Radioautographic Visualization of Differences in the Pattern of \([^{3}H]Uridine\) and \([^{3}H]Orotic Acid\) Incorporation into the RNA of Migrating Columnar Cells in the Rat Small Intestine

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ABSTRACT The epithelium of rat small intestine was radioautographed to examine whether RNA is synthesized by the salvage pathway as shown after \([^{3}H]uridine\) injection or by the de novo pathway as shown after \([^{3}H]orotic acid\) injection. The two modes of RNA synthesis were thus investigated during the migration of columnar cells from crypt base to villus top, and the rate of synthesis was assessed by counting silver grains over the nucleolus and nucleoplasm at six levels along the duodenal epithelium—that is, in the base, mid, and top regions of the crypts and in the base, mid, and top regions of the villi.

Concomitant biochemical analyses established that, after injection of either \([5-{^{3}H}]uridine\) or \([5-{^{3}H}]orotic acid\): (a) buffered glutaraldehyde fixative was as effective as perchloric acid or trichloroacetic acid in insolubilizing the nucleic acids of rat small intestine; (b) a major fraction of the nucleic acid label was in RNA, that is, 91% after \([^{3}H]uridine\) and 72% after \([^{3}H]orotic acid\), with the rest in DNA; and (c) a substantial fraction of the RNA label was in poly A+ RNA (presumed to be messenger RNA).

In radioautographs of duodenum prepared after \([^{3}H]uridine\) injection, the count of silver grains was high over nucleolus and nucleoplasm in crypt base cells and gradually decreased at the upper levels up to the villus base. In the rest of the villus, the grain count over the nucleolus was negligible, while over the nucleoplasm it was low but significant. After \([^{3}H]orotic acid\) injection, the number of silver grains over the nucleolus was negligible at all levels, whereas over the nucleoplasm the number was low in crypt cells, but high in villus cells with a peak in mid villus.

The interpretation is that, except for a small amount of label incorporated into DNA from either precursor by crypt cells, the bulk of the label is incorporated into RNA as follows. In the crypts, cells make almost exclusive use of uridine, that is, of the salvage pathway, for the synthesis of ribosomal RNA in the nucleolus and of messenger and transfer RNA in the nucleoplasm. However, when cells pass from crypt to villus, they mainly utilize orotic acid—i.e., the de novo pathway—for the synthesis of messenger and transfer RNA within the nucleoplasm.

The epithe\(l\)ium of small intestine undergoes continuous renewal, as cell production in the crypts is balanced by cell loss at the extremity of the villi (1, 2). The columnar cells, which constitute ~90% of the epithelial cells (3), complete their migration in 2–3 d in the rat, while showing the gradual changes summarized in Table I (2–6). The cells arise in the crypt base from embryonic-looking proliferating stem cells, migrate to the crypt mouth, and start ascending the villus; upon reaching the mid villus region, they complete differentiation into "absorptive cells" and produce the brush border.
enzymes (4, 7), but on arrival at the villus top, they show signs of degeneration (4, 6) and are extruded to the lumen. The most dramatic changes are observed in the nucleoli, which are large and reticulated in crypt base cells, but small and compact in villus cells (5). We therefore decided to examine the functional activity of nucleoli by measuring the rate of RNA synthesis at the various levels of the intestinal epithelium.

Previous investigations of the rate of RNA synthesis in the intestine were carried out by biochemical means (8, 9) or radioautography (10, 11) in animals killed soon after administration of a labeled RNA precursor. Using the biochemical approach with [32P]phosphate as precursor, Morrison and Porteous (9) fractionated the epithelium of rat small intestine into four groups of cells roughly corresponding to lower and upper crypt and lower and upper villus, respectively; they concluded that ribosomal RNA (rRNA) was only produced in crypt cells and poly A+ RNA—presumed to be messenger RNA—was only produced in villus cells. Using the radioautographic approach, Amano et al. (11) killed mice soon after an injection of [3H]cytidine, a precursor of both DNA and RNA, and observed in sections of small intestine from which DNA had been extracted by DNase that the silver grains indicative of RNA synthesis were numerous over crypt cell nuclei and scanty over villus cell nuclei in a ratio of 7:1.

In the present investigation, the six levels of intestinal epithelium listed in Table I were examined by radioautography to locate the newly formed RNA appearing after injection of labeled precursors. Ideally, a precursor should be selected from the circulating substances that the organism uses for RNA synthesis. It should also be available at high specific activity, so that it could be given as a "tracer" dose low enough not to increase the circulating amount beyond physiological fluctuations. Two substances, [3H]uridine and [3H]orotic acid, fulfill these conditions. Moreover, they are available with the tritium label restricted to carbon-5, a condition that maximizes their specificity for RNA (12). In fact, [5-3H]uridine has been commonly used as an RNA precursor for radioautography (13-18), but [5-3H]orotic acid has only been used occasionally (19-21). In the work about to be described, both precursors will be used for radioautography, since, as detailed below, they may serve as markers for the two modes of RNA synthesis; that is, [3H]uridine follows the salvage pathway and [3H]orotic acid follows the de novo pathway.

It has been shown by biochemical method that RNA in various tissues may be derived from labeled uridine (22-28) and labeled orotic acid (22, 23, 29, 30). In the small intestine of the rat, however, Raisonnier et al. (31) reported incorporation of [14C]uridine, but of only traces of [14C]orotic acid, which implies that orotic acid was not a suitable precursor for studies of RNA in rat small intestine. A preliminary experiment was therefore designed in which the incorporation of labeled uridine and orotic acid into small intestinal RNA was reexamined using a recent method for the extraction of RNA.

