ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 188, No. 1, May, pp. 194–205, 1978

Regulation of Pyrimidine Biosynthesis by Purine and Pyrimidine Nucleosides in Slices of Rat Tissues[1]

DAVID E. CRANDALL, CAROL J. LOVATT, AND GEORGE C. TREMBLAY

Department of Biochemistry and Biophysics, University of Rhode Island, Kingston, Rhode Island 02881

Received November 4, 1977; revised January 27, 1978

Evidence of the primary sites for the regulation of *de novo* pyrimidine biosynthesis by purine and pyrimidine nucleosides has been obtained in tissue slices through measurements of the incorporation of radiolabeled precursors into an intermediate and end product of the pathway. Both purine and pyrimidine nucleosides inhibited the incorporation of [^{14}C]-$NaHCO_3$ into orotic acid and uridine nucleotides, and the inhibition was found to be reversible upon transferring the tissue slices to a medium lacking nucleoside. The ammonia-stimulated incorporation of [^{14}C]$NaHCO_3$ into orotic acid, which is unique to liver slices, was sensitive to inhibition by pyrimidine nucleosides at physiological levels of ammonia, but this regulatory mechanism was lost at toxic levels of ammonia. Adenosine, but not uridine, was found to have the additional effects of inhibiting the conversion of [^{14}C]orotic acid to UMP and depleting the tissue slices of PRPP. Since PRPP is required as an activator of the first enzyme of the *de novo* pathway, CPSase II, and a substrate of the fifth enzyme, OPRTase, these results indicate that adenosine inhibits the incorporation of [^{14}C]$NaHCO_3$ into orotic acid and the incorporation of [^{14}C]orotic acid into UMP by depriving CPSase II and OPRTase, respectively, of PRPP. Uridine or its metabolites, on the other hand, appear to control the *de novo* biosynthesis of pyrimidines through end product inhibition of an early enzyme, most likely CPSase II. We found no evidence of end product inhibition of the conversion of orotic acid to UMP in tissue slices.

Studies employing cell-free extracts of mammalian tissues have demonstrated that several of the enzymes of the orotate pathway for the *de novo* biosynthesis of pyrimidines are subject to regulation by end products or intermediates of the pathway. The glutamine-dependent carbamoylphosphate synthetase (CPSase II)[2] is inhibited by UTP (1) and activated by 5-phosphoribosyl-1-pyrophosphate (PRPP) (2); dihydroorotase and dihydroorotate dehydrogenase are inhibited by orotic acid (3–5); and orotidine-5′-phosphate decarboxylase (ODCase) is inhibited by UMP and CMP (6, 7). Attempts to determine which of these enzymes has physiological importance as a regulatory site include several recent studies employing intact cells. Work performed in our laboratory has shown that uridine inhibits the incorporation of [^{14}C]$NaHCO_3$, but not [^{14}C]carbamoylphosphate (CP) or [^{14}C]carbamoylaspartate, into orotic acid in slices of several rat tissues (8, 9), and Ito and Uchino have found that uridine inhibits the incorporation of [^{14}C]$NaHCO_3$ into carbamoylaspartate in human lymphocytes (10). In addition, the incorporation of [^{14}C]$NaHCO_3$, but not [^{14}C]CP, into uridine nucleotides was stimulated in perfused liver after UTP levels had been depleted by treatment with galactosamine (11). These results are consistent with the interpretation that the regulation of pyrimidine biosynthesis observed in the intact cells of

[1] This investigation was supported by Public Health Service Research Grant No. CA17131 from the National Cancer Institute.

[2] Abbreviations used: CPSase I, ATP:carbamate phosphotransferase (dephosphorylating; EC 2.7.2.5); CPSase II, ATP:carbamate phosphotransferase (dephosphorylating, amido transferring; EC 2.7.2.9); PRPP, 5-phosphoribosyl-1-pyrophosphate; CP, carbamoylphosphate; OPRTase, orotidine-5′-phosphate: pyrophosphate phosphoribosyltransferase (EC 2.4.2.10); ODCase, orotidine-5′-phosphate decarboxylase (EC 4.1.1.23); ACTase, aspartate carbamoyltransferase (EC 2.1.3.2); uv, ultraviolet.

0003-9861/78/1881-0194$02.00/0

mammalian tissues occurs at CPSase II.

We have recently demonstrated that CP produced from ammonia by CPSase I within liver mitochondria constituted at least 80% of the CP incorporated into orotic acid when liver slices were incubated with ammonia and ornithine at physiological concentrations (12). Since CPSase I was thus revealed to be an important source of CP for hepatic pyrimidine biosynthesis, we sought evidence for regulation of the CPSase I-catalyzed production of CP or of the export of CP from liver mitochondria, or both, by end products of the *de novo* pathway.

An additional mechanism of control, at a site other than CPSase II, has been suggested by the observation of Hoogenraad and Lee (13) that incubation of rat hepatoma cells with uridine resulted in a rapid loss of orotate phosphoribosyltransferase (OPRTase), but not any of the other enzymes of the orotate pathway; this result led the authors to conclude that OPRTase is also a principal site of control in the *de novo* biosynthesis of pyrimidines. Earlier measurements showing that OPRTase may be the rate-limiting enzyme in the orotate pathway of Ehrlich ascites cells (14) and the observation that a mild orotic aciduria occurs during normal human pregnancy (15) also indicate that OPRTase may be a site of regulation, or at least of interruption, of the *de novo* biosynthesis of pyrimidine nucleotides. In order to assess the relative physiological importance of regulation at a site early in the *de novo* pathway (CPSase II) or late in the pathway (OPRTase), we tested the effect of uridine on the incorporation of [^{14}C]$NaHCO_3$ and [6-^{14}C]orotic acid into uridine nucleotides in slices of rat liver and spleen. The results of these measurements, reported below, support the interpretation that feedback regulation of *de novo* pyrimidine biosynthesis occurs primarily at an early point in the pathway, most likely at the reaction catalyzed by CPSase II.

The observation that the addition of the purine nucleoside adenosine to cultures of mammalian cells causes pyrimidine starvation with resulting cell death (16, 17) prompted us to examine the influence of purine nucleosides on the *de novo* pathway for pyrimidine biosynthesis. Work in our laboratory has demonstrated that purine nucleosides inhibit the incorporation of [^{14}C]$NaHCO_3$ into orotic acid in the chick oviduct and in the rat brain and mammary gland (9, 18), but the mechanism of inhibition of pyrimidine biosynthesis by purines has remained a matter for conjecture. In the present paper, we report results which show that adenosine inhibits both the incorporation of bicarbonate into orotic acid and the conversion of orotic acid to uridine nucleotides. Since the metabolism of adenosine consumes PRPP and since PRPP is involved in the *de novo* biosynthesis of pyrimidine nucleotides as an activator of CPSase II and as a substrate for OPRTase, we tested the possibility that inhibition of pyrimidine biosynthesis by adenosine might be associated with a depletion of the tissue levels of PRPP. Our findings, reported herein, show that adenosine, but not uridine, reduces the PRPP concentrations in tissue slices; thus adenosine most likely inhibits the *de novo* biosynthesis of pyrimidines by removing the PRPP needed for the reactions catalyzed by CPSase II and OPRTase.

