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The Substitution of 1-Methylhydrazine for Ammonia in the Glutamine Synthetase System*

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ABSTRACT: 1-Methylhydrazine was found to serve as a substrate for sheep brain glutamine synthetase. The Michaelis constant determined for 1-methylhydrazine is approximately 100 times larger than that for ammonia, although the maximum velocity is the same. Paper chromatography of deproteinized incubation mixtures revealed a major and a minor ninhydrin-positive, hydrazine-containing substance in the complete mixture, but absent from controls.

The enzymic products were separated from glutamic acid, glutamine, and 5-glutamylmethylamide in two solvent systems. A chromatographic reference material was prepared by condensation of pyrrolidonecarboxylic acid with 1-methylhydrazine. Two ninhydrin-positive, hydrazine-containing substances were obtained which were not separated from the corresponding major and minor components of the enzymic product. Experiments with [1-^{14}C]methylhydrazine showed that both the major and minor enzymic products contained radioactivity.

The metabolism of hydrazine and of alkylhydrazines is of current interest since these compounds are used as rocket fuels. Information concerning the biochemistry of hydrazines may be of value in the design of effective antidotes against the toxic effects of these substances (O'Brien *et al.*, 1964). At the time this study was begun little was known about the metabolism of 1-methylhydrazine. Recently, experiments with rats have shown that 45% of the administered radioactivity of an intraperitoneal dose of [1-^{14}C]methylhydrazine was expired as methane and another 40% of the radioactivity appeared as unidentified urinary products in a 24-hr period (Dost *et al.*, 1965). Since hydrazine is known to participate in the glutamine synthetase system (Speck, 1949), it was of interest to determine whether 1-methylhydrazine substitutes for ammonia to form an analog of glutamine.

Materials and Methods

The following reagents were obtained from commercial sources:[1] L-glutamine and ADP[2] (Sigma Chemical Co.), ATP (P-L Biochemicals, Inc.), L-glutamic acid (Calbiochem), 1-methylhydrazine (free base), methylamine and 1,1-dimethylhydrazine (99% pure) (Matheson Coleman and Bell), 1-methylhydrazine sulfate (Eastman Organic Chemicals), and [1-^{14}C]methylhydrazine (Nuclear Research Chemicals, Inc., Orlando, Fla.).

Recrystallization of 1-Methylhydrazine Sulfate. A yellow contamination develops on the surface of crystals of 1-methylhydrazine on standing (McKennis and Yard, 1954). The salt was recrystallized, when necessary, by the following procedure. 1-Methylhydrazine sulfate (10 g) was dissolved in approximately 20 ml of warm water and the solution was filtered rapidly by suction in the hood. Warm methanol (approximately 40 ml) was added to the cloud point and the preparation was stored under N_2 in the cold overnight. The crystals were collected by vacuum filtration, washed with cold methanol–water (2:1, v/v), and dried *in vacuo* over P_2O_5. The yield is 6–7 g.

Determination of 1-Methylhydrazine. 1-Methylhydrazine was determined colorimetrically by the method

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[1] The mention of firm names or trade products does not imply that they are endorsed or recommended by the U. S. Air Force over other firms or similar products not mentioned.

[2] Abbreviations used: ADP, adenosine diphosphate; ATP, adenosine triphosphate.

of McKennis and Yard (1954) and titrametrically as described by Kolthoff (1924).

Enzyme Preparation. The enzyme was prepared from acetone powders of sheep brain by the method of Pamiljans *et al.* (1962). The preparation was carried through the heat step and the subsequent isoelectric precipitation (fraction 7 of Pamiljans *et al.* 1962) and was then lyophilized to give a powder that was stable for several months. The powder was dissolved in 0.005 M 2-mercaptoethanol as required. A unit of enzyme activity is the amount required to generate 1 μmole of P_i from ATP/min in the standard incubation system described by Pamiljans *et al.* (1962). The specific activity of the enzyme preparations used in this study varied from 2.4 to 2.9 units/mg of protein. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Boiled Enzyme. A portion of the enzyme solution used in a given experiment was heated in a homogenizer tube in a boiling water bath for 15 min and cooled rapidly. Any water lost during the heating process was replaced and coagulated protein was resuspended by hand homogenization.

