Influence of Hydrazine on the Distributions of Free Amino Acids in Mouse Liver.* (31858)

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Previous work in this laboratory led to the finding of several metabolites (arginine, alanine, and α-ketoglutaric, oxalacetic, and glutamic acids) and diamino compounds (ornithine, 1,2-diaminoethane, 1,3-diaminopropane, 1,4-diaminobutane, and α,γ-diaminobutyric and α,β-diaminopropionic acids) which, when administered singly or in combinations to mice prior to injections of hydrazine, showed protection against lethal effects of the acute toxicity of unsubstituted hydrazine(1-4). Preliminary new findings suggested that the toxic action of hydrazine and the protective action exerted by several substances cannot be attributable entirely to direct biochemical effects upon parenchymal cells in the central nervous system (CNS). Thus, no significant changes from controls were found in our experiments in contents of free amino acids of brain tissue of mice when the animals were dying in seizure after hydrazine administration; nor were changes found in brain amino acids after administration of protective levels of arginine, glutamic acid, or a combination of the latter two amino acids. Previous work has shown that administration of hydrazine causes marked increases in content of γ-aminobutyric acid (γABA) in brains of mice and rats within 1 hour after administration(5), the maximal increases occurring 12 hours after administration of hydrazine. Since the contents of some of the major free cerebral amino acids (glutamic and aspartic acids, glutamine, γABA, and alanine) are known to be related to the function of the tricarboxylic acid cycle and since changes in levels of these substances are known to occur when glycolysis or respiration are inhibited, it was concluded tentatively that the acute toxic effects of hydrazine *in vivo* probably do not result from a major general effect on energy-yielding processes in the whole of brain tissue.

Materials and methods. The free amino acid patterns of liver tissue were followed by the use of 2-dimensional paper chromatography (descending technic). Solvent systems were phenol-water-ammonia and lutidine-water; the color reagent consisted of ninhydrin dissolved in butanol (see(6) for details of methods).

Groups of male mice of an inbred Swiss stock, approximately 25 g in weight, were treated with saline or a test solution, and then one-half of the animals in each group received hydrazine while the others did not. At various intervals the livers were removed, homogenized in 80% ethyl alcohol and the alcoholic extracts were evaporated to dryness under a bank of lights in a stream of air. The residues were taken up in water-saturated picric acid solution, lipids were removed by extraction with water-saturated ether, and suitable aliquots were used for chromatographic study.

Citrulline was isolated by a column chromatographic method(7) and determined colorimetrically(8).

Other technical details of the procedures employed can be discussed most conveniently together with the results obtained by them.

Results and discussion. General amino acid studies. In one experiment, groups of male Swiss mice of an inbred stock, 25 g in weight, were given intraperitoneal injections of saline, arginine, alanine, glutamic acid, or a mixture of equal parts of the latter 3 amino acids. In each instance each amino acid was given at a dose of 4 mmoles/kg (0.1 ml of neutral solution/25 g). Exactly 30 minutes after the injection 5 mice of each group were sacrificed and livers removed for analysis; the remaining mice received injections of hydrazine at a level of 3 mmoles/kg (0.1 ml of neutral solution/25 g). Thirty minutes after the hydrazine injection, a time at which approximately a 50% mortality occurred, 5 mice

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from the second group of animals were sacrificed, the livers removed, and each liver was processed separately for chromatographic study. Sufficient numbers of animals were used for the second half of the experiment so that at least 5 of the mice in each group survived for 30 minutes after hydrazine treatment. Livers of 5 untreated mice were used as controls.

Typical results are shown in Fig. 1-11. In chromatograms of extracts of liver of untreated mice (Fig. 1), taurine (#1) was present in highest concentration and relatively large amounts of glutamic acid (#2), glycine (#3), alanine (#4), and glutamine (#5), were noted. Also detected were aspartic acid (#15), cystine (as cysteic acid) (#6), glutathione (#7), ethanolamine phosphate (#8), serine (#9), citrulline and/or β-alanine and/or glycerylphosphorylethanolamine† (#10), valine (#11), leucine and/or isoleu-

† In the normal animals this spot contains largely glycerylphosphoryl-ethanolamine and β-alanine; the increases in intensity of this spot in hydrazine-treated animals is attributable almost entirely to an accumulation of citrulline.

