and excreted from the body during the first 2–4 days after dosing, the concentrations of radioactivity remaining in the plasma during the 2–14-day interval after dosing are assumed to represent largely metabolites of fluphenazine rather than the parent compound. However, this point could not be established experimentally. On the other hand, in the case of the esters of fluphenazine base, because of their slow-release characteristics, the esters themselves, or fluphenazine base, the product of their hydrolysis, may be released continuously into the circulation for a much longer period.

For all intact dogs given intravenous doses (1 mg/kg) of ^{14}C-fluphenazine or of its enanthate or decanoate ester, virtually identical concentrations of radioactivity were present in the circulation. In addition, the enanthate and decanoate esters of ^{14}C-fluphenazine were rapidly converted to ^{14}C-fluphenazine base and other unidentified metabolites. Thus, slow-release characteristics were not produced by the intravenous administration of the esters of ^{14}C-fluphenazine base.

A number of other lines of evidence point to the fact that the slow-release characteristics produced by the esterified fluphenazine base are a function both of esterification *per se* and of intramuscular administration. In an earlier study (1), a comparison was made, during a 35-day period, of the relative rates of release of ^{14}C-fluphenazine enanthate and ^{14}C-fluphenazine decanoate. The rates of excretion of total radioactivity in urine and feces after administration of either of these two esters intramuscularly in sesame oil were dissimilar; however, the radioactivity was excreted at the same rate in *both* urine and feces for each ester but more slowly for the decanoate ester than for the enanthate ester. This finding is consistent with the idea that the rate-limiting step for the release of either ester is its diffusion into the circulation from the injection site.

Yet another experimental situation points to the necessity of administering ^{14}C-fluphenazine enanthate intramuscularly, although not necessarily in the presence of sesame oil, to produce slow-release characteristics. In that situation, a dog whose bile duct had been cannulated was given a 1-mg/kg im dose of ^{14}C-fluphenazine enanthate in absolute ethanol (4). During the 8-hr test, concentrations of radioactivity in the plasma increased consistently. Only about 0.55% of the dose was excreted during the 8 hr, the major portion of the dose (88%) remaining at the site of injection. Thus, the administration of ^{14}C-fluphenazine enanthate intramuscularly, but not necessarily in sesame oil, was capable of producing slow-release characteristics.

Studies *in vitro* have shown that ^{14}C-fluphenazine enanthate (4) and ^{14}C-fluphenazine decanoate[7] can be hydrolyzed slowly by plasma esterases of the dog. The rates of hydrolysis of these two esters in plasma, however, appeared to be much slower than those occurring in intact dogs when the compounds were injected intravenously. This observation suggests that other organs, such as the liver, may have a much greater enzymatic capacity than does the plasma to cleave these esters. The observation that most of an intravenous dose of ^{14}C-fluphenazine enanthate or ^{14}C-fluphenazine decanoate (1 mg/kg) can be hydrolyzed within 30 min suggests that the hydrolytic capabilities of the dog are much greater than the rates at which these two esters, formulated in sesame oil, are usually released from their intramuscular injection sites.

All of these observations point to the conclusions that:

1. The slow-release characteristics produced by the enanthate and decanoate esters of fluphenazine base are a consequence of their relative rates of diffusion into the circulation from an injection site.
2. Cleavage of these esters in the circulation occurs rapidly and plays little, if any, role in the observed differences between their relative rates of release.

Once either of these two esters is released into the circulation of dogs, it is rapidly hydrolyzed to fluphenazine base and further metabolized according to the metabolic pathways previously described for ^{14}C-fluphenazine (5).

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dc Polarographic Assay of Tetracyclines

L. G. CHATTEN [x], R. E. MOSKALYK, R. A. LOCOCK, and K-S. HUANG

Abstract □ The optimum conditions for the polarographic reduction of some tetracycline antibiotics were studied. A boric acid–sodium borate buffer provided the best conditions for the electroreduction of tetracycline, oxytetracycline, chlortetracycline, and demeclocycline. Conditions are described for the quantitative determination of these tetracyclines in various dosage forms.

