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De Novo Purine Biosynthesis in Intact Cells of *Cucurbita pepo*[1]

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ABSTRACT

The capacity of intact cells of roots excised from summer squash plants (*Cucurbita pepo* L. cv Early Prolific Straightneck) to synthesize purine nucleotides *de novo* was investigated. Evidence that purine nucleotides are synthesized *de novo* included: (a) demonstration of the incorporation of [1-^{14}C]glycine, [2-^{14}C]glycine, $NaH^{14}CO_3$, and $H^{14}COONa$ into total adenine nucleotides; (b) observation that the addition of azaserine or aminopterin, known inhibitors of *de novo* purine synthesis in other organisms, blocked the incorporation of these precursors into adenine nucleotides; and (c) demonstration that the purine ring synthesized from these precursors was labeled in a manner consistent with the pathway for *de novo* purine biosynthesis found in microorganisms and animal tissues. Under optimal conditions, the activity of this pathway in roots excised from 2-day-old squash plants was 244 ± 13 nanomoles (mean ± standard error, n = 17) $NaH^{14}CO_3$ incorporated into ΣAde (the sum of the adenine nucleotides, nucleoside and free base) per gram tissue during the 3-hour incubation period.

The possible occurrence of alternative enzymic reactions for the first steps of *de novo* purine biosynthesis was also investigated. No conclusive evidence was obtained to support the operation of alternative enzymic reactions in the intact cell of *C. pepo*.

Knowledge of the capacity of plant cells to synthesize purine nucleotides *de novo* is limited (for a recent review, see 26). Available evidence indicates that in plants, purine nucleotides are synthesized *de novo* via the same basic pathway known to function in microorganisms and mammalian species. An alternative first step of *de novo* purine biosynthesis which has been identified in microorganisms and animal tissues (15, 19, 23, 24) has been suggested to also function in higher plants (13). In this reaction, PRA[2] synthesis occurs by the direct amination of ribose-5-P by ammonia. In addition, enzymic synthesis of PRA from PRPP and asparagine by an asparagine-dependent amidotransferase was demonstrated in wheat embryo extracts (13). Knowledge of the capacity of plant cells to utilize NH_4^+ or the amide group of asparagine for the *de novo* biosynthesis of purine nucleotides is essential to an understanding of the physiological importance, if any, of alternative pathways for supplying purines *de novo* to plant cells and to an understanding of higher plant nutrition and nitrogen assimilation.

In this study, the operation of the complete pathway for *de novo* biosynthesis of purine nucleotides was demonstrated in intact cells of *Cucurbita pepo* (L. cv Early Prolific Straightneck), and the activity of this pathway under optimal conditions was assessed in roots excised from 2-d-old squash plants. In addition, the ability of asparagine or NH_4^+ to replace glutamine via alternative pathways for the first steps of *de novo* purine biosynthesis was investigated in both intact cells and cell-free extracts of roots excised from *C. pepo*.

MATERIALS AND METHODS

Chemicals. All radiolabeled chemicals were purchased from ICN Pharmaceuticals, Inc. Hydrofluor (liquid scintillation cocktail) was purchased from National Diagnostics. Mineral salts (Fisher Scientific Co.) for Shive's nutrient solution were of analytical reagent quality. All other chemicals were purchased from Sigma Chemical Co. except DONV, which was generously provided by R. E. Handschumacher.

Determination of Radioisotope Content. To determine the content of radioisotope, samples were prepared as described in the text. In all cases, the samples (4 ml final volume) were subsequently diluted with 13 ml of Hydrofluor and the activity of radioisotope measured by using a Beckman LS 100 liquid scintillation spectrometer.

Plant Material. Seeds of summer squash (*Cucurbita pepo* L. cv Early Prolific Straightneck), courtesy of the Joseph Harris Co., Inc., were imbibed in distilled H_2O at room temperature. After 24 h, the seeds were rinsed three times with distilled H_2O, spread evenly between two sheets of paper toweling placed in a plastic box (33 × 23 × 9.5 cm), and moistened with 7 ml of distilled H_2O. The covered box was placed in a growth chamber where the seeds germinated in the dark for 48 h at 30°C. At the end of the germination period, the roots (generally 2–4 cm long) were excised, pooled, and immediately weighed into specific aliquots which were maintained between sheets of wet paper towels until weighing was completed. Approximately seven to eight roots were needed to obtain 150 mg fresh weight tissue.

Incorporation of Radiolabeled Precursors into Adenine Nucleotides, Nucleoside, and Free Base (ΣAde). Samples consisting of 150 mg fresh weight of excised roots were routinely preincubated for 2 h at 30°C in 5 ml Shive's nutrient solution [5 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 2 mM K_2SO_4, 1 mM KH_2PO_4, 1 mg Fe/l, 0.13 mg Cl/l, 1 mg Mn/l, 0.1 mg Zn/l, 0.1 mg B/l, 0.01 mg Cu/l, and 0.01 mg Na/l] adjusted to pH 7.4. At the end of the preincubation period, the nutrient solution was decanted, and the roots were immediately transferred to fresh Shive's nutrient solution supplemented with one of the following radiolabeled precursors at the final concentration and specific radioactivity indicated: 2 mM [1-^{14}C] or [2-^{14}C]glycine, 4000 dpm/nmol; 25 mM $H^{14}COONa$, 1800 dpm/nmol; 2 mM DL-[3-^{14}C] serine, 1730 dpm/nmol; or 25 mM $NaH^{14}CO_3$, 2640 dpm/nmol; and any other additives in a final volume of 5 ml, pH 7.4. Reaction

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[2] Abbreviations: PRA, phosphoribosylamine (5-phospho-β-D-ribosylamine); PRPP, 5-phosphoribosyl-1-pyrophosphate; DONV, 5-diazo-4-oxo-L-norvaline; ΣAde, sum of the adenine nucleotides, adenosine and adenine, obtained by converting the adenine nucleotides and adenosine to adenine by acid hydrolysis at 100°C; ΣUMP, sum of the total uridine nucleotides converted to UMP by acid hydrolysis at 100°C; GAR, 5′-phosphoribosyl-glycinamide; FGAR, 5′-phosphoribosyl-*N*-formyl-glycinamide; MSO, methionine sulfoximine.

