Table VI—Ratios of Polyoxyethylene-20-oleyl Ether-Promoted Absorption to Normal Absorption Efficiencies in the Rat

Time of Sacrifice	Vitamin B_{12} Dose, ng. 400	2000	6000
Ligated stomach animals			
2 hr.	5.6	14.8	11.3
Fasted intact animals			
2 hr.[a]	3.3	2.8	7.1
6 hr.	1.3	1.4	1.6
24 hr.	1.7	2.3	2.4
Average	1.5	1.8	2.0
Fed intact animals			
6 hr.	1.7	2.2	3.3
16 hr.	1.6	1.9	3.8
24 hr.	—	2.9	5.3
Average	1.6	2.3	4.1

[a] Excluded from averages.

At a higher concentration of the vitamin, the intrinsic factor-assisted absorption contributes little and the efficiency of absorption falls and approaches a value characteristic of the uncomplexed vitamin in the "hyperabsorptive" intestine. This value is above 4.0% of the dose absorbed whether the animal is fed or fasted.

Previous work in these laboratories demonstrated a hyperabsorptive state of the dog stomach which resulted specifically from a surfactant-organ interaction (6). This hyperabsorptive state subsequently returned to a normal absorptive state after removal of the promoter. Unpublished studies in these laboratories also demonstrated similarly hyperabsorptive states of segments of the GI tract of dogs, rats, and chickens. The present study indicates that the ligated stomach and the intact GI tract of the rat becomes hyperabsorptive for uncomplexed vitamin B_{12} under the influence of polyoxyethylene-20-oleyl ether.

It can be concluded from Tables II–IV that the absorption in intact animals continued beyond 16 hr., because the fraction of the dose absorbed continued to increase between 16 and 24 hr. Roughly one-half of the total absorption occurred during the first 6 hr. A check of fecal and urinary radioactivity indicated that at 6 hr. very little excretion had occurred, while by 16 hr. most of the unabsorbed radioactivity had passed out of the GI tract.

The ratios of efficiency of promoted *versus* normal absorption in the intact intestine (Table VI) vary with dose and time of sacrifice. The highest ratios are for the fed animals at high dose and for the animals sacrificed at 2 hr. The 2-hr. results may well include some of the influence of the high ratio of promoted *versus* normal absorption characteristic of the stomach. It was concluded that the effect of the absorption promoter, polyoxyethylene-20-oleyl ether, is to render the stomach and intestine hyperabsorptive, improving the efficiency of absorption of only the uncomplexed portion of the vitamin. The ability of the endogenous intrinsic factor to enhance the absorption of small doses of vitamin B_{12} is not impaired by the presence of polyoxyethylene-20-oleyl ether.

It is not intended in this paper to consider or to imply the therapeutic usefulness of promoted absorption in vitamin B_{12} therapy. Data from clinical reports were cited because much of the knowledge of vitamin B_{12} absorption was obtained from clinical experience. The surprising effectiveness of polyoxyethylene-20-oleyl ether in promoting absorption of vitamin B_{12} of molecular weight 1355 suggests that passive absorption mechanisms may extend to substances of even higher molecular weights.

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In Vitro Metabolism of Certain Nornuciferine Derivatives

ROBERT V. SMITH* and S. PAUL SOOD

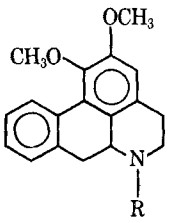
Abstract □ The metabolism of nornuciferine and seven of its derivatives by rat, rabbit, and guinea pig liver microsomes was studied. Nornuciferine was found as a metabolite of the *N*-alkylated analogs. A sensitive method for analyzing nornuciferine was developed based on reverse TLC fluorimetry. By using this method, relative *N*-dealkylation rates were studied with guinea pig microsomes. The conversion of nornuciferine to the oxoaporphine, lysicamine, was observed photolytically and metabolically with rat and rabbit microsomes. Chromatographic and spectral analyses of lysicamine are discussed.

