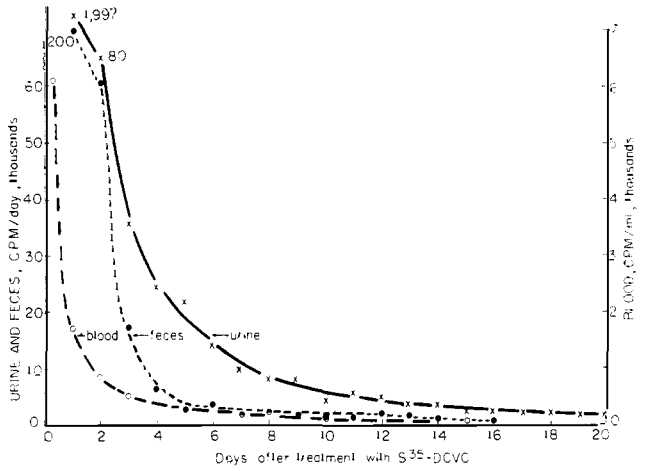

Fig. 1.—Radioactivity (CPM) of blood, urine and feces of rat treated intraperitoneally with S^{35}-DCVC.

essentially the same in both rats. In contrast, the total urinary excretion of S^{35} by the orally treated rat was, during the first 24 and 72-hour intervals, only 40% of that excreted by the parenterally treated animal. The radioactive components in the urine included inorganic sulfate, DCVC and its N-acetyl derivative as reported elsewhere.[10] The fact that in spite of a high rate of clearance of DCVC through the kidneys, excretion of the S^{35} isotope continued for a long period indicates that DCVC or a sulfur compound derived from it combined with components of tissues and thus escaped the initial rapid clearance from the blood stream.

Experimental

S^{35}-Benzyl Mercaptan (I).—The procedure of Tarver and Schmidt[11] was adapted for use of BaS^{35} (30 mc.)[12] and scaled down to obtain preparations of high specific activity. The reactions were carried out under a slow stream of nitrogen in a closed system from which emerging gas passed through a solution of ethanolic 2% $HgCl_2$.

S^{35}-Benzyl-L-cysteine (III).—Methyl 2-amino-3-chloro-L-propionate was prepared according to Fischer and Raske[13] except that diethyl ether was substituted for petroleum ether to wash the product. This ester (800 mg.) was hydrolyzed with 135 ml. of 5.8 *N* HCl and the solution concentrated to dryness *in vacuo*. The residue of 2-amino-3-chloro-L-propionic acid hydrochloride (II) was dissolved in 5 ml. of absolute ethanol and injected with a long needle and syringe through the vented stopper into the flask containing S^{35}-benzyl mercaptan (I).[14] The mixture was stirred by a continuous flow of nitrogen which escaped through the vent into a 2% ethanolic solution of $HgCl_2$. Sodium ethoxide (0.5 g. of Na/10 ml. of absolute ethanol) was added dropwise until the thymolphthalein indicator turned blue and 0.4 ml. more was added. The flask was then heated at 72° for 2 hr. and more sodium ethoxide and/or ethanol added as needed. After cooling, the precipitates of NaCl and KCl were centrifuged, the supernatant removed, and the precipitate washed 3 times with absolute ethanol. The supernatant and washings were evaporated to dryness under a stream of nitrogen while warming the flask to 40°. The vapors were passed through an ethanolic $HgCl_2$ trap. The residue was dissolved in 30 ml. of distilled water and allowed to stand overnight at room temperature. The aqueous solution was extracted 5 times with a total of 40 ml. of ether to remove dibenzyl sulfide and disulfide which were present as solids or oils. The ether extracts were combined and extracted 3 times with water, the first washing being done with water containing a drop of *N* NaOH. The aqueous extracts were combined with the extracted aqueous solution, adjusted to pH 5.5 with HCl (pH paper) and concentrated by drawing air across the surface with slight warming. The concentrated solution was cooled in a refrigerator to obtain crystalline III. It was filtered, washed with cold 50% ethanol, ethanol, ether, and dried *in vacuo* over P_2O_5. The supernatant and washings were concentrated to yield a second crop of crystals. S^{35}-Benzyl-L-cysteine (III) was isolated in crystalline form, m.p. 209–211° dec. (corr.) from four different preparations in yields from 35 to 50%.