MATERIALS AND METHODS

Chemicals. All radiolabeled chemicals and Aquasol (liquid scintillation cocktail) were purchased from New England Nuclear Corp., Boston, Massachusetts. The mixed enzymes orotidylate phosphoribosyltransferase and orotidylate decarboxylase (prepared from yeast) were obtained from Sigma Chemical Co., St. Louis, Missouri. Acids, bases, and salts were purchased from Fisher Scientific Co., Boston, Massachusetts, and all other chemicals were purchased from Sigma Chemical Co., St. Louis, Missouri.

Preparation of tissues. Tissues from adult male rats weighing 200–250 g were employed in these studies; these animals were obtained from the Charles River Colony, Boston, Massachusetts. The animals were killed by decapitation and the tissues of interest were quickly excised and placed in ice-cold saline. Tissue slices were prepared using a Stadie–Riggs tissue slicer (Arthur H. Thomas Co.), blotted on filter paper, weighed, and transferred to flasks containing Krebs Improved Ringer II solution (19) to which various intermediates and inhibitors of pyrimidine biosynthesis had been added as indicated.

Measurement of the incorporation of ^{14}C-labeled precursors into orotic acid. Studies on the incorpo-

ration of [^{14}C]$NaHCO_3$ into orotic acid were performed using 500 mg of tissue slices incubated at 37.5°C for 3 h in 20 ml of Krebs Improved Ringer II solution (19) which was supplemented with 6-azauridine (10 mM) to inhibit the conversion of orotic acid to UMP (20). The [^{14}C]orotic acid synthesized during the 3-h incubation was isolated from the reaction mixture by cocrystallization with carrier monosodium orotate as described previously (12).

Measurement of the incorporation of ^{14}C-labeled precursors into uridine nucleotides. Tissue slices weighing 500 mg were incubated for 3 h at 37.5°C in 5 ml of Krebs Improved Ringer II solution made 30 mM in [^{14}C]$NaHCO_3$ (1100 dpm/nmol). Each reaction flask was sealed with a rubber cap fitted with a plastic center well (Kontes Glassware, Vineland, New Jersey) and a filter-paper wick. At the end of the incubation period, 0.5 ml of 6 N KOH was injected through the rubber cap into the center well; the reaction was then terminated by the injection of 1 ml of 1.5 N $HClO_4$ into the reaction medium. Following an additional 15-min incubation at 37.5°C to allow $^{14}CO_2$ from the acidified reaction mixture to distill into KOH, the flasks were opened and the center wells, containing radioactive waste, were appropriately discarded. The contents of the reaction flask were homogenized using a Kinematica Polytron tissue homogenizer (Brinkmann Instruments, Westbury, New York) and the acid-insoluble material was removed by centrifugation. The acid-soluble fraction so obtained was incubated at 100°C for 1 h to convert pyrimidine nucleotides to the monophosphate form; after cooling, the acid-soluble fraction was adjusted to pH 7.2–7.4 by the addition of KOH and the precipitate of $KClO_4$ was removed by centrifugation. The ^{14}C-labeled uridine nucleotides synthesized during the incubation period were isolated as [^{14}C]UMP by cocrystallization with carrier UMP as follows: A 2-ml aliquot of a solution containing 375 mg of UMP (disodium salt)/ml was added to the neutralized acid-soluble fraction and the total volume was brought to 10 ml with H_2O. Following thorough mixing, an aliquot of 50 μl was removed and diluted to 100 ml in 0.01 N HCl, and the absorbance of the dilution was measured at 260 nm. The acid-soluble fraction containing carrier UMP was made 67% in ethanol by the addition of 20 ml of ethanol and the precipitate of UMP so formed was dissolved by heating the solution to 70°C in a water bath. The [^{14}C]UMP synthesized from [^{14}C]$NaHCO_3$ was isolated by cocrystallization with carrier UMP as the ethanolic solution cooled slowly to 4°C. The crystals were collected by suction filtration, washed on the filter with ice-cold 67% ethanol, and dissolved in water. The absorbance at 260 nm of a suitable dilution of the solution of [^{14}C]-UMP was measured to allow calculation of the recovery of carrier UMP, and the quantity of radioisotope in a 2-ml aliquot of the solution of [^{14}C]UMP was measured in a Searle Isocap 300 liquid scintillation spectrometer. The remaining [^{14}C]UMP was recrystallized from 67% ethanol, as above, to constant specific activity, which rarely required more than two recrystallizations. The specific activity of the [^{14}C]-UMP obtained from the final recrystallization was used to calculate the incorporation of [^{14}C]$NaHCO_3$ into uridine nucleotides during the 3-h incubation period. This method, employing Krebs Improved Ringer II solution made 30 mM in $NaHCO_3$, was also used to measure the incorporation of [6-^{14}C]orotic acid into uridine nucleotides.

Preparation of [^{14}C]uracil from [^{14}C]UMP. A sample of [^{14}C]UMP was isolated by cocrystallization with carrier UMP from the acid-soluble fraction of spleen slices which had been incubated with [^{14}C]$NaHCO_3$ (3667 dpm/nmol), and the [^{14}C]UMP was recrystallized to constant specific activity. An aliquot of 5 mg of recrystallized [^{14}C]UMP was hydrolyzed in 12 N $HClO_4$ at 100°C for 1 h, and the resulting [^{14}C]uracil was isolated by adsorption to and elution from charcoal, followed by descending paper chromatography in isobutyric acid:0.5 N NH_4OH (10:6) (21). The [^{14}C]-uracil was located by viewing the chromatogram under ultraviolet light, with reference to a sample of commercial uracil developed on the same chromatogram. The uv-absorbing band was cut out, the [^{14}C]uracil was eluted with water, and its specific activity was determined by measuring the A_{260} of the eluant and assaying its content of radioisotope in a liquid scintillation spectrometer.