For the radioautographic detection of the RNA formed from labeled precursors, it was first necessary to examine whether this RNA was insolubilized by the glutaraldehyde used for fixation. Hence, in another preliminary experiment, the insolubilization of RNA by glutaraldehyde was compared with that obtained with the perchoric acid and trichloroacetic acid commonly used in biochemical tests.

For the actual radioautographic localization, a single injection of [3H]uridine or [3H]orotic acid was given to rats, which were killed 20 min later. The duodenum was then processed for light and electron microscopic radioautography, and the rate of incorporation of the precursors into RNA was assessed by counts of silver grains. It was thus found that [3H]uridine and [3H]orotic acid could both be incorporated by columnar cells, but usually at different levels of the epithelium.

### MATERIALS AND METHODS

**Incorporation of [3H]Uridine and [3H]Orotic Acid into the RNA of Intestinal Mucosa**: A dose of 0.5 mCi/100 g body weight of [3H]uridine (New England Nuclear, Boston, MA; sp act 26 Ci/mmole) was injected intravenously under chloral hydrate anesthesia into an 82-g male rat, while a second animal of the same weight received 0.5 mCi/100 g body weight of [3H]orotic acid (New England Nuclear; sp act 20 Ci/mmole). The animals were killed 10 min later by perfusion with ice-cold Ringer's lactate solution containing 5 μg cycloheximide/ml. The duodenum and jejunum were placed in an ice-cold solution of 4 M guanidinium thiocyanate containing 1 M β-mercaptoethanol and taken to the cold room for the scraping of the mucosa. The mucosal scrapings were then homogenized in 10% wt/vol of this guanidinium thiocyanate solution and centrifuged over cesium chloride for RNA extraction by the method of Chirgwin et al. (32), while RNA content was determined by the method of Schmidt and Thannhauser (33), both as modified by F. Power and J. J. M. Bergeron (manuscript in preparation) (Table II).

In addition, a 150-g male rat received [3H]uridine and another received

### TABLE I

| Level          | Approximate number of cell positions* | Stage of cell development | Proliferative activity | Grain count per cell after [3H]-fucose† | Nucleolar area‡ | Nucleolar appearance |
|----------------|--------------------------------------|---------------------------|------------------------|----------------------------------------|----------------|---------------------|
| Crypt base     | 5                                    | Stem cell                 | +                      | —                                      | 3.1            | Reticulated         |
| Mid crypt      | 4-10                                 | Differentiating           | —                      | —                                      | 2.4            | Reticulated but     |
| Crypt top      | 5                                    | Differentiating           | —                      | —                                      | 1.7            | condensing          |
| Villus base    | 10                                   | Mature                    | —                      | 7                                      | 1.4            | Compact             |
| Mid villus     | 30-50                                | Terminal                  | —                      | 34                                     | 0.9            | Compact and         |
| Villus top     | 10                                   | Terminal                  | —                      | 15                                     | 0.5            | segregated         |

* This refers to the successive numbers of cells at each level on a side of crypt or villus.  
† These values are from grain counts at the various levels of duodenum of 35-40-g rats killed 90 min after [3H]fucose injection (see Fig. 16). The counts provide an index of the rate of glycoprotein synthesis; the glycoproteins involved have been identified as intestinal brush border enzymes (4).  
‡ These values are from measurements on the jejunum of 150-g rats (5); a similar pattern is present in duodenum.
[3H]orotic acid in the same doses as above. The animals were killed 20 min after injection and their tissues were processed as above. While the distribution of [3H]uridine in experiment I was consistent with previous observations using [3H]orotidine (11), the use of [3H]orotic acid in experiment II yielded such a different pattern that confirmation was required. The experiment was repeated by injecting 0.4 mCi of [3H]orotic acid into two groups of 35-40-g male Sherman rats: a group of three in which the injection was completed within a few seconds (experiment III) and a group of four in which the injection was given slowly over a 2-min period (experiment IV). The animals were all killed after 20 min, except one of those in experiment III, which was killed after 2 h. The pieces of duodenum were handled as described above. Finally, in experiment V, two 200-g rats were given 0.2 mCi intraperitoneally (2 μCi/g body weight) and killed 1 h later; the duodenum in this case was fixed in Carnoy and embedded in paraffin.

Radioautographic Procedures: For light microscopic radioautography, semithin sections from experiments I-IV were coated with NTB3 emulsion (37) and usually exposed 3 wk after [3H]uridine and 8 wk after [3H]orotic acid injection, except in experiment V, where exposure was prolonged for 8 mo.

Quantitation of radioautographic reactions was carried out in crypts and villi were cut along their long axis. In the crypts, the nuclei along one side of the lumen were divided into three equal segments (corresponding only approximately to the classification in crypt base, mid crypt, and crypt top used in Table I). The counts of silver grains over nuclei were pooled for each segment. If the number of nuclei was not divisible into thirds, adjustments were made by attributing the appropriate proportion of the grains of any border nucleus to the adjacent segments (38). The results for the three levels were reported on the abscissa of the graphs as 0-33%, 34-66%, and beyond 66%, respectively (Figs. 2 and 11). In the villi, the nuclei on one side were divided into equal intervals (which did not correspond to the classification into villus base, mid villus, and villus tip used in Table I). The counts of silver grains over nuclei were pooled for each segment. If the number of nuclei was not divisible into thirds, adjustments were made by attributing the appropriate proportion of the grains of any border nucleus to the adjacent segments (38). The results for the three levels were reported on the abscissa of the graphs as 0-33%, 34-66%, and beyond 66%, respectively (Figs. 2 and 11). The data in these figures were averages from counts of at least eight crypt and eight villi in each of the two animals given [3H]uridine and each of two given [3H]orotic acid.