Chromatography in 1-butanol:acetic acid:water (12:3:5 v./v.) was done on Whatman No. 1 paper, descending, and detection of the compound through the iodoplatinate, or ninhydrin reactions or through scanning for radioactivity. Quantitative measurement of the radioactivity of 1 cm. segments of the chromatograms revealed that more than 99% of the activity was localized in one spot. The R_f and m.p. were identical with those of a non-radioactive specimen prepared from disodium cysteinate and benzyl chloride in liquid ammonia.[15]

S^{35}-(Dichlorovinyl)-L-cysteine (IV).—For synthesis of this compound by a modification of earlier procedures,[5,6] III was dissolved in about 10 ml. of liquid ammonia in a 100 ml. cylindrical centrifuge tube which was cooled in a bath of trichloroethylene-solid carbon dioxide. It was reduced to dibenzyl and disodium S^{35}-L-cysteinate by the addition, with stirring, of small amounts of sodium until a blue color remained for at least 2 min. Trichloroethylene (0.35 ml.) was added, all at once, and the reaction mixture stirred for 45 min. The cooling bath was removed, and the contents of the tube were evaporated to dryness under slight vacuum. The residue was dissolved in 5 ml. of hot water, and transferred to a 25 ml. erlenmeyer flask. The pH was adjusted to 5.4 with HCl (pH paper) and the volume reduced by heating slightly on a hot plate while drawing air across the surface. An equal volume of absolute ethanol was added and the solution was cooled to −5° to induce crystallization. The product was filtered, washed with 50% ethanol, ethanol, and ether; it was dissolved in water, treated with a small amount of Darco G60, filtered and recrystallized from 50% ethanol. Concentration of the filtrates afforded a second crop of crystals.

The radioactive DCVC was obtained in yields up to 37.4%, based on 2.07 m*M* KOH used in reaction (2) above. The product of each of 4 different preparations had m.p. 160° dec. (corr.) and moved as a single component in chromatography using the procedures referred to above. No radioactive impurities could be detected. The specific activities for 4 preparations ranged from 7.44 to 58.8 × 10^6 DPM/mg. as measured with a Nuclear-Chicago D-47 detector fitted with a micromil window and having a counting efficiency of 30.8%.

(10) For identification of the major S^{35}-containing compounds as inorganic sulfate, DCVC and N-acetyl DCVC, paper chromatography, paper electrophoresis and dilution analysis of the eluted fractions were used. In addition, inorganic sulfate was identified through precipitation as barium sulfate and benzidine sulfate. The 2,4-dinitrophenyl derivative of DCVC-S^{35} from urine was identical with an authentic specimen (m.p. 160°). Authentic specimens of N-acetyl DCVC (m.p. 108–109°) and its *m*-toluidide (m.p. 150–151°) served to identify N-acetyl-DCVC-S^{35} from urine. Details of the methods used are described elsewhere (R. F. Derr, Ph.D. Thesis, University of Minnesota, 1960; R. F. Derr and M. O. Schultze, *Biochem. Pharmacol.*, in press).

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N-(γ-L-Glutamyl)aminobenzoic Acids

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Of a group of N-(phenylalkyl)glutamines which were recently prepared and examined for antimicrobial activity, N-benzyl-L-glutamine was an effective antagonist of the natural amino acid.[2] In addition, other