Kinetic Study. High concentrations of hydrazine and 1-methylhydrazine interfere with the phosphate analysis by reducing the phosphomolybdate complex before addition of aminonaphtholsulfonic acid. Thus, an internal standard was employed at each concentration of 1-methylhydrazine. The standard was prepared by adding a known amount of P_i to a portion of the boiled enzyme control. Kinetic data were treated[3] by the statistical method of Wilkinson (1961).

Preparations. L-Pyrrolidonecarboxylic acid (mp 152–154, uncor) and 5-glutamylmethylamide (mp 182–183) were synthesized and recrystallized as described by Lichtenstein and Gertner (1942).

5-Glutamylmonomethylhydrazine. A reference solution for chromatographic studies was obtained by incubating L-pyrrolidonecarboxylic acid (0.003 mole) and 1-methylhydrazine (0.03 mole, free base) with 1.25 ml of water in a closed tube at 37° for 20 days. The reaction mixture was evaporated to a clear syrup to remove most of the 1-methylhydrazine and water. The following steps were employed to remove excess L-pyrrolidonecarboxylic acid, residual 1-methylhydrazine, and any L-glutamic acid. The remainder of this procedure is based on an unpublished method (B. Levenberg, 1965, personal communication) used to prepare 5-glutamylmonomethylhydrazine for studies with a γ-glutamyltransferase from *Agaricus bisporus* (Gigliotti and Levenberg, 1964). The syrup was dissolved in 5 ml of water, adjusted to pH 7.4, and passed through 2.3 × 8 cm column of Dowex 1-X12 acetate form. Aliquots of fractions of the effluent fluid were spotted on paper and were checked for 1-methylhydrazine with a two-spray procedure (Goldenberg *et al.*, 1949). This method employs a $K_3Fe(CN)_6$ spray followed by a $Fe_2(SO_4)_3$–H_3PO_4 spray and reveals the presence of either free or acylated hydrazines. The positive fractions were combined and passed over a 2.3 × 8 cm column of Dowex 50-X8, NH_4^+ form, to remove 1-methylhydrazine. The hydrazine-positive fractions were pooled and reduced to a syrup by flash evaporation at 37°. The clear, but slightly yellow, syrup was used as the reference material in the chromatographic studies described below. Attempts at crystallization with excess methanol or ethanol or with mixtures of these alcohols with ethyl acetate were unsuccessful. Treatment with cold acetone gave rise to separation of a viscous, off-white oil which contained product plus impurities. The syrup contained traces of glutamic acid and 1-methylhydrazine, both of which increased in content with time of storage in the cold. In addition to the main, ninhydrin-positive, hydrazide-positive substance described by Gigliotti and Levenberg (1964) a minor component with the same reactivity as the major product was observed on heavily loaded chromatograms. The minor component had a lower R_F value than the major product in solvent I and was not observed after 18 hr of chromatography in solvent II (see Table III).

Paper Chromatography. Descending paper chromatograms were run on Whatman No. 4 paper in 1-butanol–acetic acid–water (4:1:5, upper phase, v/v, solvent I) for 6 hr or in 1-butanol–ethanol–0.5 N ammonium hydroxide (4:1:1, v/v, solvent II) for 18 hr. Amino acids were detected by treatment with "Nin-Spray" (Nutritional Biochemicals Corp.). Free and acylated hydrazines were detected with the two-spray system described above or with acidic *p*-dimethylaminobenzaldehyde. The latter reagent is useful for distinguishing between free and acylhydrazine since the bound hydrazine requires several hours for maximum reac-

TABLE I: Comparison of 1-Methylhydrazine Disappearance with Appearance of Inorganic Phosphate.[a]

	1-Methylhydrazine (μmoles)			P_i (μmoles)	
Expt	90 min	Cor	Change	90 min	Change
Boiled enzyme	9.60	12.50		0.98	
Complete	4.91	6.40	−6.10	7.06	6.08

[a] The reaction mixture was the same as for Figure 1 except for the content of 1-methylhydrazine (12.5 μmoles) and enzyme (0.51 unit, 0.33 mg of protein). The reaction mixture was incubated for 90 min at 37° and was stopped by the addition of cold 20% trichloroacetic acid. The 1-methylhydrazine and phosphate concentrations were determined on six samples as described in Methods. The factor 12.50/9.60 was used to correct the values in column 2 to those given in column 3 (see text).

