In Vitro Incorporation of Orotic Acid by Spleen and Liver Cells of Rats (37002)

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Orotic acid has been demonstrated to be an effective precursor of nucleic acid pyrimidines in animal tissues (1). After the injection of orotic acid into the rat, approximately 35% is excreted unchanged in the urine, about 35% is taken up by the liver, about 5% is found in the viscera, and the remainder is distributed throughout the rest of the body (2). Within 30 min of the injection there is no detectable metabolic pool of orotic acid in blood or liver since it is rapidly converted quantitatively to other compounds soluble in cold trichloroacetic acid or perchloric acid such as UMP, UDP (2).

It has been reported also that rats fed 1% orotic acid in their diet develop fatty liver and low to zero levels of plasma beta-lipoprotein (3). This condition is analogous to that seen in the human genetic disorder, abetalipoproteinemia; however, in the human condition long chain fatty acids are not absorbed *via* the lymph, and chylomicron formation does not occur as it does in the rat model. The relationship of this condition and the preferential uptake of orotic acid by the liver is not clear. This relationship does suggest however, that liver metabolizes orotic acid in a different way than do other visceral organs.

In attempts to define the metabolism with cell-free homogenates, Hurlbert and Reichard (4) found that the conversion of orotic acid to pyrimidine nucleotides was relatively active in the liver, pancreas, and spleen while less active in kidney and heart muscle. These authors suggested that many tissues have the enzymic capacity to utilize orotic acid efficiently but factors such as circulation and absorption greaty affect the amount of uptake and incorporation observed *in vivo*.

In the present study, we chose to examine the incorporation of orotic acid in cell suspensions of liver and spleen from normal and immunized rats. Although both tissues incorporated the label into acid-insoluble neucleic acid, there was a marked difference in the rate and the extent of incorporation in the two cell suspensions. It was also found that the amount incorporated into spleen cells could be increased significantly when the cells were obtained from immunized animals.

Materials and Methods. Cell suspensions. Spleens and livers were excised from 4 to 6 mo old male Charles River CD strain rats (Charles River Mouse Farms, Inc., Wilmington, MA). The tissues were washed in Tyrode's solution or medium 199, blotted dry, weighed and then teased gently with mincing forceps into medium 199 to release and suspend the cells. This cell suspension was passed sequentially through hypodermic needles of decreasing diameters, namely 16, 18 and 20 gauge, to break up any aggregates. The cells were pelleted by centrifugation at 700*g* for 15 min, washed once with tissue cuture medium and finally suspended in 6 ml/g of wet tissue in medium 199 containing 10% fresh rat serum and 5 m*M* $NaHCO_3$.

The suspensions were incubated for 15 min at 37° in a Dubnoff shaking water bath under an atmosphere of 95% O_2 and 5% CO_2. After this incubation, either 2 μCi of ^{14}C-orotic acid or 5 μCi of ^{3}H-orotic acid was added/6 ml of medium, equivalent to 1 g of original tissue. After varying periods of time samples were removed and the radioactivity incorporated into nucleic acid was determined.

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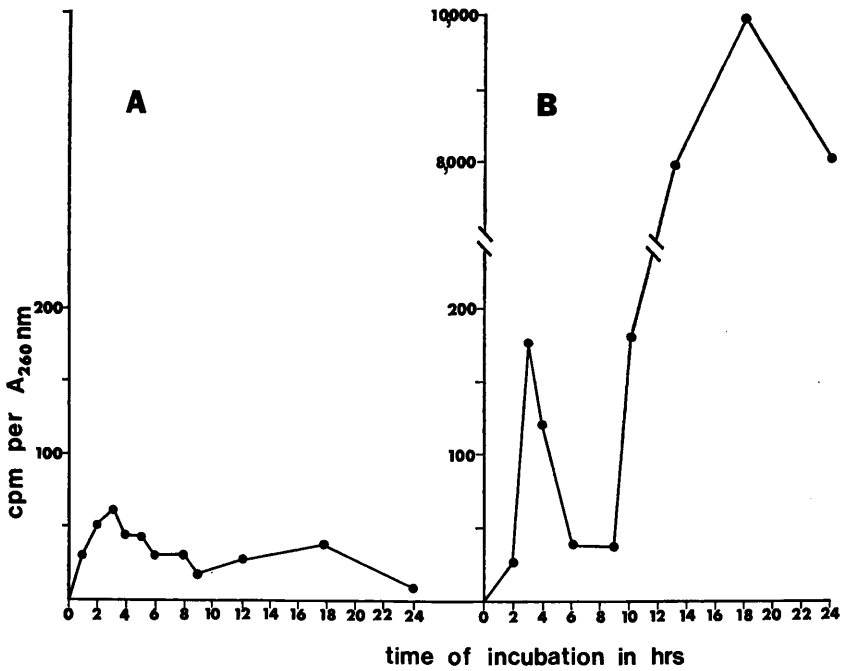

FIG. 1. The incorporation of ^{3}H-orotic acid into RNA of cell suspensions of rat: (A) spleen, (B) liver.