mixtures were incubated for 3 h at 30°C in a water bath-shaker. Incubations were carried out in 25-ml Erlenmeyer flasks sealed with rubber stoppers. When the decarboxylation of [^{14}C]glycine was being measured or $NaH^{14}CO_3$ was employed as the precursor, the rubber stoppers were fitted with a plastic center well (Kontes Glassware, Vineland, NJ) containing a filter-paper wick. In the glycine cleavage assay, the center wells contained 0.3 ml of 20% KOH during the incubation. For $NaH^{14}CO_3$, 0.5 ml of 6 N KOH was injected into the plastic center wells at the end of the incubation period. In all cases, reactions were terminated by injecting 1 ml of 6 N $HClO_4$ into the main chamber of the flask. When $^{14}CO_2$ was being trapped, it was allowed to distill from the acidified incubation mixture into the KOH in the center well for an additional 10-min incubation at 30°C. To quantitate the amount of $^{14}CO_2$ generated from [^{14}C]glycine, the center wells were transferred to scintillation vials containing 3.7 ml of H_2O, and the content of radiolabel was determined as described above. The contents of the main chamber were homogenized with a Polytron homogenizer (PCU-2, Brinkman Instruments), and the insoluble material was removed by centrifugation at 10,000*g* for 10 min at 0°C. The adenine nucleotides and adenosine synthesized by the roots from radiolabeled precursors were converted by acid hydrolysis to adenine by heating the acid-soluble supernatant fraction at 100°C for 1 h prior to neutralization with KOH. Total adenine was isolated from the neutralized acid-soluble fraction by co-crystallization with carrier adenine by the method of Wendler and Tremblay (28), except that samples were recrystallized to a constant specific radioactivity per 20 mg of crystals dried to a constant weight at 80°C. This modification eliminated the need to measure the optical density of the samples in order to determine the quantity of adenine present in each.

Identity of the Metabolite of $NaH^{14}CO_3$ that Co-Crystallized with Carrier Adenine. The identity of the metabolite of $NaH^{14}CO_3$ that co-crystallized with carrier adenine was determined by descending paper chromatography (Whatman 3 MM) employing three different solvent systems: (a) solvent system A, 95% ethanol:1 M ammonium acetate (pH 7.5) (75:30, v/v); (b) solvent system B, isobutyric acid:0.5 N NH_4OH (pH 3.6) (10:6, v/v); and (c) solvent system C, 0.1 M phosphate buffer (pH 6.8):saturated ammonium sulfate:*n*-propanol (100:60:2, v/v/v) (4). Marker adenine, run in parallel with the samples, was located with a UV light, and the corresponding areas from the chromatograms of the samples were cut out and eluted with 10 ml of 0.5 N HCl for determination of the content of adenine and radioisotope. Adenine concentration was determined by measuring the *A* at 260 nm of a 1:60 dilution in 0.01 N HCl. The content of radioisotope in 4 ml of eluant was determined as described above.

Glycine Synthesis. The incorporation of $NaH^{14}CO_3$ into glycine was assayed under the same conditions as described for the incorporation of $NaH^{14}CO_3$ into ΣAde with the exception that the acid-soluble fraction was not subjected to acid hydrolysis before neutralizaton. The reactants and products in the neutralized acid-soluble fraction were separated by TLC on sheets of PEI cellulose F (E. M. Reagents, Inc., Cincinnati, OH), using 1-butanol:acetone:water:diethanolamine (20:20:10:3, v/v/v/v) as the developing solvent (5). This procedure successfully separated glycine and serine which were visualized by 0.4% ninhydrin in 1-butanol and located alongside their respective commercial standards. The area of the chromatogram that co-chromatographed with glycine was cut out of the chromatogram and transferred to a scintillation vial containing 4 ml of H_2O and 13 ml of Hydrofluor. The content of radiolabel was determined as described above.

Distribution of ^{14}C in the Adenine Ring Synthesized from Various Radiolabeled Precursors by *C. pepo*. The incorporation of radiolabeled precursors into ΣAde and isolation of adenine were carried out as described above. In all cases, 20 mg of adenine crystals, recrystallized to a constant specific radioactivity, were reacted in a 25-ml Erlenmyer flask sealed with a rubber stopper from which was suspended a plastic center well containing 0.3 ml of 20% KOH and filter-paper wick. At the end of an experiment, the center well and its contents were transferred to a scintillation vial containing 3.7 ml of H_2O; the amount of radioisotope released as $^{14}CO_2$ and distilled into the KOH was determined.

Release of C_2. The method of Proiser and Serenkov (22) was used to release the position 2 carbon of the adenine ring as CO_2. Adenine crystals were heated in a sealed flask at 85°C in a water bath in the presence of 0.5 ml of 1 N KOH. After 30 min, 2 ml of 0.5 N H_2SO_4 was injected into the main chamber of the flask, and the sample was heated for an additional 30 min. At the end of the digestion period, 2 ml of 0.1 M $NaHCO_3$ was injected into the main chamber of the flask, and the sample was gently agitated at room temperature. After 30 min, the amount of radioisotope trapped in the center well was determined.

Release of C_2, C_6, C_8. The collective release of the carbons from positions 2, 6, and 8 of the adenine ring as CO_2 was accomplished according to the method of Cavalieri *et al.* (3). Adenine crystals dissolved in 4 ml of H_2O containing 0.2 ml of concentrated H_2SO_4 were heated in a sealed flask in a boiling water bath. After 30 min, 1.9 ml of 0.4 M $KMnO_4$ was added dropwise, and the samples were heated in a boiling water bath for an additional 20 min. The amount of radioisotope collected in the center wells was determined.

Release of C_4, C_5. Adenine was degraded to glyoxylic acid, and carbons in positions 4 and 5 of the adenine ring were released from glyoxylic acid using the method of Edson and Krebs (7). The adenine ring was initially degraded by the method of Proiser and Serenkov (22) for the removal of carbon 2 as described above, followed by oxidation with 0.6 ml of 0.4 M $KMnO_4$ at 85°C for 1 h. The $KMnO_4$ was decomposed with 2 ml of 3% H_2O_2 added dropwise with constant stirring. The sample was transferred to a centrifuge tube, and the Erlenmyer flask was rinsed with three 0.35-ml aliquots of 90°C H_2O, which were subsequently added to the centrifuge tube. The sample was spun at 10,000*g* for 5 min. The pellet was washed with 0.5 ml of H_2O and centrifuged a second time. The supernatant fractions from the two centrifugations were combined in a 25-ml Erlenmeyer flask and made alkaline with 0.4 ml of 2 N NaOH. The sample containing glyoxylic acid was heated for 15 min in a boiling water bath, cooled, and adjusted to pH 7.0 with 1 N acetic acid. To remove urea present in the sample, 0.2 ml of H_2O containing five units of urease Type III (Sigma) was injected into the main chamber of the flask. The sample was incubated for 60 min at 30°C in a water bath-shaker. The reaction was stopped with 0.25 ml of 12 N H_2SO_4. The flask was sealed as described above, and the glyoxylic acid was oxidized with 0.6 ml of 0.4 M $KMnO_4$ injected into the main chamber of the flask. The sample was incubated for 3 h at 37°C in a water bath-shaker. At the end of the incubation period, 0.2 ml of 0.1 M $NaHCO_3$ was injected into the main chamber of the flask. After 30 min in the water bath-shaker, the amount of radioisotope trapped in the center well was determined.