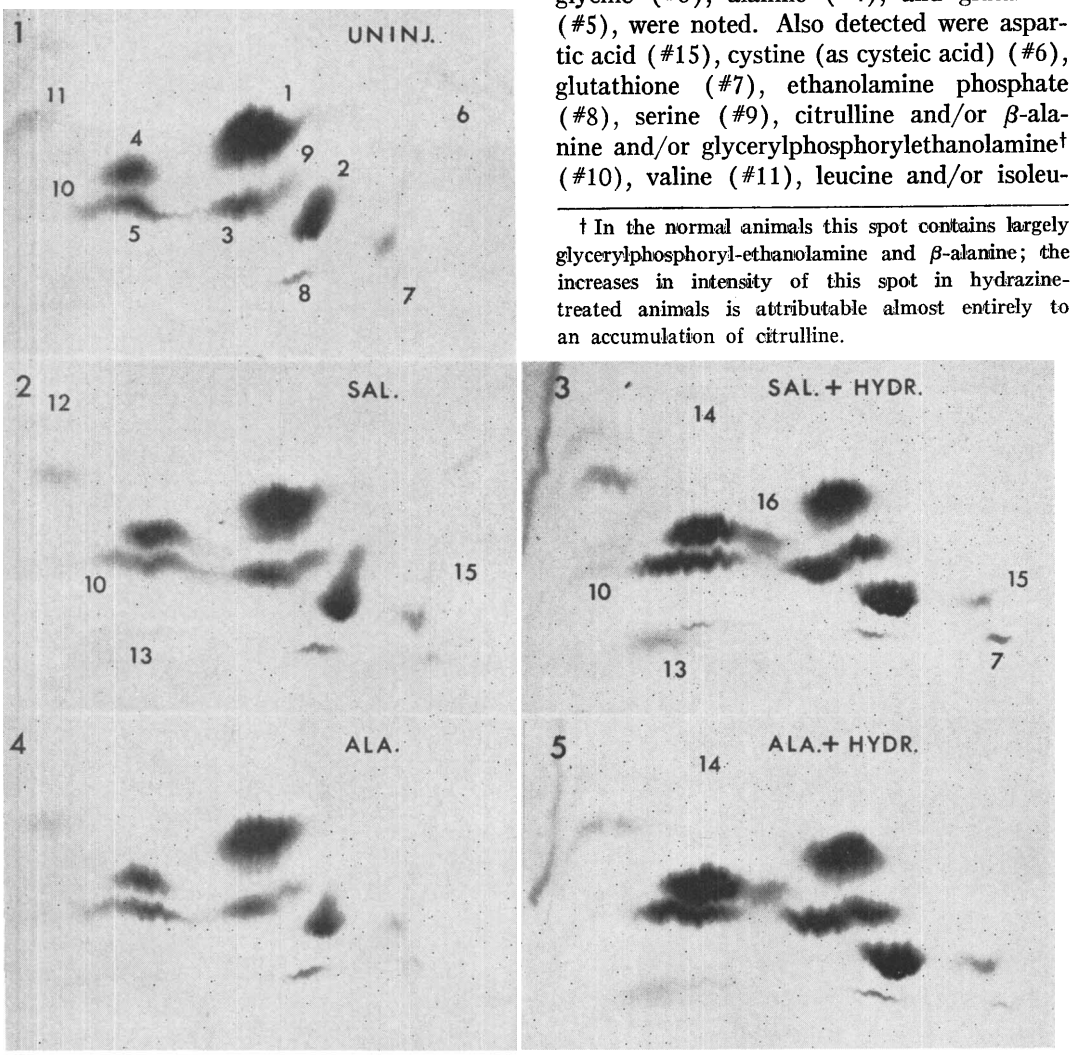