Keyphrases □ Nornuciferines—*in vitro* metabolic and photolytic conversions to lysicamine □ Lysicamine—metabolic and photolytic conversion from nornuciferines, identification □ Aporphine biotransformations—nornuciferine metabolism □ TLC fluorimetry, reverse—analysis, nornuciferines □ Photolysis—conversion of nornuciferines to lysicamine □ TLC—identification, lysicamine □ Mass spectroscopy—identification, lysicamine

The metabolism of apomorphine was studied by Kaul *et al.* (1–4). Besides these investigations, however, no studies have appeared relative to the biological fate of aporphine alkaloids. This is somewhat surprising since aporphines occur widely in the plant kingdom (5, 6). In addition, naturally derived aporphines, as well as many synthetic analogs, possess interesting biological activities (6–10). Of particular significance are reports

that apomorphine and its congeners may be useful in the treatment of Parkinsonism (11–13) and chronic manganism (13). Evidence of the dopinergic activity of aporphines (14–18) and use of apomorphine as an experimental probe of the CNS (19) underline the importance of further elucidation of aporphine metabolism.

A systematic study of aporphine biotransformations was commenced in these laboratories with an investigation of the *in vitro* metabolism of the nuciferine analogs, I–VIII, by rat, rabbit, and guinea pig liver microsomes. Results of these investigations are described in this report.



Compound	R
I	—H
II	—CH_3
III	—CH_2CH_3
IV	—$CH_2CH_2CH_3$
V	—CH_2— 
VI	—$CH_2CH{=}CH_2$
VII	—$CH_2C{\equiv}CH$
VIII	—$CH_2C_6H_5$

EXPERIMENTAL[1]

Disodium glucose 6-phosphate, glucose 6-phosphate dehydrogenase, and the sodium salt of nicotinamide adenine dinucleotide phosphate were used as purchased[2]. The nornuciferine derivatives employed were described previously (20).

1,2-Dimethoxydibenzo[*de*,*g*]quinoline-7-one (Lysicamine)—This compound was prepared according to Tomita *et al.* (21) and purified by preparative TLC using Solvent System 1, m.p. 208–209° dec. [lit. (21) m.p. 208–210° dec.].

TLC—Silica gel GF_{254} layers (250 μ), prepared by a method described previously (20) or obtained commercially[3], were used throughout. Plates scored into 1–2-cm. channels were developed 10 cm. in the following solvent systems: 1, chloroform–methanol (85:15); 2, chloroform–methanol–acetic acid (8:1:1); 3, cyclohexane–chloroform–piperidine (8:1:1); 4, benzene–piperidine (9:1); 5, benzene–ethyl acetate–piperidine (6:3:1); and 6, chloroform–acetic acid (3:1). Detection was *via* quenching of 254-nm. radiation or by fluorescence in 320–400-nm. radiation.

Reverse TLC Fluorimetry—Silica gel GF_{254} plates (scored into 2-cm. channels), spotted with samples containing 1.0–5.0 mcg. of nornuciferine, were developed (10 cm.) in Solvent System 1. Plates were scanned in the direction of the solvent development. The fluorescence quench mode was employed with the following conditions: $\lambda_{exc.}$, 254 nm.; span, 300; gain, ×100; neutral density filter, 3.00; slit width, 1 mm., length, 0.65 cm.; scanning rate, 3.2 cm./min.; and recorder speed, 1.9 cm./min. Area measurements were obtained with the formula, $A = h \cdot w_{1/2}$, where A = area, h = peak height, and $w_{1/2}$ = width at half-height.

GC—The conditions reported previously were employed (20).

Liver Preparations and Incubations—Livers from exsanguinated rats (Holtzman males), rabbits (New Zealand males)[4], and guinea pigs (Albino males) were washed with 0.25 *M* sucrose solution and homogenized[5] (at 0°) with four volumes of the latter.

Homogenates were centrifuged at $g\times10{,}000$ for 30 min. The $g\times10{,}000$ supernatant (1 ml. ≡ 1 g. liver) was used as such or centrifuged further at $g\times100{,}000$ (for 1.5 hr.), depositing microsomes which were suspended in 0.25 *M* sucrose solution (1 ml. ≡ 1.25 g. liver).