Nucleic acid fractionation. The cell suspensions were centrifuged at 700*g* for 10 min, the supernatant fluid was discarded and the cells were resuspended in a medium containing 150 m*M* NaCl, 1 m*M* EDTA (pH 7.5) 1 m*M* β-mercaptoethanol and 1 mg/ml of washed bentonite. The cells were homogenized in a Model C conical ground glass tissue homogenizer (Kontes Glass Co., Vineland, NJ). After homogenization, an equal volume of water-saturated phenol was added and the mixture was shaken for 30 min at 22°. The samples were then centrifuged at 20,000*g* for 20 min, the aqueous phase was collected, 0.1 vol of 20% potassium acetate (pH 5.3) was added, and the nucleic acids were precipitated with 3 vol of 95% ethanol at —20° for 16 hr. The precipitate was collected and dissolved in a solution containing 50 m*M* NaCl, 5 m*M* $MgCl_2$, 1 m*M* EDTA (pH 7.5), and 1 m*M* β-mercaptoethanol. For the separation of RNA fractions, this solution was made 1 *M* with respect to NaCl to precipitate ribosomal RNA, and 3 vol of 95% ethanol were added to the supernatant fluid to precipitate the remaining nucleic acids. The precipitate was dissolved in 10 m*M* Tris–HCl (pH 7.5), 1 m*M* $MgCl_2$ and applied to a DEAE-cellulose column for chromatography (5). Transfer RNA was eluted from the column at 0.4 to 0.6 *M* NaCl while DNA and mRNA eluted of 1 *M* NaCl concentration.

Radioactivity measurement. Acid precipitable ^{14}C was assayed by the filter paper disc method (6). ^{3}H was assayed by the method of Siekevitz (7) in which the final pellet was dissolved in 80% formic acid and aliquots were added to a scintillation cocktail containing methyl Cellosolve and toluene in a ratio of 10:6. The efficiency of counting ^{3}H was determined with appropriate standards and found to be 11.8% in a Packard Tri-Carb spectrometer.

Results. The incorporation of ^{3}H-orotic acid into RNA of spleen and liver cell suspensions is presented in Fig. 1. Two peaks of radioactivity were observed, one at 3 hr and one at 18 hr. It is evident that the first peak in the two tissues is analogous and probably related to incorporation of orotic

acid into so-called nuclear RNA (2) although the kinetic characteristics for the two cell suspensions are different. The second peak which is related to the incorporation of label into cytoplasmic RNA (2) differs in magnitude in the two tissues by more than 200-fold.

It has been suggested (4) that differences in orotic acid metabolism noted in *in vivo* studies between liver and spleen could be due to permeability factors and/or availability of orotate in the circulation. Cell suspensions of the two tissues were prepared here in a similar fashion so that orotic acid would presumably be accessible to both types of cells. It could reasonably be expected that incorporation would be similar if all the enzymic capabilities are present in both cell types. This does not appear to be true however, particularly for the period from 12 to 24 hr. This experiment was repeated a number of times with the same results. The findings with cell suspensions suggest that either the permeability of spleen and liver cells in suspension is different, or some other factor(s) related to the suspension technique prevents orotic acid uptake into spleen cells. This is based on the fact that cell-free homogenates from spleen and liver metabolize orotic acid similarly (4).

The spleen is one of the primary organs involved in the immune response. To stimulate the animals to increase the rate of nucleic acid syntheses in the spleen, cell suspensions were prepared from rats that had been injected intraperitoneally with 1 ml of a 2% suspension of sheep erythrocytes as antigen. After incubation with ^{14}C-orotic acid, RNA was prepared from the spleen cells and the amount of incorporation into cellular RNA determined. The results are presented in Fig. 2. The amount of incorporation increases during immunization compared to nonimmunized controls and is maximal in cell suspensions from animals receiving antigen 2 days previously. The significant increase was noted only for periods up to 12 hr, however. The level was higher at 3 hr than was found in liver at the same time interval.