Measurement of the $^{14}CO_2$ generated from [carboxy-^{14}C]orotic acid. The decarboxylation of [*carboxy*-^{14}C]orotic acid was measured by trapping $^{14}CO_2$ liberated during the conversion of orotic acid to UMP. Tissue slices weighing 500 mg were incubated at 37.5°C for 3 h in 5 ml of Krebs Improved Ringer II solution made 30 mM in $NaHCO_3$ and supplemented with 5 mM [*carboxy*-^{14}C]orotic acid (31 dpm/nmol); the reaction flask was sealed with a rubber cap fitted with a plastic center well containing 0.4 ml of 20% KOH and a filter-paper wick. The reaction was terminated by the injection of 1.0 ml of 1.5 N $HClO_4$ into the reaction mixture, and $^{14}CO_2$ was allowed to distill into the KOH during an additional incubation at 37.5°C for 1 h. The center well was then removed and placed in a scintillation vial, its contents were diluted with 1.6 ml of water and 6.5 ml of Aquasol, and the quantity of $^{14}CO_2$ liberated by the decarboxylation of [*carboxy*-^{14}C]orotic acid was measured in a Searle Isocap 300 liquid scintillation spectrometer.

Measurement of the activities of OPRTase and ODCase in cell-free extracts of liver and spleen. The combined activities of OPRTase and ODCase were assayed by measuring the quantity of $^{14}CO_2$ liberated by the decarboxylation of [*carboxy*-^{14}C]orotic acid. The reaction was carried out in a closed vessel sealed with a rubber cap fitted with a plastic center well containing 0.4 ml of 20% KOH and a filter-paper wick. The complete reaction mixture contained, in a volume of 1.5 ml, the following components at the indicated

concentrations: phosphate buffer, pH 7.4, 50 mM; $MgCl_2$, 3 mM; PRPP, 1 mM; [*carboxy*-^{14}C]orotic acid (142 dpm/nmol), 0.5 mM; and 0.2 ml of a 10% homogenate of tissue prepared in isotonic saline. Following incubation at 37°C for 30 min, the reaction was terminated by the injection of 0.2 ml of 25 mM $NaHCO_3$ through the rubber cap, followed immediately by the injection of 0.5 ml of 1.5 N $HClO_4$; the bicarbonate was added to ensure complete flushing of $^{14}CO_2$ from the reaction mixture upon subsequent acidification. Distillation of $^{14}CO_2$ into the center well was allowed to proceed during an additional 30-min incubation at 37°C. The center well was then removed and placed in a scintillation vial, and its contents were diluted with 1.6 ml of H_2O followed by 6.5 ml of Aquasol; the content of radioisotope was measured in a Searle Isocap 300 liquid scintillation spectrometer.

Determination of the concentration of PRPP in tissue slices. The content of PRPP in tissue slices was assayed by a modification of the method of May and Krooth (22), which measures the PRPP-dependent, enzyme-catalyzed decarboxylation of [*carboxy*-^{14}C]orotic acid. Following a 3-h incubation in Krebs Improved Ringer II solution (19), slices were removed with forceps and immediately frozen by dropping them into a plastic test tube containing liquid nitrogen. A 2.5-ml aliquot of ice-cold 0.4 N $HClO_4$ was added and the tissue was homogenized with a Polytron tissue homogenizer; the homogenizer was rinsed with 2 ml of ice-cold H_2O and the rinsing was added to the acidified homogenate. Acid-insoluble material was removed by centrifugation; the centrifuge was stopped rapidly and the acid-soluble fraction was decanted into a test tube containing 1.0 ml of 1.0 N KOH and one drop of phenolphthalein indicator solution. The solution was quickly adjusted to a faint-pink phenolphthalein end point with KOH or HCl and decanted into a 25-ml flask containing the other components of the assay. The assay mixture, in a volume of 6.5 ml, contained the following components; $MgCl_2$, 42 μmol; Tris–HCl, pH 8.0, 335 μmol; tissue extract as prepared above; OPRTase and ODCase (mixed enzymes, prepared from yeast), 2 U (1 U catalyzes the conversion of 1 μmol of orotic acid to UMP per hour at 25°C); [*carboxy*-^{14}C]orotic acid (920 dpm/nmol), 1 μmol. The flask was capped with a rubber seal fitted with a plastic center well containing 0.4 ml of 20% KOH and a filter-paper wick, and the reaction mixture was incubated for 2 h at 25°C. The elapsed time between acidification of the tissue slices and capping of the reaction flask was 5 min. At the end of the incubation period, the reaction mixture was made 3 mM in bicarbonate by the injection of a solution of $NaHCO_3$ through the rubber cap, and the reaction was then terminated by injection of 1.0 ml of 1.5 N $HClO_4$ through the rubber cap into the reaction mixture; $^{14}CO_2$ was allowed to distill into the center well during an additional incubation for 1 h at 25°C. The center well was then removed and placed in a scintillation vial; its contents were diluted with 1.6 ml of water followed by 6.5 ml of Aquasol, and the content of radioisotope was assayed in a Searle Isocap 300 liquid scintillation spectrometer. Each assay of PRPP was performed in parallel with identical samples to which known quantities of PRPP (10 or 20 nmol) had been added just prior to acidification; the measured loss of PRPP from these recovery standards was used to correct for losses of PRPP during the preparation of the tissue extracts.

RESULTS

Effects of Pyrimidine and Purine Nucleosides on the Incorporation of [^{14}C]-$NaHCO_3$ into Orotic Acid

Evidence for regulation of the *de novo* biosynthesis of orotic acid by pyrimidine and purine nucleosides was obtained in experiments measuring the influence of added nucleoside on the incorporation of [^{14}C]-$NaHCO_3$ into orotic acid by tissue slices.[3] In both the spleen and the liver, the incorporation of [^{14}C]$NaHCO_3$ into orotic acid was inhibited by uridine and cytidine; the purine nucleoside, adenosine, was also an effective inhibitor of the incorporation of [^{14}C]$NaHCO_3$ into orotic acid in the spleen (Table I). The liver, however, is unique among the tissues we have examined in its response to adenosine [(9, 18); this publication]; adenosine stimulated the incorporation of [^{14}C]$NaHCO_3$ into orotic acid sixfold in the liver (Table I). We also found, in two separate experiments, that this stimulation by adenosine was reduced by an average of 44% by the addition of 5 mM ornithine. Since this response to adenosine and ornithine mimics the response to ammonia and ornithine which we have previously reported to be unique to mammalian liver (12), we suspect that adenosine is serving as a source of ammonia, through adenosine deaminase, and thereby stimulating the participation of the hepatic CPSase I in pyrimidine biosynthesis. Having established that both purine and pyrimidine nucleosides are effective inhibitors of an early step in the *de novo* biosynthesis of orotic acid [(8, 9, 18); Table I], we next sought evidence for regulation of the conversion of orotic acid to uridine nucleotides in tissue slices.

[3] Properties of the system for measuring the incorporation of [^{14}C]$NaHCO_3$ into ortic acid have been reported previously (12).