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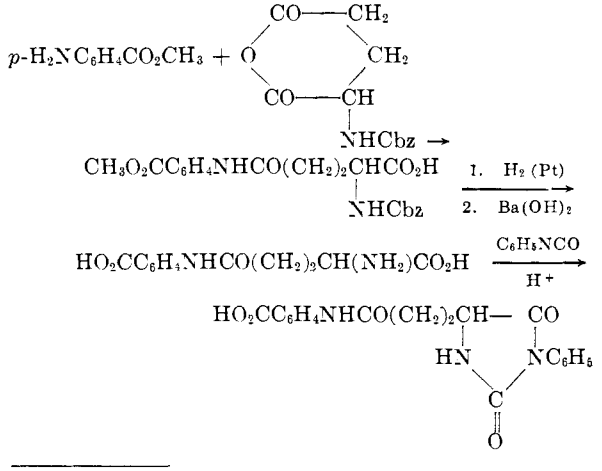
γ-glutamyl derivatives have been isolated either from natural sources[3,4] or prepared synthetically and found to inhibit the growth of microörganisms.[5,6] In an effort to study the effect of the presence of moderately reactive groupings in augmenting the toxicity of this type of metabolite analog, a series of N-phenyl derivatives of glutamine were prepared which contain an *o*-, *m*- and *p*-carboxyl group on the aromatic nucleus. In addition, because of the presence of the naturally occurring *o*- and *p*-aminobenzoic acid moieties in the corresponding glutamine condensation products, these two analogs were also examined as potential intermediary metabolites in a number of assay systems. This was further suggested by the fact that glutamine contributes a nitrogen in the biosynthesis of both anthranilic acid and *p*-aminobenzoic acid from shikimic acid.[7]

As indicated in the equations, the syntheses of the N-(γ-L-glutamyl)aminobenzoic acids were accomplished through a series of reactions involving the initial condensation of N-carbobenzoxy-L-glutamic anhydride with the appropriate methyl aminobenzoate to give the corresponding methyl ester of N-carbobenzoxy-γ-L-glutamylaminobenzoic acid. In order to obtain an acceptable yield of the *meta* derivative, it was necessary to extend the reaction time for this isomer approximately eight-fold over that required for the *ortho* and *para* analogs. Hydrogenolysis of the condensation products yielded the corresponding γ-L-glutamylaminobenzoic acid methyl esters which were then hydrolyzed with barium hydroxide to form the desired products. Using an ascending paper chromatographic technique, and developing the chromatograms with ninhydrin reagent, each of the products was demonstrated to be chromatographically pure in two different solvent systems.

Since the interaction of N-carbobenzoxyglutamic anhydride with the various aminobenzoic acids could yield the amide linkage through either of the two carboxyl groupings in the glutamyl moiety, the chemical structures of these analogs were subsequently demonstrated through the formation of hydantoin derivatives. Using the general procedure of Ware,[8] each of the glutamylaminobenzoic acids interacted with phenyl isocyanate, and in each case a hydantoin was formed, indicating the presence of a free α-amino group adjacent to a carboxyl moiety.



The biological activities of the glutamylaminobenzoic acids were uniformly disappointing. In contrast to the inhibitory activity of N-benzylglutamine,[2] none of the three analogs was toxic to growth of *Streptococcus lactis*, *Lactobacillus arabinosus* or *Escherichia coli*, at their limits of solubility in the assay media (about 500 μg./ml.). Because of the presence of the naturally occurring anthranilic and *p*-aminobenzoic acid moieties in the condensation products, the compounds were also examined for growth promoting activity. A number of studies were carried out using *L. arabinosus* in an attempt to replace a tryptophan response in this organism by N-(γ-glutamyl)-*o*-aminobenzoic acid; however, no growth-promoting effects were observed. Subsequently, using an *E. coli* mutant which requires the aromatic amino acids plus *p*-aminobenzoic acid, unsuccessful attempts were made to replace the tryptophan and *p*-aminobenzoic acid requirement by N-(γ-L-glutamyl)-*o*-aminobenzoic and N-(γ-L-glutamyl)-*p*-aminobenzoic acids, respectively. The inhibitory and growth-promoting effects of these derivatives were also examined in some botanical systems, *e.g.*, using floating sections of both apical and subapical segments of *Avena sativa* L.[9] The *o*- and *p*-(γ-L-glutamyl)aminobenzoic acids were relatively ineffective; however, the N-(γ-L-glutamyl)-*m*-aminobenzoic acid was somewhat inhibitory at about 0.1 mg./ml.