[3] The author thanks Mr. Walter P. Knowles of the Biometrics Unit, U. S. Air Force School of Aerospace Medicine, for establishing the computer program used to treat the kinetic data in these studies.

tion, whereas free hydrazine gives a yellow or yellow-orange spot immediately (McKennis and Yard, 1959).

Chromatography of the Enzymic Reaction Product. The reaction mixture was the same as that given in the footnote to Table I, except 50 μmoles of 1-methylhydrazine was employed. The reaction was stopped after 60 min by the addition of cold trichloroacetic acid to a final concentration of 10%. A quantity of P_i equal to 15% of the added 1-methylhydrazine was formed. Portions of 25 μl of deproteinized solution were taken for paper chromatographic analysis. Samples were run alone and in mixture with suitable reference solutions, which were prepared in 10% trichloroacetic acid.

p-Dimethylaminobenzaldehyde Treatment of Enzymic Reaction Product. A 0.2-ml portion of the deproteinized solution used for detection of the product was streaked on Whatman No. 3 paper and chromatographed in solvent II for 18 hr. A 2-mm strip was cut through the center of each application streak to the distal end of the paper. The enzymic product was located on this test strip with Nin-Spray. An area 2-cm wide and long enough to include the streak plus 0.5 cm on each side was cut out of the paper in the area of the product. As a control, an area the same size was cut from the corresponding position of a chromatogram of the boiled enzyme sample. The strip was cut into small pieces and extracted with three 2-ml portions of water, with intermediate filtration into a 25-ml round-bottomed flask. The water was removed by lyophilization and the residue was taken up in 0.5 ml of water for analysis with *p*-dimethylaminobenzaldehyde. Portions of 25 μl of this solution alone or with an internal standard of 1-methylhydrazine were treated with 50 μl of 2 N H_2SO_4 and diluted to a final volume of 100 μl with water. The samples were either treated immediately with 0.1 ml of 2.5% *p*-dimethylaminobenzaldehyde in methanol, or they were hydrolyzed for 1 hr at 100° before color development. After exactly 30 min the volume was brought to 1.0 ml with glacial acetic acid. The spectra were determined on a Beckman Model DK-2 ratio recording spectrophotometer.

[1-^{14}C]Methylhydrazine Experiment. The reaction mixture contained 2.78 units of glutamine synthetase (sp act. 2.5, 1.11 mg of protein) or boiled enzyme, 64 μmoles of [1-^{14}C]methylhydrazine (2.03×10^4 cpm/μmole), plus the remaining components listed in the footnote to Figure 1, but in double amounts, in a final volume of 2.0 ml. After a 60-min incubation at 37° the reaction was stopped by the addition of 2.0 ml of cold 20% trichloroacetic acid. The deproteinized supernatant solution obtained by centrifugation was analyzed for P_i and for ^{14}C-labeled compounds. Portions of 50 μl of the complete and boiled enzyme samples were chromatographed alone and mixed with 5-glutamylmethylhydrazine in solvent I for 8 hr. The chromatogram was cut into strips and analyzed for radioactivity in the Vanguard Autoscanner 880. This low-background instrument was coupled to the Vanguard automatic data system, which integrates and records the counts under the peaks. Triplicate strips were developed with either ninhydrin, acidic *p*-dimethylaminobenzaldehyde spray, or with the ferricyanide–ferric sulfate reagent.