TABLE I

EFFECTS OF URIDINE, CYTIDINE, AND ADENOSINE ON THE INCORPORATION OF [^{14}C]$NaHCO_3$ INTO OROTIC ACID IN RAT LIVER AND SPLEEN[a]

Additions (mM)	Incorporation of [^{14}C]$NaHCO_3$ into orotic acid (% of corresponding control)	
	Spleen, 125.0 ± 6.1 (24)[b]	Liver, 27.0 ± 2.4 (26)[b]
None (control)	100	100
Uridine (10)	24.7 ± 1.1 (18)	29.4 ± 2.6 (20)
Cytidine (10)	46.5 ± 2.4 (3)	61.2 ± 6.4 (7)
Adenosine (2.5)	63.3 ± 5.1 (4)	660.6 ± 126.9 (5)

[a] The experimental method employed in measuring the incorporation of [^{14}C]$NaHCO_3$ into orotic acid has been described previously (12). Each value for the incorporation of [^{14}C]$NaHCO_3$ into orotic acid obtained when a nucleoside was present in the incubation medium is presented as a percentage of its respective control ± standard error, with the number of observations included in each value listed in parentheses.

[b] These control values are presented as averages ± standard errors, in nanomoles of [^{14}C]$NaHCO_3$ incorporated into orotic acid per gram of tissue during a 3-h incubation; the number of observations for each value is listed in parentheses.

Characterization of the Method Employed for the Isolation of [^{14}C]Uridine Nucleotides as [^{14}C]UMP

To ensure that hydrolysis of the acid-soluble fraction in 0.25 N $HClO_4$ at 100°C for 1 h was sufficient for the quantitative conversion of uridine nucleotides to UMP, a recovery experiment was performed in which a sample of commercial [^{14}C]UTP was added to an incubation mixture just prior to acidification. Following hydrolysis of the acid-soluble fraction at 100°C for 1 h, nucleotides were extracted by adsorption to and elution from charcoal (23) and fractionated by descending paper chromatography in 1 M ammonium acetate:95% ethanol (3:7) (21); more than 95% of the radioisotope recovered from the chromatogram was found to cochromatograph with UMP. In two experiments in which a sample of [^{14}C]UMP was added to a typical incubation mixture just prior to acidification, 98.2 and 94.5% of the radioisotope were recovered upon subsequent cocrystallization with carrier UMP. In addition, when [^{14}C]orotic acid and [^{14}C]orotidine monophosphate were tested in similar experiments as possible contaminants of [^{14}C]UMP isolated by cocrystallization, no detectable radioisotope was isolated with the carrier UMP. The metabolite of [^{14}C]-$NaHCO_3$ which cocrystallizes with carrier UMP was identified as [^{14}C]UMP by descending paper chromatography on Whatman 3 MM paper in 95% ethanol:1 M ammonium acetate (75:30), isobutyric acid:0.5 N NH_4OH (10:6), and isopropanol: concentrated HCl:H_2O (170:41:39) (21); in each of these solvent systems, more than 96% of the radioisotope cocrystallizing with carrier UMP was found to cochromatograph with the carrier.

Some Properties of the System Used for Measuring the Incorporation of [^{14}C]-$NaHCO_3$ and [6-^{14}C]Orotic Acid into Uridine Nucleotides

Mild homogenization of slices of liver or spleen before incubation with [^{14}C]-$NaHCO_3$ or [6-^{14}C]orotic acid resulted in a loss of 88% or more of the amount of radioisotope isolated by cocrystallization with carrier UMP. Thus, the observed incorporation of ^{14}C-labeled precursors into uridine nucleotides in tissue slices is a measure of pyrimidine biosynthesis by intact cells, and not an artifact of our procedure or incorporation by a small number of ruptured cells.

The possibility that the observed incorporation of [^{14}C]$NaHCO_3$ into uridine nucleotides might reflect incorporation into the ribose moiety rather than the pyrimidine ring was ruled out by comparing the specific activity of the [^{14}C]UMP isolated from the acid-soluble fraction with that of the [^{14}C]uracil derived from the same sample of [^{14}C]UMP by hydrolysis of the *N*-glycosidic bond. The specific activity of the original sample of [^{14}C]UMP was 259 dpm/μmol, and that of the [^{14}C]uracil derived from this sample of [^{14}C]UMP by hydrolysis of the *N*-glycosidic bond was found to be 277 dpm/μmol; this result indicates that all of the radioisotope was located in the pyrimidine ring, and that little or no incorporation of [^{14}C]$NaHCO_3$ into the ribose moiety of the nucleotide had occurred. On the basis of the results presented above, our method was judged to be

a simple and reliable means for measuring the incorporation of radiolabeled precursors into the pyrimidine moiety of uridine nucleotides.

Effects of Uridine and Adenosine on the Incorporation of [^{14}C]NaHCO$_3$ into Uridine Nucleotides

The incubation of spleen slices with uridine or adenosine produced, in both cases, a significant inhibition of the incorporation of [^{14}C]NaHCO$_3$ into uridine nucleotides (Fig. 1). In addition, uridine (10 mM) was also found to inhibit the incorporation of [^{14}C]NaHCO$_3$ into uridine nucleotides by 52 ± 7% (average ± SE; N = 3) in similar experiments with liver slices.[4] These results are consistent with those reported above (Table I) showing inhibition by uridine and adenosine of the incorporation of precursor into orotic acid in spleen slices, and inhibition by uridine in liver slices.

In making such comparisons between the results obtained from measurements of the incorporation of precursor into orotic acid and the incorporation of precursor into UMP, attention is drawn to the differences in the two procedures. The incorporation of precursor into orotic acid is measured in the presence of 6-azauridine, a drug which is quite effective in preventing the further metabolism of orotic acid in tissue slices and minces (9, 12). For want of a suitable drug, no such measure is taken to prevent the further metabolism of uridine nucleotides. Thus a comparison of the rate of incorporation of precursor into orotic acid with the rate of incorporation of precursor into uridine nucleotides would suggest a wider disparity than actually exists, owing to differences in the rates at which the newly synthesized end products are being depleted by their further metabolism. Similarly, a comparison of the sensitivity of each incorporation to end product inhibition requires additional consideration. The addition of uridine to effect end product inhibition also expands the pool size of uridine nucleotides, thus trapping the isotopically labeled uridine nucleotides being synthesized *de novo,* albeit at a lower rate. Since no such interference by uridine occurs in measurements of the incorporation of precursor into orotic acid, a comparison of the results of both measurements would suggest a difference in sensitivity to end product inhibition which may not exist. Thus, while the absolute values reported herein can only be directly compared with others obtained by the same experimental procedure, each of these procedures is adequately sensitive to detect regulation of the *de novo* pathway in the intact cell and to confirm the interpretation of the results obtained by the other.

