Glutamine and Glutaminase in Blood

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RECENTLY THE NEED of a method for the determination of glutamine and glutaminase in the blood and urine of humans led to the evaluation and adaptation of a single system for both. Methods of glutamine assay available depend on the determination of the ammonia liberated by mild hydrolysis with dilute hydrochloric, sulfuric, or trichloroacetic acid, or alkali, heat at neutral *p*H, or the action of the specific enzyme, glutaminase (1). Other methods measure the decreased reactivity with nitrous acid or ninhydrin that occurs when glutamine undergoes hydrolysis and condensation to ammonium pyrrolidone carboxylate. Krebs (2) demonstrated that the cortex of kidneys of sheep, guinea pig, pig, and rabbit contained an enzyme, glutaminase, which hydrolyzes glutamine to ammonia and glutamic acid. Archibald (3) has described the preparation and standardization of kidney glutaminase in a form which makes the enzyme readily applicable for microestimation of glutamine in blood and other biologic materials. Seegmiller, Schwartz, and Davidson (4) have described a glutaminase preparation of bacterial origin. It has been observed in rats that renal glutaminase activity increases fourfold during adaptation to ammonium chloride acidosis (5). This led us to choose rats previously treated with NH_4Cl as the source of glutaminase preparation.

PREPARATION OF GLUTAMINASE EMULSION

Adult rats (250–300 Gm.) are made acidotic by giving them as drinking water a 2% NH_4Cl solution for 7 days. At the end of this period the animals are sacrificed by a blow on the head. The kidneys are removed and homogenized at 5° in a Waring Blendor in sufficient 0.9% saline to give a final concentration of 5 per cent. The homogenate is treated with

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5 ml. of 0.12M sodium cyanide and frozen for future use. This enzyme preparation is used for glutamine assay in blood.

ASSAY OF GLUTAMINASE ACTIVITY

Reagents and Equipment

1. 0.2M 2-amino-2-(hydroxymethyl)-1,3-propanediol
2. 0.6M Na_2HPO_4
3. 0.12M NaCN
4. 50% $HClO_4$
5. 0.2M glutamine solution
6. NH_3-free water
7. 2M H_3PO_4
8. 5-ml. Pyrex tubes with glass stoppers
9. Incubator set at 38°

Procedure

This procedure is that of Rector, Seldin, and Copenhaver (5) with modifications. To two 5-ml. Pyrex test tubes equipped with glass stoppers, add to each 0.30 ml. of 2-amino-2-(hydroxymethyl)-1,3-propanediol, 0.40 ml. of Na_2HPO_4 solution, and 0.15 ml. of NaCN solution. The *p*H of this mixture is adjusted to 7.5 with 2M H_3PO_4. Add to one tube 1 ml. of serum, plasma, or whole blood, 0.20 ml. of glutamine solution, and 0.20 ml. of NH_3-free H_2O. Add 1 ml. of serum, plasma, or whole blood and 0.40 ml. of NH_3-free water to the second tube. The tube contents are mixed well and incubated for 4 hours at 38°. After incubation add 0.15 ml. of $HClO_4$ to each tube, centrifuge, and take aliquots for NH_3 analysis. Glutaminase activity is therefore a function of the NH_3 liberated from the glutamine solution corrected for free NH_3 in the blank, and expressed as μM of NH_3 liberated per 100 ml. of plasma.

The glutaminase activity of the enzyme preparation is measured as above using 1.00 ml. of homogenate preparation in the place of blood and incubating the tubes for 1 hour at 38°. An aliquot is removed for NH_3 analysis. An aliquot of the homogenate preparation is dried at 100° in a tared tube and weighed. The glutaminase activity is expressed as μM of NH_3 per 100 mg. of dry tissue per hour.

ASSAY OF GLUTAMINE IN BLOOD

Reagents and Equipment

1. Same as those for glutaminase activity.
2. Glutamine standards (1, 2, and 3 μM/ml.).

Procedure

To each of two 5-ml. Pyrex test tubes equipped with glass stopper add 0.30 ml. of 2-amino-2-(hydroxymethyl)-1,3-propanediol, 0.40 ml. of Na_2HPO_4 solution, and 0.20 ml. of NaCN solution. Adjust the *p*H of this mixture to 7.5 with 2M H_3PO_4. Add 1 ml. of plasma, serum, or whole blood to the two tubes. Add 0.50 ml. of kidney homogenate to the sample tube, and 0.50 ml. of NH_3-free water to the blank tube. The contents of the tubes are mixed and incubated for 4 hours at 38°. An aliquot is removed for NH_3 analysis. An additional blank is prepared containing 1 ml. of H_2O and 0.5 ml. of kidney homogenate. This blank is to correct for any additional NH_3 arising from the homogenate during incubation. Glutamine standards of 1, 2, and 3 μM/ml. are analyzed with each run.