Keyphrases □ Tetracycline—polarographic analysis, pharmaceutical formulations □ Oxytetracycline—polarographic analysis, pharmaceutical formulations □ Chlortetracycline—polarographic analysis, pharmaceutical formulations □ Demeclocycline—polarographic analysis, pharmaceutical formulations □ Polarography—analysis, tetracycline, oxytetracycline, chlortetracycline, and demeclocycline in pharmaceutical formulations □ Antibiotics—tetracycline, oxytetracycline, chlortetracycline, demeclocycline, polarographic analysis in pharmaceutical formulations

Despite their well-known disadvantages, microbiological assays remain as the official methods of analysis for the tetracyclines (1, 2). However, the search for more accurate alternatives continues. Recently, two reviews (3, 4) dealt with the many methods reported for this important class of antibiotics.

Polarographic analyses of tetracyclines were first reported in the early 1950's (5, 6), and numerous studies

Table I—Selected pH and Applied Potential at Which Total Diffusion Current is Read

Antibiotic	pH	Applied Voltage
Tetracycline hydrochloride	7.75	−1.70
Oxytetracycline hydrochloride	8.20	−1.60
Chlortetracycline hydrochloride	7.95	−1.66
Demeclocycline hydrochloride[a]	7.75	−1.70

[a] A 1% gelatin solution (0.13 ml) was added with a calibrated dropper.

on this subject have appeared since then (3). However, none of these studies has been concerned specifically with the potential of this technique for the quantitative analysis of the various tetracyclines in pharmaceutical dosage forms. Furthermore, although considerable work has been reported on the elucidation of the mechanism of reduction at the dropping mercury electrode (7–9), the exact nature and sites of reduction for these drugs remain in doubt.

The purposes of this present study were to systematically investigate the effect of pH and buffer systems on the reduction of these antibiotics at the dropping mercury electrode and then to apply the optimum conditions to their quantitative determination in pharmaceutical dosage forms. The results on the mechanism of reduction of the tetracyclines at the dropping mercury electrode will be the subject of a subsequent publication.

EXPERIMENTAL

Apparatus—All polarograms were recorded on a polarograph[1] equipped with a 30-ml H-type cell, maintained at 25 ± 0.2°, a saturated calomel electrode, and a dropping mercury electrode. A pH meter[2] fitted with a glass–calomel electrode system was used for all pH measurements and for the potentiometric titrations. Titration lamps and a magnetic stirring apparatus were also used.

Reagents and Solutions—All chemicals were either ACS or reagent grade. The following were used: acetic acid; 0.05 *N* perchloric acid in dioxane, standardized against primary standard potassium acid phthalate; 0.5% gentian violet in acetic acid; 6% mercuric acetate in acetic acid; 0.2 *M* boric acid stock solution; 0.05 *M* sodium borate stock solution; freshly prepared 1% gelatin solution; hydrochloric acid; and disodium ethylenediaminetetraacetic acid.

Reference Materials—Tetracycline hydrochloride (100.1%), oxytetracycline hydrochloride (96.8%), chlortetracycline hydrochloride (97.9%), and demeclocycline hydrochloride (100.6%) were used without further purification. Their purity was determined by the method of Sideri and Osol (10).

Optimum Buffer System and Optimum pH—The following buffer systems were examined: Britton–Robinson (pH 2.6–11.8), glycine–hydrochloric acid (pH 2.2–3.6), lithium acetate–acetic acid (pH 3.6–5.6), phthalate–sodium hydroxide (pH 4.2–6.0), barbital (pH 6.8–9.2), phosphate (pH 5.7–8.0), boric acid–sodium borate (pH 7.6–9.2), and sodium borate–sodium hydroxide (pH 9.3–10.0). All buffers were approximately 0.05 *M* (final dilution). Solutions of each buffer were prepared at intervals of 0.2 pH unit. After a 10-min deaeration with nitrogen, each buffer solution was scanned over an applied potential range of from 0 to −2.0 v.

Solutions of approximately 1×10^{-4} *M* for each tetracycline salt were prepared in each individual buffer solution, and a polarogram was obtained. The optimum buffer system was then selected, and a further study of the influence of pH was conducted at intervals of 0.1 pH unit. The pH was determined for all solutions before and after electrolysis.