As part of experiment IV, a piece of duodenum was removed from a glutaraldehyde-perfused animal, postfixed in Bouin, and embedded in paraffin. Parasagittal sections were then incubated in a solution of 10 mg ribonuclease A (Sigma Chemical Co., St. Louis, MO) per milliliter of saline for 6 h at 37°C and pH 5.3. Finally, after a 15-min passage through cold 5% trichloroacetic acid, the slides were stained and subjected to radioautography as described earlier.

For electron microscopic radioautography, thin (gold) sections taken out of blocks from the two animals of experiments I and II were processed by the method of Kopriwa (39). After a 6-mo exposure, the sections were developed in Epon-ascorbic acid (40). Under these conditions, the grains resulting from the activation of a silver bromide crystal could appear as single or multiple particles. In counting, any group of two to four closely associated particles was treated as a single silver grain. Counts were carried out over the nucleolus and the rest of the nucleus, referred to as the nucleoplasm, at the six levels defined in Table I by the position of the cells (Tables V and VI).

RESULTS

Incorporation of [3H]Uridine and [3H]Orotic Acid into RNA of Intestinal Mucosa

The total radioactivity of the mucosa, as well as that of the nucleic acids and particularly RNA, was greater after injection of [3H]uridine than [3H]orotic acid (Table II). The proportion of label in RNA and DNA was, respectively, 91 and 9% after [3H]uridine and 72 and 28% after [3H]orotic acid. Moreover, a substantial amount of radioactivity was present...
Localization of Uridine Label

Light microscopic radioautographs of duodenal-epithelium from the 35-40-g rats and the 150-g rat injected with \([3^3\text{H}]\)uridine (experiment I) revealed that nearly all silver grains were over nuclei, being abundant in the lower crypt region, much less numerous near the crypt mouth, and scanty along the villus (Fig. 1). The grain counts per nucleus in 35-40-g rats (Fig. 2) showed a peak in the lowest segment of the crypt, followed by a decreasing count higher up, while the values remained at a steady low level along the villus.

In electron microscopic radioautographs (Figs. 3-9), silver grains were seen over the nucleoplasm and nucleolus, but were in greater concentration over the latter. In spite of

in the poly A\(^+\) form with both precursors (Table III). While the amount of RNA as poly A\(^+\) was relatively small (2.0\% after \([3^3\text{H}]\)uridine and 2.1\% after \([3^3\text{H}]\)orotic acid), its specific activity was higher than that of poly A\(^-\) RNA by a factor of ~12 after \([3^3\text{H}]\)uridine and 24 after \([3^3\text{H}]\)orotic acid.

Suitability of Glutaraldehyde Fixative for RNA Insolubilization

After \([3^3\text{H}]\)uridine injection, the radioactivity of intestinal pieces immersed into buffered glutaraldehyde did not differ significantly from that of pieces immersed into trichloroacetic or perchloric acid (Table IV). Following \([3^3\text{H}]\)orotic acid injection, glutaraldehyde retained significantly more label than trichloroacetic acid but not more than perchloric acid (Table IV). Hence, glutaraldehyde fixation insolubilized the tissue label arising from \([3^3\text{H}]\)uridine or \([3^3\text{H}]\)orotic acid at least as effectively as the reagents commonly used to do so and, therefore, was suitable as a fixative prior to radioautography with either precursor.

FIGURE 1 Radioautograph of duodenal mucosa from a 35–40-g rat killed 20 min after injection of 0.5 mCi of \([3^3\text{H}]\)uridine (experiment I). Exposure, 21 d. The horizontal bar at center left indicates the limit between crypt and villus. This bar is 30 nm long. Silver grains are almost exclusively over nuclei. The heaviest concentration is over the nuclei in the base of the crypt. There is a decline toward the top of the crypt. In the villus, silver grains are rare. X 600.
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Figure 2. Incorporation of [3H]uridine in duodenal cells. Counts of silver grains per cell nucleus at various levels of duodenal crypt and villus in [3H]uridine radioautographs prepared as in Fig. 1, except that the exposure was 18 d (experiment I). Grain counts decrease from the lowest to the uppermost segment of the crypt and are low along the length of the villus.

Variation from cell to cell, the reaction was usually heavy in the crypt base (Figs. 8 and 9), somewhat less intense in the mid crypt (Figs. 6 and 7), fairly low in crypt top (Fig. 5), and even lower in villi (Figs. 3 and 4). Within crypt nucleoli, the pars fibrosa usually showed more silver grains than the pars granulosa (e.g., Fig. 7), whereas within the nucleoplasm, pale areas displayed more grains than chromatin masses (41). The counts of silver grains (Table V) confirmed the abundance of radioactivity in the nucleoli of crypt base and mid crypt, with the large standard error in the former reflecting individual variation; the number of grains over nucleoli sharply decreased in crypt top and villus base, and was not significantly different from background in mid villus and villus top. The counts over the nucleoplasms (Table V) were high in crypt base and mid crypt, decreasing in crypt top and villus base, and low but above background in mid villus and villus top.

Localization of Orotic Acid Label

The light microscopic radioautographs of duodenal epithelium from 35-40-g rats given [3H]orotic acid (experiment II) were exposed three to six times as long as after injection of [3H]uridine (experiment I) to provide reactions of comparable intensity (Fig. 10 vs. Fig. 1). Silver grains were again largely restricted to the nuclei; they were sparse over crypt cell nuclei and fairly abundant over villus cell nuclei (Fig. 10). The grain counts per nucleus (Fig. 11) were low in the crypt, rose in the lowest segments of the villus, and reached a peak in mid villus. In the uppermost segments, counts decreased to a low level.