NH_3 ANALYSIS

Reagents and Equipment

1. Hydrochloric acid, stock solution: Standardized 0.100N HCl. Working sample: Dilute stock standard 1:20 to give 0.0050N HCl.
2. Barium hydroxide: From a saturated solution of $Ba(OH)_2$ prepare a working standard. Dilute stock 1:20 for approximately 0.03N $Ba(OH)_2$.
3. Microtitration table with mounted Scholander buret (7).
4. Beckman *p*H meter with standard probe electrodes.
5. Conway's microdiffusion cells.
6. Saturated K_2CO_3.

Procedure

The measurement of ammonia formed by glutaminase action on glutamine in blood requires an accurate microdetermination of ammonia. Conway's microdiffusion technic (6) was employed with modifications. One milliliter of 0.0050N HCl is placed in the center well. The outer well contains aliquots for NH_3 analysis and 1 ml. of saturated K_2CO_3. The cell is sealed and incubated for 45 minutes at 38°. The HCl from the center well is transferred quantitatively to a 3-ml. vial, using approximately 1 ml. of NH_3-free water to assist in the transfer. This solution is then titrated to *p*H 7.5 with $Ba(OH)_2$, using an electrometric titration assembly as described by Seligson (7).

RESULTS AND DISCUSSION

Each homogenate preparation is analyzed for glutaminase activity and free NH_3. It has been noted that the enzyme preparation progressively shows a decrease in glutaminase activity and an increase in free NH_3.

Table 1 gives glutaminase activity and free NH_3 values over a period of 1 month.

Table 1. Glutaminase Activity and Free-NH_3 Rate

Day	μM of NH_3/100 mg. of dry tissue per hour	Free NH_3 (μG)
Initial	176	3.0
0	176	3.0
4	168	4.5
11	155	5.0
23	118	15.0
31	97	25.0

If the free-NH_3 value exceeded 15 μg. per 0.5 ml. of homogenate, the use of this mixture was discontinued because high NH_3 values decreased the accuracy of the determination. The glutaminase activity listed here is lower than that reported by Rector, Seldin, and Copenhaver (5). In this procedure incubation was at *p*H 7.5 for 1 hour, instead of *p*H 8.0 for 30 minutes. These differences account for the lower activity; however, this preparation does give enzymatic action suitable for the determination of glutamine in blood. Plasma from normal humans did not show any glutaminase activity using this method.

Table 2 shows the per cent conversion of glutamine to glutamic acid plus NH_3. This range represents the values obtained from at least five daily analyses over the period of one month. Commercial glutamine is known to contain impurities, therefore, correction for NH_3 liberated without the addition of the enzyme preparation was subtracted from the amount produced with the enzyme. The 1- and 2-μM/ml. standards are of the order of glutamine in blood and show a conversion range of 95–105 per cent. Standards must be analyzed with each run to determine variations in the effect of the enzyme as a result of preparation, age, or incubation time.

Table 2. Per Cent Conversion of Glutamine Standards

Glutamine Standards	Glutamine mg./100 ml.	% Conversion (Range)
1 μM/ml.	14.6	95–105
2 μM/ml.	29.2	95–98
3 μM/ml.	43.8	75–90
4 μM/ml.	57.4	60–80
5 μM/ml.	72.0	50–70

The level of glutamine in 100 ml. of fasting dog or human plasma is 5–12 mg., as reported by Hamilton and Archibald (1) using different

procedures. Hamilton used the ninhydrin–carbon dioxide method, and Archibald used a glutaminase method. The method described herein gave results ranging from 8.0 to 11.1 mg. per 100 ml. of plasma for normal humans. Hamilton concluded also that red cells have approximately the same concentration of glutamine as the surrounding plasma. This was confirmed using this method. Table 3 shows comparison of the two sources.

Table 3. GLUTAMINE IN MG./100 ML.