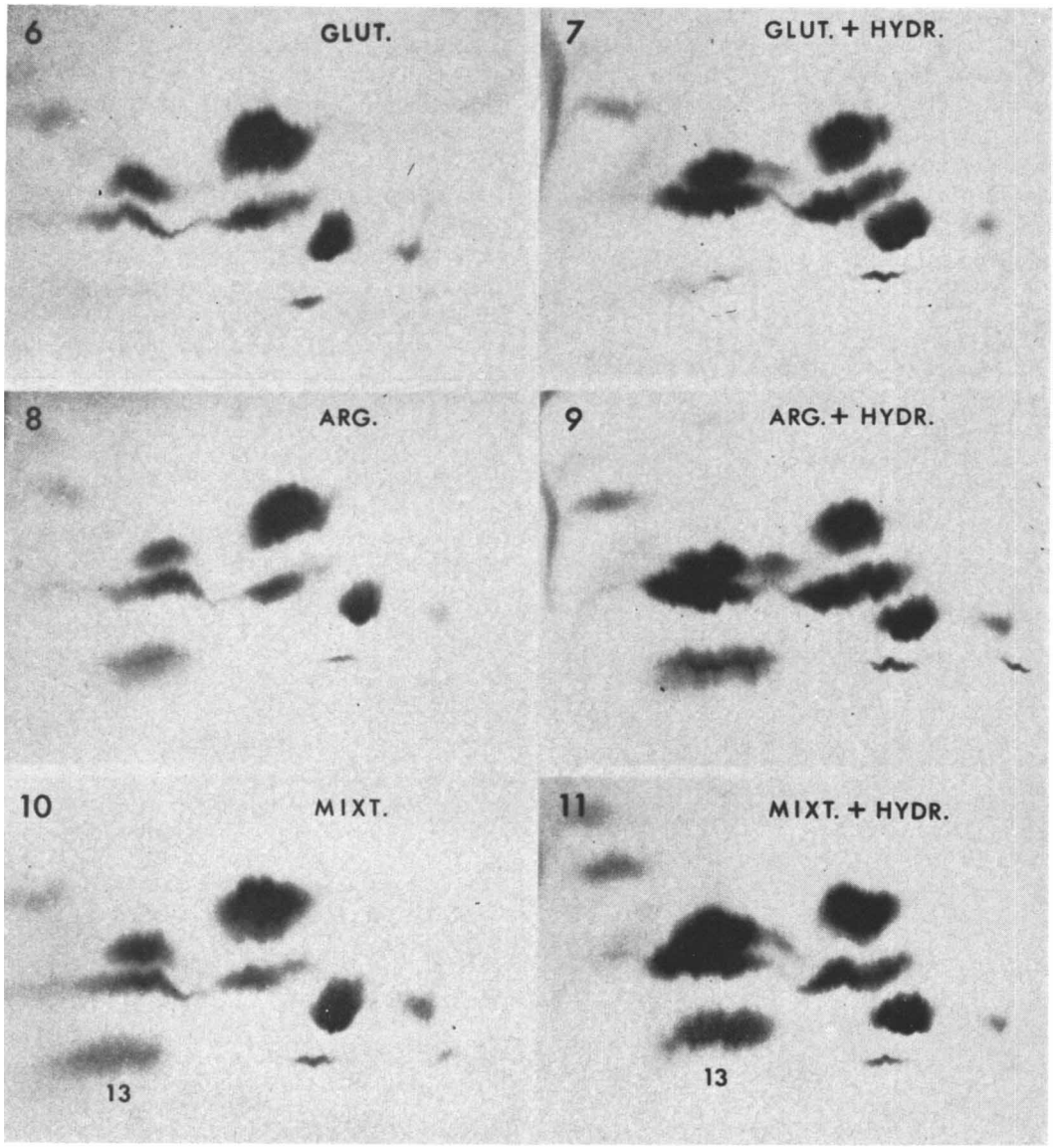