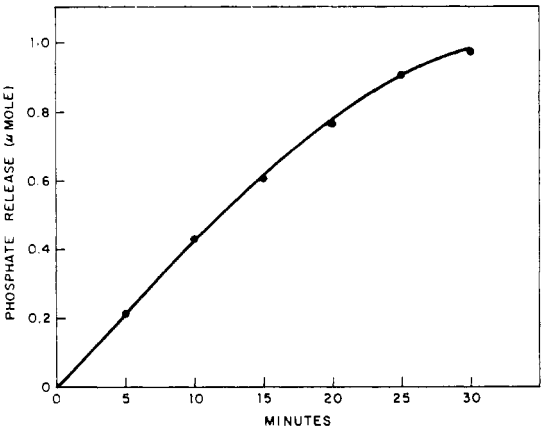


FIGURE 1: Time course of P_i release. The reaction mixture contained 25 μmoles of 2-mercaptoethanol, 10 μmoles of ATP, 50 μmoles each of $MgCl_2$, sodium L-glutamate, 1-methylhydrazine sulfate, and imidazole–HCl buffer, all at pH 7.2, plus 0.041 unit of enzyme (sp act. 2.4, 17 μg of protein) in a final volume of 1.0 ml. Incubation was carried out for the given time intervals and the reaction was stopped by the addition of 1.0 ml of 20% trichloroacetic acid. Inorganic phosphate was determined by the method of Fiske and Subbarow (1925) on a 1.0-ml portion of the resulting solution. Each point represents the mean of six samples.

Results

It was important to ascertain the purity of the material used in this study, since contamination of 1-methylhydrazine with other amino compounds, such as methylamine or ammonia, would give rise to P_i production in the incubation mixture. Six samples of the recrystallized sulfate salt of 1-methylhydrazine were titrated with potassium iodate in HCl (Kolthoff, 1924) and a value for the mean plus or minus the standard error of the mean of 99.42 ± 0.25% was observed. These data represent the reducing agent content and define the upper limit for nonreducing impurities in the 1-methylhydrazine.

When 50 μmoles of 1-methylhydrazine was used as the amino compound, the appearance of P_i was found to be a linear function of time up to 15 min (Figure 1). The amount of phosphate released in 30 min in this experiment represents only 2% of the added 1-methylhydrazine. In order to rule out the possibility that the observed activity was due to a 2% impurity, a larger amount of enzyme was incubated with only 12.5 μmoles of 1-methylhydrazine. In this experiment both the appearance of phosphate and the disappearance of free 1-methylhydrazine were measured. The results are summarized in Table I. Note that some loss of

1-methylhydrazine occurred in the boiled enzyme controls during the 90-min incubation period.

Assuming that the loss of 1-methylhydrazine from the complete system is proportional to that observed in the boiled enzyme sample, one may correct the 90-min 1-methylhydrazine values by multiplying with the factor 12.50/9.60 (column 2, Table I). The difference between the corrected 1-methylhydrazine values is given in the third column of Table I. Thus, under conditions in which 6.08 μmoles of P_i was released, a corresponding decrease of 6.10 μmoles of 1-methylhydrazine occurred. This is the expected result for a glutamine synthetase catalyzed condensation of L-glutamic acid and 1-methylhydrazine.

The effect of varying the concentration of 1-methylhydrazine on the rate of the enzymic reaction was studied. The reaction mixture was the same as that given in the legend of Figure 1, except for the quantities of 1-methylhydrazine (12.5–200 μmoles, variable) and enzyme (0.06 or 0.16 unit, sp act. 2.9). The Michaelis constant of 1-methylhydrazine was found to be 1.66×10^{-2} M. The maximum velocity V_{max} was 0.056 μmole of phosphate released/min with 19.4 μg of protein and 0.152 μmole of phosphate released/min with 52 μg of protein. The specific activities calculated from these data are 2.89 and 2.92, in agreement with the value 2.9 obtained with hydroxylamine in the standard incubation mixture of Pamiljans *et al.* (1962). Thus, the V_{max} with 1-methylhydrazine is the same as that with hydroxylamine when L-glutamic acid is used in the sheep brain glutamine synthetase system.