In the standard incubation procedure, 1 μmole of nornuciferine derivative (hydrochloride salt) was mixed with 2.5 ml. of $g\times10{,}000$ supernatant or microsomal preparation, 1 ml. of 0.1 *M* tromethamine–HCl buffer (pH 7.6), 1 μmole of nicotinamide adenine dinucleotide phosphate, 5 μmoles of disodium glucose 6-phosphate, and 2.0 units of glucose 6-phosphate dehydrogenase; the total volume of the incubation mixture was 4.3 ml. Control mixtures were prepared in the same way but with a liver preparation which had been heated on a steam bath for 2 min. A second blank was prepared without substrate. Mixtures were incubated in a shaker-type water bath at 37.0 ± 0.1° in the air and in the absence of light. The effect of magnesium ion on enzyme activity was studied with magnesium chloride (5 mmoles) added to the incubation mixture prior to the addition of the microsomal preparation. Optimum pH was determined with 0.1 *M* tromethamine–HCl buffers of pH 6.0, 6.5, 7.0, 7.4, 7.6, and 8.0. For rate studies, the total volume of the incubation mixture was 12.9 ml., and 2.1-ml. aliquots were removed at appropriate time intervals.

Extraction of Incubation Mixtures—After heating on a steam bath for 2 min., the incubation mixtures were cooled and then extracted with three 2-ml. portions of 25% isoamyl alcohol in *n*-heptane. The extracts were combined, washed twice with water, dried over anhydrous sodium sulfate, and evaporated to dryness with a current of cold air in the absence of light. The residue was dissolved in 0.3 ml. of chloroform for TLC.

Photolytic Conversion of Nornuciferines to Lysicamine—Nornuciferines (1 μmole) were dissolved in 1-ml. portions of chloroform. The solutions were exposed to sunlight for 4 hr. at room temperature and then subjected to TLC. The effect of solvent variation on lysicamine formation was studied with solutions in ethanol, benzene, and *tert*-butyl alcohol. The effect of light on the conversion of lysicamine was further studied by bubbling air through chloroform and *tert*-butyl alcohol solutions of nornuciferine (and its hydrochloride salt) in the dark.

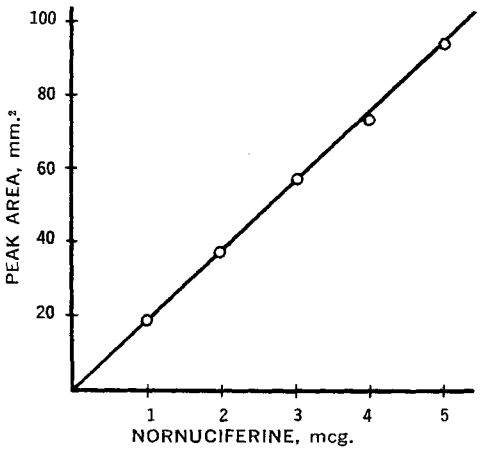


Figure 1—*Standard curve for reverse TLC fluorimetry of nornuciferine.*

RESULTS AND DISCUSSION

Quantitation of Nornuciferine—Initial metabolism experiments revealed the need for a sensitive quantitative method for nornuciferine (I). The GC method previously described (20) was suitable for mixtures of I and the *N*-alkylnornuciferines (II–VIII) where the former was present in significant proportion. However, where I composed 5% or less of the aporphine mixture, quantitation was difficult since the retention times of I and some of its derivatives (*e.g.*, II and III) were close, leading to insufficient resolutions.

[1] Melting points were obtained with a Thomas-Hoover apparatus and are corrected. IR spectra were recorded on Beckman IR-5A and 10 instruments. UV and visible spectra were obtained with Beckman DK-2 and Gilford-240 spectrophotometers. GC was performed with a Hewlett-Packard 5750B gas chromatograph equipped with dual flame-ionization detectors. Reverse TLC fluorimetry was accomplished with a Nester-Faust Uniscan 900 scanner connected to a Texas Instruments, model PS01W6A, 1-mv. recorder.

[2] Sigma Chemical Co., St. Louis, Mo.

[3] Analtech, Newark, Del.