The predominance of orotic acid label in villus cells was confirmed under various conditions. Whether the [3H]orotic acid was injected slowly (experiment IV; Fig. 16) or rapidly (experiment III), whether the animal was young or old (experiment II), whether or not the dose was reduced (experiment V), and whether the animals were killed at 20 min or later, when many grains were over the cytoplasm (experiments III and V), the overall pattern of villus cell reaction was the same. Within the experiments, however, individual variation could be observed in the size and location of the villus peak. Thus, it approximately extended from the 30% to the 50% segment in Fig. 11, but from the 20% to the 30% segment in Fig. 16.

When paraffin sections of the duodenum (experiment IV) were incubated with ribonuclease A prior to radioautography, 82-87% of the silver grains were removed from the nuclei of villus cells, while the few silver grains present over the nuclei of crypt cells persisted.

Electron microscopic radioautographs after labeled orotic acid injection revealed that silver grains were rare or absent over the nucleolus, but could be present over the nucleoplasm, as seen in Figs. 12-15 (from specimens exposed as long as the [3H]uridine ones in Figs. 3-9). Nucleoplasmic grains were sparse over crypt nuclei (Fig. 15), occurred more frequently over the nuclei of villus base and the first cells of mid villus (Fig. 14), and seemed most numerous in the lower half of the mid villus (Fig. 13); their frequency decreased over the nuclei of upper mid villus and villus top (Fig. 12). The counts of silver grains (Table VI) confirmed that nucleolar values were very low at all levels of crypt and villus, whereas nucleoplasmic values were low in crypt, but fairly high in villus with higher values along the mid villus. Within the mid villus, the lower half was more strongly labeled than the upper half, as shown by respective nucleoplasmic counts of 8.9 ± 0.9 and 4.0 ± 1.0.

DISCUSSION

Behavior of Uridine

Suitability of Uridine as RNA Precursor: Uridine gives rise to the pyrimidine residues of RNA through a sequence of phosphorylations yielding [3H]uridine mono-, di-, and triphosphate (UMP, UDP, and UTP), the last one of which is incorporated into RNA through the action of a polymerase (42-44). The sequence is known as salvage pathway since it uses the circulating [3H]uridine (45-48) arising from food and nucleic acid breakdown in tissues (43). In the rat, the blood uridine turns over with a half-life of ~3 min (48, 49) and has a concentration reported as 0.24 (48), 0.95 (47), and 7.92 µg/ml (50). In the present investigation, the injected dose of labeled uridine—usually 4.8 µg—was of a comparable order of magnitude. By performing the injection slowly over a 2-min period, the labeled uridine was likely to blend with the circulating uridine without increasing its level beyond that of normal fluctuations. Hence, the labeled uridine functioned as a "tracer," reflecting the behavior of uridine under physiological conditions. Moreover, to minimize the variations in precursor pool that have been observed in animals fasted overnight or in cells grown in culture (13), we used healthy animals fed ad libitum. Under these physiological conditions, the amount of incorporated label could be used as an index of the rate of RNA synthesis from uridine. It should be kept in mind, however, that a small fraction of the label, 9%, appeared in DNA, presumably in crypt cells synthesizing DNA prior to division.

While glutaraldehyde is commonly used for fixation prior to [3H]uridine radioautography (12, 13, 16), the ability of this fixative to retain RNA has been questioned (51). However, the experiment described in Table IV demonstrated that cacodylate-buffered glutaraldehyde insolubilized as much [3H]uridine label as the perchloric and trichloroacetic acid commonly used for nucleic acid precipitation. Moreover, the uridine label retained in sections by glutaraldehyde was local-
Figures 3-9  Electron microscope radioautographs of nuclei at various levels of the duodenal epithelium 20 min after injection of 0.5 mCi of [5-3H]uridine (experiment I). Exposure, 6 mo. Fig. 3: Villus top. No silver grains are over the small condensed nucleolus (n). Two are over the nucleoplasm. c, chromatin. Fig. 4: Mid villus. Again the small nucleolus (n) shows no grains. Fig. 5: Crypt top. Rare grains are over the nucleolus (n). One is over the nucleoplasm. Figs. 6 and 7: Mid crypt. The nucleoli show a fair amount of silver grains, which are mainly located over the dense cords (pars fibrosa). The two figures illustrate individual variation. Note the presence of grains over the nucleoplasm. Figs. 8 and 9: Crypt base. In spite of individual variation, crypt base cells show a greater reaction over both nucleoli and nucleoplasm than at other levels. While grains predominate over dense regions of the nucleolus, there seems to be a fair number over the light cords (pars granulosa), which suggests more rapid evolution from pars fibrosa to granulosa than in mid crypt. Bar, 1 μm. X 18,000.
Radioautograph of duodenal mucosa from a 35-40-g rat killed 20 min after injection of 0.4 mCi of [5-3H]orotic acid (experiment II). Exposure, 90 d. The horizontal bar, 30 nm long, indicates the limit between crypt and villus. Silver grains are almost exclusively over nuclei. A few are over the nuclei of the crypt. More are over the first few nuclei of the villus. Maximal grain concentration is observed over nuclei higher up in the villus (mid villus level). × 600.

FIGURE 11 Incorporation of [3H]orotic acid in duodenal cells. Counts of silver grains per cell nucleus at various levels of duodenal crypt and villus in [5-3H]orotic acid radioautographs prepared as in Fig. 10, except that the exposure was 54 d (experiment II). A few silver grains are present in the crypt segments. The counts rise from the lowest villus segment to a short plateau toward the middle segments and drop to a low level in the uppermost segments.

