Comparison of Aspirin and Copper Aspirinate with Respect to Gastric Mucosal Damage in the Rat

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Abstract □ The copper salt of aspirin has been compared with aspirin in terms of damage to mucosal tissue. Using a protein-bound dye to highlight erosions, it has been found that copper aspirinate is at least as damaging as aspirin itself. This finding is not in agreement with previously published claims. Copper aspirinate produced more widespread superficial erosions and slightly less deep erosions than aspirin alone. Mixtures of the copper(II) ion and aspirin produced results similar to copper aspirinate, suggesting that the hydrolysis products of copper aspirinate, copper(II) ion and aspirin, together may be especially damaging to the mucosa. Copper alone was not damaging, but aspirin alone yielded intermediate results. Short incubation times produced only erosions (no ulcers), which were clearly differentiated into two classes by depth of color. Histological examination verified this classification into superficial and deep erosions.

Keyphrases □ Copper aspirinate—gastric mucosal damage in the rat, protein-bound dye, histology, comparison with aspirin □ Aspirin—gastric mucosal damage in the rat, protein-bound dye, histology, comparison with copper aspirinate □ Ulceration—gastric mucosa, rat, aspirin and copper aspirinate, comparison using protein-bound dye and histology

Gastric mucosal damage induced by the administration of aspirin has been studied for many years. The animal model most often used has been the rat, in which, following exposure to aspirin, numerous ulcers have been found in the corpus (with a few in the antrum) (1, 2). It has been demonstrated that aspirin is absorbed from the corpus and antrum, but not the rumen (3, 4). Buffered aspirin has been shown to produce fewer lesions than aspirin alone (1, 5–7), presumably because the buffered aspirin is not absorbed from the stomach but from the intestine.

In 1976 Sorenson (8, 9) reported that copper aspirinate [tetrakis(acetylsalicylato)-μ-dicopper(II)], the copper salt of acetylsalicylic acid, had a strong antiulcer effect compared with aspirin alone when administered during the period of ulcer development in the Shay rat (10). On the basis of his observations, Sorenson suggested that the copper complex of aspirin may be the pharmacologically active form of this drug. Numerous other researchers then investigated the lesion-producing ability of copper aspirinate in the rat model (11–16). Although some conclusions were drawn supporting Sorenson's claim, these studies leave considerable uncertainty concerning the relative ulcerogenic potential of copper aspirinate and aspirin.

The results presented here are an attempt to make a quantitative comparison of the effect of aspirin and copper aspirinate on the gastric mucosa of the rat, using a protein-bound dye to highlight lesions (17) and histological examination of the tissue to validate the interpretation of the dye observations. With respect to the copper aspirinate, experiments were included to differentiate between the effects of the complex and its hydrolysis products, aspirin and the copper(II) ion. A moderate (pharmacological) dose and short incubation period were used in all experiments. In a separate set of experiments, all compounds were given with a buffer system to assess the effect of the buffer when the drug is prevented from leaving the stomach by ligation of the duodenum.

EXPERIMENTAL

Since lesion formation due to aspirin is altered by the presence of food in the stomach (5, 17), all animals (male Sprague–Dawley-derived rats, 150–300 g body weight) were fasted 24 hr prior to the experiments. Anesthesia was induced with ether and maintained with an intraperitoneal injection of sodium pentobarbital[1] (50 mg/kg of body weight). An endotracheal cannula was inserted through a midline neck incision. The abdomen was opened and the duodenum ligated, care being taken not to obstruct the vascular supply to the stomach. An infant feeding tube was inserted through the esophagus into the stomach, and the stomach was rinsed with 10–15 ml of saline to remove bits of food and other foreign matter. The test suspension (0.5 ml/100 g of body weight) was instilled, and the tube removed. Preliminary experiments indicated that this volume of fluid resulted in an intragastric pressure of <4 cm H_2O when initially administered. The abdomen was closed with wound clips.