[4] Pel-Freez Biologicals, Rogers, Ark.

[5] Virtis-45 homogenizer.

Table I—Reverse TLC Fluorimetry of Nornuciferine

Amount Developed, mcg.	Amount Found, mcg.	Percent Recovery
1	1.01	101.0
1	0.98	98.0
1	0.97	97.0
2	1.90	95.0
2	2.04	102.0
2	1.95	97.5
3	2.90	96.6
3	2.94	98.0
4	3.82	95.5
4	3.88	97.0
5	4.78	95.6
5	4.87	97.4
5	4.84	96.8

Mean = 97.5 (n = 13)
Relative standard deviation = ±1.9%

The use of a quantitative TLC technique seemed attractive since excellent resolution of I from II–VIII could be achieved through TLC (20). The procedure of reverse TLC fluorimetry was readily employed and provided the requisite sensitivity. Figure 1 shows a standard curve for quantitating I following development in Solvent System 1. Consistently linear relationships were obtained over the range of 1–5 mcg. Curves embracing quantities beyond this range tended to become hyperbolic (22).

In a typical experiment, two amounts of I were developed, and peak areas derived from these were used in calculating the quantity of I in samples developed on the same plate. Thus, up to eight samples could be analyzed per plate (20 cm. wide), with a resulting analysis time of approximately 10 min./sample. Results of experiments designed to evaluate accuracy and precision of the method described are given in Table I. Overall, the recovery values are good while the precision is better than those reported with similar techniques (23, 24).

***In Vitro* Metabolism**—Extracts obtained from incubation mixtures of nuciferine (II) with rat and rabbit liver preparations (g× 10,000 supernatant) were submitted to TLC in Solvent Systems 2, 3, and 4. Compound I was observed along with an unknown metabolite referred to here as I*a*. The formation of I and I*a* was estimated to be less than 1%. The degree of conversion was not increased by using the microsomal (g×100,000) preparation or by addition of magnesium chloride (25). Compounds III and IV showed even less conversion to I, while *N*-dealkylation of V through VIII could not be detected. The absence of I*a* following microsomal incubation of III and IV seemed to be related to low *N*-dealkylation activity. Indeed, when I was incubated with rat and rabbit liver preparations, relatively greater quantities of I*a* were formed.

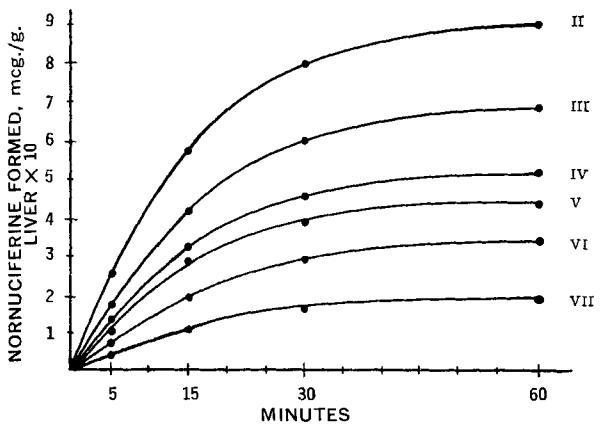
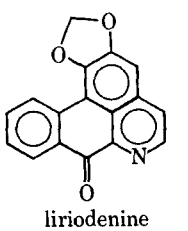
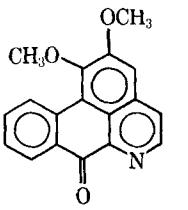


Figure 2—N-*Dealkylation of Compounds II–VII by guinea pig microsomes. Each point represents an average of from three to four determinations.*

