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SODIUM SULPHOMETHYL DERIVATIVES OF POLYMYXINS

BY

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Over the past years attempts have been made to reduce or modify the properties or undesirable side effects of certain drugs possessing free amino groups by use of the so-called "sulphomethylation" technique where free amino groups are treated with formaldehyde followed by sodium bisulphite to give a sulphomethyl derivative, thus:

$$R - NH_2 + HCHO \longrightarrow R - N = CH_2 + H_2O$$
$$R - N = CH_2 + Na^+HSO_3^- \longrightarrow R - NH - CH_2 - SO_3^-Na^+$$

Reviews discussing this process in general and in its application to antibiotics have been made by Logemann & Miori (1955) and Pindell & Lein (1964).

Parenteral administration of the parent colistin and other polymyxins can give rise to pain at the site of injection and other undesirable side effects. Controlled sulphomethylation has been shown to minimize these side effects without destroying antibacterial activity (Stansly, Shepherd & White, 1947; Koyama, 1957; Shoji, Hamada, Watanabe, Chiba, Kurozawa & Koyama, 1959; Barnett, Bushby & Wilkinson, 1964).

Barnett *et al.* (1964) showed that increases in microbiological activity occurred when solutions of sulphomethyl derivatives of various polymyxins were heated at 37° and suggested that this was due to conversion to the parent antibiotics. They suggested that sulphomethylation of more than one free amino group in each molecule results in loss of antibacterial activity and that any observed activity of more fully substituted derivatives was due to preliminary hydrolysis of these groups and that this hydrolysis occurs in the body.

After intramuscular administration of colistin as its hydrochloride in man excretion in the urine is retarded for 2–3 hr, high peak levels being attained within 7 hr (Forni & Guidetti, 1956). Pulaski & Rosenberg (1949) found similar results with polymyxin B sulphate, urinary levels remaining low for the first 12 hr after injection before rising to high values. Barnett *et al.* (1964) reported low urinary levels in the first 6 hr after parenteral administration of polymyxins B and E. CSMS and sulphomethylated derivatives of other polymyxins are readily excreted in the urine, high levels being reached within 30 min of intramuscular administration (Wright & Welch, 1960; Boger & Gavin, 1961; McMillan, Price, MacLaren & Scott, 1962; Barnett *et al.*, 1964).

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This paper describes investigations made into the possible hydrolysis of CSMS and a derivative of polymyxin B, described as penta-N-sulphomethyl polymyxin B, in both simple aqueous, and biological systems, and also records changes in microbiological activity caused by such hydrolysis. In addition, a comparison has been made of the incidence of pain upon injection and side effects encountered with both drugs in man.

METHODS

Chemicals

Commercial samples of the antibiotics were obtained as follows: colistin sulphomethate sodium as Colomycin methanesulphonate (CSMS), Lot A2092, and colistin sulphate as Colomycin sulphate (CS), Lot A1326 (Pharmax Ltd.); sodium polymyxin B methanesulphonate as Thiosporin, sodium penta-N-sulphomethylpolymyxin B (SPBMS), Lot 15866, and polymyxin B sulphate (PBS) as Aerosporin, Lot 15984 (Burroughs Wellcome and Co.).

Detection of changes in antibacterial activity in vitro

To measure possible increases in activity produced by heating solutions of CSMS and SPBMS, expressed as changes in the Minimum Inhibitory Concentration, it is desirable to adopt a tube dilution method allowing very rapid growth of the test organism. This reduces any superimposed change in activity, due to further hydrolysis occurring in the broth at 37° before the stage where the bacterial population has increased, to a level beyond which further changes in activity would not influence the visual bacteriostatic end-point. *Escherichia coli* 95 I.S.M. (Institut Société de Microbiologie) was selected as the test organism, since it grew rapidly in Sensitivity Test Broth (Oxoid), was very sensitive to the two antibiotics, and visible growth could be detected readily after 2 hr incubation at 37°.

Sterile solutions containing 200 μg/ml. CSMS or SPBMS in 0.1 M phosphate buffer, pH 7, were incubated at various temperatures for up to 24 hr. At selected intervals aliquots were removed and serial dilutions made in Sensitivity Test Broth. The tubes were inoculated with 0.2 ml. of a growing broth culture of *E. coli* and incubated for 2 hr at 37°, when the Minimum Inhibitory Concentration was estimated by observing the lowest concentration to inhibit growth and the highest to allow it in the same series of tubes. The bacteriostatic end-point for any solution of CSMS or SPBMS did not change after incubating for 1½, 2, 3, 6 or 9 hr and the sensitivity of the *E. coli* cultures did not appear to change significantly during the experiment as adjudged by examination of control tubes.