The compounds or mixtures employed in these experiments were ground to fine powders, and test suspensions in 0.1% aqueous nonionic surfactant[2] were prepared by sonicating the appropriate amount of the test compound or mixture for 10 min. A nonionic suspending agent was chosen in order to avoid decomposing the copper complex (18). Molar equivalents of the copper(II) ion and aspirin were used in the experiments, as shown in Table I. The suspension volume administered each animal was determined from its body weight (0.5 ml/100 g of body weight). In each case the dose was equivalent to 100 mg aspirin/kg. The suspensions tested were aspirin, copper aspirinate (19), copper sulfate plus aspirin, and copper alone (in the form of copper sulfate). All suspensions were given with and without a buffer. The buffered suspensions contained 3.0 mg/ml of dihydroxyaluminum glycinate[3] and 6.0 mg/ml of magnesium carbonate. This buffer system is the same as that used in a commonly available proprietary product (20).

The suspensions were allowed to incubate in the stomach for 120 min. Ten minutes prior to the end of the incubation period, the animals were given an intravenous (femoral vein) injection of 5% protein-binding dye[4] in saline (17). At 120 min of incubation, the animals were sacrificed. The stomachs were immediately removed, opened along the lesser curvature, and washed with ~10 ml of saline. When necessary, a camel's hair brush was used to remove the mucous layer. The stomachs were pinned over rubber stoppers and examined under a dissecting microscope. Lesions could easily be separated into two groups with well-defined boundaries depending on the intensity of the dye in the tissue. Each of the two groups (one a light blue, the other very dark) was scored by counting the number of lesions (x) in each of five size classes (y). The classes were defined according to Guth *et al.* (2, 21): $y = 1$ (pinpoint lesions), $y = 2$ (lesions <1 mm in diameter), $y = 3$ (lesions 1–2 mm in diameter), $y = 4$ (lesions 2–4 mm in diameter), and $y = 5$ (lesions >4 mm in diameter). A lesion index was computed by:

$$\text{Lesion Index} = \sum_{i=1}^{5} x_i y_i$$

Mean, standard deviation, and standard error were calculated for each group of rats; the number of rats per group ranged from 6 to 17. The stomachs were stored in 10% formalin in saline and recounted after 24

[1] Diabutal; Diamond Labs, Des Moines, Iowa.
[2] Tween 80; ICN Pharmaceuticals, Inc., Cleveland, Ohio.
[3] Dihydroxyaluminum glycinate was generously donated by the Bristol-Meyers Company.
[4] Pontamine Sky Blue 6BX; Pfaltz and Bauer, Stamford, Conn.

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Table I—Test Suspensions of Drug in 0.1% Aqueous Suspending Medium [a]

Compound	Compound, mg	Aspirin, mmole	Copper, mmole
Aspirin	20.0	0.111	—
Copper aspirinate	23.6	0.111	0.0558
Copper sulfate	13.9	—	0.0558
Copper sulfate/aspirin	13.9/20.0	0.111	0.0558

[a] Per milliliter of suspension. Buffered suspensions also contained 3.0 mg/ml of dihydroxyaluminum glycinate and 6.0 mg/ml of magnesium carbonate (20).

hr. The average of the two values was recorded as the lesion index. The Student's t test (unpaired) was applied as a test of significance ($p < 0.05$).

Representative samples of each type of lesion were taken from the stomach, embedded in paraffin, and stained (hematoxylin–eosin) for histological evaluation. The blocks were sectioned in a serial manner so that the lesions were not missed. The evaluation of the histological material was done under a double-blind procedure.

RESULTS

The lesions outlined by the blue dye were separated into two classes based on two distinct colors of damaged mucosal tissue. Histological examination revealed that the lighter of these were superficial erosions (penetrating less than halfway through the mucosa) and the darker were deep erosions (penetrating more than halfway through the mucosa). No true ulcers were produced in any of these systems. There is excellent agreement between the two methods for rats tested with copper aspirinate or a mixture of copper(II) ion and aspirin, but poor agreement in the experiments involving aspirin alone (Table II). In terms of the lesion index, agreement is good where the index is large, but poor where the index is small.