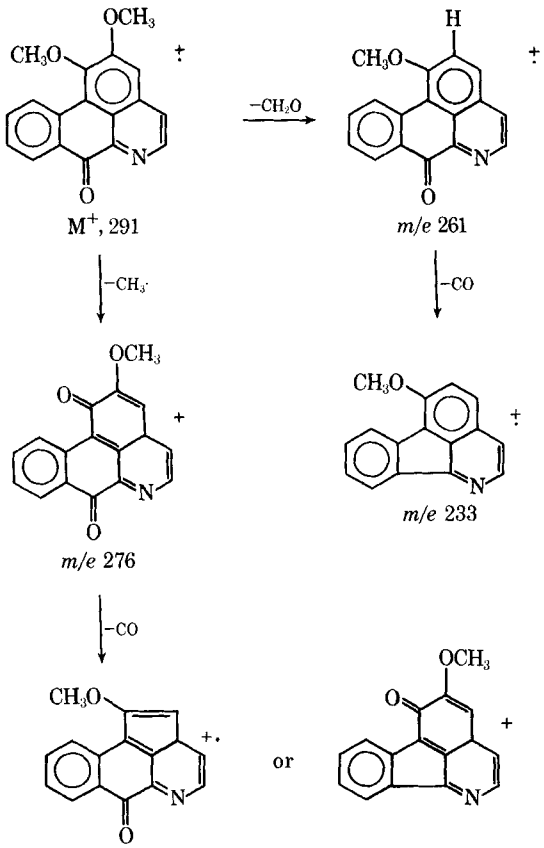

liriodenine


lysicamine

In contrast to these results, guinea pig liver microsomal preparations showed considerably enhanced *N*-dealkylation activity. When studied over the pH range of 6.0–8.0, optimum activity was found between 7.4 and 7.6. Interestingly, I*a* was not observed as a metabolite with this species.

The greater *N*-dealkylation activity observed with guinea pig microsomes permitted study of relative rates. The results of this investigation are depicted in Fig. 2. In every instance, the reaction was linear for 15–20 min., with sharp departure from linearity after 30 min. The deviation from linearity was not due to limiting amounts of substrate or known cofactors (26–28). The *N*-dealkylation rates of II–V were inversely related to the size and complexity of *N*-alkyl groups (29). The *N*-dealkylation rates of Compounds VI–VII showed decided differences from that of II, while *N*-dealkylation of VIII was too low to be quantitated. These results may be a function of decreases in lipid solubilities of Compounds VI–VIII because of their more polar *N*-alkyl groups. McMahon (30) noted that there is a good correlation between lipid solubilities and *N*-dealkylation rates. That is, as lipid solubility decreases, the rate of *N*-dealkylation decreases. This was observed and quantitated by Hansch *et al.* (31) and Lien and Hansch (32) for a number of microsomal transformations and appears to be related to the fact that microsomal enzymes are coated with a lipid layer. Thus, lipid solubility can be a rate-limiting factor because of its effect on substrate availability at enzyme sites.

Other than Compound I*a* (*vide infra*), no other metabolites were observed following incubation of Compounds I–VIII with rat,



Scheme I

Table II—TLC and Spectral Data of Ia and Lysicamine

System	TLC $R_f \times 10^2$ Ia	TLC $R_f \times 10^2$ Lysicamine	UV/Visible[a] $\lambda_{max.}$, nm. (ϵ) Ia	UV/Visible[a] $\lambda_{max.}$, nm. (ϵ) Lysicamine	IR[b] ν C=O, cm.$^{-1}$ Ia	IR[b] ν C=O, cm.$^{-1}$ Lysicamine	Mass Spectra[c] m/e (Relative Abundance) Ia	Mass Spectra[c] m/e (Relative Abundance) Lysicamine
1	65	65	235 (26,900)	235 (29,510)	1662	1668	291 (100)	291 (100)
2	50	50	270 (22,100)	270 (25,700)	—	—	276 (4)	276 (2)
3	8	8	307 (5,700)	307 (5,750)	—	—	261 (1)	261 (1)
4	27	27	400 (7,300)	400 (8,700)	—	—	248 (57)	248 (41)
5	37	37	453 (3,200)[d]	453 (3,800)[d]	—	—	233 (7)	233 (5)
6	15	15	—	—	—	—	—	—

[a] Ethanol solutions. [b] Spectra were nearly superimposable; solid films. [c] See Scheme I for structural assignments; peaks at m/e less than 233 were of relatively low abundance and showed no differences in position. [d] Visible band after addition of one drop of 0.1 N HCl.

rabbit, or guinea pig liver preparations. Of particular interest was the apparent absence of *O*-dealkylation, which was an anticipated biotransformation (20).