Solution stability was determined for each tetracycline by obtaining polarograms on each solution at 0.5-hr intervals after preparation.

Calibration Curves—All calibration curves were made by averaging five determinations, and all current readings were corrected to account for the percent purity of the reference material.

A quantity of the tetracycline hydrochloride equivalent to about 50 mg of the antibiotic was accurately weighed, dissolved in boric acid–sodium borate buffer of optimum pH, transferred to a 100-ml volumetric flask, and diluted to volume with buffer (0.5 mg/ml). Working standard solutions were prepared to contain 0.010, 0.025, 0.0375, 0.050, 0.075, 0.100, and 0.125 mg/ml by appropriately diluting the stock solution with the buffer. A 25-ml aliquot of a standard solution was pipetted into the polarographic cell already equilibrated at 25° and then deaerated.

The applied potential range was from 0.00 to −2.00 v, and the sensitivity scale was set at 0.15 μamp/mm. The rate of mercury flow at the applied potential at which the diffusion current was measured was 2.95 mg/sec; the drop time, *t*, was 2.5 sec; and the mercury column height was 65.0 cm. Optimum conditions of pH and applied potential are presented in Table I.

Procedures for Dosage Forms—*Tablets and Capsules*—A suitable quantity of the powdered material was accurately weighed into a 150-ml beaker, 90 ml of the specified buffer solution was added, and the mixture was stirred for 10 min. The slurry was filtered under suction through a medium-porosity sintered-glass filter. The clear filtrate was quantitatively transferred to a 100-ml volumetric flask and diluted to volume with the specified buffer (concentration of stock solution ~0.5 mg/ml).

A 15-ml aliquot of the stock solution was quantitatively transferred to a 100-ml volumetric flask and diluted to volume with the buffer solution. A 25-ml aliquot of this solution was transferred to the polarographic cell. Deaeration was carried out, and the polarogram was obtained as previously described.

Ointments—The following modification was required. A stock solution was prepared by successively heating, stirring, cooling, and filtering an accurate weight of ointment with four separate portions of specified buffer (90, 50, 30, and 20 ml). The accumulated filtrate was transferred to a 200-ml volumetric flask and made up to volume with buffer. The procedure for tablets and capsules was then followed, beginning with: "A 15-ml aliquot of the stock solution . . . ."

Syrups, Suspensions, and Pediatric Drops—The liquid preparation was thoroughly mixed, and then a suitable quantity was pipetted into a 100-ml volumetric flask and diluted to volume with the optimum buffer (concentration of stock solution ~0.5 mg/ml). If the tetracycline existed as an insoluble form such as the free base or complex, an appropriate amount of 3 *M* HCl was added to ensure dissolution. The procedure for tablets and capsules was then followed, beginning with: "A 15-ml aliquot of the stock solution . . . ."

In the presence of a calcium–oxytetracycline complex, it was necessary to add 3 moles of disodium ethylenediaminetetraacetic acid/mole of complex.

RESULTS AND DISCUSSION

Doskočil and Vondrácek (5) examined the polarographic behavior of chlortetracycline in phosphate buffer from pH 5.0 to 8.2. They stated that the optimum condition for quantitative work was pH 6.0. The behavior of tetracycline, chlortetracycline, and oxytetracycline in phosphate buffers was investigated (11), and the optimum pH was 5.91 for tetracycline, 5.70 for chlortetracycline, and 6.0 for oxytetracycline. The author showed well-resolved second waves for each of these antibiotics and a good relationship between concentration in micrograms per milliliter and diffusion current. A reasonably well-resolved second wave for tetracycline hydrochloride in phosphate buffer at pH 6.2 was reported (12), and a plot of diffusion current *versus* concentration was linear. However, there was no reported attempt to apply the method to the analysis of pharmaceutical products.

In the present investigation into the use of phosphate buffers as media for the polarographic reduction of the tetracyclines, the results reported previously (5, 11, 12) could not be reproduced.

Maxima appeared in the polarograms for all antibiotics over the entire range studied in the phosphate buffer. Figure 1 illustrates the polarograms obtained for tetracycline in this buffer system and is representative for the other substances used. Only a small maximum occurred at pH 5.86; but when 1 drop of 1% gelatin (0.13 ml) was added, a poorly resolved wave resulted. In one study, Doskočil (6)

[1] Sargent model XV.
[2] Corning model 10.