Figure 1 displays lesion index values calculated by the equation for superficial and deep erosions. In addition to differences in the depth of dye color (due to extent of leakage of the dye–protein complex from

Table II—Agreement of Lesion Grading by Technique [a]

Sample	Treatment	Dye Evaluation	Histological Evaluation [b]	Agree Disagree
1	Copper aspirinate	Superficial	Superficial	Agree
2		Deep	Deep	Agree
3		Superficial	No Lesion	Disagree
4		Superficial	Superficial	Agree
5		Deep	Deep	Agree
6		Superficial	Deep	Disagree
7		Superficial	Superficial	Agree
8		Deep	Deep	Agree
9		Deep	Deep	Agree
10	Copper sulfate plus aspirin	Deep	Deep	Agree
11		Superficial	Deep	Disagree
12		Deep	Deep	Agree
13		Superficial	Superficial	Agree
14		Deep	Deep	Agree
15		Superficial	Superficial	Agree
16	Aspirin	Deep	Superficial	Disagree
17		Deep	Superficial	Disagree
18		Deep	Deep	Agree
19		Deep	Deep	Agree
20		Superficial	None	Disagree
21		Superficial	None	Disagree
22		Deep	Deep	Agree
23		Deep	Superficial	Disagree
24		Superficial	Superficial	Agree
25	Aspirin plus buffer	Deep	Deep	Agree
26		Superficial	None	Disagree
27		Deep	None	Disagree
28		Superficial	Deep	Disagree
29		Deep	Deep	Agree
30		Superficial	Deep	Disagree

[a] In the dye evaluation, assignment was made on the basis of color; in the histological evaluation, on the basis of penetration into the mucosal surface. [b] Superficial lesions extended less than one-half the mucosal thickness; deep lesions penetrated more than one-half the thickness. No lesion penetrated the muscularis mucosa.