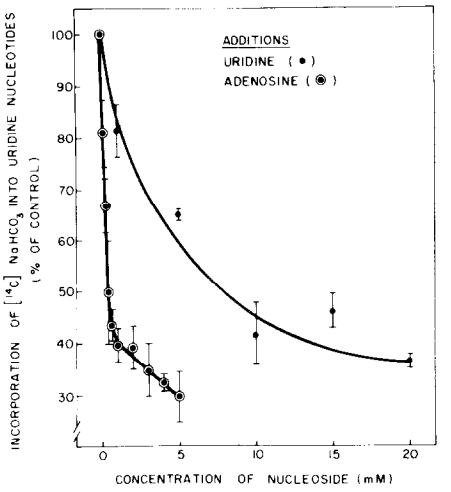

FIG. 1. The effect of uridine and adenosine on the incorporation of [^{14}C]NaHCO$_3$ into uridine nucleotides in rat spleen. The experimental method employed in measuring the incorporation of [^{14}C]NaHCO$_3$ into uridine nucleotides is described under Materials and Methods. Each value is presented as a percentage of the control value ± standard error, with a minimum of three observations represented by each point shown. The control value is 66.8 ± 3.7 (average ± SE; N = 20) nmol of [^{14}C]NaHCO$_3$ incorporated into uridine nucleotides per gram of spleen during the 3-h incubation period.

Effects of Uridine and Adenosine on the Incorporation of [6-^{14}C]Orotic Acid into Uridine Nucleotides

We tested the possibility that OPRTase may have a regulatory role in the *de novo* biosynthesis of pyrimidines in slices of rat liver and spleen by measuring the effects of

[4] The control value for the liver was 19.1 ± 3.7 (average ± SE; N = 4) nmol of [^{14}C]NaHCO$_3$ incorporated into uridine nucleotides per gram of liver during 3 h of incubation.

uridine and adenosine on the incorporation of [6-^{14}C]orotic acid into uridine nucleotides. We found that the addition of 10 mM uridine to the incubation medium was without effect on the incorporation of [6-^{14}C]-orotic acid into uridine nucleotides in either tissue (Fig. 2); the results were essentially the same over a 100-fold range in substrate concentrations. The addition of 5 mM adenosine, however, inhibited the incorporation of [6-^{14}C]orotic acid into uridine nucleotides by 80% in the liver and 38% in the spleen. In eight experiments employing slices of liver and spleen, the addition of 10 mM 6-azauridine, a known inhibitor of the conversion of orotic acid to UMP (20), reduced the incorporation of [6-^{14}C]orotic acid into uridine nucleotides by an average of 88.5% (Fig. 2), thus demonstrating that our experimental system is adequately sensitive to detect inhibition of the conversion of orotic acid to UMP in the intact cell. These results

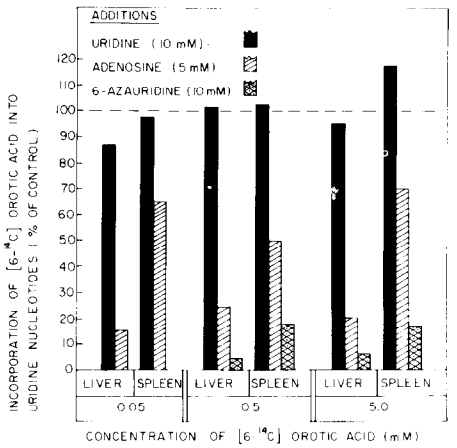

FIG. 2. The effect of uridine, adenosine, and 6-azauridine on the incorporation of [6-^{14}C]orotic acid into uridine nucleotides in rat liver and spleen. The experimental method employed in measuring the incorporation of [6-^{14}C]orotic acid into uridine nucleotides is described under Materials and Methods. Each value is presented as a percentage of its corresponding control and represents the average of two to four observations. The control values for the liver averaged 44 ± 11, 73 ± 13, and 127 ± 16, and those for the spleen averaged 13 ± 4, 56 ± 8, and 104 ± 11, when the concentrations of [6-^{14}C]orotic acid were 0.05, 0.5, and 5.0 mM, respectively. These values are given in nanomoles of [6-^{14}C]orotic acid incorporated into uridine nucleotides per gram of tissue·3 h ± standard error; each value is an average of three to four assays.

were corroborated in another assay system which did not depend on the isolation of [^{14}C]UMP, but which instead measured the $^{14}CO_2$ liberated from [*carboxy*-^{14}C]orotic acid during its conversion to UMP. In two separate experiments, the addition of 10 mM uridine to the incubation medium produced no inhibition of the decarboxylation of [*carboxy*-^{14}C]orotic acid, while the addition of 10 mM 6-azauridine inhibited the decarboxylation of [*carboxy*-^{14}C]orotic acid by 93%; these results are virtually identical to those obtained by measuring the incorporation of [6-^{14}C]orotic acid into uridine nucleotides and argue strongly that the conversion of orotic acid to UMP is not affected by uridine or its metabolites in the intact cells of mammalian liver or spleen.

In addition to the above studies employing intact cells, we also tested directly for a possible uridine-promoted depletion of the levels of one or both of the last two enzymes in the *de novo* pathway by assaying the combined activities of OPRTase and ODCase in cell-free extracts of slices of liver and spleen following incubation with this pyrimidine nucleoside. In an initial experiment using liver slices incubated without uridine, the combined activities of OPRTase and ODCase declined sharply, reaching 18 ± 2% (average ± SE; $N = 5$) of the activity of freshly prepared slices after 3 h of incubation; since this rapid loss of activity occurred in the absence of uridine, we judged liver slices to be an inappropriate system for testing for a uridine-promoted depletion of the levels of OPRTase and ODCase. In contrast to the liver, OPRTase and ODCase in spleen slices declined only slightly after a 3-h incubation without uridine, to 85 ± 6% (average ± SE; $N = 3$) of the value of freshly prepared slices, while incubation with 10 mM uridine resulted in a decline to 72 ± 7% (average ± SE; $N = 3$) of the control level; these values are not significantly different, indicating that incubation with uridine has no effect on the combined activity of OPRTase and ODCase in slices of spleen.

We also tested the ability of spleen slices to recover from inhibition by uridine in an experiment which extended the incubation period from 3 to 6 h. We found that the

rates of incorporation of [^{14}C]$NaHCO_3$ into uridine nucleotides over a 3-h incubation period were the same whether the radioisotope was added at zero time or at 3 h by transfer of the slices to fresh medium; similarly, the rates of incorporation measured in the presence of uridine also remained constant at reduced levels over the entire 6-h incubation period. In two experiments designed to test for recovery from inhibition, slices were transferred after 3 h of incubation in medium containing uridine to fresh medium lacking uridine, and the incorporation of [^{14}C]$NaHCO_3$ into uridine nucleotides was measured during a second 3-h incubation period. The incorporation measured after transfer to the uridine-free medium was 1.9 to 2.0 times the value obtained during the first incubation period in the presence of uridine; since 2.4 times the inhibited value would have constituted full recovery, the observed reversal of inhibition was almost complete.