Glutamine synthetase is also known to act as a γ-glutamyltransferase (see Meister, 1962); *i.e.*, it catalyzes the formation of γ-glutamylhydroxamate and ammonia from hydroxylamine and glutamine. Interference with hydroxamate formation by the addition of various amino compounds has been taken as an indication of their participation in the reaction. It was found that a high concentration of 1-methylhydrazine does inhibit the formation of γ-glutamylhydroxamate (Table II).

To define further the nature of the glutamine synthetase catalyzed reaction between L-glutamate and 1-methylhydrazine, experiments were carried out to characterize the product. A summary of the results of paper chromatographic analysis of the products is presented in Table III. All of the incubation mixtures contained L-glutamic acid, which served as an internal chromatographic standard. The product was separated from glutamine and from 5-glutamylmethylamide, but was not separated from the reference 5-glutamylmethylhydrazine. The yellow designation for the hydrazide area refers to the condition of an "aged" chromatogram after ninhydrin spray. Initially, the spots are purple, as expected for an α-aminocarboxylic acid, but the purple color fades and the yellow color increases over a period of 7–10 days. The product was not detected in zero-time controls. The product was absent as well from boiled enzyme and nonenzyme controls and from mixtures lacking either sodium glutamate, ATP, or 1-methylhydrazine. To rule out the possibility that the product was merely 5-glutamylhydrazine, samples of the deproteinized incubation mixtures and authentic 5-glutamylhydrazine, hydrazine, and monomethylhydrazine were chromatographed in solvent II. 1-Methylhydrazine and hydrazine re-

TABLE II: 1-Methylhydrazine Inhibition of γ-Glutamyltransferase Activity.[a]

1-Methylhydrazine Added (μmoles)	Glutamyl Transferse Activity E_{500} 15 min	Std Dev
None	0.487	0.005
100	0.238	0.009

[a] The incubation mixture contained 1-methylhydrazine as indicated, 100 μmoles of imidazole buffer, 1.25 μmoles of $MnCl_2$, 25 μmoles of sodium arsenate, 0.125 μmole of ADP, 10 μmoles of hydroxylamine, and 50 μmoles each of 2-mercaptoethanol and L-glutamine, all at pH 7.2, plus 0.016 unit of glutamine synthetase (sp act. 2.4, 6.8 μg of protein) in a volume of 1.0 ml; temperature 37°. Each value represents the mean of six determinations.

TABLE III: Summary of Data on Chromatography of the Enzymic Products.[a]

Sample	R_G of Ninhydrin-Positive Substances: Solvent I, Major	Solvent I, Minor	Solvent II
Boiled enzyme	1.22 Y		0.46 Y
	1.0		1.0
Complete	1.20 Y		0.48 Y
	1.0		1.0
	0.65 Y	0.26 Y	3.4 Y
1-Methylhydrazine	1.22 Y		0.50 Y
L-Glutamine	0.83		2.3
5-Glutamylmethylamide	1.20		9.0
5-Glutamylmethylhydrazine	0.63 Y	0.25 Y	3.6 Y

[a] The table lists the R_G values of components detected by ninhydrin after paper chromatography of deproteinized incubation mixtures. Abbreviations used: R_G is defined as the ratio of the distance travelled by a given compound to that of glutamic acid; Y, a yellow color developed on standing after ninhydrin treatment (see text).

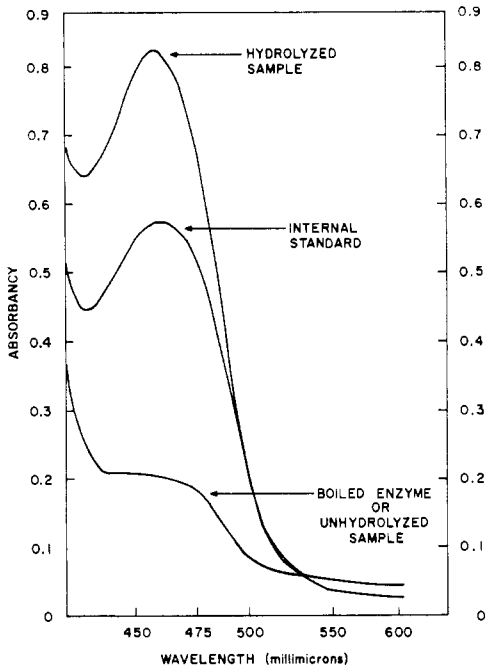


FIGURE 2: Spectra of various samples treated with *p*-dimethylaminobenzaldehyde (see Methods for details).