The lack of any significant biological activity of these compounds may be due to their inability to penetrate the cell wall; or, if they are structurally comparable to a natural intermediate, the normal biosynthetic route must involve a more complex derivative of the condensation product.

Experimental[10]

Since these derivatives were synthesized through essentially identical experimental procedures, specific details will be given for only one isomer; data on the other analogs will be presented in Table I. In each case, the N-(γ-glutamyl)aminobenzoic acid methyl esters as well as the free acids were demonstrated to be homogeneous by the ascending paper chromatographic technique in two solvent systems using ninhydrin reagent to develop the chromatograms.

***o*-(N-Carbobenzoxy-γ-L-glutamyl)aminobenzoic Acid Methyl Ester.**—A solution of 11.8 g. (0.045 mole) of N-carbobenzoxy-L-glutamic anhydride[11] and 8.1 g. (0.054 mole) of methyl anthranilate in 80 ml. of ethyl acetate was heated under reflux using anhydrous conditions for 6 hr. After cooling to room temperature, the reaction mixture was extracted with 5% sodium hydroxide solution. The resulting alkaline aqueous phase was acidified to pH 3 with concd. hydrochloric acid and then extracted with ether. After drying with anhydrous sodium sulfate, the ether was evaporated *in vacuo*, and the viscous residue was crystallized from ethanol and water. The product was filtered, washed with water and dried, yielding 16.8 g. (76%), m.p. 118–120°.

***o*-(γ-L-Glutamyl)aminobenzoic Acid Methyl Ester.**—*o*-(N-Carbobenzoxy-γ-L-glutamyl)aminobenzoic acid methyl ester (6.5 g.) was dissolved in 200 ml. of ethyl alcohol, and hydrogen was passed through the solution for 3 hr. at room temperature and

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TABLE I

o-, *m*-, AND *p*-(γ-L-GLUTAMYL)AMINOBENZOIC ACIDS AND DERIVATIVES

Compound	M.p., °C.	Yield, %	Empirical formula	Calculated, %			Found, %		
				C	H	N	C	H	N
(N-Carbobenzoxy-γ-L-glutamyl)aminobenzoic Acid Methyl Ester									
o-	118–120	76[a]	$C_{21}H_{22}N_2O_7$	60.86	5.35	6.76	60.89	5.55	6.78
m-	106–107	77[b]	$C_{21}H_{22}N_2O_7$	60.86	5.35	6.76	60.89	5.24	6.74
p-	155–156	83[a]	$C_{21}H_{22}N_2O_7$	60.86	5.35	6.76	60.72	5.19	6.69
(γ-L-Glutamyl)aminobenzoic Acid Methyl Ester									
o-	155–156	80	$C_{13}H_{16}N_2O_5$	55.70	5.75	10.00	55.65	5.68	9.84
m-	169–170	81	$C_{13}H_{16}N_2O_5$	55.70	5.75	10.00	55.40	5.68	10.01
p-	208–209	80	$C_{13}H_{16}N_2O_5$	55.70	5.75	10.00	55.41	5.75	10.15
(γ-L-Glutamyl)aminobenzoic Acid									
o-	258–259 (dec.)	74	$C_{12}H_{14}N_2O_5$	54.13	5.30	10.52	54.12	5.56	10.38
m-	162–163	62	$C_{12}H_{14}N_2O_5$	54.13	5.30	10.52	54.03	5.04	10.61
p-	297–300 (dec.)	53	$C_{12}H_{14}N_2O_5$	54.13	5.30	10.52	53.87	5.33	10.38
3-Phenylhydantoin of (γ-L-Glutamyl)aminobenzoic Acid									
o-	194–195 (dec.)	55	$C_{19}H_{17}N_3O_7 \cdot H_2O$	59.21	4.97	10.90	59.06	4.97	10.80
m-	201–202	91	$C_{19}H_{17}N_3O_7 \cdot H_2O$	59.21	4.97	10.90	59.24	5.05	11.05
p-	200–201	52	$C_{19}H_{17}N_3O_7 \cdot H_2O$	59.21	4.97	10.90	59.24	5.34	10.82

[a] Reflux time, 6 hr. [b] Reflux time, 48 hr.

at atmospheric pressure in the presence of 0.5 g. of palladium black catalyst. The reaction mixture was filtered to remove the catalyst, the filtrate was evaporated to dryness, and the residue was crystallized from ethanol and water. There was obtained 3.5 g. (80%) of product, m.p. 155–156°.