Effects of of Uridine and Adenosine on the Concentration of PRPP in Tissue Slices

Adenosine was found to be a potent inhibitor both of the incorporation of [^{14}C]-$NaHCO_3$ into orotic acid and of the incorporation of [6-^{14}C]orotic acid into uridine nucleotides (Figs. 1 and 2). Since PRPP is required in both of these reaction sequences, as an activator of CPSase II and as a substrate for OPRTase, we explored the possibility that the incubation of tissue slices with adenosine resulted in a decrease in the concentration of PRPP. Since PRPP has been shown to be unstable in acid solution (24), commercial PRPP (the purity of which had been determined in separate assays) was added to some of the tissue preparations before acidification, and several determinations of the recovery of the added PRPP were made in parallel with each assay of the concentration PRPP in tissue slices. Each determination was then corrected for the loss of PRPP as measured for that individual experiment; the average recovery of PRPP (10 or 20 nmol) added to tissue slices just prior to acidification was 31.3 ± 1.4% (average ± SE; $N = 22$). Assays of commercial PRPP revealed that the generation of $^{14}CO_2$ from [*carboxy*-^{14}C]orotate was linear with respect to added PRPP, at least up to 40 nmol. No additional generation of $^{14}CO_2$ resulted from doubling either the incubation time, the concentration of [*carboxy*-^{14}C]orotate, or the amount of the yeast enzymes, OPRTase and ODCase, in the reaction mixture. The concentration of PRPP in tissue slices after 3 h of incubation was found to be 7.2 μM in the liver and 16.5 μM in the spleen (Table II). The addition of 10 mM uridine to the incubation medium produced either no change or a slight increase in the concentration of PRPP, both in liver and in spleen. The effect of adenosine, however, was strikingly different from that of uridine; when tissue slices were incubated for 3 h with 5 mM adenosine, the concentrations of PRPP decreased to 52 and 36% of the values obtained in the absence of adenosine in the liver and spleen, respectively.

Effects of Uridine and Adenosine on the de Novo Biosynthesis of Pyrimidines in the Ammonia-Stimulated Liver

Since we had previously determined that CPSase I of hepatic mitochondria is an important source of CP for pyrimidine bio-

TABLE II

EFFECTS OF URIDINE AND ADENOSINE ON THE CONCENTRATION OF PRPP IN TISSUE SLICES[a]

Additions (mM)	Concentration of PRPP (% of corresponding control)		
	Liver	Spleen	
		Expt 1	Expt 2
None (control)	100	100	100
Uridine (10)	121.8 ± 9.7	97.6	130.9
Adenosine (5)	52.2 ± 5.9	38.1	34.6

[a] The content of PRPP was determined by measuring the PRPP-dependent, enzyme-catalyzed decarboxylation of [*carboxy*-^{14}C]orotic acid, as described under Materials and Methods. The values of PRPP concentrations in tissue slices incubated with uridine or adenosine are presented as percentages of the corresponding control. For the liver, the values are given as averages ± SE for three determinations; the results of two experiments with spleen slices are presented separately. The control values, given as micromolar concentrations of PRPP in tissue slices incubated for 3 h in the absence of nucleoside, were 7.2 ± 1.9 μM ($N = 3$) for the liver and 16.8 and 16.2 μM for two experiments with spleen slices.

synthesis in the liver (12), we explored the possibility that the synthesis of CP by CPSase I or the export of CP from the mitochondria was subject to regulation by end products of the *de novo* pyrimidine pathway. In a test for feedback regulation of CPSase I, the incorporation of [^{14}C]-$NaHCO_3$ into orotic acid in liver slices incubated with the physiological concentration (0.7 mM) of NH_4Cl was found to be sensitive to inhibition by uridine and cytidine, whether or not ornithine was added (Table III). Since the addition of 0.7 mM NH_4Cl stimulated the incorporation of [^{14}C]$NaHCO_3$ into orotic acid sixfold over the value obtained when NH_4Cl was omitted, and since this ammonia-enhanced incorporation was completely prevented by the addition of hyperphysiological (5 mM) ornithine, we interpret these results to indicate that the ammonia-enhanced activity was due to CPSase I, and that pyrimidine nucleosides or their metabolites are effective inhibitors of the production of CP by CPSase I or of the export of CP from the mitochondria. The slight stimulation of the incorporation of [^{14}C]$NaHCO_3$ into orotic acid that was observed when liver slices incubated with 0.7 mM NH_4Cl were supplemented with adenosine (Table III) can be attributed, as it was previously (Table I), to adenosine's role as ammonia donor, through adenosine deaminase, thereby increasing the production of [^{14}C]CP by CPSase I.

When liver slices were supplemented with 5 mM NH_4Cl, a concentration sufficient to stimulate fully the incorporation of [^{14}C]$NaHCO_3$ into orotic acid, the inhibitory effects of uridine and cytidine were greatly diminished or lost (Table III). The pyrimidine nucleosides were thus found to be much less effective as inhibitors of the incorporation of [^{14}C]$NaHCO_3$ into orotic acid in liver slices exposed to toxic levels of ammonia than in liver slices incubated either with physiological concentrations of ammonia or without ammonia. In contrast to the liver, the addition of 5 mM NH_4Cl to slices of the spleen, a tissue which does not possess CPSase I activity, had no effect on the rate of incorporation of [^{14}C]$NaHCO_3$ into orotic acid, nor did the presence of ammonia modify the inhibitory effects of uridine and cytidine in this tissue (Table III).

Although we found that adenosine was without effect on the incorporation of [^{14}C]$NaHCO_3$ into orotic acid in liver slices incubated with toxic concentrations of ammonia, this purine nucleoside was a powerful inhibitor of the incorporation of precursors into uridine nucleotides under the same conditions. In two separate experiments employing liver slices supplemented with 5 mM NH_4Cl, the incorporations of

TABLE III

EFFECTS OF URIDINE AND ADENOSINE ON THE INCORPORATION OF [^{14}C]$NaHCO_3$ INTO OROTIC ACID IN THE AMMONIA-STIMULATED LIVER[a]

Additions (mM)	Incorporation of [^{14}C]$NaHCO_3$ into orotic acid (% of corresponding control)			
	Liver slices			Spleen slices
	0.7 mM NH_4Cl, 164.9 ± 14.7 (14)[b]	0.7 mM NH_4Cl + 0.5 mM ornithine, 60.6 ± 12.2 (8)[b]	5 mM NH_4Cl, 481.4 ± 50.1 (12)[b]	5 mM NH_4Cl, 120.0 ± 9.0 (3)[b]
None	100	100	100	100
Uridine (10)	57.2 ± 4.6 (11)	66.9 ± 9.8 (8)	88.7 ± 10.1 (5)	30.7 ± 2.3 (3)
Cytidine (10)	73.3 ± 4.5 (7)	62.2 ± 12.8 (8)	81.2 ± 9.8 (4)	41.0 ± 4.8 (3)
Adenosine (5)	145.0 ± 10.1 (3)		89.5 ± 5.1 (3)	

[a] The experimental conditions are described under Materials and Methods. Each value for the incorporation of [^{14}C]$NaHCO_3$ into orotic acid obtained when the incubation medium was supplemented with a nucleoside is presented as a percentage of its respective control ± standard error, with the number of observations included in each value listed in parentheses.