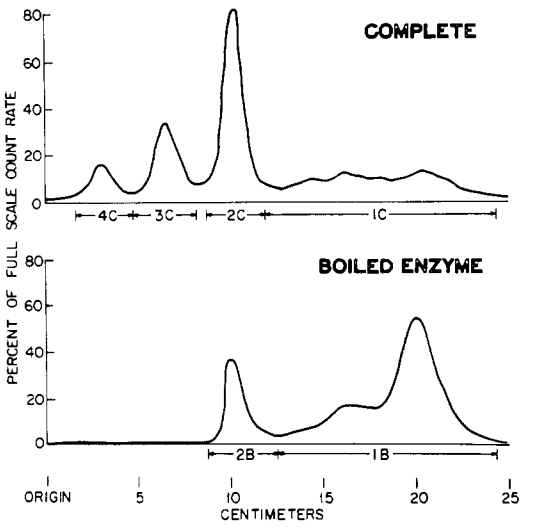


FIGURE 3: Tracing of a chromatogram of ^{14}C-labeled compounds. Each tracing is the graphical average of the strip count records of two chromatograms (see Methods for details).

mained near the origin; 5-glutamylhydrazine streaked from the origin to an area centered 2.5 cm from the origin; the enzymic product moved 6.6 cm and was clearly separated from 5-glutamylhydrazine. Furthermore, upon treatment with *p*-dimethylaminobenzaldehyde, the hydrazine-containing compounds gave a yellow product, whereas 1-methylhydrazine and the enzymic product yielded a yellow-orange derivative.

The product was recovered from a large-scale chromatogram and was analyzed for 1-methylhydrazine before and after hydrolysis in 1 N sulfuric acid. The spectra of various samples that were treated with *p*-dimethylaminobenzaldehyde are compared in Figure 2. The bottom spectrum was obtained on analysis of the extract of the boiled enzyme chromatogram, after hydrolysis. Within the limits of experimental error, the same curve was obtained for an extract of the chromatogram of the complete system before acid hydrolysis. On hydrolysis of the product a substance was released which reacted with *p*-dimethylaminobenzaldehyde to give a derivative with the spectrum shown in the top curve. An internal standard was prepared by adding 1-methylhydrazine to a portion of the extract of the boiled enzyme chromatogram. The absorption maximum of the derivative of the standard and that of the hydrolysis product was found to be 458 mμ. Glutamic acid was detected by paper chromatography in the hydrolysate of the complete system, but not in the boiled enzyme sample.

Additional evidence for the enzymic formation of 5-glutamylmethylhydrazines was obtained in experiments with ^{14}C-labeled 1-methylhydrazine. A tracing of a strip-count record of an incubation mixture chromatogram is shown in Figure 3. The components of fractions 3 and 4 cochromatographed with the reference 5-glutamylmethylhydrazine preparation. All four fractions were positive for hydrazine as tested with the ferricyanide–ferric sulfate reagent. Fractions 1 and 2 reacted with acidic *p*-dimethylaminobenzaldehyde in the manner of free hydrazines, whereas fractions 3 and 4 reacted as bound hydrazine (McKennis *et al.*, 1959). With ninhydrin, fraction 1 gave a yellow spot, typical of free methylhydrazine; fraction 2, corresponding to the R_F of glutamic acid, gave a purple spot with a yellow center; fractions 3 and 4 gave a purple spot that slowly turned yellow. A summary of the radioactivity data compiled on the automatic data system is given in Table IV. The radioactivity associated with

TABLE IV: Summary of Radioactivity Data from [^{14}C]-Methylhydrazine Experiment.[a]