Fig. 1-11. Comparisons of free amino acid patterns of livers of control mice (Fig. 1) with those of mice receiving saline (Fig. 2) and saline followed by hydrazine (Fig. 3); alanine (Fig. 4) and alanine followed by hydrazine (Fig. 5); glutamic acid (Fig. 6) and glutamic acid followed by hydrazine (Fig. 7); arginine (Fig. 8) and arginine followed by hydrazine (Fig. 9); and a mixture of the above 3 amino acids alone (Fig. 10) or followed by hydrazine (Fig. 11). Extracts obtained from 75 mg of fresh weight of tissue were employed for decending 2-dimensional chromatography (phenol, right to left; lutidine, bottom to top). Constituents on chromatograms: taurine, 1; glutamic acid, 2; glycine, 3; alanine, 4; glutamine, 5; cystine (cysteic acid), 6; glutathione, 7; ethanolamine phosphate, 8; serine, 9; citrulline and/or β-alanine and/or glycerylphosphorlyethanolamine, 10; valine, 11; leucine and/or isoleucine, 12; ornithine, 13; tyrosine, 14; aspartic acid, 15; threonine, 16.



cine (#12), and threonine (#16). Occasionally small amounts of ornithine (#13) were observed. Amino acid patterns identical to those of untreated controls (Fig. 1) were found in the saline-injected mice (Fig. 2). Changes were found in amino acid patterns of livers of saline-injected mice after hydrazine injection (Fig. 3). There were marked increases in contents of alanine, glycine, serine, threonine, glutamine, citrulline, and ornithine. Increases in valine, glutamic and aspartic acids, and glutathione also were seen in several instances. Tyrosine (#14), not seen in control samples, appeared in extracts of the livers of the hydrazine-treated animals.

The injection of alanine or glutamic acid (Fig. 4 and 6, respectively), produced no significant changes in amino acid pattern. Although it is possible that these amino acids may not have entered the liver parenchyma, the more likely possibility is that these amino acids were utilized extremely rapidly in the liver. The changes produced when hydrazine was given after either of the latter 2 amino

acids (Fig. 5 and 7) were similar to those seen in the saline-injected animals. The injection of arginine alone (Fig. 8) or of a mixture containing arginine, alanine, and glutamic acid (Fig. 10) produced an elevation of ornithine content (spot #13) in mouse liver, but no other major detectable changes. This was understandable in view of the great amount of arginase activity normally found in liver, and showed that the rate of formation of ornithine through arginase activity exceeded the rate of its removal under the conditions of the experiment. In addition to the changes observed previously after hydrazine injection, there were increases in ornithine (Fig. 9 and 11) in the livers of mice receiving hydrazine after arginine or after administration of the amino acid mixture containing arginine. In the livers of hydrazine-treated animals after pretreatment with a mixture of arginine, glutamate, and alanine there were much smaller elevations in glycine, serine, and threonine contents by comparison with the animals receiving hydrazine alone and no apparent increments in glutamic and aspartic acid contents (Fig. 11). Previous work has shown that although pretreatment of mice with any 1 of the above 3 amino acids protected mice against single doses of hydrazine to some extent, there was a remarkable enhancement of the protection when all 3 were given together(2). The changes in amino acid levels suggested that hydrazine may cause widespread effects on amino acid metabolism. The above described changes, the increases in ornithine contents, and the protective effects of administered arginine suggested the possibility that in hydrazine toxicity the Krebs ornithine cycle and several transaminases might be inhibited. The findings of elevated levels of blood ammonia in animals intoxicated with hydrazine support such a supposition(9).

Semiquantitative and quantitative studies of citrulline contents. Because of the latter findings it was of interest to study the citrulline distribution in liver and other tissues of hydrazine-treated mice. One-dimensional chromatograms (ascending technic) were used on sheets of Whatman No. 1 paper which were 8½ × 7½ inches. The solvent system consisted of butanol-acetic acid-water (3-1-1), which moved picric acid to the top of the sheet and gave an excellent separation of urea from citrulline. Sheets were dipped in Ehrlich reagent‡ for color development. Known amounts of urea and citrulline, ranging from 1 to 10 μg were used as standards and evaluation was made by visual comparison of spot intensity with simultaneously run standards.