Detection of chemical changes in vitro

Electrophoresis was performed on Whatman No. 1 filter paper with 0.05 M phosphate buffer, pH 7, for 1.5 hr using 8 mA/11 cm wide paper strip, the temperature of the paper strip not being allowed to rise above 22. Previous experiments (above) revealed that hydrolysis, adjudged by changes in M.I.C. value, was minimal under these conditions. For the electrophoretograms of aqueous solutions, antibiotic material was located by spraying the papers with ninhydrin reagent (0.2 g ninhydrin, water 5 ml., glacial acetic acid 5 ml., acetone 90 ml.) followed by heating at 100° for 15 min to produce hydrolysis of the sulphomethyl groups and allow the reaction to take place. 50–200 μg of CSMS or SPBMS could be separated and detected with this procedure. With smaller quantities of antibiotic and when serum or urine solutions were investigated, the antibiotic fractions were located by pressing electrophoretograms on to an agar plate seeded with *Bordetella bronchiseptica* ATCC 4617 (American Type Culture Collection), removing, and incubating the plate for 18 hr at 37°. The resultant autobiograms corresponded to the antibiotic fractions detected with ninhydrin on similar electrophoretograms. From 1–10 μg CSMS or SPBMS and from 0.25–2.0 μg CS or PBS in water, serum or urine gave satisfactory autobiograms by this technique.

Changes in aqueous media

Solutions containing 5 mg/ml. of CSMS or SPBMS in 0.1 M phosphate buffer, pH 7, or in 0.1 N HCl, were incubated at different temperatures for up to 20 hr in a manner similar to that shown to

produce changes in antibacterial activity, portions removed at intervals and 20 μl. aliquots (100 μg antibiotic) examined by electrophoresis.

Changes in plasma in vitro

Solutions containing 0.5 mg/ml. of CSMS or SPBMS in human citrated plasma were incubated at 37°, portions removed at intervals and 10 μl. aliquots (5 μg antibiotic) examined by electrophoresis. Reference solutions of the two antibiotics were prepared in cold plasma and placed immediately on to the paper strip to minimize any possible hydrolysis. Citrated plasma, and serum used in this and other experiments, were obtained by pooling fresh plasma or sera from 12 human volunteers.

Chemical changes in vivo

Two healthy males were given either 200 mg CSMS or 200 mg SPBMS in 2 ml. normal saline by deep intramuscular injection into the upper arm. The total urine output from each was measured and aliquots assayed for antibiotic activity, the remainder being stored at $-5°$ until required. Volumes calculated to contain 5 μg and 2.5 μg of antibiotic were applied to filter paper strips, separated by electrophoresis, and autobiograms produced.

Measurement of antibacterial levels in the urine

Urine specimens were diluted with an equal volume of 20% w/v phosphate buffer pH 6 and standard solutions of CSMS and SPBMS prepared in a mixture containing equal volumes of the 20% phosphate buffer and urine obtained before the administration of antibiotic. These were assayed by the cylinder plate diffusion technique using Difco Antibiotic Assay Medium No. 9 and *Bord. bronchiseptica* A.T.C.C. 4617, as indicator organism. Although it was suspected that at least some hydrolysis of sulphomethyl groupings of CSMS and SPBMS would occur in the body, it was thought that a reasonable estimate of antibiotic concentration in the urine would be obtained by using authentic CSMS or SPBMS for preparing the standard solutions. Preliminary experiments suggested that at the 100–500 μg/ml. level the calculated concentration of antibiotic in a sample of urine containing a sulphomethyl derivative which had been completely hydrolysed to the parent antibiotic would range from $\times 1$ to $\times 1.5$ respectively of the actual value if assayed against standard solutions containing CSMS or SPBMS.

Investigation of the incidence of side effects following intramuscular injection

Twelve healthy young adults, four females, eight males, were divided into two equal groups; six were given 500,000 u. SPBMS (92 mg) and six 1,500,000 u. CSMS (120 mg) by deep intramuscular injection into the deltoid region of the right arm. Both preparations were dissolved in 2 ml. normal saline. The subjects were examined at 2, 7 and 24 hr after administration for the incidence of side effects. Four days later the same subjects were given the alternative drug in the left arm and the incidence of side effects again recorded. All injections were prepared and coded by an independent colleague before being administered to eliminate bias in assessing results.