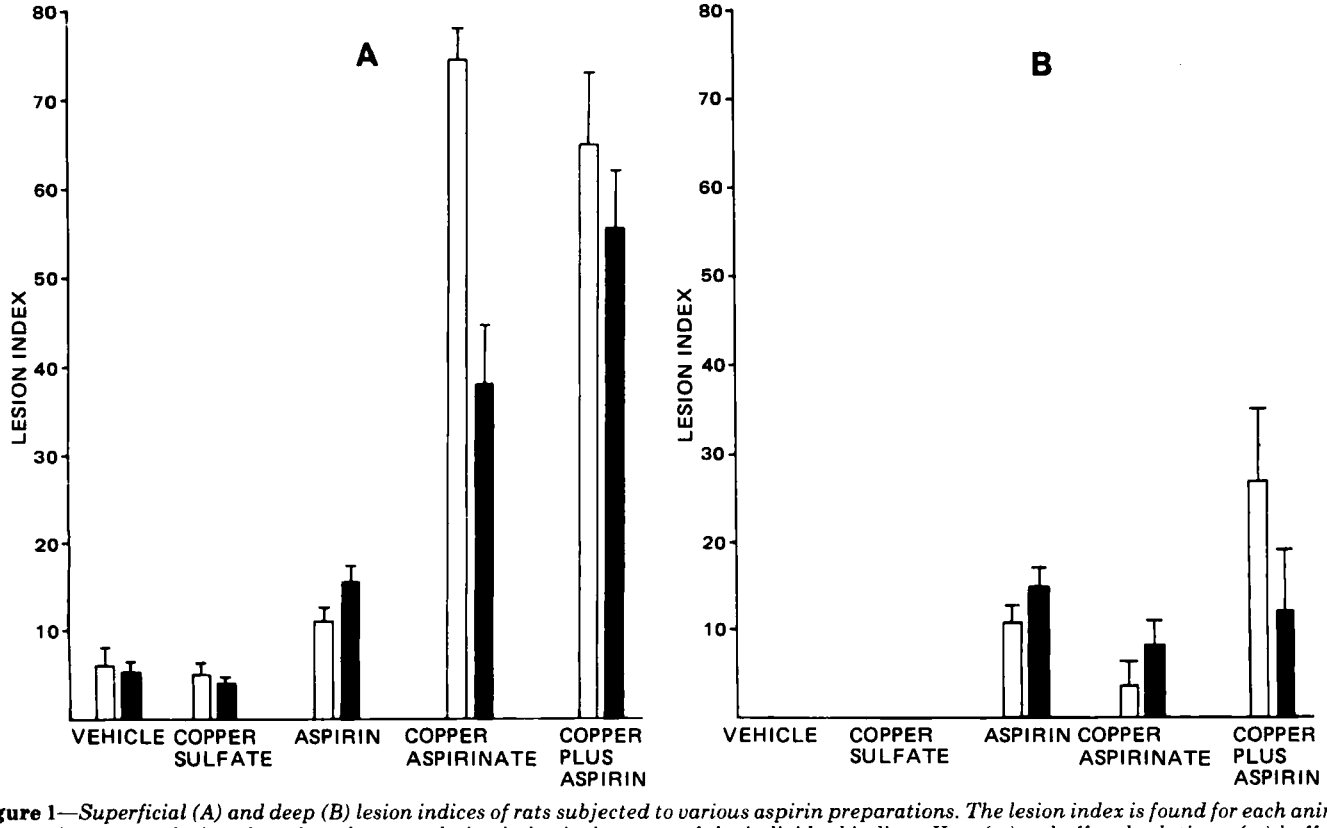

Figure 1—*Superficial (A) and deep (B) lesion indices of rats subjected to various aspirin preparations. The lesion index is found for each animal by summing over each size of erosion; the group lesion index is the mean of the individual indices. Key: (□) unbuffered solutions; (■) buffered solutions. The bars represent +1* SEM.

Table III—Distribution of Superficial Lesions by Size [a]

Test Compound	n		Number of Lesions of Each Size 1	2	3	4	5	Lesion Index
Control	7	Mean	2.36	1.86				6.07
		SEM	0.43	0.89				2.14
Control (buffered)	6	Mean	3.58	0.83				5.25
		SEM	1.23	0.33				1.38
Copper sulfate	8	Mean	3.44	0.75	0.25			5.06
		SEM	0.63	0.37	0.16			1.61
Copper sulfate (buffered)	12	Mean	2.42	1.67				4.08
		SEM	0.59	0.79				0.70
Aspirin	17	Mean	5.94	2.35	0.18			11.18
		SEM	0.95	0.53	0.13			1.89
Aspirin (buffered)	17	Mean	7.50	2.53	0.88	0.06		15.35
		SEM	2.01	0.49	0.32	0.06		2.40
Copper aspirinate	12	Mean	50+	6.58	1.92	0.71	0.62	74.75
		SEM		1.06	0.48	0.35	0.42	3.43
Copper aspirinate (buffered)	10	Mean	7.89	6.39	3.67	1.61	0.45	38.30
		SEM	1.52	1.45	0.71	0.90	0.30	6.26
Copper sulfate plus aspirin	11	Mean	9.68	6.04	3.77	2.91	4.23	65.30
		SEM	1.10	0.88	0.65	1.07	0.98	8.65
Copper sulfate plus aspirin (buffered)	17	Mean	20.26	5.79	3.97	1.74	0.91	55.53
		SEM	4.15	1.06	0.67	0.37	0.27	7.02

[a] The diameter of individual lesions determined the category into which they fell for counting. The mean represents the average number of lesions of each size, and the lesion index is the score obtained by summing.

