TABLE III.—SOLUBILITY CHANGE, PER CENT DECREASE RELATIVE TO SOLUBILITY IN WATER, FOR ACETANILIDE AND SEVERAL DERIVATIVES, IN GOING FROM WATER TO 63.4% SUCROSE SOLUTION

Solute	Solubility in Water, mg./ml.	Solubility in 63.4% Sucrose Soln., mg./ml.	Decrease, %
Acetanilide	6.38	4.25	33
p-Methylacetanilide	1.05	0.77	27
p-Ethoxyacetanilide	0.93	0.59	36
p-Hydroxyacetanilide	13.85	9.80	29
p-Aminoacetanilide	15.98	14.02	12
Acetoacetanilide	9.87	4.96	50

vantage in achieving solubility or in avoiding problems due to increased or decreased solubility.

Although there is no sweeping involvement of the dielectric constant as either the parameter of choice or the mechanism involved, it may be a useful tool to the formulator in considering solubility problems.

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Metabolism of ^{14}C-Labeled Glutamic Acid and Pyroglutamic Acid in Animals

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A useful method has been developed for the paper chromatographic separation and identification of certain amino acids found in biological fluids. A synthetic route to ^{14}C-labeled pyroglutamic acid was developed. The ^{14}C-labeled glutamic acid and pyroglutamic acid were given orally to mice and rabbits. The drug concentration in various tissues was determined utilizing a chromatogram scanner or a liquid scintillation counter. Experimental data indicated that the metabolic products formed following glutamic acid therapy were pyroglutamic acid, γ amino butyric acid, and glutamine. Animals to which the labeled pyroglutamic acid had been administered showed radioactivity present as γ amino butyric acid and glutamic acid.

GLUTAMIC ACID is an important amino acid. Although it is not essential for growth, it has been known for many years to be a major constituent of body protein and to take part in many metabolic processes. In clinical therapy the monosodium salt has been used in place of the free acid because L-glutamic acid is only slightly soluble in water and is absorbed slowly by ingestion. On the other hand the monosodium salt, which is soluble to the extent of over 70% at room temperature is readily absorbed (1, 2). Monosodium glutamate has been tested in many types of neurological and psychiatric cases with positive results (3, 4) and with negative results (5). However, glutamic acid therapy is a problem from two points of view. First, large doses are required; and second, its taste is difficult to mask.

In 1944, Ratner demonstrated the formation of D-pyroglutamic acid in rats fed DL-glutamic acid (6). Wilson and Koeppe determined labeled carbon dioxide excretion, pyroglutamic acid formation, and tissue glutamic acid concentrations after the administration of D- and L-glutamic acid-2-^{14}C and DL- or D-glutamic acid-5-^{14}C (7). They found that when labeled D-glutamic acid was administered in small doses, intraperitoneally or by stomach tube, more than 50% of the radioactivity was excreted in the urine in 24 hr., most of it as D-pyroglutamic acid. There has been a great deal of investigation of the metabolism of pyroglutamic acid in animals and man. Bethke and Steenboek found that it was converted to glutamic acid and postulated that it was an enzymatic transformation (8). However, no reports of the presence of pyroglutamic acid in brain tissue were found. Thus, it is proposed to develop a micro-method which could accurately determine the distribution of glutamic acid or a metabolic product in various tissues following oral administration of glutamic and pyroglutamic acid. If the oral administration of pyroglutamic acid shows the same compound or compounds are crossing the blood–brain barrier then possibly it can be used in place of glutamic acid to give a similar therapeutic response at a lower dosage.

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METHODS AND PROCEDURES

The paper chromatographic procedure described below is based on a method reported by Clayton and Strong (9).

Paper Chromatography.—Standard solutions were prepared of various amino acids. Known quantities of the solutions were spotted on strips of Whatman No. 1 chromatographic paper. The strips were

dried and developed by the descending techniques in chromatographic tanks containing a solution consisting of methyl ethyl ketone, propionic acid, and water (75:25:20). The strips were developed at 25° for 8 hr., removed from the tanks, and air dried. The strips were then sprayed with a 0.2% solution of ninhydrin in alcohol and activated in an oven at 80°. All of the amino acids appeared as characteristic pink to purple colored spots on the paper except for pyroglutamic acid. The strips were placed in an atmosphere of chlorine vapors for 5 min., allowed to hang in a hood overnight, and then sprayed with a starch–potassium iodide solution to show the pyroglutamic acid as a blue-black spot on a light blue background (10). The R_f values calculated for the various amino acids of interest were:

DL-Glutamine	0.22
DL-, L-, and L(+) Glutamic acid	0.29
γ Amino butyric acid	0.52
Glutathione	0.55
Pyroglutamic acid	0.68