LABELING OF NUCLEIC ACIDS BY [3H]URIDINE IN THE MUCOSA OF SMALL INTESTINE: The labeled nucleic acids of the mucosa comprised 9% DNA and 91% RNA. Of the RNA label, 19.7% was identified as poly A+ RNA, which is presumably heterogeneous RNA being processed into mRNA. A clue to the content of labeled rRNA was provided by the radioautographs, since the [3H]uridine reactions over nucleoli could be attributed to newly synthesized rRNA (16, 53, 54). The number of silver grains over nucleoli at each level (Table V) was multiplied by the corresponding number of cell positions (Table I) and the results were added up. It was thus found that 23.5% of the nuclear grains were over nucleoli, being presumably attributable to new rRNA. This figure provided an order of magnitude and so did the 19.7% value of poly A+ RNA obtained from chemical analysis. The balance of the labeled RNA, that is, over half of it, was presumed to be in transfer RNA (tRNA).

INTERPRETATION OF [3H]URIDINE RADIOAUTOGRAPHS: It was known that the nucleoli of columnar cells were large and reticulated in crypt base and gradually decreased in size at higher levels to become small and compact on the villus (5, 55). The incorporation of uridine label by nucleoli, attributed to synthesis of rRNA (16, 53, 54), varied in a comparable manner, being intense in crypt base and mid crypt, low in crypt top and villus base, and negligible in the rest of the villus. The incorporation of uridine label by the nucleoplasm was attributed to the synthesis of tRNAs and mRNAs (41, 56–59), although a fraction of the label in the proliferating cells of crypt base and mid crypt (60) could consist of newly formed DNA. Hence, the production of mRNA and/or tRNA was intense in crypt base and mid
crypt, moderate in crypt top, and low though significant along the villus.

Behavior of Orotic Acid

Suitability of Orotic Acid as RNA Precursor: Orotic acid mainly arises within cells (42-44) and, according to Hauschka (43), mixes with the exogenous orotic acid arising from food; both jointly react with 5-phosphoribosyl-1-pyrophosphate in the presence of a two-enzyme complex (orotidine-5-phosphorylase and decarboxylase) to give rise to UMP. This sequence, known as the de novo pathway, ends in the processing of UMP through UDP to RNA as in the salvage pathway (42-44). Orotic acid is present in urine (61-65) and milk (66, 67) and is therefore likely to occur in the circulation, but information on the subject is scanty. In man, the blood concentration has been reported to be below 5 \( \mu \text{g/ml} \) (68, 69) and somewhat higher in argininemia (70). In the only report available on the rat (71), orotic acid concentration is given as 6.1 \( \mu \text{g/ml} \). The dose of \(^{1}H\)orotic acid administered to our animals—3.1 \( \mu \text{g} \) in most cases—was of a comparable order of magnitude and, especially when injected slowly over a 2-min period, should function as a tracer dose that mixes with the circulating orotic acid and thus reflects its behavior under physiological conditions. Since this tracer dose was given to healthy animals fed ad libitum, the substances present along the de novo pathway should be in a steady state. Hence, the amount of incorporated label provided an estimate of the rate of RNA synthesis from orotic acid, although the passage of 28% of the label into DNA should be kept in mind.

Glutaraldehyde was at least as effective as perchloric and trichloroacetic acid in retaining the nucleic acid label acquired after \(^{1}H\)orotic acid injection (Table IV). That the label was in nucleic acids was also shown by the localization of nearly all radiolabeled materials over nuclei within the site of nucleic acid synthesis (52). Moreover, when sections of glutaraldehyde-fixed intestine were treated with RNase prior to autoradiography, the label was mostly removed from villus cells, but not crypt cells, which indicates that labeled RNA was present in villus cells and labeled DNA was present in crypt cells.

Labeling of Nucleic Acids by \(^{1}H\)Orotic Acid in the Mucosa of Small Intestine: Raisonnier et al. (31) reported that the mucosa of rat intestine contained only a negligible amount of the two-enzyme complex required for the synthesis of RNA from orotic acid; they implied that this substance was not a significant precursor of RNA in rat intestine. However, in our experience after \(^{1}H\)orotic acid injection, a substantial amount of label appeared in nucleic acids of which 72% was in RNA, and therefore the two-enzyme complex was likely to be present in the cells of the intestinal mucosa. Furthermore, about one-fifth of the RNA label was in the form of poly A+ RNA. Finally, the insignificant radiographic reactions over nuclei indicated that little or no rRNA was produced. Under these conditions, it was likely that the poly A+ RNA consisted of tRNA.

| Table VII | Influence of Glutaraldehyde on the Adsorption of \(^{1}H\)Uridine or \(^{1}H\)Orotic Acid by BSA |
|-----------|---------------------------------|
| \(^{1}H\)Uridine | Label in trichloroacetic acid precipitate % |
| Water control | 0.025 ± 0.0009 |
| 2.5% buffered glutaraldehyde | 0.041 ± 0.0051 |
| \(^{1}H\)Orotic acid | Label in trichloroacetic acid precipitate % |
| Water control | 0.025 ± 0.0056 |
| 2.5% buffered glutaraldehyde | 0.068 ± 0.0062 |

To a mixture of BSA (1 mg in 0.1 ml) and \(^{1}H\)Uridine (5 \( \mu \text{g/m} \) containing 870,000 counts) was added either 0.4 ml water for control or 0.15 ml of 5% glutaraldehyde in 0.1 M sodium cacodylate buffer, plus 0.25 ml water. Then 0.5 ml of 10% trichloroacetic acid was added to precipitate the albumin. After centrifugation and two washings in 5% trichloroacetic acid, the pellet was dried and its radioactivity was measured in the scintillation counter. The experiment was run in quintuplicate. Even though glutaraldehyde seemed to increase the radioactivity bound to the precipitate, the amounts adsorbed with or without glutaraldehyde were very small. In the experiment carried out with \(^{1}H\)orotic acid (814,000 counts), the precipitate-bound radioactivity was again negligible.