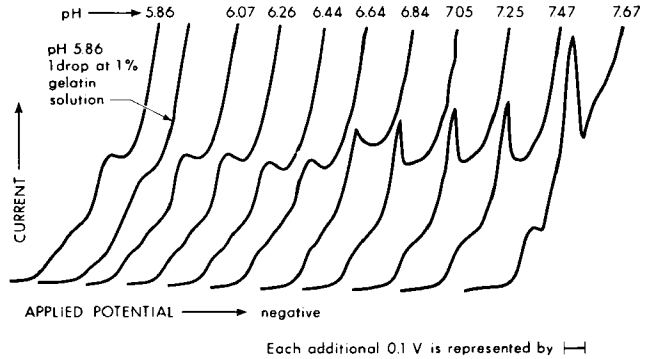

Figure 1—*Current–voltage curves of tetracycline hydrochloride in phosphate buffer. Each segment begins at −0.8 v. Concentration = 1.0 × 10⁻⁴ M.*

obtained poorly resolved waves at pH 8.3 with 0.2 *M* phosphate buffer. Silvestri (13) reported the behavior of doxycycline in phosphate buffer at pH 6, and his figures show that the two doxycycline waves were poorly resolved and could not be used quantitatively.

A method involving ac polarography was reported (14), and Hetman (15, 16) utilized differential cathode ray polarography with a twin-cell technique. While considerable advances have been made recently in ac polarography, the equipment for these two aspects is not so common that either can be employed on a routine analytical basis.

As outlined under *Experimental*, the suitability of a number of buffer systems for dc polarography was investigated. Polarograms were obtained at 0.2 pH intervals for each buffer over its entire operating range. Then solutions of the four tetracycline salts were prepared with each individual buffer solution. Only a few polarograms were found to be of analytical value.

In the Britton–Robinson buffer, the second wave for all tetracyclines was obscured by the reduction of hydrogen ion at pH values below 6. This result was generally true for all buffers studied. The glycine–hydrochloric acid buffer (pH 2.2–3.6) gave very poorly resolved polarograms for this reason. The same situation was observed with the lithium acetate–acetic acid buffer (pH 3.6–5.6) and potassium acid phthalate–sodium hydroxide buffer (pH 4.2–6.0). In the latter buffer, however, tetracycline hydrochloride gave three poorly resolved waves (pH 5.8–6.0), which were of no analytical value. Maxima were readily apparent over the entire range of the barbital sodium–hydrochloric acid buffer (pH 6.8–9.2). In the sodium borate–sodium hydroxide buffer (pH 9.3–10.0), a series of poorly resolved waves occurred for the tetracyclines.

Only in the boric acid–sodium borate buffer (pH 7.6–9.2) was it possible to achieve a well-resolved limiting current plateau for the tetracyclines (Fig. 2). The optimum pH for tetracycline hydrochloride was chosen to be 7.75, while 8.20 and 7.95 were the values for oxytetracycline and chlortetracycline, respectively. Figure 3 shows that demeclocycline gave either maxima or poorly resolved waves over the entire range of this buffer. However, the addition of 0.13 ml of 1% gelatin solution at pH 7.75 resulted in a satisfactory polarogram.

Solutions of 1×10^{-4} *M* were employed for all tetracyclines to determine diffusion dependency in the selected buffer system and at the optimum pH. The height of the mercury column was set at arbitrary intervals between 56.0 and 90.0 cm, and a polarogram was ob-

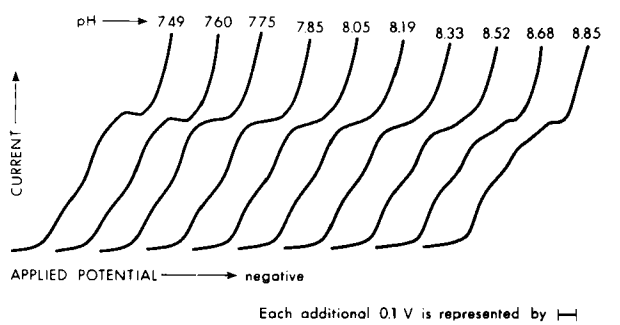

Figure 2—*Current–voltage curves of tetracycline hydrochloride in boric acid–sodium borate buffer. Each segment begins at −0.9 v. Concentration = 1.17 × 10⁻⁴ M.*