Table IV—Distribution of Deep Lesions by Size [a]

Test Compound	n		Number of Lesions of Each Size 1	2	3	4	5	Lesion Index
Control	7	Mean						0
		SEM						
Control (buffered)	6	Mean						0
		SEM						
Copper sulfate	8	Mean						0
		SEM						
Copper sulfate (buffered)	12	Mean						0
		SEM						
Aspirin	17	Mean	4.82	2.30	0.38			10.62
		SEM	1.17	0.46	0.17			1.51
Aspirin (buffered)	17	Mean	4.71	3.59	0.55	0.29		14.62
		SEM	1.96	1.26	0.33	0.21		5.44
Copper aspirinate	12	Mean	0.54	0.67	0.21	0.17		3.17
		SEM	0.29	0.40	0.11	0.11		1.05
Copper aspirinate (buffered)	10	Mean	1.44	2.00	0.89			8.11
		SEM	1.44	0.74	0.53			3.06
Copper sulfate plus aspirin	11	Mean	4.36	5.00	2.77	0.77	0.14	26.68
		SEM	1.54	1.45	1.00	0.41	0.14	7.15
Copper sulfate plus aspirin (buffered)	17	Mean	3.85	2.56	0.91	0.03		11.88
		SEM	1.04	0.56	0.32	0.02		2.57

[a] The diameter of individual lesions determined the category into which they fell for counting. The mean represents the average number of lesions of each size, and the lesion index is the score obtained by summing.

damaged blood vessels), deep lesions were small and circular or oval, whereas superficial erosions were irregular in shape and considerably larger. The difference in size is apparent from the comparative lesion indices for the two types of erosions, which are plotted on the same scale in Fig. 1A and B. In general, the presence of the buffer had no significant effect on the extent of damage produced under these experimental conditions.

Copper aspirinate and a mixture of aspirin and copper sulfate caused significant increases over controls in both the superficial and deep lesion indices. Copper aspirinate and copper(II) ion plus aspirin had significantly higher superficial lesion indices than aspirin alone ($p < 0.001$), but were not significantly different from each other. The copper aspirinate deep lesion index was significantly less than the aspirin deep lesion index in the unbuffered systems ($p < 0.001$), but not in the buffered systems.

Tables III and IV contain data from which the lesion indices were calculated. It is clear from this data that the higher superficial lesion index of copper aspirinate and copper(II) ion plus aspirin represent both more and larger erosions than are found for aspirin alone. There does not appear to be a clear difference between the deep erosion data of copper aspirinate and aspirin.

Some deep erosions in categories 3, 4, and 5 were observed to have red or brown centers to which the blue dye had not penetrated; it was initially supposed that these represented ulcers. On histological examination, however, these lesions were found to be erosions, indicating that disruption of the circulation occurs prior to ulcer development. A small number of these appeared in aspirin-treated rats, somewhat more in copper aspirinate-treated rats, and the most in copper-plus-aspirin-treated rats. When copper sulfate was used in the absence of aspirin, the lesion indices were not significantly different from the controls for either superficial or deep erosions.

DISCUSSION

Initial damage to the mucosa from absorption of aspirin is more easily and accurately assessed by the use of a protein-binding dye. Two stages prior to ulcer formation can be clearly distinguished by the loss of the dyed protein into the tissue. The darker blue lesions, as can be seen from the agreement of histological and dye diagnoses (Table II), are deep erosions, penetrating more than halfway through the mucosa. Of the 16 lesions diagnosed as deep by the dye method, 76% were in agreement using the histological diagnosis.