RESULTS

The Minimum Inhibitory Concentrations for CS, PBS, CSMS and SPBMS were found to lie between 0.04–0.06, 0.04–0.06, 0.60–0.80, and 0.13–0.15 μg/ml. respectively, the lower figures of the pairs being the highest concentration of antibiotic in the tubes of the series to allow growth and the higher figures being the lowest concentration in the same series of tubes observed to cause inhibition of growth.

Aqueous solutions of CSMS and SPBMS increased rapidly in activity when heated at 50° in buffer pH 7, at lower temperatures the changes being proportionally less (Fig. 1). Solutions of CS or PBS when heated for 6 hr at 50° in buffer pH 7 did not change in activity.

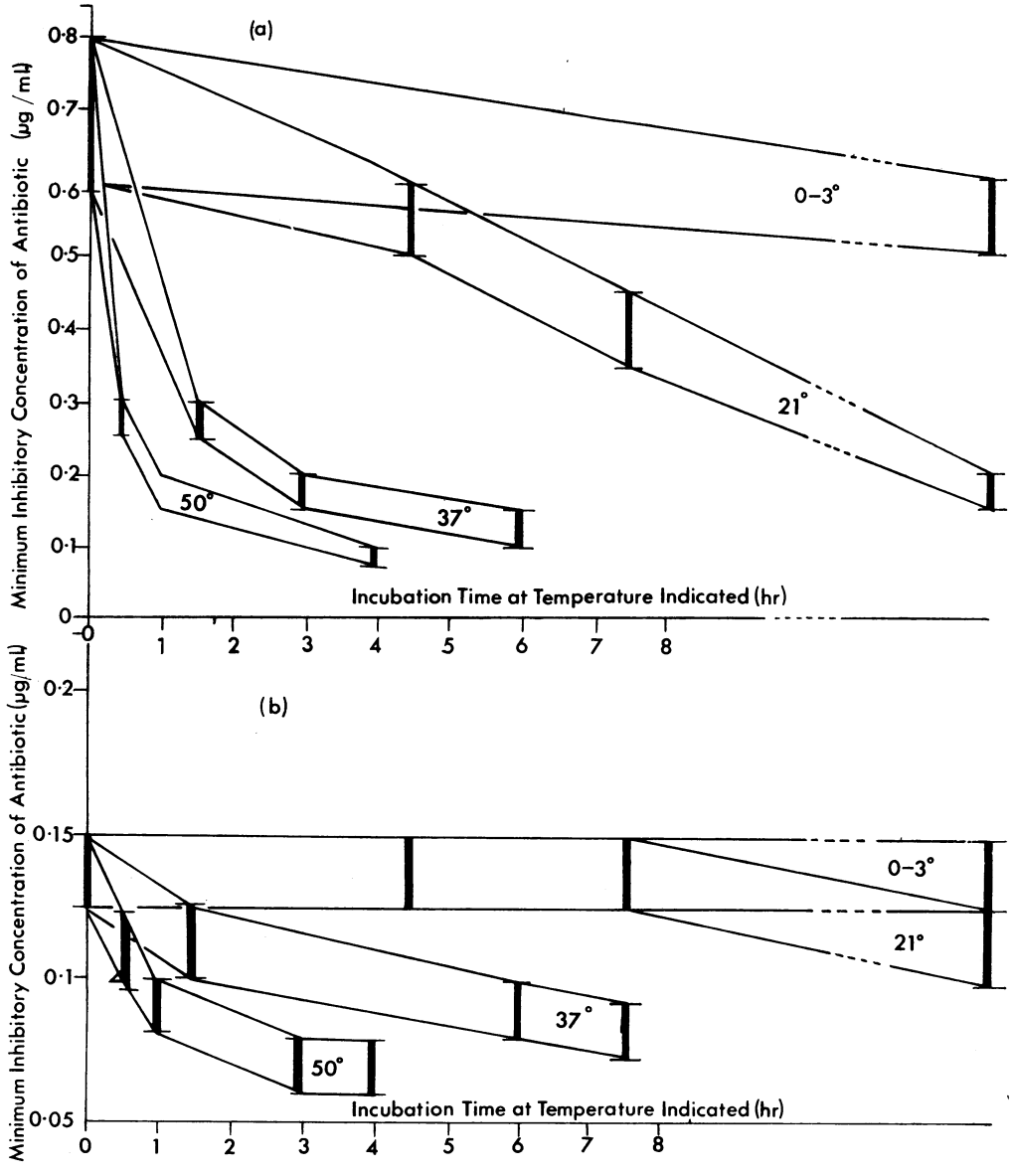

Fig. 1. The increase in antibacterial activity of CSMS and SPBMS solutions against *E. coli* following heat treatment. Solutions of CSMS and SPBMS were heated at pH 7 before dilution and measurement of their bacteriostatic activity. The upper limits of the tie lines represent the lowest concentrations of antibiotic observed to inhibit growth after its treatment for the time and temperature shown, while the lower limits represent the highest concentrations observed to allow growth after the same treatment. Similar results were obtained for the three replicate experiments. (a) CSMS, (b) SPBMS.