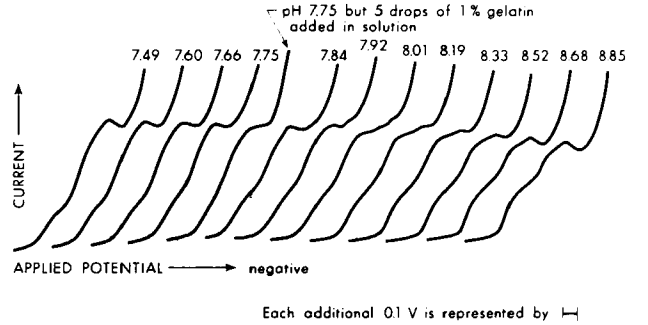

Figure 3—*Current–voltage curves of demeclocycline hydrochloride in boric acid–sodium borate buffer. Each segment begins at −0.9 v. Concentration = 1.05 × 10⁻⁴ M.*

tained at each interval. A plot was made of the current against the square root of the corrected mercury column height. The result was a straight line which, upon extrapolation, did not pass through the origin. Based upon Zuman's (17) report, it was concluded that, although the reduction process is largely diffusion dependent, kinetic factors also play a part. However, plots of total diffusion current *versus* concentration were linear for tetracycline hydrochloride and chlortetracycline hydrochloride from 0.01 to 0.125 mg/ml. For oxytetracycline hydrochloride and demeclocycline hydrochloride, linearity extended from 0.01 to 0.1 mg/ml; thereafter, the plot began to flatten.

The slopes of the respective calibration curves were as follows: tetracycline hydrochloride, 2.58; chlortetracycline hydrochloride, 2.20; oxytetracycline hydrochloride, 1.96; and demeclocycline hydrochloride, 2.96. Total diffusion current for the two waves was read in all instances, because the first wave was not sufficiently well resolved to permit separate measurements of either wave.

Tablets and Capsules—The general procedure was used without exception, even for products containing tetracycline phosphate complex (see Samples 15–18 in Table II). No calibration curve was developed for the complex because of batch-to-batch differences in the stoichiometry. Since the label of these products also declared a tetracycline hydrochloride equivalent, it was feasible to use the calibration curve for that antibiotic.

As a further check, tetracycline waves were determined in the presence of disodium as well as monosodium phosphate. No influence was observed until the phosphates were present in a quantity greatly exceeding the amount in the pharmaceutical product. Analytical results for all commercial preparations are given in Table II. As outlined under *Experimental*, 0.13 ml of 1% gelatin solution was employed for each demeclocycline hydrochloride sample.

Syrups, Suspensions, and Pediatric Drops—When the dosage form existed as the free base or as an insoluble complex, an appropriate amount of 3 *M* HCl was added to bring the antibiotic into solution. The acid would split any complex, with the resulting formation of calcium chloride. Before polarographing, this latter substance must be sequestered by the further addition of disodium ethylenediaminetetraacetic acid. In all instances, after the addition of hydrochloric acid, it was necessary to readjust the pH carefully to the optimum value by the addition of sodium borate powder. The general procedure was then followed.

Interferences—Nystatin was present in one capsule (Sample 18) and interfered with the tetracycline wave. Its presence was compensated for by running a polarogram on the amount of nystatin and subtracting its current from the total current obtained with the combined tetracycline–nystatin wave.

Polymyxin B sulfate interfered with the assay of ointments, and products containing that antibiotic could not be analyzed for their tetracycline content.

None of the commercial powders for injection could be assayed because of the presence of one or more of the following: procaine hydrochloride, magnesium chloride, and ascorbic acid. All interfered with the reduction of tetracycline, but magnesium chloride could be removed by chelating with disodium ethylenediaminetetraacetic acid.