For lesions diagnosed as superficial erosions by the dye method, agreement was poor; less than half were in agreement with the histological diagnosis. Of those that did not agree, the majority were found to have no erosion by the microscopic examination. Thus, the superficial erosions identified by the dye technique included both erosions penetrating less than halfway through the mucosa and pre-erosion lesions which allowed loss of the protein–dye complex from the gastric capillaries. The minimal pathological change necessary to allow the dye to accumulate in the mucosa would be an alteration in capillary permeability of sufficient magnitude to allow transendothelial movement of the protein–dye complex. Robins (22) has shown, by transmission electron microscopy, that the first structural change after administration of aspirin is alteration of the capillary endothelial basement membrane, which leads to the breakdown of small blood vessels before any other cytolytic effects appear. Thus, bleeding (and the loss of the protein–dye complex into the tissue) could occur without gross visible damage to the mucosal structures. Williams *et al.* observed no erosions when copper aspirinate was given to rats, but even after 18 hr, noted that bleeding had occurred (12). Their technique would not have allowed detection of these lesions.

In those experiments in which the superficial lesion index was highest, the correlation between dye and histological evaluation was remarkable. Table II indicates that in copper aspirinate experiments, agreement was 7 of 9, and in copper-plus-aspirin experiments, agreement was 5 of 6. In experiments where the superficial lesion index was low, correlation was poor: for aspirin alone, agreement was 4 of 9, and for buffered aspirin, 2 of 6. Thus, in terms of the calculated superficial lesion indices, the difference between copper aspirinate and aspirin is even larger than it appears to be from Fig. 1A. For the deep lesion index, the only disagreements in diagnosis were overdiagnosis by the dye method in the aspirin and aspirin-plus-buffer classes. Thus, the calculated deep lesion index for aspirin would be less than that indicated in Fig. 1B, whereas those for copper aspirinate and copper-plus-aspirin would be unchanged. It is clear that these data do not support the Sorenson conclusions that copper aspirate is less damaging to stomach mucosa than aspirin.

The Shay rats (10) used by Sorenson are prepared by ligating the duodenum and allowing acid to accumulate in the stomach for 16–19 hr, resulting in ulcer formation. In Sorenson's experiments (8), the aspirin or copper aspirinate was placed in the stomach during the period of ulcer formation. At the end of the experiment, an ulcer index was calculated. The substance was deemed active if the ulcer index was less than that of the controls. Under this protocol, both aspirin and copper aspirinate were reported to be active antiulcer agents, with copper aspirinate much more so than aspirin. These conclusions actually refer to the change in the rate of ulcer formation or healing in the Shay rat model. The excess acid and very long incubation times make Sorenson's results unique and of questionable value in the study of lesion formation by aspirin and related compounds under normal conditions of absorption.

With respect to the other studies in which copper aspirinate was tested, conditions varied greatly (11–16). Williams *et al.* (12) did not fast rats prior to experimentation and did not examine the stomachs for 18 hr after dosing. It has been shown that fed rats develop fewer stomach lesions from aspirin than do fasted rats (5, 17) and that spontaneous healing of lesions begins within 6 hr after damage (5). These authors tallied numbers of erosions, but did not assess size or severity of lesions. They reported that while rats treated with aspirin developed 50% more lesions (histological evaluation of lesion type was not done) than controls, those treated with copper aspirinate developed no erosions (though bleeding was observed). The significance of these data is not clear due to the aforementioned variations in the experimental protocol. Rainsford and Whitehouse (13) reported ulcers formed in groups of five fasted rats given aqueous suspensions of aspirin or copper aspirinate. Those given copper aspirinate were also subjected to cold stress. These authors concluded that since the copper aspirinate-treated rats (with cold stress) did not have significantly more mucosal damage than the aspirin-treated rats, copper aspirinate caused less damage to the mucosa. The data reported in this paper, using larger groups of rats, does not support their conclusions. Boyle *et al.* (11), reporting the percentage of rats having at least one "area of erosion," found that for aspirin alone this figure was ~98% and for copper aspirinate ~15%. Their paper refers to both erosions and ulcers, and it is not clear which were counted. The difference in results is perhaps due to the difference in assessment—the lack of a protein-binding dye to enhance preulcer lesions and a counting formula that did not include both number and size of lesions. Sorenson's results were also tested by Lewis *et al.* (14–16). The rats they used were very young, and it has been shown that juvenile rats do not have the same susceptibilities to aspirin-induced lesions as adult rats (23). Results were about the same for free aspirin as for the copper complex. Observations were made after 6 hr of incubation without magnification or the aid of a dye.