Synthesis of Labeled Pyroglutamic Acid.—A measured solution of glutamic acid-1-^{14}C, buffered to a pH of 2.0 with hydrochloric acid, was sealed in a glass ampul, and placed in an oven at 130° for 72 hr. After the ampul was cooled, it was opened and a sample subjected to paper chromatographic examination. The paper strips, after chromatography, were run through a chromatogram scanner, model RSC-160 by Atomic Accessories, Inc., at a rate of 1.5 in./min. with a constant flow of Geiger gas. The rate meter attachment was a Tracermatic by Tracer Labs. Standard curves of glutamic and pyroglutamic acid were obtained by plotting for a scan of a given number of counts per minute the area under the curve in centimeters against the number of gamma's of isotope spotted. The conversion of glutamic acid to pyroglutamic acid was nearly quantitative.

Liquid Scintillation Detection.—To increase the sensitivity for the detection of micro quantities of the amino acids in the tissue samples, the paper strips were cut into small portions and placed in scintillation fluid. The fluid used consisted of 0.1 Gm. of 1,4-di-2-(5-phenyloxazolyl)-benzene, 14 Gm. of 2,5-diphenyloxazole, 100 Gm. of naphthalene (C.P.), and sufficient dioxane to make 2 L. The liquid scintillation counter was from Baird Atomic, Inc. From the R_f values for the various amino acids, the activity found in various portions of the paper strips could be identified.

Comments on the Analytical Procedures.—It was found that the paper strip could not be chlorinated before spraying with ninhydrin if the ninhydrin-positive amino acids were to be detected. It was also noted that the chlorination step could not be speeded up without a loss of sensitivity. Thin-layer chromatography was tried. Satisfactory separation of the amino acids was not obtained.

BIOLOGICAL TESTING

Investigation with Rabbits.—A fasted rabbit was fed 200 μl. of glutamic acid-1-^{14}C, which contained 20 μc. of activity in 20 Gm. of glutamic acid carrier. After administration of the drug, 2-ml. samples of blood were obtained from the rabbit by cardiac punctures at 0.5, 1, 2, 3, 4, 6, 18, and 24-hr. intervals. One milliliter of a 10% solution of trichloroacetic acid was added to the blood sample, the suspension mixed, centrifuged, and the clear supernatant fluid separated. A 100-μl. sample of the supernatant was spotted on a strip of Whatman No. 1 chromatography paper and the strips chromatographed in the previously described developing system. The strips were allowed to develop for 6 hr., dried, and run through the chromatogram scanner with a rate meter attached. A 250-μl. sample of the supernatant fluid was added to 15 ml. of scintillation fluid in low ^{40}K vials, the mixture shaken, and counted in a liquid scintillation counter to give the total activity for the sample. Portions of the chromatographed strips were treated with scintillation fluid and the resulting solutions counted as before. From the R_f values found for the various amino acids, the source of radioactivity found in the samples could be identified.

The pyroglutamic acid-1-^{14}C, which had been prepared from the labeled glutamic acid, was fed to a 2.5-Kg. rabbit. The procedure followed was the same as for the labeled glutamic acid study except that 300 μl. of pyroglutamic acid-1-^{14}C in a 3-Gm. carrier solution of pyroglutamic acid was used as the dose.

Investigation with Mice.—Three white mice, each weighing approximately 20 Gm., were fed 1 ml. of a glutamic acid carrier solution which contained 1 μc. of glutamic acid-1-^{14}C. After 3 hr., the mice were heparinized and sacrificed. The blood from the three mice was pooled, the protein precipitated with trichloroacetic acid, the mixture centrifuged, and the supernatant fluid separated. The brain was enucleated from each mouse and washed with three portions of sterile saline solution at 5°. The brains from the three mice were pooled, homogenized with 2 ml. of cold saline solution, the mixture centrifuged, and the clear liquid collected. The kidneys of each mouse were treated in a similar manner. Control tissues were similarly prepared from untreated mice. Fifty microliters of the three different tissue samples were spotted on strips of Whatman No. 1 chromatography paper. The strips were developed in the previously described manner. The chromatographed strips after drying were run through the chromatogram scanner. No clearly defined areas of radioactivity could be detected. The strips were then cut into sections and placed in ^{40}K free glass vials, each containing 15 ml. of scintillation fluid. Radioactivity was detected in some of the vials. Knowing the area (R_f) of the

TABLE I.—SERUM LEVELS OF LABELED AMINO ACIDS FOLLOWING ADMINISTRATION OF LABELED GLUTAMIC AND PYROGLUTAMIC ACIDS TO RABBITS[a]

	Glutamic Acid[b]	Pyroglutamic Acid[c]
Glutamic acid	36[d]	60
Glutamine	28	..
γ Aminobutyric acid	..	60
Pyroglutamic acid	32	20

[a] Radioactivity obtained from a liquid scintillation counter of 100 μl. of a 3-hr. serum sample chromatographed on paper and extracted with scintillation fluid. [b] Dose of 250 μl. in 20 Gm. of carrier solution (20 μc. of activity). [c] Dose of 300 μl. in 2 Gm. of carrier solution (20 μc. of activity). [d] Average number of counts/min. for six animals.

