/mm³; NC, 2,600/mm³; reticulocytes, 8/1,000 RBC.

Liver homogenate contains an oxidizing system which will use xanthine or xanthopterin as the substrate in a Thunberg tube with methylene blue. Xanthopterin, folic acid, and teropterin were incubated with a homogenate prepared from rat livers in a Waring blendor. The effect upon cell proliferation in bone marrow culture *in vitro* is shown in Table 2.

Folic acid and teropterin did not produce a greater rate of proliferation than the liver homogenate without supplement before incubation. After incubation with the homogenate the products formed did increase the rate of cell production. The inhibiting action of 2-amino-4-hydroxy-7-methyl pteridine (7MP) was not appreciably changed by incubation with the liver homogenate. The data for 7MP are not included in the table.

TABLE 3

EFFECT OF THE PRODUCTS PRODUCED BY THE ACTION OF ENZYMES FROM RAT GASTRIC MUCOSA ON THE RATE OF CELL PROLIFERATION IN BONE MARROW CULTURE*

| Supplement/ml of suspension | Final concentration (RBC/mm³) | Increase in RBC (%) | Final concentration (NC/mm³) | Increase in NC (%) | Retic. /1,000 RBC |
|---|---|---|---|---|---|
| Enzyme only (EN)—zero time | 8,480 | 22 | 6,480 | 51 | 16 |
| Enzyme only (EN)—72 hrs | 8,300 | 20 | 6,250 | 46 | 16 |
| 2γ xanthopterin plus EN—zero time | 13,240 | 91 | 9,250 | 116 | 30 |
| 2γ xanthopterin plus EN—72 hrs | 36,500 | 430 | 14,600 | 242 | 48 |
| 5γ folic acid plus EN—zero time | 8,320 | 20 | 6,050 | 42 | 16 |
| 5γ folic acid plus EN—72 hrs | 37,500 | 440 | 16,200 | 278 | 52 |
| 2γ vitamin $B_{14}$ plus EN—zero time | 38,000 | 450 | 18,800 | 340 | 54 |
| 2γ vitamin $B_{14}$ plus EN—72 hrs | 39,200 | 460 | 17,600 | 310 | 48 |

*Time of bone marrow incubation, 5.5 hrs; concentration of initial bone marrow suspension: RBC, 6,920/mm³; NC, 4,280/mm³; reticulocytes, 11/1,000 RBC.

Gastric mucosa has been associated with hemopoiesis and with the intrinsic factor of Castle. The gastric mucosa of several rats was disintegrated in the Waring blendor with phosphate buffer at pH 7.4, centrifuged, and filtered. The extract contained an oxidase system which would use xanthine or xanthopterin as a substrate in the Thunberg tube with methylene blue. Xanthopterin, folic acid, and vitamin $B_{14}$ were incubated under toluene at 37° C with the gastric mucosa extract. A few results are shown in Table 3.

The activity of the enzyme preparation without supplement did not change during incubation. Folic acid plus the enzyme, when first mixed, gave the same activity or rate of cell proliferation as the enzyme alone. After incubation with the enzyme the products from folic acid and xanthopterin produced a rate of cell proliferation approximately equal to that produced by vitamin $B_{14}$. The activity of vitamin $B_{14}$ was not altered by incubation with the enzyme preparation.

A single injection of microgram levels of the products produced by both milk and stomach mucosa enzymes on xanthopterin and on folic acid caused a rapid rise in RBC, WBC, and reticulocytes in rats made anemic on a purified diet plus sulfathiazole, equivalent to the effect

of 1 m/kg of xanthopterin by the technique previously described (*4*).

The above experiments explain much of the confusion and conflicting results that have been obtained in the study of the extrinsic and intrinsic factors of Castle. Apparently the extrinsic factor may not be a single substance, but xanthopterin, folic acid, teropterin, and possibly other pteroyl derivatives may serve as the extrinsic factor. Enzyme systems which were present and which were very strong in the gastric mucosa extract and present in the milk xanthine oxidase preparation and the liver homogenate, served as the intrinsic factor. Vitamin $B_{14}$ and the products produced by the action of the oxidase systems on xanthopterin and folic acid appeared to be not identical compounds, although they had similar activity when measured by the techniques used. The end-products of the action of milk, liver, and gastric mucosa enzymes on a single substrate may be different. The active material from xanthopterin is not leucopterin. The chemistry of the various materials is under investigation.

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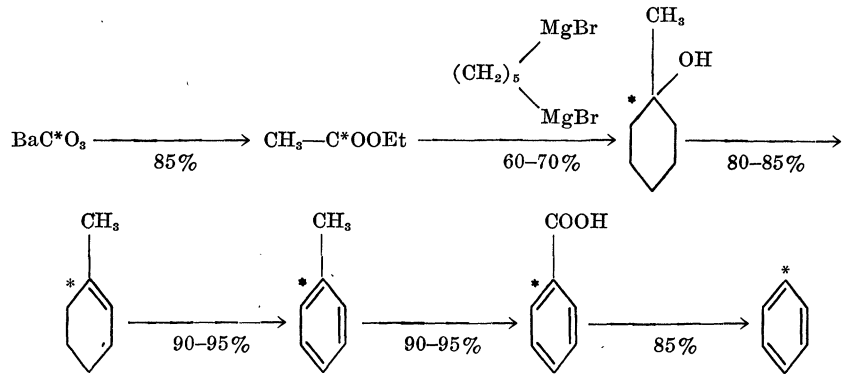
# A Synthesis of Benzene, Toluene, and Benzoic Acid Labeled in the Ring With Isotopic Carbon


**Melvin Fields, Morris A. Leaffer, and John Rohan**

*Chemical Research Laboratory,*
*Tracerlab, Inc., Boston, Massachusetts*


The development of an efficient procedure for the synthesis of benzene, toluene, and benzoic acid labeled in the ring with carbon 13 or carbon 14 is of considerable interest because of the utility of these aromatic compounds in the preparation of numerous substances of importance in organic chemistry, medicine, and biology.




While the synthesis of $C^{14}$-labeled mesitylene (*2*) affords an intermediate for the preparation of tagged benzene, this procedure suffers from the disadvantages that half of the radioactivity is converted to barium carbonate in an early step of the synthesis and the over-all yield is quite low.

We have found a satisfactory route to toluene and benzoic acid labeled on carbon 1 of the ring and to tagged benzene in the sequence of reactions illustrated above.

The synthesis of carboxyl-labeled ethyl acetate was accomplished by carbonation of methyl magnesium iodide with isotopic carbon dioxide, followed by reaction of the sodium acetate with diethyl sulfate in a modification of the procedure of Sakami, Evans, and Gurin (*4*). Preparation of 1-methylcyclohexanol-1 labeled on carbon 1 was achieved by reaction of carboxyl-labeled ethyl acetate with the Grignard reagent from pentamethylene dibromide according to the procedure of Grignard and Vignon (*1*). Dehydration of the carbinol with iodine as described by Mosher (*3*) afforded 1-methylcyclohexene, which was converted to toluene by vapor-phase dehydrogenation over platinized asbestos (*6*). The benzoic acid, prepared by oxidation of toluene with potassium permanganate (*5*), underwent smooth decarboxylation on treatment with copper oxide and quinoline. The over-all yields from barium carbonate varied from 35 to 50% for toluene, from 3% to 40% for benzoic acid, and from 25 to 40% for benzene.

A particularly attractive feature of this synthesis is the production of toluene and benzoic acid singly labeled on a specific position of the ring. Complete details of the work will be published elsewhere.

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