Both CSMS and SPBMS could be separated by electrophoresis into several component fractions possessing differing rates of migration towards the anode from the centre point of the electrophoretograms, and could be distinguished at least to some extent from CS and PBS (Fig. 2). SPBMS also contained a fraction appearing as a pale yellow slightly greasy spot on the electrophoretogram which did not react with ninhydrin.

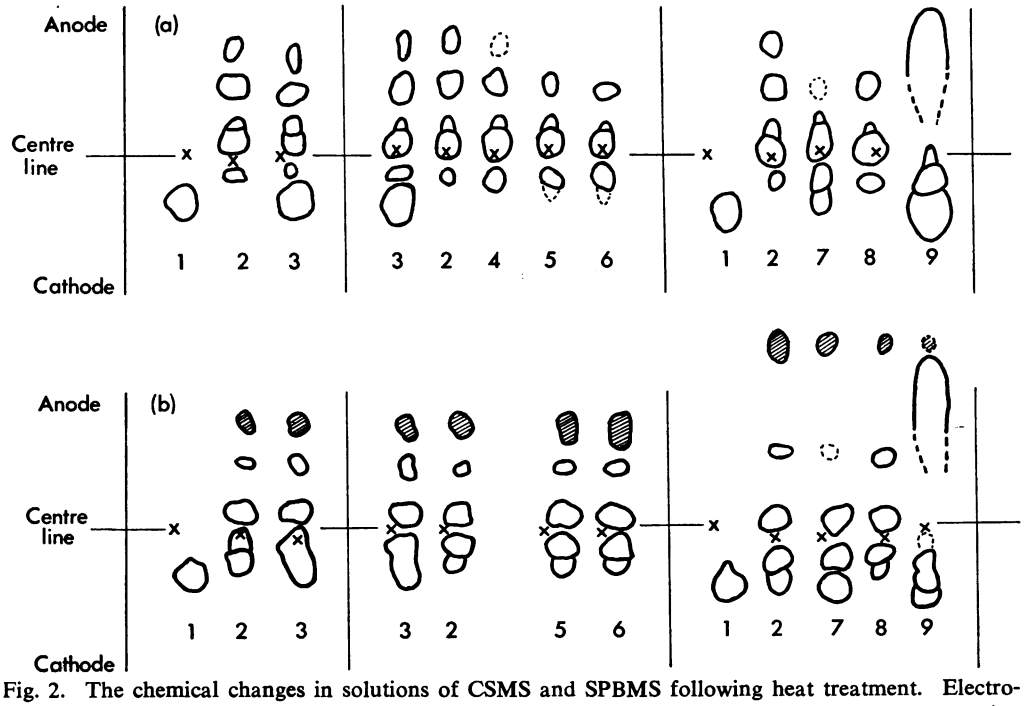

Fig. 2. The chemical changes in solutions of CSMS and SPBMS following heat treatment. Electrophoretograms of 100 μg quantities of CSMS or SPBMS from solutions heated under varying conditions: (a) three electrophoretograms of CSMS specimens; (b) three electrophoretograms of SPBMS specimens. The specimens were heated in phosphate buffer pH 7 as follows: 1=CS or PBS not heated; 2=CSMS or SPBMS not heated; 3=CSMS+CS or SPBMS+PBS not heated; 4, 5, 6=CSMS or SPBMS heated at 50° for 0.5, 4, and 20 hr respectively; 7=CSMS or SPBMS heated at 100° for 10 min; 8=CSMS or SPBMS heated at 37° for 4 hr; 9=CSMS or SPBMS heated in 2 N HCl at 37° for 2 hr. Hatched spots did not react with the ninhydrin reagent.