Studies of tissues by one-dimensional chromatography were then performed as follows. Five groups of mice were used, *viz.*, (1) untreated controls, (2) saline-injected, (3) arginine-treated, (4) hydrazine-treated, and (5) arginine- and then hydrazine-treated. Both compounds were administered intraperitoneally: arginine at 4 mmoles/kg and hydrazine at 3 mmoles/kg. In Group 5, hydrazine was administered 30 minutes after arginine. In some instances the mice were sacrificed in 30 minutes and the appropriate tissues were removed; in others, tissues were removed immediately after death from seizure (the time interval ranged from 15 to 40 minutes after injection of hydrazine). In each experiment from 3 to 5 mice were used and the tissues processed separately for each animal. Extracts of liver, kidney, brain, muscle, heart and lung were prepared. Samples containing the equivalent of 2.5, 5.0, and 10 mg of fresh tissue, respectively, were examined. In the hydrazine-treated mice, citrulline was detected only in the liver; all other tissues gave negative results. Citrulline was not detected in any of the tissues of the controls or in mice receiving arginine alone. Arginine enhanced the effect of hydrazine in the accumulation of citrulline. The concentration of citrulline in the livers of arginine-hydrazine-treated mice was increased many times over that in the livers of hydrazine-treated mice. Urea was found in considerable quantities in all tissues examined, both in control and hydrazine-treated animals. Citrulline was not found in the urines of either group of mice.

Quantitative determinations then were

‡ One g of p-dimethylamino-benzaldehyde was dissolved in 10 ml of concentrated HCl and 90 ml of acetone was added. The solution was prepared just before use.

TABLE I. Influence of Hydrazine Injection on Citrulline Content of Liver.*

Group	Treatment†	No. of mice	Citrulline content, μg/g fresh wt
1	Saline	8	21
2	"	8	7
3	Arginine	8	19
4	AGKO‡	8	101
5	Saline + hydrazine	8	267
6	" "	12	179
7	Arginine + hydrazine	8	1176
8	" "	10	1800
9	AGKO + hydrazine	9	2430

* All hydrazine-treated animals received intraperitoneal injections of 3 mmoles/kg and the livers were excised 30 min after the injection. Results shown are averages of closely checking replicate analyses from pooled samples obtained from the numbers of animals shown in Table.

† Those animals which were pretreated with protective substances received them by intraperitoneal injection 30 min before being given hydrazine. The animals in these groups not receiving hydrazine were sacrificed at 60 min after injection.

‡ Mixture containing arginine, glutamate, α-ketoglutarate, and oxalacetate. Each animal pretreated with 4 mmoles/kg.

made of the citrulline contents of extracts of pooled livers of normal mice injected 1 hour prior to sacrifice with saline, arginine, or a previously studied optimal protective mixture containing isomolar amounts of arginine, glutamate, α-ketoglutarate, and oxalacetate(3). Measurements also were made on extracts of livers of mice which were injected with the above substances, given hydrazine 30 minutes later, and sacrificed 30 minutes after the injection of hydrazine. The results are shown in Table I. Low levels of citrulline were found in the livers of the saline-injected animals (Groups 1 and 2). Administration of arginine (Group 3) did not produce a significant increase over the control level of citrulline or arginine (see preceding section), although increases in ornithine content were found (see Fig. 8 and 10). Argininosuccinic acid was below the level of detection on all chromatograms. The above findings suggest that the large amounts of arginine (4 mmoles/kg) administered to normal mice were disposed of rapidly. The increase in ornithine content of the liver after arginine injection may be attributable to a greater rate of formation of ornithine from arginine than of its metabolic or excretory removal. The 5-fold increase in citrulline content over the control levels observed when arginine was given together with glutamate, α-ketoglutarate, and oxalacetate (Group 4) suggests that under these circumstances the rate of formation of argininosuccinate from citrulline and aspartate may become rate-limiting in the function of the Krebs ornithine cycle. Approximately a 20-fold increase in citrulline content over the controls was observed 30 minutes after hydrazine was injected into the saline controls (Groups 5 and 6). Since only a relatively small increase in ornithine content was produced by this treatment (Fig. 2 and 3), it must be concluded that the effect of hydrazine on the ornithine cycle must be largely at the step forming argininosuccinate. The 100-fold increase in citrulline content observed when hydrazine followed arginine administration (Groups 7 and 8) and the much smaller relative increase in ornithine content (Fig. 8 and 9) also is consistent with the above suggestion. An even greater increment in citrulline content was found when the AGKO§ mixture was given prior to the hydrazine (Group 9). The above results suggest strongly that one of the points of attack of hydrazine might be at the condensation reaction which takes place between citrulline and aspartic acid to form argininosuccinate. Experiments now are in progress to test the effect of hydrazine on this reaction and on the formation of adenylosuccinic acid, a similar condensation reaction, in isolated systems.