Detection of Enzymic Activities in Cell-Free Extracts. In all cases, enzymic activities were measured in cell-free extracts prepared from roots excised from 2-d-old *C. pepo* seedlings. Roots were homogenized with a Duall all-glass conical tissue grinder in the specified buffer to give a 20% homogenate (w/v). The homogenate was centrifuged at 10,000*g* at 0°C for 10 min, and the supernatant fraction served as the source of enzyme. In each case, the protein content of this fraction was determined by the Bradford method (2).

PRA Synthesis. Enzymic synthesis of PRA was measured by allowing its further metabolism in the presence of [1-^{14}C]glycine;

incorporation of glycine into GAR and FGAR is dependent on the availability of PRA (10). [^{14}C]GAR and [^{14}C]FGAR were separated from reactants and other products in the reaction mixture by TLC.

Three g of roots excised from 2-d-old squash plants were homogenized in 15 ml of 0.5 M Tris buffer (pH 8.0). Each incubation mixture contained the following components at the given concentrations in a final volume of 1 ml: 25 mM Tris buffer (pH 8.0); 5 mM $MgCl_2$; 5 mM ATP; 15 mM [1-^{14}C]glycine, 733 dpm/nmol; 5 mM PRPP or 15 mM ribose-5-P; 15 mM L-glutamine, 15 mM NH_4Cl, or 15 mM L-asparagine; and 0.5 ml of enzyme preparation.

After 30 min incubation at 38°C, the reactions were terminated by transfer to a boiling water bath for 2 min. Precipitated protein was removed by centrifugation, and the reactants and products contained in the supernatant liquid were separated by TLC on Eastman Kodak #13254 cellulose sheets using *n*-butanol:acetic acid:H_2O (4:1:5, v/v/v) as the developing solvent. Glycine was visualized with 0.4% ninhydrin in 1-butanol; [^{14}C]glycine was located alongisde the commercial standard. No standards of GAR or FGAR were available.

Putative GAR and FGAR were located on the chromatograms by identifying a region that was: (a) ninhydrin-positive; (b) orcinol-positive; and (c) radio-labeled. (The chromatogram was cut into sections 2.5 cm wide × 1.25 cm high which were each transferred to a scintillation vial containing 4 ml of 0.01 N HCl and 13 ml of Hydrofluor; content of ^{14}C in each section was determined as described above.) In addition, in subsequent experiments the content of radio-label was shown to be dependent upon inclusion of the cell-free extract, PRPP, and L-glutamine in the reaction mixture and to be diminished by the addition of azaserine to the assay. The identity of GAR and FGAR was confirmed by R_F values upon rechromatographing the eluate of the putative GAR-FGAR chromatographic area on PEI cellulose sheets (J. T. Baker Chemical Co.) using isopropanol:H_2O:10 N HCl (66:33:1, v/v/v) as the developing solvent (17, 27).

Asparaginase. Asparaginase activity was demonstrated in cell-free extracts of *C. pepo* by the method of Pauling and Jones (21). Ammonium ion production was measured spectrophotometrically in the presence of excess NADH, α-keto glutarate, and commercial glutamate dehydrogenase.

The plant enzyme was extracted in 40 mM phosphate buffer (pH 7.4) as described above. The complete reaction mixture contained in a final volume of 2 ml: 40 mM phosphate buffer (pH 7.4); 20 mM L-asparagine; and 1 ml of enzyme preparation.

After 30-min incubation at 25°C, the reaction was terminated by adding 0.5 ml of 10 N HCl. Precipitated protein was removed by centrifugation at 10,000*g* at 0°C for 10 min, and the supernatant fraction was neutralized (pH 7.2–7.4) with 6 N KOH. Content of free ammonium ions in a 0.4-ml sample of the neutralized acid-soluble fraction from the reaction mixture was determined in a 1-ml reaction volume of the following composition: 0.4 ml of the neutralized acid-soluble fraction; 120 mM phosphate buffer (pH 7.4); 200 μM NADH; 0.2 M α-ketoglutarate; and 12 units glutamate dehydrogenase (Sigma, Type II) (1 unit reduces 1.0 μmol of α-ketoglutarate to L-glutamate/min at pH 7.4 and 25°C in the presence of free ammonium ions). The reaction was allowed to run to completion at 25°C, and $A_{366\ nm}$ was determined. The complete reaction mixture but without the added asparagine, incubated for 30 min, served as the blank. The assay was linear for ammonium ion concentrations from 0 to 200 nmol.

Glutamine Synthetase. Glutamine synthetase activity was assayed by the formation of γ-glutamyl hydroxamate by a modification of the method of McCormack *et al.* (18). The addition of 0.5 ml of cell-free extract prepared in 0.2 M Tris-maleic buffer (pH 7.2) initiated the reaction which proceeded in a 1-ml reaction mixture of the following composition: 50 mM Tris-maleic buffer (pH 7.2), 15 mM $MgCl_2$, 5 mM ATP, 40 mM glutamate, and 10 mM hydroxylamine. Reactions were incubated for 60 min at 30°C and terminated by the addition of 0.3 ml of a solution containing equal volumes of 15% ferric chloride in 0.2 N HCl (w/v), 24% TCA, and 6 N HCl. Reactions mixtures were centrifuged at 10,000*g* for 10 min and the $A_{540\ nm}$ of the supernatants measured. An $A_{540\ nm}$ of 0.1 corresponded to 0.225 μmol γ-glutamyl hydroxamate.