Whole blood	*Plasma*
11.6	10.8
11.4	10.5
11.5	10.4

Figure 1 shows a comparison of results obtained in the plasma of normal persons, patients with liver disease, and patients with liver coma after glutamic acid infusion. Patients with liver disease showed high values

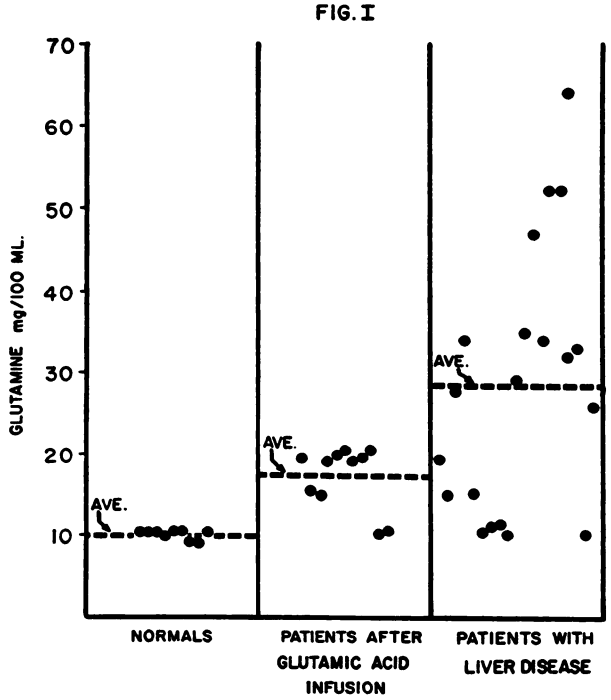

Fig. 1. Mean values ± standard deviation: Normals—10.4 ± 0.64 mg./100 ml.; Patients with severe liver disease—28.2 ± 9.8 mg./100 ml.; Patients after glutamic acid infusion—16.7 ± 2.0 mg./100 ml.

ranging from 10.8 to 60.0 mg. of glutamine per 100 ml. of plasma. These samples were analyzed after 3 months' storage in a deep freeze. Seegmiller and associates (4) obtained a mean value of 8.55 mg. of glutamine per 100 ml. of plasma for normal humans, and 10.3 mg. per 100 ml. for patients in hepatic coma. Although they concluded that patients in hepatic coma are within the normal range, 45 per cent of their patients had glutamine values outside two standard deviations of their normal range. In the present study 75 per cent of the values in liver disease were outside two standard deviations of the normal range. This discrepancy is due to at least two factors. The variation of the normal values is less in the present study (standard deviation 0.64 mg./100 ml.) than in the study of Seegmiller *et al.* (0.98 mg./100 ml.). In addition, four of the values (20 per cent) in the present study are far above the range observed for the other values or the range of Seegmiller. These determinations are on 2 patients who died of their disease within 24 hours of the study and may represent more severe disease than the others or the patients of Seegmiller.

Glutamic acid (8) and ammonia (9) are elevated in the blood in patients with liver disease and glutamine is elevated in the tissues (10). Both glutamic acid and ammonia are removed by the formation of glutamine (11). Therefore it was not unreasonable to search for an elevated plasma glutamine level in liver disease. That the glutamine was not significantly elevated after the infusion of glutamic acid was, therefore, an unexpected finding.

Asparagine was added to standard glutamine solution to determine if rat kidney glutaminase would liberate NH_3 from asparagine. Results indicate that asparagine in the range of 1–10 μM does not yield NH_3 when treated with rat-kidney glutaminase. Archibald (1) has shown that ammonia is formed from adenosine and guanosine by dog-kidney glutaminase. Such experiments were not performed with the rat preparation, but similar behavior toward the purines is to be expected. However, these substances are not present in significant quantities in normal blood.

Ultrafiltrates of blood were obtained and the glutamine measured to determine if the liberated NH_3 obtained from plasma was derived from glutamine or nonfilterable precursors. Analysis of the filtrate gave values of 12.1 to 13.0 mg. of glutamine per 100 ml. of filtrate.

Recovery of glutamine from blood gave the values listed in Table 4. The "glutamine-found" column represents the difference between total glutamine and the plasma level (11.0 mg./100 ml.).

Table 4. Recovery Experiment

Run	Glutamine Added mg./100 ml.	Glutamine Found mg./100 ml.	% Recovery
1	13.2	14.0	106
2	27.3	26.7	98
3	34.8	35.6	102
4	26.2	24.6	94

An attempt was made to determine glutamine in urine by this procedure, but high NH_3 values obtained from urine made it impractical. The procedure for measuring glutamine and glutaminase in blood as presented here is believed to be a simple and accurate procedure. The ready availability of rat-kidney homogenate makes this a convenient method.

SUMMARY

A convenient and accurate procedure for the determination of glutaminase activity and glutamine in blood has been described.

Kidney homogenate from rats made acidotic by feeding NH_4Cl was used as a glutaminase source and the Conway microdiffusion method was used to determine ammonia.

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