Sample	Fraction Number (% of total cpm)				
	1	2	3	4	3 and 4
Boiled enzyme	78.4	21.7	...	...	...
Complete	45.6	31.9	14.4	8.2	22.6

[a] The fraction numbers correspond to the numbered areas shown in Figure 3. The sum of the per cent radioactivity found in areas 3 and 4 is given in the last column.

free 1-methylhydrazine (fraction I) is reduced in the complete system as compared to the boiled enzyme control. The radioactivity found in the glutamic acid region was unexpected. One possible explanation is

that a salt or chelate complex of methylhydrazine and glutamic acid migrated like glutamic acid alone. Such an explanation is supported by the following observations. The methylhydrazine is not acylated according to the acidic *p*-dimethylaminobenzaldehyde test; no radioactivity was found in this region when [^{14}C]-methylhydrazine was chromatographed alone or after removal of glutamic acid from the deproteinized solutions by ion-exchange chromatography.

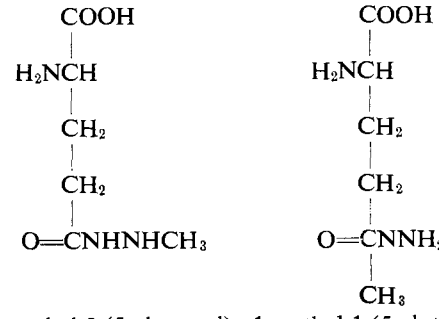
The recovery of radioactivity in fractions 3 and 4 was 22.6% of the total counts per minute detected on the strip. This value agrees well with the amount of P_i released during the course of the experiment. The incubation of 64 μmoles of 1-methylhydrazine in the system described in Methods gave rise to 15μ moles of P_i, which corresponds to 23.4% of the 1-methylhydrazine present.

Discussion

From the above results it is concluded that 1-methylhydrazine serves as a substrate in the glutamine synthetase system. It appears that two enzymic products, presumably isomers of 5-glutamylmethylhydrazine, are produced. Although only one enzymic product was expected, the observation of two products of condensation of pyrrolidonecarboxylic acid and 1-methylhydrazine might have been predicted on the basis of the work of Hinman and Fulton (1958). These investigators studied the reaction of various anhydrides and esters with methyl derivatives of hydrazine. In the case of 1-methylhydrazine, substitution at either nitrogen atom was obtained in every experiment. It is interesting to note that with anhydrides the major product was the 1-methyl-1-acyl derivative, whereas the 1-methyl-2-acylhydrazine was formed predominantly with esters as the acylating agent (Hinman and Fulton, 1958). Thus, it is possible that the two products[4] observed in this study in the enzymic reaction between glutamic acid and 1-methylhydrazine and in the condensation of pyrrolidonecarboxylic acid with 1-methylhydrazine may be the isomers shown in Chart I. Experiments are in progress to isolate quantities of the products sufficient to allow further characterization.

The apparent Michaelis constant for 1-methylhydrazine was found to be about 100 times larger than the corresponding values for hydroxylamine and ammonia reported by Pamiljans *et al.* (1962). It was found that the maximum velocity with L-glutamic acid is the same with either 1-methylhydrazine or hydroxylamine as the amino substrate. Preliminary experiments with unsymmetrical dimethylhydrazine gave a value of 0.15 M for the apparent Michaelis constant, whereas symmetrical dimethylhydrazine, in the range 0.02–0.2 M, gave no release of inorganic phosphate. Since the value obtained for the K_m of unsymmetrical dimethylhydrazine is approximately

CHART I: Two Possible Products of the Glutamine Synthetase Catalyzed Reaction between L-Glutamic Acid and 1-Methylhydrazine.