[b] Control values are presented as averages ± standard errors, in nanomoles of [^{14}C]$NaHCO_3$ incorporated into orotic acid per gram of tissue during a 3-h incubation; the number of observations for each value is listed in parentheses.

[^{14}C]$NaHCO_3$ and [6-^{14}C]orotic acid into uridine nucleotides were inhibited by averages of 87 and 77%, respectively. These observations are consistent with the interpretation that adenosine prevents the conversion of orotic acid to UMP by depleting the cells of PRPP, but does not inhibit the synthesis of orotic acid in liver slices which have been provided with ammonia because, under these conditions, CPSase I displaces CPSase II in pyrimidine biosynthesis and CPSase I is not influenced by PRPP.

DISCUSSION

Evidence presented in this paper and in previous reports (8, 9, 18) demonstrates that uridine or its metabolites are effective end product inhibitors of the *de novo* pathway for pyrimidine biosynthesis in the intact cell. The observations reported herein, that uridine inhibits the incorporation of [^{14}C]$NaHCO_3$, but not [6-^{14}C]orotic acid, into uridine nucleotides, extend these earlier studies and strengthen the interpretation that CPSase II is the primary site of regulation by uridine. In addition, the recovery of spleen slices from inhibition by uridine, as measured by the rate of incorporation of [^{14}C]$NaHCO_3$ into uridine nucleotides, was nearly complete upon transfer of the slices to uridine-free medium, a result consistent with the observation that levels of OPRTase and ODCase in the spleen were not altered by incubation with this pyrimidine nucleoside. While our experimental method readily yielded evidence of end product regulation of the *de novo* biosynthesis of pyrimidines at an early reaction in the pathway, we were unable to detect any regulatory effect by uridine or its metabolites governing the activities of the last enzymes in the pathway, OPRTase and ODCase. It is important to emphasize that these results were obtained with procedures which were well suited to the demonstration inhibition of the combined action of OPRTase and ODCase by adenosine and by 6-azauridine. Thus, we conclude that the regulatory mechanism demonstrated in rat hepatoma cells by Hoogenraad and Lee (13) does not operate in slices of rat liver or spleen.

Adenosine is thought to enter mammalian cells by a process which involves initial cleavage of the sugar moiety by a membrane-bound nucleoside phosphorylase, producing intracellular ribose-1-phosphate; subsequent uptake of the free base involves a condensation with PRPP catalyzed by adenine phosphoribosyltransferase (25). Since PRPP is required both as an activator for CPSase II (2) and as a substrate for OPRTase, reduction of cellular levels of PRPP to concentrations below those required for saturation would result in decreases in the rate of pyrimidine biosynthesis. We found that the incubation of liver and spleen slices with 5 mM adenosine decreased PRPP concentrations by 48 and 64%, respectively. Thus it is likely that the incorporation of [^{14}C]$NaHCO_3$ into orotic acid and the incorporation of [^{14}C]orotic acid into uridine nucleotides are inhibited by adenosine through the depletion of PRPP; this inhibition may, however, be peculiar to the availability of an exogenous supply of purines for salvage.[5] Since incubation with uridine did not result in a decrease in the intracellular concentrations of PRPP or inhibition of the conversion of orotic acid to UMP, the possibility that uridine acts similarly to adenosine can be excluded.

The failure of adenosine to inhibit the incorporation of [^{14}C]$NaHCO_3$ into orotic acid in liver slices incubated with NH_4Cl (Table III) is consistent both with the interpretation that CPSase I provides much of the CP for pyrimidine biosynthesis during ammonia stimulation (12) and with the interpretation that adenosine inhibits the biosynthesis of pyrimidines by reducing cellular levels of PRPP. If these interpretations are correct, one would expect adenosine not to inhibit the incorporation of [^{14}C]$NaHCO_3$ into orotic acid in liver slices supplemented with ammonia, since CPSase I, unlike CPSase II, does not require PRPP as an activator. The incorporation of

[5] The suggestion that adenosine might interfere with pyrimidine biosynthesis by competition for PRPP was offered earlier by Ishii and Green (16); Meuth and Green subsequently demonstrated that the inclusion of adenosine in the growth medium resulted in a reduction of the PRPP levels in cultured mammalian cells (personal communication).

[^{14}C]$NaHCO_3$ and [6-^{14}C]orotic acid into uridine nucleotides, however, does require PRPP as a substrate for OPRTase, and the incorporation of both of these radioactive precursors into uridine nucleotides in liver slices supplemented with 5 mM NH_4Cl was strongly inhibited by adenosine; these observations strengthen the interpretation that the effect of adenosine on the *de novo* biosynthesis of pyrimidines is due to the reduction in PRPP concentration following the administration of this purine nucleoside.

The incorporation of [^{14}C]$NaHCO_3$ into orotic acid in liver slices incubated with physiological concentrations of ammonia and ornithine was found to be sensitive to inhibition by pyrimidine nucleosides or their metabolites (Table III); this observation confirms the earlier report of Kerson and Appel (26), who demonstrated that the ammonia-dependent CPSase I of rat liver was inhibited by pyrimidine nucleotides in a cell-free assay system. Since uridine was found to be a potent inhibitor of the incorporation of [^{14}C]$NaHCO_3$ into orotic acid in liver slices incubated without NH_4Cl (Table I), a basal activity which may be attributed to CPSase II, it may be tempting to speculate that the uridine-sensitive incorporation of [^{14}C]$NaHCO_3$ into orotic acid represents the contribution of CPSase II, while the uridine-insensitive fraction represents that of CPSase I; the evidence, however, does not support this interpretation. Of the several tissues we have examined, only in mammalian liver is the *de novo* synthesis of orotic acid stimulated by ammonia, and this accelerated synthesis is progressively dampened by increasing concentrations of ornithine (12); we attribute this ammonia-stimulated, ornithine-sensitive incorporation of [^{14}C]$NaHCO_3$ into orotic acid, which is unique to mammalian liver, to the participation of CPSase I in the *de novo* biosynthesis of pyrimidines. Since the uridine-sensitive fraction of the incorporation of [^{14}C]$NaHCO_3$ into orotic acid in the presence of physiological ammonia is 2.6 times greater than the total uninhibited incorporation observed in the absence of ammonia (Tables I and III), and since the ammonia-stimulated incorporation can be completely prevented by hyperphysiological ornithine, the data support the interpretation that the uridine-sensitive incorporation in the presence of ammonia is predominantly a reflection of regulation of the contribution of CP by CPSase I. These results indicate that, under normal physiological conditons, the intramitochondrial production of CP, or the export of CP from the mitochondria, or both, are inhibited by uridine or its metabolites.