RESULTS

Demonstration of the *De Novo* Biosynthesis of Purine Nucleotides in Intant Cells of *C. pepo*. Ring carbons of purine nucleotides that are synthesized *de novo* in microorganisms and animal tissues are provided in the following manner: carbons 2 and 8 originate in formate, carbon 6 is supplied by CO_2, and carbons 4 and 5 are from glycine incorporated as a single molecule. Evidence for the *de novo* synthesis of the purine ring in this manner in intact cells of excised squash roots was provided by: (a) demonstration of the incorporation of [1-^{14}C]glycine, [2-^{14}C] glycine, $NaH^{14}CO_3$, and $H^{14}COONa$ into adenine nucleotides (Table I); (b) observation that the incorporation of carbons 1 and 2 of glycine is equal and, therefore, consistent with the incorporation of glycine as an intact molecule (Table I); and (c) demonstration that the addition of known inhibitors of *de novo* purine biosynthesis in microorganisms and animal tissues (azaserine, a glutamine analog, or aminopterin, an inhibitor of tetrahydrofolate formation) reduced the incorporation of these precursors into adenine nucleotides in *C. pepo* (Table I). Significance should not be attached to the variations in sensitivity of precursor incorporation to the presence of inhibitor. Such variations probably arise from differences in the rate of appearance of label in the adenine ring relative to the rate of uptake of the inhibitor. In addition, it was demonstrated that the purine ring of the adenine nucleotides synthesized by squash roots from these precursors was labeled in a manner consistent with the pathway for *de novo* purine biosynthesis found in microorganisms and animals (Table II). Evidence was provided by experiment 1 in Table II that carbon 2 of the purine ring synthesized by *C. pepo* originated in formate. In experiment 2, where carbon 8 of the purine ring was released in addition to carbon 2, there was a corresponding increase in the amount of radiolabel recovered from adenine crystals labeled with [^{14}C]formate. In this experiment, carbon 6 of the adenine ring was also released, and a corresponding increase in the per cent of radiolabel recovered from $NaH^{14}CO_3$-labeled adenine was observed that did not occur in experiment 1 or 3. The relatively high per cent of radiolabel recovered in experiment 2 from both the [^{14}C]glycine-labeled

Table I. *Incorporation of Precursors of De Novo Purine Biosynthesis into Adenine Nucleotides in Intact Cells of Squash Roots*

Conditions for the incorporation of 25 mM $NaH^{14}CO_3$ (2640 dpm/nmol) were optimal; no attempts were made to determine optimal conditions for the incorporation of 2 mM [1 or 2-^{14}C]glycine (4000 dpm/nmol) or 25 mM $H^{14}COONa$ (1800 dpm/nmol).

Precursor	Precursor Incorporated into ΣAde	Incorporation of Precursor into ΣAde in the Presence of Inhibitor	
		Aminopterin (10 mM)	Azaserine (1 mM)
	nmol/g tissue·3 h	*% inhibition*	
[1-^{14}C]Glycine	212	37	78
[2-^{14}C]Glycine	214	31	83
$NaH^{14}CO_3$	244	24	97
$H^{14}COONa$	107	10	54

Table II. *Distribution of ^{14}C in the Purine Ring of Adenine Nucleotides Synthesized from Various [^{14}C] Precursors by C. pepo*

Conditions for the incorporation of 25 mM $NaH^{14}CO_3$ (2640 dpm/nmol) were optimal; no attempts were made to determine optimal conditions for the incorporation of 2 mM [1 or 2-^{14}C]glycine (4000 dpm/nmol) or 25 mM $H^{14}COONa$ (1800 dpm/nmol). Adenine nucleotides and adenosine synthesized from [^{14}C]-labeled precursors by *C. pepo* were converted to adenine by acid hydrolysis at 100°C. Adenine was isolated from the neutralized acid-soluble fraction by co-crystallization with carrier adenine. Adenine crystals, recrystallized from H_2O to a constant specific activity, were degraded as described in "Materials and Methods."

Precursor	Radioactivity Isolated with Carrier Adenine	$^{14}CO_2$ Recovered from Degradation of Adenine	
	dpm/20 mg	*dpm*	%
Experiment 1: C_2 Position			
$NaH^{14}CO_3$	3208	769	24
$H^{14}COONa$	1209	921	76
[1-^{14}C]Glycine	3565	399	11
[2-^{14}C]Glycine	5513	505	9
Experiment 2: C_2, C_6, and C_8 Positions			
$NaH^{14}CO_3$	3773	2550	68
$H^{14}COONa$	1390	1566	113
[1-^{14}C]Glycine	3565	1930	54
[2-^{14}C]Glycine	5513	3440	62
Experiment 3: C_4, C_5 Positions			
$NaH^{14}CO_3$	2675	729	27
$H^{14}COONa$	1209	74	5
[1-^{14}C]Glycine	3565	1600	45
[2-^{14}C]Glycine	5513	1647	30

and $NaH^{14}CO_3$-labeled adenine crystals is consistent with the known labeling of carbons 2 and 8 by the α carbon of glycine through the formation of methenyltetrahydrofolate (26) by the glycine cleavage reaction. During the formation of methenyltetrahydrofolate, the carboxyl group of glycine is released as CO_2 (14). To estimate the activity of the glycine cleavage enzyme in 2-d-old squash roots, the decarboxylation of [1-^{14}C]glycine was measured under the same assay conditions as the incorporation of glycine into ΣAde. During the 3-h incubation, 4.5 μmol of glycine were cleaved per gram tissue. Thus, the labeling of carbons 2, 6, and 8 of the adenine ring by glycine would be anticipated. Experiment 3 provided evidence that carbons 4 and 5 of the adenine ring in plants are incorporated as glycine. The high level of radiolabel recovered from $NaH^{14}CO_3$-labeled adenine suggests the synthesis of glycine from $NaH^{14}CO_3$. Under the same assay conditions used to measure the incorporation of $NaH^{14}CO_3$ into ΣAde, the incorporation of $NaH^{14}CO_3$ into glycine was confirmed; 622 nmol $NaH^{14}CO_3$ were incorporated into glycine per gram roots during the 3-h incubation.

Serine is an effective donor of carbon for the synthesis of N^5, N^{10}-methenyltetrahydrofolic acid by well-known reversal of the reaction catalyzed by hydroxymethyl transferase (26). The capacity of squash roots to utilize the β carbon of serine in the *de novo* biosynthesis of purine nucleotides was confirmed by measuring the incorporation of [3-^{14}C] serine into adenine nucleotides: 261 ± 7 nmol (mean ± SE, $n = 4$) [3-^{14}C]serine were incorporated into ΣAde per gram tissue during the 3-h incubation period. Despite the high rates of glycine decarboxylation, glycine synthesis, and the incorporation of the β carbon of serine into adenine nucleotides, the results of this labeling study provide additional evidence demonstrating that the synthesis of purine nucleotides *de novo* in plants is consistent with the pathway known to operate in microorganisms and mammals.