Interpretation of \(^{1}H\)Orotic Acid Radioautographs: While orotic acid did not seem to be involved in the production of rRNA by the nucleolus, it was incorporated by the nucleoplasm. The weak reactions observed in the nucleoplasm of crypt base and mid crypt cells, whose divisions implied DNA synthesis, were attributed to newly formed DNA. The nucleoplasmatic reactions in villus cells, which were known not to synthesize DNA (60), were attributed to production of mRNA and tRNA.

The pattern of \(^{1}H\)orotic acid incorporation along the villus (Fig. 11) resembled the pattern of distribution of intestinal brush border enzymes, as determined by enzyme analysis (7) and \(^{3}H\)fucose radioautography (72). When graphs of the distribution of \(^{1}H\)fucose-labeled brush border enzymes of \(^{1}H\)orotic acid incorporation were superimposed (Fig. 16), the ascending portion of the \(^{1}H\)orotic acid curve (from experiment IV) was associated with, or slightly preceded, the rise in fucose incorporation. This observation raised the possibility that the incorporation of orotic acid into the nucleoplasm of villus cells provided mRNAs and tRNAs for the synthesis of brush border enzymes.

Variation in RNA Synthesis during Migration of Columnar Cells

Radioautography reveals that the crypt cells, which are cells recently produced by mitosis, chiefly use uridine—that is, the salvage pathway—for the synthesis of their RNA. After the cells migrate to the villus, however, they mainly use orotic acid—that is, the de novo pathway—for RNA synthesis. (Counts of silver grains indicate that villus cells incorporate about four times as much orotic acid as uridine.) On the
average, cells leave the crypt a day after they arise from mitosis, so that in such a time, the uridine-using crypt cells become orotic acid-using cells. The marked change might be due to a drastic modification of the transport of uridine (73) and orotic acid (74) into and out of the cells, or the two-enzyme complex required by the de novo pathway might only be produced when cells approach maturity on the villus.

The pattern change is not complete. Thus, when orotic acid incorporation rises to a peak in the nucleoplasm of mid villus nuclei, there is still a low but steady incorporation of uridine. Thus, RNA is being synthesized in the nucleoplasm at different rates along the salvage and de novo pathways, even though the last steps in both consist of the same UMP-UDP-UTP series. It thus appears that in mid villus cells, the precursors derived from uridine mix little or not at all with those derived from orotic acid, perhaps by being restricted to distinct compartments in the nucleoplasm. Each pathway could be associated with a different nucleoplasmic structure in such a way as to allow the reaction chain to occur with only minimal diffusion of the products (75).

The sites of synthesis of the RNA types can be deduced from the localization of the reactions. Thus, nucleolar reactions indicate that rRNA arises from uridine at a rate that is high in crypt base cells and decreases in parallel with the decline of nucleolar size as cells migrate to the mid villus, where synthesis of this RNA seems to cease.

Nucleoplasmic reactions indicate that mRNA and tRNA are actively produced from uridine by the stem cells of the crypt base and their daughter cells in the mid crypt. These RNAs are likely to be involved in the active protein synthesis that is known to occur at these levels (76), presumably for the needs of the cells arising from local mitoses. As cells pass through crypt top and villus base, the synthesis of RNAs from either uridine or orotic acid is low, even though cells are differentiating or their synthesis of protein reaches a peak (76). Presumably these cells utilize the RNAs elaborated at lower levels. When the cells reach the mid villus region, their RNA arises mainly from orotic acid with a peak of incorporation in lower mid villus. The peak is associated with the production of brush border enzymes and is therefore attributed to the production of mRNAs and tRNAs for the synthesis of these enzymes. Finally at the villus top, little RNA is produced from either precursor, while the cells show signs of degeneration prior to being lost to the lumen.