The high values obtained with several liquid dosage forms can be attributed to undeclared, reducible preservatives, solubilizers, and stabilizers in the syrups and suspensions. If the nature and quantity of these substances were known to the analyst, it would be a simple

Table II—Analysis of Tetracycline Antibiotics

Sample	Dosage Form	Labeled Strength, %	
		Polarographic[a]	Manufacturer Assay
	Tetracycline Hydrochloride		
1	250-mg capsule	108.9 ± 0.6	Not available
2	250-mg capsule	107.2 ± 0.5	112.0
3	250-mg capsule	107.4 ± 0.7	109.0[b] 107.5[c]
4	250-mg capsule	101.3 ± 0.5	103.2[b]
5	250-mg capsule	99.9 ± 1.0	98.2[b] 101.0[c]
6	250-mg tablet	104.2 ± 0.2	106.0[b]
7	250-mg tablet	96.7 ± 0.5	102.5[b] 97.0[c]
8	1% ophthalmic ointment	110.2 ± 0.2	106.6[b] 115.8[c]
9	3% topical ointment	108.8 ± 0.2	103.5[d]
10	125-mg/5 ml suspension	134.7 ± 0.9	Not available
11	25-mg/ml syrup	110.7 ± 0.5	102.9[b] 116.0[c]
12	25-mg/ml suspension	124.5 ± 0.2	Not available
13	25-mg/ml syrup	107.3 ± 0.4	112.8[b]
14[e]	25-mg/ml suspension	134.6 ± 0.1	113.1
15[f]	100-mg capsule	109.6 ± 0.3	108.0[b]
16[f]	250-mg capsule	102.1 ± 0.1	106.4[b]
17[f]	500-mg capsule	100.8 ± 0.8	102.0[b]
18[f]	250-mg capsule	100.2 ± 0.2	108.8[b]
	Chlortetracycline Hydrochloride		
19	3% topical ointment	100.7 ± 1.1	107.8[b] 112.8[c]
20	1% ophthalmic ointment	89.6 ± 0.4	94.8[b] 106.8[c]
21	250-mg capsule	106.7 ± 0.8	108.0[b] 108.9[c]
	Demeclocycline Hydrochloride		
22	300-mg tablet	106.4 ± 0.2	110.0[b] 103.7[c]
23	150-mg capsule	104.8 ± 0.4	110.0[b] 105.9[c]
	Oxytetracycline		
24[g]	25-mg/ml syrup	127.2 ± 0.7	114.0[b]
25[g]	100-mg/ml drops	96.5 ± 0.4	113.0
26[h]	250-mg capsule	103.4 ± 0.8	103.9
27[h]	250-mg capsule	98.3 ± 0.3	99.4[b] 98.3[c]

[a] Average of five determinations ± *SD*. [b] Microbiological assay. [c] Spectrophotometric assay. [d] Chromatographic assay. [e] Tetracycline base. [f] Tetracycline phosphate. [g] Calcium salt. [h] Oxytetracycline hydrochloride.

matter to compensate for them by performing an appropriate blank.

Table II also contains the assay values provided by the manufacturers. In many instances, two methods of assay were employed, a spectrophotometric and a microbiological, and frequently their values agreed only moderately well. For most capsules and tablets, the proposed method agreed within 2% with one result or the other. Since the lack of accuracy by the microbiological method is well documented, it is not surprising to find more frequent agreement between the results of the polarographic and spectrophotometric methods.

CONCLUSION

The technique of dc polarography was applied to the analyses of a number of pharmaceutical products containing one of four tetracyclines. In general, satisfactory results were obtained with most dosage forms. However, interference resulted from the presence of certain other antibiotics as well as other electroreducible substances such as procaine hydrochloride and ascorbic acid. Optimum results for the tetracyclines were obtained with a boric acid–sodium borate buffer system.

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Inhibitory Effect of Dioctyl Sodium Sulfosuccinate on Pepsin Activity

GURCHARAN S. JODHKA, SAID A. KHALIL [x], and M. WAFIK GOUDA [x]

Abstract □ The inhibitory effect of dioctyl sodium sulfosuccinate on hog pepsin activity was investigated over the pH 1.5–3.0 range. The inhibitory effect was studied using a natural substrate, hemoglobin, and a synthetic substrate, *N*-acetyl-L-phenylalanyl-L-diiodotyrosine. The mechanistic studies revealed that a substrate–inhibitor interaction was the major mechanism of inhibition with hemoglobin. However, some direct enzyme inhibition also was involved. With the synthetic substrate, the inhibition was due to a competition between the substrate and the inhibitor molecules for the enzyme. The possible therapeutic significance of the inhibitory effect of the medicinal surfactant is discussed.