Incubation of CSMS and SPBMS solutions under similar conditions to those shown to produce marked changes in microbiological activity resulted in limited gross changes in their electrophoretic patterns (Fig. 2). After heating at 50° for 4 hr the electrophoretic pattern of CSMS showed a disappearance of the faster moving fractions with some increase in the slower moving fractions. Even after heating a solution of SPBMS at 50° for 20 hr only slight loss of the faster moving fractions occurred as suggested by changes in colour intensity of the separated spots. Boiling the solutions for 10 min resulted in more pronounced changes. Incubation in 2 N HCl for 2 hr at 37° resulted not only in almost complete hydrolysis of the sulphomethyl groups with the release of the parent antibiotics but also the breakdown of the peptide rings and release of the constituent amino acids.

Autobiograms prepared from solutions containing 500 μg/ml. CS, PBS, CSMS, or SPBMS in cold fresh plasma, serum, or urine and separated as quickly as possible, did not reveal that the pattern of separation of the antibiotic fractions was grossly different from that of aqueous solutions. Thus, at this concentration the degree of protein binding or other possible interference was not sufficient to alter markedly the electrophoretic patterns. Control plasma or serum did not produce zones of inhibition in the autobiograms with the volumes involved in this experiment.

Incubation of CSMS or SPBMS in fresh plasma at 37° produced appreciable changes in their electrophoretic pattern with rapid disappearance of the faster moving fractions and accumulation of the slower moving fractions (Fig. 3).

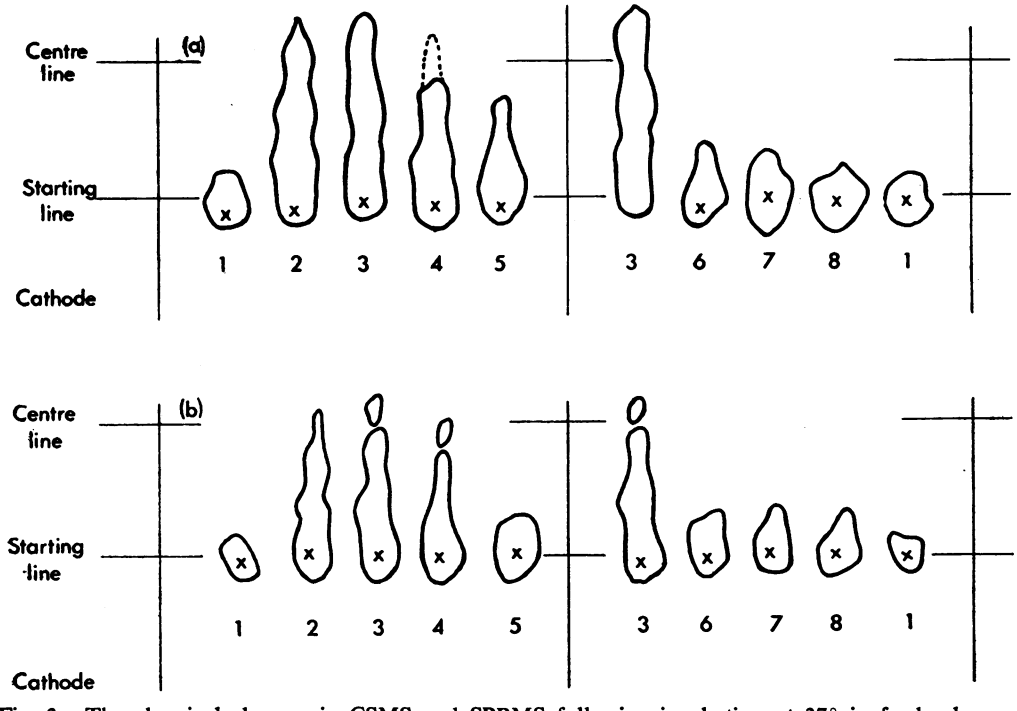

Fig. 3. The chemical changes in CSMS and SPBMS following incubation at 37° in fresh plasma. Autobiograms of 5 μg (10 μl. solution) quantities of CSMS or SPBMS incubated for varying times in plasma: (a) two autobiograms of CSMS specimens; (b) two autobiograms of SPBMS specimens: 1=CS or PBS (0.5 μg) not incubated in plasma; 2=CSMS or SPBMS not incubated in plasma; 3=CSMS+CS or SPBMS+PBS not incubated in plasma (1, 2 and 3 were however made up in plasma); 4, 5, 6, 7, and 8=CSMS or SPBMS incubated in plasma at 37° for 1 hr, 3 hr, 5 hr, 7 hr and 24 hr respectively.