The experiments reported in this paper eliminated as many of these variables as possible. Using a protein-binding dye and summing over both number and sizes of lesions, clear differences appeared between the effects of copper aspirinate and aspirin. Figure 1B shows that copper aspirinate produced fewer deep erosions than aspirin alone although there was apparently a greater disruption of blood flow from copper aspirinate as indicated by the absence of dye in the center of some of these erosions. The difference in the deep lesion indices for these two systems was not significant when buffer was present. The copper aspirinate yielded larger numbers of superficial lesions than aspirin alone (Fig. 1A). The combination of copper plus aspirin caused more deep lesions than either aspirin or copper aspirinate alone, suggesting that it is the hydrolysis products of the copper aspirinate that are the most potent lesion-producing system. The smaller deep lesion index of copper aspirinate *versus* aspirin may be due, in part, to the slow hydrolysis of the complex. The concentration of the hydrolysis products (copper and aspirin) may be sufficient to produce large numbers of superficial lesions, but not enough to produce as many deep lesions as the aspirin alone.

With the exception of one experimental group, in the experimental protocol employed here, the addition of buffer did not result in a significant decrease in lesion formation. Only in the group of animals receiving copper aspirinate did the addition of buffer result in a significant decrease in the formation of deep erosions (Fig. 1B). This should not be interpreted as contradicting the known fact (24) that buffered aspirin is less damaging to stomach mucosa than aspirin alone. In these experiments, the stomach was isolated from the intestinal tract so that the drug was held in the stomach for the duration of the experiment.

The data presented allow several conclusions. First, if one employs protein-bound dye to mark or outline gastric lesions, the severity or type of lesion must be verified by histological methods. The appearance of the dye on the mucosal surface can result from prelesion capillary endothelial damage, erosions, or ulcers. The protocol employed here did not produce ulcers, and it was possible to differentiate between deep and superficial erosions by the color characteristics of the extravasated dye. The dye method includes pre-erosion capillary endothelial damage with superficial erosions. Secondly, the administration of copper aspirinate produced larger numbers and sizes of superficial erosions than did aspirin alone. The frequency of deep erosions was slightly less for copper aspirinate than for aspirin alone. Thirdly, the combination of copper(II) ions and aspirin resulted in a greater effect (a larger deep lesion index) than the copper complex, suggesting that it may be the hydrolysis products of the copper aspirinate complex which cause the more severe gastric mucosal damage. Finally, the addition of a buffer to the preparation resulted in a decrease in lesion formation only in the case of copper aspirinate and only as evaluated from superficial erosions.

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Hemolysis Caused by Cetomacrogol 1000: Evidence for Hydroxyl Radical Participation

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Abstract □ The mechanism of cetomacrogol 1000-induced hemolysis was investigated. Previous conclusions that peroxides are involved in the hemolytic process were confirmed. The possibility that hydrogen peroxide, superoxide, hydroxyl radical, or singlet oxygen, which are known to induce hemolysis, are involved in cetomacrogol 1000-induced hemolysis was tested by using specific inhibitors and inactivators. The hydroxyl radical ($OH^{\cdot}$) was shown to be the only apparent oxygen species involved in cetomacrogol 1000-induced hemolysis. Its contribution to the hemolytic potency of the surfactant is ~30%.