Keyphrases □ Dioctyl sodium sulfosuccinate—mechanism of inhibition of pepsin activity □ Pepsin activity—mechanism of inhibition by dioctyl sodium sulfosuccinate □ Digestive enzymes—pepsin, mechanism of inhibition by dioctyl sodium sulfosuccinate □ Surfactants—dioctyl sodium sulfosuccinate, mechanism of inhibition of pepsin activity

Peptic ulcers can be defined as acute or chronic ulcerations of the digestive tract, occurring in an area accessible to gastric secretions. In spite of much recent effort, this disease continues to be an incompletely understood and a rather common disorder. Although little is known about the definite etiology of the disease, the interrelationship of a number of mucosal defensive and aggressive factors seems to determine an individual's susceptibility to ulcers (1, 2). Whatever the causative factors, it has now been unequivocally established that, through some unknown mechanism, the normal resistance of the gastric mucosa to acid and pepsin is compromised in all peptic ulcers (1).

Much experimental evidence is available indicating the importance of pepsin in the pathogenesis of the disease (3–6). In humans, the role of this enzyme in gastroduodenal ulceration was indirectly evidenced by the antiulcer activity shown by some macroanions, which inhibited peptic hydrolysis (7, 8). Oral administration of pepsin inhibitors such as degraded carrageenan, amylopectin sulfate, and a sulfated glycopeptide resulted in significant prevention and treatment of peptic ulcers in humans (9, 10). It was reported that the antipeptic effect of these sulfated macromolecules is related to the negative charge of the sulfate group and their high molecular weight (11–14).

Dioctyl sodium sulfosuccinate, an anionic surfactant that is widely used medicinally as a fecal softener, is a molecule with a relatively high molecular weight. Its inhibitory effect on tryptic activity was reported previously (15), and it strongly suppressed ulcer formation in restrained rats when given intraduodenally (16). These findings led to the present investigation, which dealt with the inhibitory effect of dioctyl sodium sulfosuccinate on the peptic activity of pepsin from hog stomach mucosa.

EXPERIMENTAL

Materials—Pepsin[1], hemoglobin[2], *N*-acetyl-L-phenylalanyl-L-diiodotyrosine[3], dioctyl sodium sulfosuccinate[4], and ninhydrin[5] were used as received. All other chemicals were either USP or reagent grade.

Methods for Antipeptic Activity—Two different methods for determining the antipeptic activity were used.

Method I—In this method, denatured hemoglobin was digested under standard conditions. The undigested hemoglobin was precipitated with trichloroacetic acid, and the amount of unprecipitated protein was estimated spectrophotometrically. The method used was a modification of Rajgopalan *et al.* (17).

The substrate (0.4–1.0 mg/ml), enzyme (10–60 μg/ml), and inhibitor (50–400 μg/ml) were prepared in hydrochloric acid (pH 1.8) or distilled water. All solutions were freshly prepared, adjusted to pH 1.8 immediately after preparation, and brought to 37° before mixing. One milliliter of the enzyme (pepsin) solution (I) was pipetted into each flask, already maintained at 37° in a metabolic water bath shaker[6]. Then 1 ml of the inhibitor (dioctyl sodium sulfosuccinate) solution (II) was added to each flask, followed by 2 ml of the substrate (hemoglobin) solution (III).

[1] From hog stomach mucosa, 3× crystallized and lyophilized, Nutritional Biochemicals Corp., Cleveland, Ohio.

[2] Denatured, standardized for protease assay, Nutritional Biochemicals Corp., Cleveland, Ohio.

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

[4] Aerosol O. T. 100%, Sargent-Welch Scientific Co., Skokie, Ill.

[5] J. T. Baker Chemical Co., Philipsburg, N.J.

[6] Model G-77, New Brunswick Scientific Co., New Brunswick, N.J.