When the concentration of ammonia was increased to toxic levels, where the incorporation of [^{14}C]$NaHCO_3$ into orotic acid in liver slices is increased 20-fold, the sensitivity to pyrimidine nucleosides was lost (Table III). Our observations are consistent with the interpretation that, when ammonia levels are high, uridine or its metabolites no longer inhibit the production of CP by CPSase I or the export of CP from the mitochondria, and the detoxification of ammonia via the synthesis of orotic acid supersedes the regulation of pyrimidine biosynthesis. Support for this interpretation is provided by Pausch *et al.* (27), who found that the perfusion of liver with 15 mM NH_4Cl resulted in a 20-fold increase in the incorporation of [^{14}C]bicarbonate into uridine nucleotides and that this ammonia-stimulated incorporation could not be further increased by the administration of galactosamine. Although Pausch *et al.* suggested that the effect of ammonia was to abolish the regulatory effect of uridine nucleotides on CPSase II, we found no evidence for this mechanism. The addition of 5 mM NH_4Cl to slices of spleen, a tissue containing only CPSase II, did not alter the potent inhibition by uridine of the incorporation of [^{14}C]$NaHCO_3$ into orotic acid in this tissue (Table III). Thus, lack of feedback regulation of CPSase I during ammonia toxicity is a more likely explanation of their observation. In a more recent investigation employing whole animals, Pausch *et al.* (28) found that repeated hourly injections of ammonium acetate resulted in large increases in the concentrations of hepatic uridine nucleotides, an observation which is consistent with both an increased contribution by CPSase I to the orotate pathway and a lack of regulation of this contribution

by uridine nucleotides at high concentrations of ammonia. The additional observation that hepatic ammonia concentrations had returned to the normal range 30–60 min after the injection of ammonium acetate does not alter our interpretation that the increased synthesis of uridine nucleotides reflects mitochondrial production and export of CP in response to transient increases in ammonia concentrations. Thus, since their study was based on responses to high doses of ammonia, the experimental system employed does not permit either an estimate of the relative contributions of CP from CPSase I and CPSase II to the *de novo* pathway or a statement about feedback regulation of CPSase I under normal physiological conditions.

REFERENCES

1. TATIBANA, M., AND ITO, K. (1969) *J. Biol. Chem.* **244,** 5403–5413.
2. TATIBANA, M., AND SHIGESADA, K. (1972) *Biochem. Biophys. Res. Commun.* **46,** 491–497.
3. KENNEDY, J. (1974) *Arch. Biochem. Biophys.* **160,** 358–365.
4. KENNEDY, J. (1973) *Arch. Biochem. Biophys.* **157,** 369–373.
5. CHEN, J., AND JONES, M. E. (1976) *Arch. Biochem. Biophys.* **176,** 82–90.
6. BLAIR, D. G. R., AND POTTER, V. R. (1961) *J. Biol. Chem.* **236,** 2503–2506.
7. CREASEY, W. A., AND HANDSCHUMACHER, R. E. (1961) *J. Biol. Chem.* **236,** 2058–2063.
8. SMITH, P. C., KNOTT, C. E., AND TREMBLAY, G. C. (1973) *Biochem. Biophys. Res. Commun.* **55,** 1141–1146.
9. TREMBLAY, G. C., JIMENEZ, U., AND CRANDALL, D. E. (1976) *J. Neurochem.* **26,** 57–64.
10. ITO K., AND UCHINO, H. (1973) *J. Biol. Chem.* **248,** 4782–4785.
11. PAUSCH, J., WILKENING, J., NOWACK, J., AND DECKER, K. (1975) *Eur. J. Biochem.* **53,** 349–356.
12. TREMBLAY, G. C., CRANDALL, D. E., KNOTT, C. E., AND ALFANT, M. (1977) *Arch. Biochem. Biophys.* **178,** 264–277.
13. HOOGENRAAD, N. J., AND LEE, D. C. (1974) *J. Biol. Chem.* **249,** 2763–2768.
14. SHOAF, W. T., AND JONES, M. E. (1973) *Biochemistry* **12,** 4039–4051.
15. WOOD, M. H., AND O'SULLIVAN, W. J. (1973) *Amer. J. Obstet. Gynecol.* **116,** 57–61.
16. ISHII, K., AND GREEN, H. (1973) *J. Cell Sci.* **13,** 429–439.
17. GREEN, H., AND CHAN, T. (1973) *Science* **182,** 836–837.
18. GULEN, S., SMITH, P. C., AND TREMBLAY, G. C. (1974) *Biochem. Biophys. Res. Commun.* **56,** 934–939.
19. DAWSON, R. M. C. (1969) *in* Data for Biochemical Research (Dawson, R. M. C., Elliott, D. C., Elliott, W. H., and Jones, K. M., eds.), 2nd ed., p. 507, Oxford University Press, New York/Oxford.
20. HANDSCHUMACHER, R. E., AND PASTERNAK, C. A. (1958) *Biochim. Biophys. Acta* **30,** 451–452.
21. CIARDI, J. E., AND ANDERSON, E. P. (1968) *Anal. Biochem.* **22,** 398–408.
22. MAY, S. R., AND KROOTH, R. S. (1976) *Anal. Biochem.* **75,** 389–401.
23. KUSAMA, K., AND ROBERTS, E. (1963) *Biochemistry* **2,** 573–576.
24. HISATA, T. (1975) *Anal. Biochem.* **68,** 448–457.
25. LI, C., AND HOCHSTADT, J. (1976) *J. Biol. Chem.* **251,** 1181–1187.
26. KERSON, L. A., AND APPEL, S. H. (1968) *J. Biol. Chem.* **243,** 4279–4285.
27. PAUSCH, J., WILKENING, J., GROTELUSCHEN, H., AND DECKER, K. (1975) *Digestion* **12,** 337.
28. PAUSCH, J., KEPPLER, D., AND GEROK, W. (1977) *Eur. J. Biochem.* **76,** 157–163.