Identification of Metabolite Ia—TLC of extracts obtained after incubation of I and II with rat and rabbit liver preparations revealed the presence of a yellow-colored compound (Ia) with similar mobility to I and II in neutral and acidic solvent systems (1, 2, and 6) but lower mobility in basic solvent systems (3–5).

During these investigations, it was observed that chloroform and chloroform–methanol solutions of Compounds I–VIII, but especially I, developed a yellow color when exposed to light. Following TLC examination, it was shown that the yellow component of these solutions possessed identical mobility to Ia. Photochemically produced Ia was purified and isolated by preparative TLC. A UV-visible spectrum of Ia showed four bands (in ethanol): $\lambda_{max.}$ = 235, 270, 307, and 400 nm. The 400-nm. band did not shift in alkali (ethanolic sodium hydroxide) but did shift to 453 nm. when Ia was treated with hydrochloric acid.

Compound Ia showed no OH or NH stretching bands in the IR but did possess a band at 1662 cm.$^{-1}$, which could be indicative of a highly conjugated carbonyl function. A search of the literature revealed that these data were very close to those of the naturally occurring oxoaporphines, liriodenine (33, 34), and lysicamine (35).

Spectral and TLC comparison of Ia with an authentic sample of lysicamine (21) showed that the two were identical (Table II). Mass spectra[6] of Ia and lysicamine contained three principal peaks, as indicated in Table II; ionization and fragmentations giving rise to these peaks are proposed in Scheme I. The fragmentations depicted are well documented (36, 37), while equivalent peaks were reported for several liriodenine analogs (38, 39).

Cava and Dalton (40) reported that liriodenine could be produced in 30% yield by passage of air through a *tert*-butyl alcohol solution of anonaine, the methylenedioxy homolog of I. *tert*-Butyl alcohol solutions of I were similarly treated to yield lysicamine; however, in the absence of light, no conversion was detected by TLC. The photolytic conversion of I to lysicamine was most efficient in chloroform, although it also occurred in ethanol and benzene. Compounds II–VIII could also be photolytically converted to lysicamine, but to a much lesser extent, and other products were detected by TLC. Under laboratory fluorescent lighting, chloroform solutions of I were shown (by visible spectroscopy) to be quantitatively converted to lysicamine in 7 days. Attempts were made to standardize the lighting requirement, for instance, by using mercury vapor lamps with principal emission at 254 nm. or 320–400 nm. These conditions, however, gave mixtures of reaction products.

Since lysicamine could arise as an artifact and not an actual metabolite of I, experiments were designed to investigate this possibility. Colorimetric analysis of Ia in the presence of I or II was possible since the latter do not absorb at 400 nm. Thus, extracts obtained from incubations of I and II with rat liver preparations were analyzed using blanks obtained from I and II incubated with denatured enzyme. Following TLC identification, Ia was quantitated. A 1.20% conversion of I to Ia and a 0.50% conversion of II to Ia were observed. These data tend to confirm the proposed intermediacy of I in the formation of Ia from II.

[6] The authors are indebted to Dr. William L. Budde and the Purdue University Mass Spectrometry Center for these determinations.

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* To whom all correspondence should be addressed.

Dialysis and Circular Dichroism Study of the Binding of Sulfaethidole to Crystalline and Fraction V Bovine Serum Albumin

H. B. KOSTENBAUDER*, M. J. JAWAD*, J. H. PERRIN†, and V. AVERHART†

Abstract □ Sulfaethidole was found to exhibit similar binding to both crystalline and Fraction V bovine serum albumin. Binding of sulfaethidole to both crystalline and Fraction V bovine serum albumin results in considerable induced optical activity, with no evidence of change in the α-helical structure. Although equilibrium dialysis studies indicate one primary site and three secondary binding sites for sulfaethidole, only the binding at the primary site is detectable from circular dichroism studies.