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REFERENCES

1. Leblond, C. P., and B. Messier. 1958. Renewal of chief cells and goblet cells in the small intestine as shown by radioautography after injection of thymidine-3H into mice. Anat. Rec. 132:247-259.
2. van den Osten, J. M., W. J. Viner, W. T. Daems, and H. Gajda. 1976. The relation between cell proliferation, differentiation and ultrastructural development in rat intestinal epithelium. Cell Tiss. Res. 174:183-199.
3. Cheng, H., and C. P. Leblond. 1974. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. 1. Columnar cells. Am. J. Anat. 141:461-480.
4. Leblond, C. P. 1981. The life history of cells in renewing systems. Am. J. Anat. 160:113-158.
5. Altmann, G. G., and C. P. Leblond. 1982. Size and structure of the nucleoli of columnar cells at various levels of crypt and villus in the rat jejunum. J. Cell Sci. 61:83-99.
6. Falcons, N. R. 1982. Ultrastructural changes during maturation of villus columnar cells of the rat jejunum. Ph.D. Thesis. University of Western Ontario, London, Ontario.
7. Nordström, C., A. Dahlqvist, and L. Josefsson. 1968. Quantitative determination of enzymes in different parts of the villi and crypts of rat small intestine. J. Histochem. Cytochem. 15:713-721.
8. Shafritz, D. A., and J. R. Senior. 1967. Synthesis of pyrimidine nucleotide precursors in humana and rat small intestinal mucosa. Biochem. Biophys. Acta. 141:332-341.
9. Morrison, A., and J. W. Porteous. 1980. Changes in the synthesis of ribosomal ribonucleic acid and poly(A) containing ribonucleic acid during the differentiation of intestinal epithelial cells in the rat and in the chick. Biochem. J. 188:609-618.
10. Shorter, R. G., and B. Cremer. 1962. Ribonucleic acid and protein metabolism in the gut. I. Observations in gastro-intestinal cells with rapid turnover. Gut. 3:118-128.
11. Armanino, M., C. P. Leblond, and N. J. Nadler. 1965. Radioautographic analysis of nuclear RNA in mouse cells. Exp. Cell Res. 38:314-340.
12. Hayhoe, F. G. J., and D. Quaglini. 1965. Autoradiographic investigations of RNA and DNA metabolism of liver homogenates cultured with photobacterium luminous: uridine-3H as a specific precursor of RNA. Nature (Lond.). 205:151-156.
13. Baserga, R., and D. Malamud. 1969. Autoradiography, Techniques and Applications. Harper and Row, New York. 181-202.
14. Jacobs, J. 1967. An electron microscope radioautographic study of the site of initial synthesis of RNA in the nucleus of Smittia. Exp. Cell Res. 48:276-282.
15. Kacazaki, S. 1968. The ultrastructure and RNA metabolism in nuclei of early sea urchin embryos. Exp. Cell Res. 52:13-26.
16. Fakan, S., and W. Bernhard. 1971. Localization of rapidly and slowly labelled nuclear RNA as visualized by high resolution autoradiography. Exp. Cell Res. 67:129-141.
17. Tres, L. L. 1975. Nucleolar RNA synthesis of meiotic prophase spermatocytes in the human testis. Chromosoma (Berl.). 53:141-151.
18. Mcle, C., and A. Stahl. 1981. Ultrastructural organization sites of transcription and distribution of fibrillar centers in the nucleolus of the mouse oocyte. J. Cell Sci. 48:105-126.
19. Nocoduz, N. A., and J. C. H. Max. 1966. RNA synthesis in rat and mouse hepatic cells as studied with light and electron microscope radioautography. J. Cell Biol. 30:655-663.
20. Patterson, C. M., and B. J. Kruger. 1975. An autoradiographic study on the effect of a range of fluoride doses on the utilization of 3H-orotate by ameloblasts in the rat. Arch. Oral Biol. 20:149-151.
21. Martinez-Ramon, A., and S. Grisolia. 1980. Increased incorporation of aspartate and decreased incorporation of orotate in fibroblasts from Lesch-Nyhan patients as revealed by autoradiography. Biochem. Biophys. Res. Commun. 90:1-10.
22. Witschi, H. 1972. A comparative study of in vivo RNA and protein synthesis in rat liver and lung. Cancer Res. 32:1686-1694.
23. Ord, I. G., and L. A. Stocken. 1974. Uptake of orotate and thymidine by normal and regenerating livers. Biochem. J. (Tokyo). 132:47-54.
24. Zardi, L., and R. Baserga. 1974. Ribosomal RNA synthesis in WI-38 cells stimulated to proliferate. Exp. Mol. Pathol. 11:118-127.
25. Grummt, I., V. A. Smith, and F. Grummt. 1976. Amino acid starvation affects the initiation frequency of nuclear RNA polymerase. Cell. 7:439-445.
26. Morselli, U., G. Kramer, K. Harms, and H. Hilz. 1970. Separate pyrimidine-ribonucleotide pools for messenger RNA and ribosomal RNA synthesis in HeLa S3 cells. Eur. J. Biochem. 46:535-540.
27. Cortes, F., N. W. Levin, F. Diemler, K. Venkatachalam, C. P. Verghese, and J. Bernstein.
1979. Incorporation of exogenous precursors in uridine nucleotides and ribonucleic acid. Biochem. J. (Tokyo). 182:677-686.
28. Yagita, T. 1977. Metabolism of $^5$S uridine in mouse and specificity for labeling of liver ribonucleic acid. Int. J. Biochem. 8:395-401.
29. Archer, S. J., and J. C. West. 1973. In vitro incorporation of uric acid by spleen and liver cells of rats. Proc. Soc. Exp. Biol. Med. 142:262-265.
30. Lewan, L., N. Petersen, and T. Yangner. 1975. Incorporation of orotic acid into nucleotides and RNA in mouse organs during 60 minutes. Hoppe-Seyler's Z. Physiol. Chem. 356:425-429.
31. Raisonnier, A., M. E. Bouma, C. Salvat, and R. Infante. 1981. Metabolism of uracil acid lack of orotic phosphoribosyltransferase in rat intestinal mucosa. Eur. J. Biochem. 118:565-569.