1-methyl-2-(5-glutamyl)-hydrazine (I)

1-methyl-1-(5-glutamyl)-hydrazine (II)

1000 times that of ammonia, the possibility exists that the observed activity was due to a small impurity of an active amine. Further experimentation is required to determine whether a 5-glutamyl derivative of unsymmetrical dimethylhydrazine is formed in the glutamine synthetase system. Kinetic studies with hydrazine were hampered by interference of high concentrations of hydrazine with the method of P_i analysis. Deviations from linearity, suggesting substrate activation, were apparent in plots made according to Lineweaver and Burk (1934). Although an apparent Michaelis constant was not obtained, it is obvious from the data taken at low hydrazine concentration that hydrazine is a better substrate than 1-methylhydrazine, but poorer than ammonia. Thus, the following amino substrate activity series obtains: ammonia > hydrazine > 1-methylhydrazine > unsymmetrical dimethylhydrazine (*vide supra*).

Several further experiments are suggested by the observation that 1-methylhydrazine substitutes for ammonia in an *in vitro* glutamine synthetase system. It is essential to determine whether 5-glutamylmethylhydrazine is also formed *in vivo*. Studies on the effects of this compound on enzymes that utilize glutamine for biosynthesis of essential metabolites are also of interest. In addition, toxicological studies should be conducted with 5-glutamylmethylhydrazine to determine the toxicity of this compound relative to that of 1-methylhydrazine.

Acknowledgments

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[4] It is recognized that several other possibilities exist since ring formation could occur by reaction of the 1-carboxyl group of the given isomers with a nitrogen atom of the hydrazine moiety.

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Purification and Properties of a D(−)-β-Hydroxybutyric Dimer Hydrolase from *Rhodospirillum rubrum**

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ABSTRACT: An enzyme which catalyzes the hydrolysis of the dimeric ester of D(−)-β-hydroxybutyric acid to 2 equiv of the monomer has been purified 70-fold from *Rhodospirillum rubrum*. It exhibits a high degree of specificity for β-hydroxybutyric dimer possessing the D(−) configuration. Evidence is presented which suggests that the dimer may not be the physiological substrate for the enzyme. Thus, the hydrolase attacks a low molecular weight oligomer of β-hydroxybutyric acid, presumably the trimer, at much faster rates than the dimer. The enzyme shows no activity with other esters tested.

Poly-β-hydroxybutyrate (PHB),[1] the major lipid reserve material of many types of bacteria, occurs in the cell as discrete granules. Recent investigations have demonstrated that the enzymatic depolymerization of native PHB granules isolated from *Bacillus megaterium* is dependent on the successive action of two soluble factors (tentatively designated as activator and depolymerase) isolated from extracts of *Rhodospirillum rubrum* (Merrick and Doudoroff, 1964). In addition to the soluble enzyme system, a labile particulate factor intimately bound to the granules also contributes to the depolymerization of PHB. Evidence has been presented which suggests that this labile factor may be associated with the membrane of the PHB granules (Merrick *et al.*, 1965; Merrick, 1965). The products of depolymerization are D(−)-β-hydroxybutyrate (80–85%) and a soluble ester (15–20%). The soluble ester has been isolated and its identification as the dimeric ester of D(−)-β-hydroxybutyric acid (3-*O*-D-(3-D-hydroxybutyryl)hydroxybutyric acid) is reported in this paper.

An extracellular enzyme isolated from *Pseudomonas lemoignei* is also capable of digesting PHB, but the mechanism of hydrolysis differs from the enzyme system described above (Merrick *et al.*, 1962; Merrick and Doudoroff, 1964; Delafield *et al.*, 1965b). The exoenzyme attacks purified PHB but not native PHB granules, while the activator and depolymerase fractions from *R. rubrum* do not digest the purified polymer. Further, the action of the extracellular depolymerase on the polymer results in the formation of the dimeric ester of β-hydroxybutyric acid as the principal product, although some monomer is also produced.

Thus, the dimer appears to be an important metabolite in the breakdown of purified PHB or PHB contained in native granules. The further metabolism of the dimer to its monomeric constituents can readily be demonstrated with *R. rubrum* extracts. The present communication describes the purification and proper-

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[1] Abbreviations: PHB, poly-β-hydroxybutyrate; NAD, nicotinamide-adenine dinucleotide; DFP, diisopropylphosphorofluoridate.