Identity of the Metabolite of $NaH^{14}CO_3$ that Co-Crystallizes with Carrier Adenine. The reliability of the procedure for the recovery of [^{14}C]adenine by co-crystallization with carrier was verified by paper chromatography in three different solvent systems. The radiolabeled metabolite of $NaH^{14}CO_3$ isolated by co-crystallization with carrier adenine co-chromatographed with commercial adenine. In addition, chromatography of the carrier adenine containing the metabolite of $NaH^{14}CO_3$ synthesized by the squash roots did not result in a change in its specific radioactivity. The specific radioactivity of adenine recrystallized to a constant specific radioactivity was 29,451 dpm/A_{260nm} before chromatography and 29,052, 29,359, and 30,884 dpm/A_{260nm} after chromatography in solvent systems A, B, and C, respectively.

Optimal Conditions for Measuring the Incorporation of $NaH^{14}CO_3$ into ΣAde in Intact Cells. Assays routinely employed 150 mg of excised roots in 5 ml of Shive's nutrient solution, pH 7.4. Incorporation of $NaH^{14}CO_3$ was linear with time for up to 6 h. Preincubation of excised squash roots at 30°C in Shive's nutrient solution for up to 3 h had no effect on the incorporation of $NaH^{14}CO_3$ into ΣAde during the subsequent 3-h incubation period. Therefore, a 2-h preincubation period in Shive's solution alone followed by a second incubation period of 3 h in fresh Shive's solution supplemented with the radiolabeled precursor and other additives, as indicated, was routinely employed in all incorporation studies reported here. These preincubation and incubation times are identical to those that are optimal for measuring the incorporation of $NaH^{14}CO_3$ into ΣUMP (16) and enable the simultaneous measurement of the activities of the pathways for *de novo* pyrimidine and purine biosynthesis. Maximal incorporation was obtained with 25 mM $NaHCO_3$ (Fig. 1). *De novo* purine biosynthesis was not significantly stimulated by providing the roots with a nitrogen source, in addition to the NO_3^- routinely supplied in Shive's nutrient solution. Addition

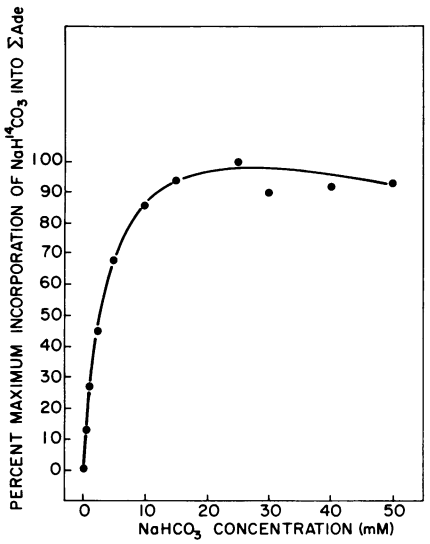

FIG. 1. Incorporation of $NaH^{14}CO_3$ (2640 dmp/nmol) into ΣAde per gram tissue during the 3-h incubation period as a function of $NaHCO_3$ concentration. The data are the average results of two separate experiments expressed as a per cent of the maximum incorporation in each experiment. Maximum incorporation was 3884 and 3732 dpm/20 mg adenine crystals in the two experiments, each employing 150 mg of excised roots.

of glutamine, asparagine, urea, or NH_4Cl up to a final concentration of 10 mM did not stimulate the incorporation of $NaH^{14}CO_3$ into ΣAde, demonstrating that nitrogen was not limiting to purine synthesis under the assay conditions employed. Addition of 0.1 M sucrose or 0.1 M α-D-glucose did not significantly stimulate the incorporation of $NaH^{14}CO_3$ into ΣAde, indicating that roots did not require substrates for respiration during the 3-h incubation. Supplying exogenous glycine, formic acid, aspartic acid, or D-ribose did not significantly stimulate the incorporation of $NaH^{14}CO_3$ into ΣAde, demonstrating that these compounds were not limiting to *de novo* purine biosynthesis in intact cells of 2-d-old squash plants when this protocol was used to assay the activity of the pathway.

Specific Requirement for Glutamine for *De Novo* Purine Biosynthesis in *C. pepo*. The direct enzymic synthesis of PRA from ribose-5-P and ammonia was demonstrated in cell-free extracts prepared from roots excised from 2-d-old squash plants. This alternative enzymic reaction was distinguished from the enzymic synthesis of PRA by L-glutamine-PRPP amidotransferase on the basis of nitrogen source specificity and sensitivity to inhibition by azaserine (Table III). L-Glutamine was utilized far more effectively than L-asparagine or NH_4Cl as a nitrogen source in the presence of PRPP, and the incorporation of [^{14}C]glycine into the combined GAR-FGAR fraction was inhibited by azaserine. In the presence of ribose-5-P, ammonia and asparagine served as nitrogen sources for PRA synthesis. Incorporation of [^{14}C]glycine into the combined GAR-FGAR fraction in the presence of ribose-5-P and L-glutamine was not significantly greater than the no-enzyme control. In addition, azaserine caused the accumulation of the radiolabel from [^{14}C]glycine in GAR and FGAR when ammonia was provided as the nitrogen source with ribose-5-P. Evidence for the enzymic synthesis of PRA by a pathway that does not derive its nitrogen from glutamine is provided by the observation that PRA synthesis was not dependent upon the prior incorporation of ammonia into glutamine. When NH_4Cl or L-asparagine was supplied with ribose-5-P, the incorporation of [^{14}C]glycine into GAR and FGAR was not reduced by the addition of 1 mM MSO, a known irreversible inhibitor of glutamine synthetase in plants and animals (Table III) (6, 25). Glutamine synthetase from squash roots was shown to be inhibited more than 50% by 1 mM MSO (Table IV).

Reliability of this method for isolating GAR and FGAR was confirmed by rechromatographing the eluate of the putative GAR-FGAR area of the chromatogram developed in *n*-butanol:acetic acid:H_2O (4:1:5, v/v/v) (Fig. 2) on PEI cellulose

Table III. *Phosphoribosylamine Synthesis from PRPP and Ribose-5-Phosphate*

Each reaction mixture contained the following components at the given concentrations in a final volume of 1 ml: 25 mM Tris buffer, pH 8.0; 5 mM $MgCl_2$; 5 mM ATP; 15 mM [1-^{14}C]glycine, 733 dpm/nmol; 5 mM PRPP or 15 mM ribose-5-P; 15 mM L-glutamine or 15 mM NH_4Cl or 15 mM L-asparagine; and 0.5 ml of enzyme preparation. When added, azaserine and MSO were provided at a final concentration of 1 mM. GAR and FGAR were isolated by TLC using *n*-butanol:acetic acid:H_2O (4:1:5, v/v/v). Data are the results of two to four experiments with the number of experiments given in parentheses. Where appropriate, the data are presented as mean nmol product ± SE.