32. Chirpwin, J. M., A. W. Prybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonucleic acid. Biochemistry. 24:3294-3299.
33. Schmidt, G., and S. J. Thannhauser. 1945. A method for the determination of disoxyribonucleic acid, ribonucleic acid and phosphoproteins in animal tissues. J. Biol. Chem. 161:83-89.
34. Aviv, H., and P. Leder. 1972. Purification of biologically active globin messenger RNA by chromatography. Proc. Natl. Acad. Sci. USA. 69:1408-1412.
35. Rachubinski, R. A., D. P. S. Verma, and J. J. M. Bergeron. 1980. Synthesis of rat liver microsomal cytochrome b 5 by free ribosomes. J. Cell Biol. 84:705-716.
36. Munro, H. W., and A. Fleck. 1966. Recent developments in the measurement of nucleic acids in biological materials. Analyst. 91:78-88.
37. Kopriwa, B. M., and C. P. Leblond. 1962. Improvements in the coating technique for electron microscopic radioautography. Histochemie. 37:1-17.
38. Kit, S. 1970. Nucleotides and nucleic acids. In Metabolic Pathways 1970. D. M. Greenberg, editor. Academic Press, Inc., New York. 69-275.
39. Munro, H. W., and A. Fleck. 1966. Acrylamide gel electrophoresis of HeLa cell nucleolar RNA. J. Histochem. Cytochem. 10:269-284.
40. Sanger, F., and E. F. Baldwin. 1977. DNA sequencing. Proc. Natl. Acad. Sci. USA. 74:5463-5467.
41. Littau, V. C., V. G. Allfrey, J. H. Frenster, and A. E. Mirsky. 1964. Active and inactive nucleolar areas in mammalian metaphase chromosomes. J. Cell Biol. 21:177-197.
42. Kopriwa, B. M. 1975. A comparison of various procedures for fine grain development in electron microscopic radioautography. Histochemie. 44:201-224.
43. Clark, J. H. 1975. Electrophoresis of HeLa cell nucleolar RNA. Proc. Natl. Acad. Sci. USA. 72:1088-1095.
44. Jordan, E. G. 1981. The Nucleolus. Carolina Biol. Readers, J. J. Head, editor. Burlington, NC. 1-16.
45. Adamstone, F. B., and A. B. Taylor. 1972. Nucleolar reorganization in epithelial cells of the jejunum of the rat. J. Morphol. 146:131-152.
46. Karle, J. M., L. W. Anderson, C. Erlichman, and R. L. Cysyk. 1980. Serum uridine levels in patients receiving N-(phosphonacetyl)-L-aspartate. Cancer Res. 40:2938-2940.
47. Hatchwell, L. C., and J. A. Milner. 1978. Factors affecting amino acid induced orotic aciduria in rats. J. Nutr. 108:1976-1982.
48. Jordan, E. G. 1981. The Nucleolus. Carolina Biol. Readers, J. J. Head, editor. Burlington, NC. 1-16.
49. Perry, R. P. 1962. The cellular sites of synthesis of ribosomal and 45 RNA. Proc. Natl. Acad. Sci. USA. 48:2179-2186.
50. Perry, R. P. 1976. Processing of RNA. Annu. Rev. Biochem. 45:605-629.
51. Langenberg, W. G. 1980. Glutaraldehyd nonfixation of isolated viral and yeast RNA's. J. Histochem. Cytochem. 28:311-315.
52. de Robertis, E. D. P., and E. M. F. de Robertis. 1980. Cell and Molecular Biology. Saunders College Press, Philadelphia, PA.
53. Weisburt, R. A., U. Loening, M. Willems, and S. Penman. 1981. 1-Acetylglutamyl dehydrogenase of Hela cell nuclear RNA. Proc. Natl. Acad. Sci. USA. 78:1088-1095.
54. Jordan, E. G. 1978. The Nucleolus. Carolina Biol. Readers, J. J. Head, editor. Burlington, NC. 1-16.
55. Perry, R. P. 1962. The cellular sites of synthesis of ribosomal and 45 RNA. Proc. Natl. Acad. Sci. USA. 48:2179-2186.
56. Perry, R. P. 1976. Processing of RNA. Annu. Rev. Biochem. 45:605-629.
57. Kopriwa, B. M., and C. P. Leblond. 1962. Improvements in the coating technique for electron microscopic radioautography. J. Histochem. Cytochem. 10:269-284.
58. Bailey, J. A., and B. L. Karlin. 1978. Incorporation of exogenous precursors in uridine nucleotides and ribonucleic acid. Biochem. J. (Tokyo). 182:869-876.
59. Kari, J. M., L. W. Anderson, C. Erlichman, and R. L. Cysyk. 1980. Serum uridine levels in patients receiving N-(phosphonacetyl)-L-aspartate. Cancer Res. 40:2938-2940.
60. Hatchwell, L. C., and J. A. Milner. 1978. Factors affecting amino acid induced orotic aciduria in rats. J. Nutr. 108:1976-1982.
61. Fekete, I. 1978. Effects of alloxan on orotic acid and glycogen content in various vertebrate species. Experientia. 34:827-828.
62. Rhode, E. H., and E. F. Baldwin. 1977. DNA sequencing. Proc. Natl. Acad. Sci. USA. 74:5463-5467.
63. Greenberg, editor. Academic Press, Inc., New York. 69-275.
64. Hatchwell, L. C., and J. A. Milner. 1978. Factors affecting amino acid induced orotic aciduria in rats. J. Nutr. 108:1976-1982.
65. Becroft. 1978. Absence of orotic aciduria in adenosine deaminase deficiency and purine nucleoside phosphorylase deficiency. Clin. Exper. Immunol. 34:42-45.
66. Hatchwell, L. C., and J. A. Milner. 1978. Factors affecting amino acid induced orotic aciduria in rats. J. Nutr. 108:1976-1982.
67. Kosier, R. 1965. The effect of ammonia administration on orotic acid excretion in rats. J. Biol. Chem. 240:1729-1724.
68. Perry, R. P. 1976. Processing of RNA. Annu. Rev. Biochem. 45:605-629.
69. Kopriwa, B. M. 1975. A comparison of various procedures for fine grain development in electron microscopic radioautography. Histochemie. 44:201-224.
70. Kopriwa, B. M. 1975. A comparison of various procedures for fine grain development in electron microscopic radioautography. Histochemie. 44:201-224.
71. Kopriwa, B. M. 1975. A comparison of various procedures for fine grain development in electron microscopic radioautography. Histochemie. 44:201-224.