Keyphrases □ Sulfaethidole binding to crystalline and Fraction V bovine serum albumin—studied by dialysis and circular dichroism □ Dialysis—sulfaethidole binding to bovine serum albumin, determination □ Circular dichroism—sulfaethidole binding to bovine serum albumin, determination

It was recently noted that sulfaethidole exhibits similar binding to both crystalline and Fraction V bovine serum albumin, while salicylate ion shows markedly lower binding to Fraction V than to crystalline bovine serum albumin (1). It was further observed that the addition of 10 mg.% sulfaethidole to 4.55% bovine serum albumin dispaced salicylate from binding to crystalline bovine serum albumin but markedly enhanced the binding of salicylate to Fraction V bovine serum albumin. It was suggested that sulfaethidole might induce a subtle conformational change in Fraction V bovine serum albumin; studies were, therefore, undertaken to determine if such conformational change could be detected by circular dichroism (CD) measurements.

EXPERIMENTAL[1]

Dialysis Procedure—Ten milliliters of a 5.00% w/v serum albumin (7.25×10^{-4} *M*) solution in 0.054 *M* phosphate buffer, pH 7.4, adjusted with sodium chloride to be isotonic, was placed in dialysis bags made by knotting the ends of the dialysis tubing. Bags were placed in 120-ml., wide-mouth, screw-capped bottles containing 10 ml. of buffered sulfaethidole solution, and the containers were agitated for 12 hr. at 37°. Prior to analysis, the volumes of inside and outside solutions were carefully measured, and protein concentrations were corrected for volume change (final protein concentration was 4.55% w/v). Total sulfaethidole concentrations in the protein compartment at equilibrium ranged from approximately 3.2 to 73 mg.% (1.1×10^{-4}–25.7×10^{-4} *M*). Buffer solutions and glassware were sterilized, and the aseptic technique was used in preparing solutions and dialysis bags. Dialysis tubing was boiled in three changes of distilled water and finally in buffer solution.

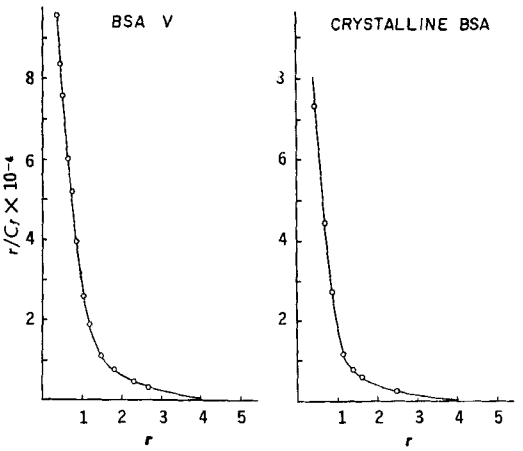

Figure 1—*Scatchard plot for binding of sulfaethidole by bovine serum albumin in 0.054* M *phosphate buffer, pH 7.4, 37°. For bovine serum albumin V, the solid line corresponds to* $n_1 = 1$, $K_1 = 1.5 \times 10^5$, $n_2 = 3$, *and* $K_2 = 1.6 \times 10^3$. *For crystalline bovine serum albumin, the solid line corresponds to* $n_1 = 1$, $K_1 = 1.2 \times 10^6$, $n_2 = 3$, *and* $K_2 = 1.0 \times 10^3$.

Analytical Procedures—Sulfaethidole concentration was determined for both inside and outside solutions following equilibrium, utilizing the Bratton–Marshall (2) procedure. Inside and outside solutions from a sample containing no sulfaethidole were utilized as blanks. Absorbances were determined at 535 nm. using a spectrophotometer[2].

[1] Visking dialysis tubing, 27/32, Union Carbide Corp., Chicago, Ill.; Fraction V bovine serum albumin, Armour Pharmaceutical Co., Chicago, Ill.; and crystalline bovine serum albumin, Nutritional Biochemicals Corp., Cleveland, Ohio, were used as received from the supplier. Sulfaethidole [N^1-(5-ethyl-1,3,4-thiadiazol-2-yl)sulfanilamide] was recrystallized from water, m.p. 185–186°; all other chemicals were reagent grade.

[2] Cary 15, Cary Instruments, Monrovia, Calif.