Additions or Deletions	GAR-FGAR Synthesized	
	PRPP	Ribose-5-P
	nmol/mg protein·30 min[a]	
No enzyme	17 ± 3 (4)	16 ± 2 (3)
No glutamine	24 (2)	
+ Glutamine	114 ± 38 (4)	23 ± 7 (3)
+ NH_4Cl	30 ± 4 (3)	40 ± 7 (3)
+ Asparagine	30 ± 4 (3)	41 ± 10 (3)
+ Azaserine, + glutamine	≤25 (2)	
+ Azaserine, + NH_4Cl		≥90 (2)
+ NH_4Cl, + MSO	≤37 (2)	≤57 (2)
+ Asparagine, + MSO	≤25 (2)	≤65 (2)

[a] Average mg protein/g 2-d-old squash roots was 8.25 ± 0.1 (n = 7).

Table IV. *Inhibition of Glutamine Synthetase Activity in Cell-Free Extract by Methionine Sulfoximine*

The complete reaction mixture contained the following components at the final concentrations indicated in a total volume of 1 ml: 50 mM Tris-maleic buffer, pH 7.2; 15 mM $MgCl_2$; 5 mM ATP; 40 mM glutamate; 10 mM hydroxylamine; and 0.5 ml of enzyme preparation. When added, MSO, azaserine, and DONV were provided at the final concentrations given. The reaction was terminated by the addition of 0.3 ml of a solution containing equal parts of 10% ferric chloride in 0.2 N HCl (w/v), 24% TCA, and 6 N HCl. A_{540} of the supernatant fraction was determined. The complete reaction mixture to which the stop mix was added immediately upon the addition of enzyme preparation (zero time blank) served as the reaction blank. The zero time blank relative to the no-enzyme blank was 699 and 383 nmol/mg protein·60 min for experiments 1 and 2, respectively.

Additions or Deletions	Glutamyl Hydroxamate Synthesized	
	Experiment 1	Experiment 2
	nmol/mg protein·60 min[a]	
Complete (control)	1589	1939
− Glutamate	671	973
− ATP, − $MgCl_2$	287	507
+ MSO, 1 mM	438	877
+ MSO, 2 mM	739	891
+ MSO, 10 mM	725	685
+ Azaserine, 1 mM	1301	1809
+ DONV, 1 mM		2000

[a] Average mg protein/g 2-d-old squash roots was 8.25 ± 0.1 (n = 7).

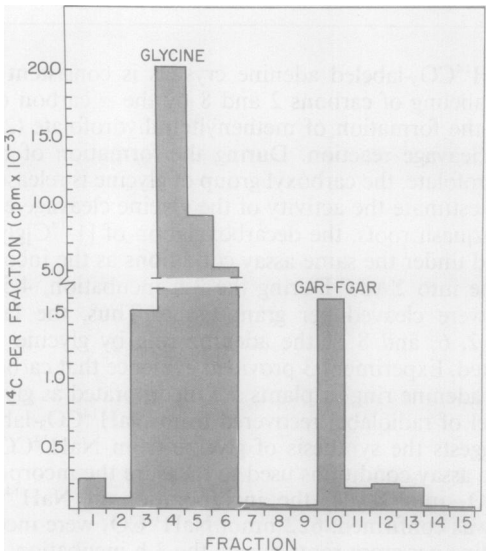

FIG. 2. Chromatographic separation of GAR and FGAR from glycine by TLC on Eastman Kodak #13254 cellulose sheets developed in *n*-butanol:acetic acid:H_2O (4:1:5, v/v/v). The reaction contained in a 1-ml volume: 25 mM Tris buffer (pH 8.0); 5 mM $MgCl_2$; 5 mM ATP; 15 mM [1-^{14}C]glycine, 733 dpm/nmol; 5 mM PRPP; 15 mM L-glutamine; and 0.5 ml of the supernatant liquid of a 20% homogenate of 2-d-old squash roots in 500 mM Tris buffer (pH 8.0). After incubation for 30 min at 38°C, the reaction was terminated by transfer to a boiling water bath, and an aliquot of the deproteinized reaction mixture was chromatographed.

sheets using isopropanol:H_2O:10 N HCl (66:33:1, v/v/v) as the developing solvent as described by Warren and Buchanan (27) and Martin and Owen (17). When the putative GAR-FGAR from two separate experiments was rechromatographed, 67% and 46% of the radiolabel in each experiment was recovered at R_F 0.4, the R_F value of authentic GAR (17, 27), and 51% and 41% was recovered at R_F 0.75, that of authentic FGAR (17, 27). The recovery of radiolabel at R_F 0.4 and 0.75 was not influenced by whether the substrates were L-glutamine and PRPP or NH_4Cl and ribose-5-P.

To investigate the possible role of L-asparagine in supporting *de novo* purine synthesis in intact cells, the L-asparagine analog DONV was added to the preincubation and incubation mixtures. DONV is known to inhibit asparaginase (9), but it is not known if it inhibits other L-asparagine-utilizing enzymes. Asparaginase activity in cell-free extracts of 2-d-old squash roots was 920 ± 127 nmol (mean ± SE, n =3) per mg protein in 30 min and was completely inhibited by 1 mM DONV. The addition of 1 mM DONV to assays employing intact cells inhibited the incorporation of $NaH^{14}CO_3$ into ΣAde approximately 50% (maximum inhibition of 70% was achieved with concentrations of 5 mM or higher). L-Glutamine completely reversed this inhibition in intact cells (Table V), indicating that DONV does not inhibit the synthesis of PRA by L-glutamine-PRPP amidotransferase nor any other steps in the *de novo* biosynthesis of adenine nucleotides. NH_4Cl or L-asparagine did increase *de novo* purine synthesis in the presence of DONV (25% and 30%, respectively) but failed to completely reverse this inhibition; aspartic acid had little effect (Table V). The failure of aspartic acid to reverse the inhibition by DONV implies that asparagine does not serve as an important source of aspartic acid for the synthesis of adenine nucleotides. These data taken together suggest two possibilities: (a) that L-asparagine may be used directly as a source of nitrogen for the *de novo* biosynthesis of purine nucleotides via an enzyme that is inhibited by DONV, or (b) that L-asparagine provides NH_3 for purine synthesis or glutamine synthesis through the action of asparaginase which was demonstrated to be inhibited by DONV.