Upon fractionation of the ribonucleic acid in the cell, as described, it was found that no significant amount of radioactivity could be detected in ribosomal RNA (1 *M* NaCl insoluble ribonucleic acid). The label was observed both in the tRNA and other RNA fractions eluted from DEAE-cullulose with the same characteristics as that given in Figs. 1 and 2.

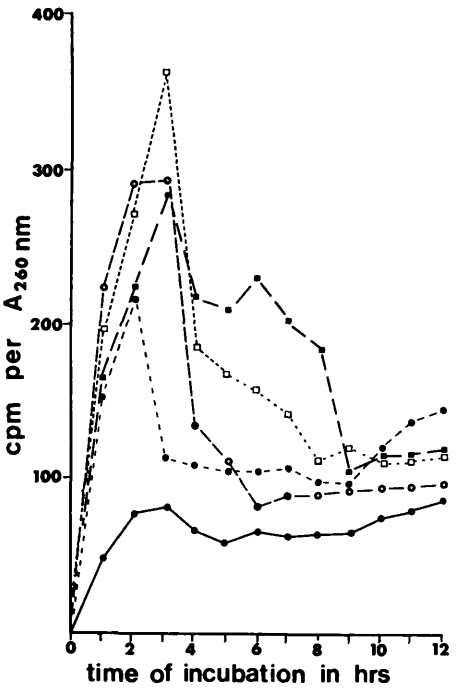

FIG. 2. The incorporation of ^{14}C-orotic acid into RNA of spleen cell suspensions from rats that had received 1 ml of a 2% sheep erythrocyte suspension as antigen at varying times prior to the preparation of cell suspensions. (●) Nonimmunized spleen cell suspensions; (○) antigen given 1 day before preparation of spleen cells; (□) antigen given 2 days before preparation of spleen cells; (■) antigen given 4 days before preparation of spleen cells; (●) antigen given 8 days before preparation of spleen cells.

Discussion. Orotic acid is a key intermediate for the synthesis of UMP. Following the injection of orotic acid there is a rapid conversion to UMP, then to uridylic acid and cytidylic acid. No orotic acid can be detected in liver within 30 min after the injection (2, 8). Since orotic acid is immediately transformed in the cell to pyrimidine nucleotides, the amount of radioactivity that will be incorporated into ribonucleic acid will depend upon the size of the cellular pool of the pyrimidine nucleotides, and not merely on the

uptake of orotic acid itself (9). Owing to this rapid conversion, there is utilization of labeled nucleotides from a pool of intermediates (10). Reutilization of these nucleotides also occurs, which indicates that the nucleotides are in the 5′-phosphorylated form (11). Despite the recycling of the label that originates with the orotic acid, Blobel and Potter (10) were able to determine half-life values for various RNA fractions, and Yu and Fiegelson (12) suggest that orotic acid is most useful for long-term incorporation studies.

Incorporation of orotic acid into acid insoluble ribonucleic acid of rat liver was found to be diphasic with an early rapid uptake into so-called nuclear RNA and a later incorporation into so-called cytoplasmic RNA (2). In mice, also, two peaks of incorporation of radioactivity into RNA of liver and kidney have been found after injection of ^{3}H-orotic acid (13). In the present study, two peaks of incorporation were also found when cell suspensions were used. The early peak at 3 hr appeared in suspensions of both liver and spleen cells while the second peak was most pronounced in liver and inconsistently present in the spleen. The relative amount of incorporation into RNA of the spleen was influenced by immunization and increased to a maximal level when cells were prepared at 2 days after the injection of antigen. At no time, however, did the second peak of radioactivity in the spleen compare to that found in liver.

These findings suggest that although conversion of orotic acid to pyrimidine nucleotides may occur as efficiently in cell-free homogenates of liver as in those of spleen (4), the differential permeability and/or metabolism of orotic acid may differ extensively between intact liver cells and intact spleen cells.

Summary. Cell suspensions of liver and spleen from rats incorporated labeled orotic acid into RNA when incubated in medium 199 containing 10% rat serum. Two peaks of incorporation were noted in each tissue—one at 3 hr and one at 18 hr of incubation. The rates and the extent of incorporation, however, differed markedly between the two suspensions. Cells from the liver showed a minor early peak and the most pronounced level of incorporated label at 18 hr of incubation, exceeding that of spleen for that time interval by 200-fold. Incorporation at the 3 hr period was consistently lower in cells from spleen than those from liver. This incorporation could be increased in spleen if cells were obtained from animals that had been immunized.

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