The autobiograms of portions of urine from the two males injected with CSMS or SPBMS revealed an increasing change of pattern (Fig. 4). Urine collected 1 hr after injection contained almost all the original antibiotic fractions but subsequent specimens showed a progressive disappearance of the faster moving fractions with an increase in the slower moving fractions. The quantites of antibiotic excreted in the urine of the two subjects are shown in Table 1.

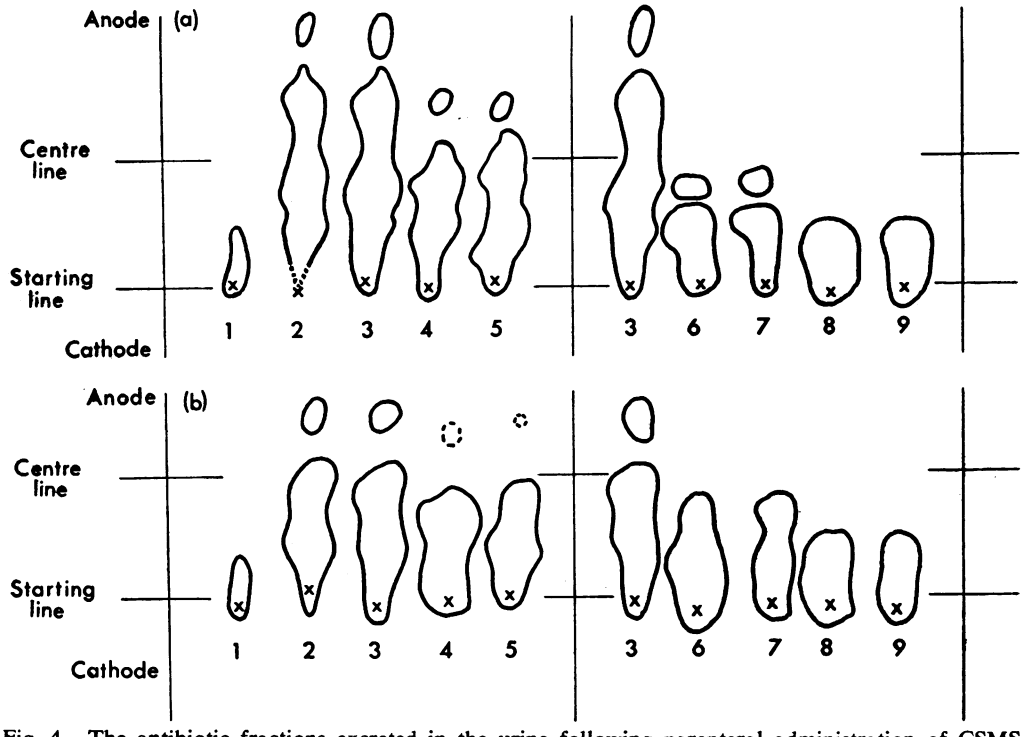

Fig. 4. The antibiotic fractions excreted in the urine following parenteral administration of CSMS and SPBMS. Autobiograms of urine specimens calculated to contain 5 μg or 2.5 μg of antibiotic. Reference compounds were prepared in control urine: (a) two autobiograms of urine specimens following CSMS administration; (b) two autobiograms of urine specimens following SPBMS administration: 1=reference compounds CS or PBS (0.5 μg); 2=reference compounds CSMS or SPBMS (5 μg); 3=reference compounds CSMS (5 μg)+CS (0.5 μg) or SPBMS (5 μg)+PBS (0.5 μg); 4 and 5=urine specimens collected 1 hr after administration, 5 μg and 2.5 μg respectively; 6 and 7=urine specimens collected 3 hr after administration, 5 μg and 2.5 μg respectively; 8 and 9=urine specimens collected 7.5 hr after administration, 5 μg and 2.5 μg respectively.