***o*-(γ-L-Glutamyl)aminobenzoic Acid.**—A solution of 1.0 g. of *o*-(γ-L-glutamyl)aminobenzoic acid methyl ester in 100 ml. of water was adjusted to pH 12 with saturated barium hydroxide solution, and heated on a steam bath for about 1 hr. The reaction mixture was kept at pH 12 by the addition of more barium hydroxide as required. After the pH of the reaction mixture became constant, it was cooled to room temperature, and carbon dioxide was passed through the solution until it became acid to litmus paper. Precipitation of the remaining barium ions was completed by the addition of 10% sulfuric acid. The resulting mixture was adjusted to pH 8 with 10% sodium hydroxide solution and filtered through a Celite mat. The filtrate was acidified to pH 5 with sulfuric acid, cooled, and the crystallized product which formed was filtered and dried over phosphorus pentoxide to give 0.7 g. (74%) of material which was recrystallized from water, m.p. 258–259° dec.

N-(2,4-Dioxo-3-phenyl-5-imidazolidine-L-propanoyl)-*o*-aminobenzoic Acid [3-Phenylhydantoin of *o*-(γ-L-Glutamyl)aminobenzoic Acid].—According to the general procedure of Ware[8] for the preparation of hydantoins, 148 mg. (10% molar excess) of phenyl isocyanate was added dropwise to a hot stirred solution of 300 mg. of *o*-(γ-L-glutamyl)aminobenzoic acid and 120 mg. of sodium carbonate in 15 ml. of dioxane and water (1:1). The reaction mixture was heated on a steam bath for 30 min. and kept at about pH 8 through the addition of sodium carbonate. Sufficient concd. hydrochloric acid was added to the cooled reaction mixture to give a solution of about pH 1, and this was then heated on a steam bath for an additional 15 min. After cooling in an ice bath, the precipitated product was filtered, washed with water and dried. The resulting material was recrystallized from ethanol and water to give 240 mg. of product, m.p. 194–195° dec., which gave a negative reaction to ninhydrin reagent.

Microbial Assays.—Previously reported procedures[12,13] were used for general toxicity studies with *S. lactis* 8039 and *L. arabinosus* 17-5. The assays with *E. coli* 9723 were carried out using an inorganic salts–glucose medium[14] and a previously reported assay technique.[15] In addition to the inhibition studies, the ability of *o*-(γ-L-glutamyl)aminobenzoic acid to replace anthranilic acid or tryptophan for *L. arabinosus* 17-5 was examined in a medium[12] which was modified by omitting tryptophan and which had a total glutamic acid concentration of 20 μg./ml. In comparable studies with the *E. coli* mutant 83-1,[16] the basal medium was supplemented with 10 μg./ml. each of L-phenylalanine, L-tyrosine and L-tryptophan, and various concentration levels of *p*-(γ-L-glutamyl)aminobenzoic acid were added in an effort to induce growth. Alternately, L-phenylalanine, L-tyrosine and *p*-aminobenzoic acid (0.01 μg./ml.) were added to be basal medium, and the effect of increasing concentration levels of *o*-(γ-L-glutamyl)aminobenzoic acid (which could potentially serve as a precursor of tryptophan) was determined.

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Agents Affecting Lipid Metabolism. II. Analogs of Mevalonic Acid[1]

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Since the discovery that mevalonic acid is one of the key intermediates in the biosynthesis of cholesterol, many attempts have been made to find antimetabolites of this compound.[2-12] With the exception of the work of Daeniker and Druey,[8] none of these potential antimetabolites has involved modification of the functional

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