Keyphrases □ Cetomacrogol 1000—induction of hemolysis, peroxidation, hydroxyl radical participation, erythrocytes □ Surfactants—cetomacrogol 1000-induced hemolysis, peroxidation, hydroxyl radical participation □ Peroxides—role in surfactant-induced hemolysis, cetomacrogol 1000, hydroxyl radical participation

Polyoxyethylene-derived surfactants are widely used as pharmaceutical aids, although their tendency to form peroxides is well known (1–4) and their deleterious effects on various drugs have been proven (5–7). Like other surface-active compounds, the polyoxyethylene surfactants cause hemolysis when they come in contact with red blood cells (7–9). Results recently published (10) indicate that the hemolytic activity of polyoxyethylene surfactants may be ascribed to their tendency to form peroxides. It should, however, be emphasized that hemolysis is only one form of cytotoxicity.

Peroxidation is believed to be one of the main mechanisms that give rise to membrane damage and cellular death (11–16). Destruction of normal tissue as a consequence of inflammatory reactions is also ascribed to oxygen radicals. The superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide, the hydroxyl radical ($OH^{\cdot}$), and the oxygen radicals ($^1O_2$) are highly reactive and can damage most types of cellular polymers. They have been shown to oxidize proteins, peroxidize fatty acids, and cleave polysaccharides *in vitro* (15). All were found to induce lysis of various types of cells (17–19). The abundance of polyunsaturated fatty acids in cell membranes and the facility with which these undergo oxidation are assumed to be the cause of peroxide-induced lysis (20).

Because of the widespread use of these surfactants and the awareness of the pernicious effect of free radicals and especially peroxides, we decided to study the mechanism by which these compounds induce peroxidation, with the ultimate aim of finding potential antidotes to these harmful effects. Their simple structure, easy availability, and the facility with which cell damage can be estimated by means of hemolysis make erythrocytes the ideal model for such research. Cetomacrogol 1000 (polyethylene glycol 1000 monocetyl ether) was chosen as the model polyoxyethylene surfactant in this research.

EXPERIMENTAL

Materials—Cetomacrogol 1000, polyethythelene glycol 1000 monocetyl ether[1], is a solid and, thus, not sensitive to autooxidation (4). Solutions of cetomacrogol 1000 were freshly prepared. Histidine[2], tryptophan[3], mannitol[3], thiourea[4], and digitonin[2] were commercial samples. Hydroquinone[5] was crystallized from benzene prior to use. Catalase[6] was dialyzed against water (specific activity 1.57×10^6 U/ml), superoxide dismutase[3] had a specific activity of 2650 U/mg of protein. Saponin-A was obtained from *Styrax officinalis* (21).

Determination of Hemolytic Activities—All experiments were performed on citrated blood freshly drawn from albino rats. The erythrocytes, freed of plasma by three washings in cold isotonic saline, were diluted with isotonic buffer (3.95 g of $Na_2HPO_4 \cdot 2H_2O$, 0.76 g of KH_2PO_4, 7.2 g of NaCl, *q.s.* to 1000 ml with water; pH adjusted to 7.4) to give a 1% suspension. Varying quantities of hemolysin, dissolved in buffer, were added to 2 ml of the erythrocyte suspension, and the volume was increased to 4 ml with buffer. The components were added in the following order: erythrocyte suspension, buffer, and the hemolyzing agent. The mixtures were incubated for 60 min at 37° in a shaking water bath, and then were centrifuged at $1000 \times g$; the absorbance of the supernatant was determined at 540 nm. The percentage of hemolysis was determined by comparison with a sample in which 100% hemolysis was obtained by the addition of digitonin.

Effect of Inhibitors on Surfactant-Induced Hemolysis—Two sets

[1] Cetomacrogol 1000, A.B.M. Chemical Ltd., England.
[2] E. Merck, Darmstadt, West Germany.
[3] Sigma Chemical Co., St. Lous, Mo.
[4] Riedel-De Haen AG. Seelze-Hannover.
[5] Photographic grade; May and Baker Ltd., Dagenham, England.
[6] Sigma—1000.

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