Comment. Hydrazine can affect the function of a variety of vitamin B_6 enzymes (see ref.(10) for detailed discussion of actions of carbonyl-trapping agents). It is, therefore, not surprising that the contents of several of the free amino acids of liver that are known to undergo transamination (alanine, glutamic and aspartic acids, ornithine, and glycine) or are otherwise degraded by B_6 enzymes (serine and threonine) show increases in content in the livers of hydrazine-treated animals. A decreased rate of operation of the ornithine

§ This mixture contains arginine, glutamate, α-ketoglutarate and oxalacetate in isomolar amounts. The animals were given a single injection at a dose level of 4 mmoles/kg.

cycle by partial blockade of argininosuccinate synthesis could account for the increased diversion of ammonia to glutamine synthesis in the liver (Fig. 1-11) and increases in ammonia content in the blood(9) in hydrazine-treated animals.

It is not readily apparent how the above findings in liver could explain in any way the typical tonic-clonic convulsions observed in animals intoxicated with hydrazine. Extracts were prepared from livers of normal and hydrazine-treated mice by heat coagulation at pH 5.5, filtration, and concentration, and comparable graded amounts of the extracts were injected into mice. In no instance was there an indication of the presence of toxic seizure-producing material in such extracts.

It may tentatively be suggested that hydrazine entering the brain in small amounts may act directly on some neural elements or possibly on glial or endothelial cells in the CNS producing an effect which results in hyperexcitability but which does not result in major changes in nitrogen or carbohydrate metabolism. A preliminary indication that the structural properties of capillaries might be affected by hydrazine was the finding that lung hemorrhages were observed by us often in animals that had died as a result of tonic-clonic convulsions induced by hydrazine, but not in animals in which similar convulsions were produced by other means such as electro-shock or Metrazol. Hydrazine-treated mice killed just prior to the anticipated onset of seizures also showed no evidence of lung hemorrhage. The above results are consistent with the interpretation that the strength of the capillaries may be decreased by hydrazine and that the tremendously increased pressure in the lung occurring during the seizures may cause the rupture of the weakened vessels (see ref. 10 for suggestion that hydrazine might affect collagen structure). Needless to say, if structural alterations were to be produced in cerebral capillaries, there would be expected to be serious physiological derangements.

Summary. Two-dimensional paper chromatographic methods were employed to study the changes produced in amino acid distribution in tissues of mice treated with hydrazine and in mice which had been pretreated with protective substances prior to the administration of hydrazine. The data indicate that the time of occurrence of the symptoms of acute hydrazine poisoning (tonic-clonic convulsions) coincided with significant changes in free amino acid distribution in liver but not with any major effects in brain. In all animals which had been injected with hydrazine increases in citrulline content were observed in liver, but not in other tissues or urine. When injection of arginine alone or arginine together with other substances was followed by hydrazine, remarkable increases in liver citrulline levels were found. There was no indication of the accumulation of argininosuccinic acid. The data suggested that in livers of hydrazine-poisoned animals the condensation reaction of citrulline with aspartic acid to give argininosuccinic acid might become rate-limiting in the operation of the ornithine cycle and that the function of several other amino-acid metabolizing systems also might be disturbed. It is not yet clear whether or not any relationship exists between metabolic changes in liver and the often-fatal convulsions. Incidental observations also suggested the possibility that hydrazine might have effects on capillary fragility and permeability.

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