TABLE 1

THE QUANTITIES OF ANTIBIOTIC EXCRETED IN THE URINE FOLLOWING INTRAMUSCULAR INJECTION OF SPBMS AND CSMS

Time of specimen collection after injection (hr)	CSMS			SPBMS		
	Volume of urine passed (ml.)	Antibiotic titre (μg/ml.)	Quantity of antibiotic excreted (μg)	Volume of urine passed (ml.)	Antibiotic titre (μg/ml.)	Quantity of antibiotic excreted (μg)
0	58	0	—	20	0	—
1	63	525	33,075	24	135	4,240
2	70	457	31,990	40	138	5,520
3	59	575	33,925	34	140	4,760
4	—	—	—	52	138	7,176
6	92	240	22,050	54	129	6,966
7·5	73	174	12,702	40	66	2,640
10	102	41	4,151	60	63	3,780
13	129	9	1,198	70	50	3,500
22	230	2	529	200	70	14,000
24	—	—	—	45	10	450

Fewer side effects were noticed following intramuscular injection of CSMS than following injection of SPBMS. SPBMS was found to produce severe pain lasting for at least 2 hr in the injected arms of nine subjects and lasting for at least 7 hr in three subjects, while CSMS produced only slight localized pain in two subjects. There were five cases of paraesthesiae and four of headache, dizziness or nausea following SPBMS administration, with none being observed for CSMS. The incidence of severe pain, the incidence of side effects other than pain, and the incidence of the combined side effects, was greater with SPBMS than CSMS, χ^2 values for these being 7.11, 4.17 and 5.80 respectively (for a null hypothesis, $\chi^2=3.84$ when $n=1$, $P=0.95$). After 24 hr all symptoms had disappeared from the majority of subjects.

DISCUSSION

Colistin and polymyxin B have related, but different, chemical formulae, each possessing five free amino groups belonging to L α,γ-diaminobutyric acid residues. Since both antibiotics possess five free amino groups it should be theoretically possible to prepare five progressively sulphomethylated derivatives. Electrophoresis of CSMS and SPBMS did in fact result in their separation into a series of component fractions and these corresponded to the fractions possessing potential antibacterial activity as shown by the autobiograms obtained. It is reasonable to suggest that these components represent fractions varying in the degree of substitution of the amino groups in the parent antibiotics and indicate that neither product is fully substituted.

Comparison of the results shown in Figs. 1 and 2 suggests that only minor changes in the chemical state of CSMS and SPBMS were responsible for major changes in the antibacterial activity of the two products. The results suggest that these changes in antibacterial activity are due to selective hydrolysis of sulphomethyl groups. It would appear to be necessary for only partial hydrolysis with the production of lower substituted derivatives to produce activity approaching that of the parent antibiotics. Barnett *et al.* (1964) suggested that the activity of sulphomethyl derivatives of colistin and other polymyxins is dependent upon the initial hydrolysis of sulphomethyl groups to at least the mono substituted derivatives or the parent antibiotics. This deduction was based on their observations that the acetylation of polymyxin B beyond the monoacetyl derivative produced inactive material (resistant to hydrolysis under the conditions such as used for the examination of CSMS and SPBMS in our paper); the monoacetyl derivative itself being considerably less active than the parent antibiotic, polymyxin B. They showed that by heating solutions of CSMS and other sulphomethyl derivatives of polymyxins at 37°, pH 7, for 24 hr marked increases in antibacterial activity occurred and suggested that this was caused by hydrolysis of the majority of the sulphomethyl groups on each molecule, but offered no direct experimental support for this latter suggestion. It would appear from these observations that sulphomethylation does not inactivate colistin or polymyxin B to the same extent as acetylation but does markedly reduce antibacterial activity if substitution is taken beyond a certain degree.

Changes in the antibacterial activity of CSMS and SPBMS occur at 37° in solution, and thus during the determination of the minimum inhibitory concentration against a particular organism some increase in activity would take place in the culture medium during the interval between the commencement of incubation and point where the

bacterial population has begun to divide and reached a size beyond which further slight changes in activity could not influence the bacteriostatic end point. With *E. coli* this interval must have been very short or the changes in microbiological activity produced by heating the solutions would have been swamped by the subsequent changes occurring during the interval.

Since the hydrolysis of the sulphomethyl groups of CSMS and SPBMS occurred at a considerably faster rate in plasma *in vitro* than in buffer, it is concluded that the plasma contained some component possessing the specific ability to hydrolyse the sulphomethyl groups with the liberation of free amino groups.