To examine these possibilities, glutamine synthetase was inhibited by the addition of 1 mM MSO. A concentration of 1 mM or greater maximally inhibited the activity of glutamine synthetase in cell-free extracts prepared from 2-d-old squash roots (Table IV). Maximum inhibition of the incorporation of $NaH^{14}CO_3$ into ΣAde in intact squash roots was also achieved with 1 mM MSO. The reduction in incorporation was consistently only 10% to 15%. Increasing the concentrations of MSO to 10 mM did not increase the per cent inhibition (Table VI).

L-Glutamine completely reversed the inhibition of *de novo* purine synthesis by MSO and significantly stimulated the incorporation of $NaH^{14}CO_3$ into ΣAde, providing further evidence that the enzymes of the *de novo* purine pathway are not inhibited by MSO. Therefore, if L-asparagine or NH_3 is used directly for the synthesis of purine nucleotides, the presence of MSO should not compromise its ability to support the *de novo* synthesis of adenine nucleotides. On the other hand, if L-asparagine and NH_4Cl simply provide a source of NH_3 for the synthesis of glutamine, the addition of MSO will inhibit their ability to support *de novo* purine synthesis. In the presence of MSO, L-asparagine and NH_4Cl failed to reverse the reduced incorporation of $NaH^{14}CO_3$ into ΣAde. Thus, L-asparagine is most likely a source of ammonia for the synthesis of glutamine used for the *de novo* biosynthesis of purine nucleotides in *C. pepo*.

Table V. *Inhibition of the Incorporation of $NaH^{14}CO_3$ into ΣAde by DONV in Intact Cells*

Incorporation of $NaH^{14}CO_3$ (25 mM, 2640 dpm/nmol) into ΣAde in the control was 244 ± 13 nmol (mean ± SE, n = 17) per gram tissue for the 3-h incubation period. L-Glutamine, L-asparagine, NH_4Cl, and L-aspartic acid were all provided at a final concentration of 10 mM. When added, DONV was provided at a final concentration of 1 mM. The data are the average results of two to four experiments.

Additions	Incorporation of $NaH^{14}CO_3$ into ΣAde	
	− DONV	+ DONV
	% control	
Control	100	55
+ Glutamine	112	108
+ Asparagine	103	80
+ NH_4Cl	114	87
+ Aspartic acid	117	64

Table VI. *Reversal of Methionine Sulfoximine Inhibition of De Novo Purine Biosynthesis in Intact Cells*

Incorporation of $NaH^{14}CO_3$ (25 mM, 2640 dpm/nmol) into ΣAde in the control was 244 ± 13 nmol (mean ± SE, n = 17) per gram tissue for the 3-h incubation period. L-Glutamine, L-asparagine, and NH_4Cl were all provided at a final concentration of 10 mM. When added, MSO was provided at a final concentration of 1 mM. The data are the average results of two to four experiments.

Additions	Incorporation of $NaH^{14}CO_3$ into ΣAde	
	− MSO	+ MSO
	% control	
Control	100	89
+ Glutamine	112	155
+ Asparagine	103	74
+ NH_4Cl	114	86

DISCUSSION

Squash roots possess a full complement of enzymes for the *de novo* biosynthesis of adenine nucleotides. The occurrence of the classical pathway for the *de novo* biosynthesis of purine nucleotides was suggested by demonstration of the incorporation of several radiolabeled precursors into adenine nucleotides, inhibition of this incorporation by known inhibitors of *de novo* purine biosynthesis in microorganisms and animal tissues, and demonstration that the purine ring is labeled by these precursors in a manner consistent with the known pathway for *de novo* purine synthesis in microorganisms and animals.

The capacity of 2-d-old squash roots to synthesize purine nucleotides *de novo* was assessed by measuring the activity of the *de novo* purine pathway in intact cells under optimal conditions; 244 ± 13 nmol (mean ± SE, n = 17) $NaH^{14}CO_3$ were incorporated into ΣAde per gram roots during the 3-h incubation period. In this same tissue, the level of *de novo* biosynthesis of pyrimidine nucleotides under optimal conditions was 144 ± 10 nmol (mean ± SE, n = 15) $NaH^{14}CO_3$ incorporated into ΣUMP per gram roots during the 3-h incubation period (16). In comparison, Atkins *et al.* (1) observed rates of *de novo* purine biosynthesis in root nodules of 1.1 nmol/h·g fresh weight of nodule tissue from *Lupinus albus*, a legume that exports asparagine as the major product of nitrogen fixation, and 600 nmol/h·g fresh weight of nodule tissue from *Vigna unguiculata*, a ureide-producing legume.

The first step committed to *de novo* biosynthesis of purine nucleotides by the classical pathway has been established to be the formation of phosphoribosylamine from PRPP and glutamine, catalyzed by the enzyme L-glutamine-PRPP amidotransferase (EC 2.4.2.14) (8). The existence of this enzyme in biological systems appears to be ubiquitous. Evidence of an alternative pathway for the synthesis of phosphoribosylamine from ammo-

nia and ribose-5-P is accumulating. Although, in some cases, the results are compromised by the nonenzymic formation of PRA from ribose-5-P and ammonia (20), enzymic synthesis of PRA by 5′-phosphoribosylamine synthetase (EC 6.3.4.7) has been clearly demonstrated in the cell-free extracts prepared from the liver of several avian species (19, 23), *E. coli* (15), *Aerobacter aerogenes* (20), mammalian cells in culture (24), and wheat germ (13). The results of the present study provide evidence that suggests the presence of an enzyme in cell-free extracts prepared from squash roots that utilizes ribose-5-P and either ammonia or L-asparagine for the synthesis of PRA.

While there is substantial evidence supporting enzymic synthesis of PRA by 5′-phosphoribosylamine synthetase in cell-free extracts, there is no conclusive evidence either demonstrating or disallowing the operation of this enzyme in intact cells (11, 12).

Cell-free extracts prepared from a mutant line of Chinese Hamster ovary cells lacking L-glutamine-PRPP amidotransferase were shown to possess significant phosphoribosylamine synthetase activity. However, these cells in culture could not support elevated rates of FGAR synthesis with added ammonia under conditions which stimulated FGAR synthesis in normal cells (24). Thus, while the synthesis of phosphoribosylamine from ammonia and ribose-5-P was demonstrated in cell-free extract, the activity of this enzyme was insignificant in its contribution toward meeting the intact cell's demand for purine nucleotides.