TABLE II.—TISSUE LEVELS OF LABELED AMINO ACIDS FOLLOWING ADMINISTRATION OF LABELED GLUTAMIC AND PYROGLUTAMIC ACID TO MICE[a]

	Glutamic Acid[b]			Pyroglutamic Acid[b]		
	Serum	Brain	Kidney	Serum	Brain	Kidney
Glutamic acid	42[c]	40	46	. . .	29	. .
Glutamine	. .	28	36	. . .	. .	. .
γ Aminobutyric acid	. .	. .	. .	. . .	. .	82
Pyroglutamic acid	30	. .	. .	310	. .	. .

[a] Radioactivity obtained from a liquid scintillation counter of 100 μl. of a 3-hr. tissue sample chromatographed on paper and extracted with scintillation fluid. [b] Dose of 1 ml. of glutamic or pyroglutamic acid carrier with 1 μc. of activity. [c] Average number of counts/min. for three groups of three animals/group.

strip associated with the vials having radioactivity, it was possible to predict the amino acids involved.

The entire procedure was repeated with groups of three mice being fed pyroglutamic acid-1-^{14}C. The dosage was the same as for the glutamic acid study.

RESULTS AND DISCUSSION

In the synthesis of the labeled pyroglutamic acid from the labeled glutamic acid, it was found that the 72-hr. heating at 130° gave better than a 98% conversion of glutamic acid to pyroglutamic acid. Heating for 24 hr. at the same temperature gave about a 95% conversion, while heating at 100° for 24 hr. gave only about an 80% conversion.

Tables I and II summarize the distribution of the different labeled amino acids found in the various tissues after the oral administration of labeled glutamic and pyroglutamic acid to rabbits and mice. No attempt was made to determine absorption rates. However, when the total concentration of labeled pyroglutamic acid and labeled glutamic acid in the tissues studied are compared it can be seen that at the 3 hr. sampling time, despite a lower dose being given, the pyroglutamic acid is more completely absorbed.

The paper strips which contained the chromatographed samples from the blood of the rabbits fed labeled glutamic acid showed only the presence of glutamic acid and pyroglutamic acid when they were run through the chromatogram scanner. The acids were identified by their R_f values on the paper, as well as their color following spraying of the strips with the described indicator solutions. The presence of labeled glutamine in the blood samples was not detected until the strips were cut into sections and examined with the liquid scintillation counter. The highest radioactivity levels in the blood were found between 2 and 4 hr. after oral administration of the labeled glutamic acid. These activity levels were found to be equivalent to approximately 4% of the administered dose of labeled drug. The chromatogram scanner did not differentiate the radioactivity source on paper strips prepared from the blood of rabbits fed the labeled pyroglutamic acid. It was possible, however, to differentiate the amino acids containing the radioactivity using the liquid scintillation counter.

In the mice, the highest tissue concentration of radioactivity was found to be between 2 and 4 hr. after administration of the labeled acids. In all of the studies with mice, the chromatogram scanner was not able to differentiate the sources of radioactivity in the tissues examined. After 3 hr., the mice which had been fed the labeled glutamic acid were found to have the greatest concentration of radioactivity in the kidney, while after pyroglutamic acid adminstration the highest activity was found in the blood.

Attempts to obtain autoradiograms from contact of the chromatographed strips with X-ray film were not successful. Various concentrations of the tissue fluids were chromatographed as well as various development times were tried for the X-ray film. It was thought that the autoradiograms might give a second method for checking R_f values for the labeled amino acids.

SUMMARY

It has been found that the metabolism of labeled glutamic acid and pyroglutamic acid in mice can be followed using a combination of paper chromatography and liquid scintillation counting. Labeled pyroglutamic acid was found to be absorbed after oral administration and converted to glutamic acid. The labeled glutamic acid was further found to be present in brain tissue while the pyroglutamic acid was not found in the brain tissue at any time. Thus, it might be possible to obtain a therapeutic response similar to that of glutamic acid after oral administration of pyroglutamic acid.

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