Barnett *et al.* (1964) determined the concentration of various sulphomethyl polymyxin derivatives and CSMS in the blood and urine following intramuscular administration by diffusion assay techniques using the parent antibiotics PBS or CS as standards. They also measured the bactericidal activity of the same specimens by a tube dilution method. The differences between the values obtained with these two methods were taken to indicate that the various sulphomethyl derivatives were present in the blood partly in the unchanged form and that the rate of hydrolysis of the sulphomethyl groups was lower than that which they assumed to take place *in vitro* at 37° in simple aqueous solutions. Using a similar argument, they also suggested that the antibiotic material excreted in the urine was mainly in the unchanged form but that a small but significant portion had been hydrolysed to the parent antibiotics. However, as suggested previously the estimation of partially hydrolysed sulphomethyl derivatives of colistin and polymyxin B by the diffusion assay technique using the parent antibiotics or CSMS and SPBMS for preparing the standard solutions will result in errors due, at least in part, to differences in the diffusion characteristics of the sulphomethyl derivatives and their parent antibiotics. While such approximations are satisfactory for obtaining some idea of the antbiotic levels in a particular biological fluid, attempts to use the results to decide on the degree of hydrolysis of the various derivatives could be open to criticism.

Barnett *et al.* (1964) also examined the urine specimens by electrophoresis and reported that only components corresponding to the antibiotic fractions injected were detected. In contrast, the results shown in Fig. 4 show that there was a progressive reduction in the more fully substituted fractions of CSMS and SPBMS with an increase in the lower substituted fractions and possibly parent antibiotics in the urine samples collected following injection of CSMS or SPBMS. These results cannot be attributed to the initial selective excretion of the higher sulphomethylated fractions by the kidneys followed by the later excretion of the lower substituted fractions since the 1 hr specimens contained almost all the fractions. However, in the case of SPBMS at least, the proportion of parent antibiotic (if any) in the urine might initially be less than that in the body because of its reported delayed excretion. The most probable explanation is that progressive hydrolysis of the sulphomethyl groups had taken place in the body with the production of lower substituted fractions and possibly the final emergence of parent antibiotics. Comparison of the results depicted in Figs. 2, 3 and 4 suggests that hydrolysis of the sulphomethyl groups of CSMS and SPBMS occurs to a greater rate in plasma and in the body than in a simple aqueous system at 37°, pH 7.

SPBMS was found to produce a high incidence of side reactions, including severe pain in the arm, following intramuscular injection while CSMS was virtually free from such

reactions. The quantities of each drug used for the estimation bear a relationship to usual therapeutic doses since SPBMS is recommended to be given as a 600,000 u. (92 mg) dose four times daily and CSMS as a 1,500,000 u. (120 mg) dose three times daily. The results obtained with CSMS are in agreement with published reports where a low incidence of side effects and the absence of pain was found (Edgar & Dickinson, 1962; Taylor & Allison, 1962; McMillan *et al.*, 1962).

Barnett *et al.* (1964) suggest that the clincal results obtained with CSMS have been disappointing. However, an appraisal of the recent British work alone will show this statement to be unfounded. CSMS has been found to be very successful in comparison with other available antibiotics in the treatment of systemic and urinary tract infections caused by microorganisms sensitive to colistin (Taylor & Allison, 1962; McMillan *et al.*, 1962; Marsden & Hyde, 1962; Edgar & Dickinson, 1962; Dawson-Edwards, 1963; Taylor, 1963; Colley & Frankel, 1963; Speirs, Selwyn & Nicholson, 1963; Murdoch, 1964).

SUMMARY

1. Commercial colistin sulphomethate sodium (CSMS) and sodium polymyxin B methanesulphonate (SPBMS) were shown by electrophoresis to be composed of various fractions probably differing in the degree of sulphomethylation of the free amino groups.

2. CSMS and SPBMS were less active weight for weight than the parent antibiotics *in vitro* but activity increased when solutions at neutral pH were heated, the increases being related to the temperature and length of heating. Electrophoresis suggested that selective hydrolysis of sulphomethyl groups had taken place to cause these increases in activity.

3. This hydrolysis occurred more rapidly in human plasma at 37° and examination of the antibiotic fractions excreted in the urine suggested that this hydrolysis also took place in the body to a greater extent than in simple aqueous solutions.

4. In contrast to CSMS, injection of SPBMS caused a high incidence of severe localized pain and other undesirable side effects.

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