The question of the potential contribution, if any, made by alternative enzymes for the synthesis of PRA toward the provision of purine nucleotides in the intact cells of *C. pepo* was addressed in the present study. Use of DONV in assays employing intact roots excised from 2-d-old squash plants provided evidence suggesting that L-asparagine was a potential source of nitrogen utilized in the *de novo* synthesis of purine nucleotides in this tissue. While the synthesis of phosphoribosylamine from L-asparagine or ammonia and ribose-5-P was indicated in cell-free extracts prepared from 2-d-old squash roots, neither L-asparagine nor NH_4Cl could reverse the reduced incorporation of $NaH^{14}CO_3$ into ΣAde in the presence of MSO. These observations taken together suggest that PRA synthesis by alternative enzymes or the use of a nitrogen source other than L-glutamine is of insufficient magnitude to contribute significantly toward the *de novo* biosynthesis of purine nucleotides in *C. pepo*.

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LITERATURE CITED

1. ATKINS CA, A RITCHIE, PB ROWE, E MCCAIRNS, D SAUER 1982 *De novo* purine synthesis in nitrogen-fixing nodules of cowpea (*Vigna unguiculata* [L.] Walp.) and soybean (*Glycine max* [L.]). Plant Physiol 70: 55–60
2. BRADFORD MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
3. CAVALIERI LF, JF TINKER, GB BROWN 1949 Degradation in the purine series studied with isotopes of nitrogen and carbon. J Am Chem Soc 71: 3973–3976
4. CIARDI JE, EP ANDERSON 1968 Separation of purine and pyrimidine derivatives by thin layer chromatography. Anal Biochem 22: 398–408
5. COSSINS EA, SK SINHA 1966 The interconversion of glycine and serine by plant tissue extracts. Biochem J 101: 542–548
6. DIMARTINO RV, V VONA, A FUGGI, C RIGANO 1982 Effect of L-methionine sulfoximine, a specific inhibitor of glutamine synthetase, on ammonium and nitrate metabolism in the unicellular alga *Cyanidium caldarium*. Physiol Plant 54: 47–51
7. EDSON NL, HA KREBS 1936 Micro-determination of uric acid. Biochem J 30: 732–735
8. GOLDTHWAIT DA 1956 5-Phosphoribosylamine, a precursor of glycinamide ribotide. J Biol Chem 222: 1051–1068
9. HANDSCHUMACHER RE, CJ BATES, PK CHANG, AT ANDREWS, GA FISHER 1968 5-Diazo-4-oxo-L-norvaline: Reactive asparagine analog with biological specificity. Sciene (Wash DC) 161: 62–63
10. HARTMAN SC, B LEVENBERG, JM BUCHANAN 1956 Biosynthesis of purines: XI. Structure, enzymatic synthesis, and metabolism of glycinamide ribotide and (α-*N*-formyl)-glycinamide ribotide. J Biol Chem 221: 1057–1071
11. HENDERSON JF 1963 Dual effects of ammonium chloride on purine biosynthesis *de novo* in Ehrlich ascites tumor cells *in vitro*. Biochim Biophys Acta 76: 173–180
12. HENDERSON JF, MKY KHOO 1965 On the mechanism of feedback inhibition of purine biosynthesis *de novo* in Ehrlich ascites tumor cells *in vitro*. J Biol Chem 240: 3104–3109
13. KAPOOR M, ER WAYGOOD 1962 Initial steps of purine biosynthesis in wheat germ. Biochem Biophys Res Commun 9: 7–10
14. KIKUCHI G 1973 The glycine cleavage system: composition, reaction mechanism and physiological significance. Mol Cell Biochem 11: 169–187
15. LEGAL ML, Y LEGAL, J ROCHE, J HEDEGAARD 1967 Purine biosynthesis: Enzymatic formation of ribosylamine-5-phosphate from ribose-5-phosphate and ammonia. Biochem Biophys Res Commun 27: 618–624
16. LOVATT CJ, LS ALBERT, GC TREMBLAY 1979 Regulation of pyrimidine biosynthesis in intact cells of *Cucurbita pepo*. Plant Physiol 64: 562–569
17. MARTIN JR DW, NT OWEN 1972 Repression of and depression of purine biosynthesis in mammalian hepatoma cells in culture. J Biol Chem 247: 5477–5485
18. MCCORMACK DK, KJF FARNDEN, MJ BOLAND 1982 Purification and properties of glutamine synthetase from plant cytosol fraction of lupin nodules. Arch Biochem Biophys 218: 561–571
19. NIERLICH DP, B MAGASANIK 1961 Alternative first steps of purine biosynthesis. J Biol Chem 236: PC32–PC33
20. NIERLICH DP, B MAGASANIK 1961 Phosphoribosylglycinamide synthetase of *Aerobacter aerogenes*. J Biol Chem 240: 366–374
21. PAULING KD, GE JONES 1980 Asparaginase II of *Saccharomyces cerevisiae*: Dynamics of accumulation and loss in rapidly growing cells. J Gen Microbiol 117: 423–430
22. PROISER E, GP SERENKOV 1963 Caffeine biosynthesis in tea leaves. Biokhimiya 28: 857–861
23. REEM GH 1968 Enzymatic synthesis of 5′-phosphoribosylamine from ribose 5-phosphate and ammonia, an alternative first step in purine biosynthesis. J Biol Chem 243: 5695–5701
24. REEM GH 1977 Purine biosynthesis in mutant mammalian cells. CIBA Found Symp 48: 105–122
25. RONZIO RA, WB ROWE, A MEISTER 1969 Studies on the mechanism of inhibition of glutamine synthetase by methionine sulfoximine. Biochemistry 8: 1066–1075
26. ROSS C 1981 Biosynthesis of nucleotides. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants, Vol 6. Academic Press, New York, pp 169–205
27. WARREN L, JM BUCHANAN 1957 Biosynthesis of the purines. XIX. 2-Amino-*N*-ribosyl-acetamide 5′-phosphate (glycinamide ribotide) transformylase. J Biol Chem 229: 613–626
28. WENDLER PA, GC TREMBLAY 1980 Quantitative isolation of radiolabeled metabolites without chromatography: Measurement of biosynthesis of purines, pyrimidines, and urea in isolated hepatocytes. Anal Biochem 108: 406–418