under the presidentship of Sir Ardeshir Dalal, member for planning and development. Following the conference in London in October of last year, attended by representatives of Britain, the United States, Canada, Australia and Free France and specialists from South Africa and India, with the advice of Sir Alexander Fleming, the discoverer of penicillin, an international agreement on a worldwide uniform standard and unit of penicillin was reached by the Health Committee of the League of Nations. Announcing this decision, Sir Henry Dale, president of the Royal Society, stated that British manufacturers would pool all their information and give all their results to the British Medical Research Council and make the manufacture of penicillin a genuinely national effort.

SPECIAL ARTICLES

MODIFICATION OF GRAMICIDIN THROUGH REACTION WITH FORMALDEHYDE

THE toxicity of tyrothricin and of its components, gramicidin and tyrocidine, has been a limiting factor in the general applicability of these antibacterial agents in medicine.[1] In connection with other work in progress in this laboratory,[2,3] an attempt was made to reduce this toxicity by means of reaction with formaldehyde. Since the toxicities of these materials are at least partly due to their hemolytic action,[4] *in vitro* assays of the latter property were used as a tentative measure of changes in toxicity of modified preparations, although no strict correlation between these two properties appears to have been demonstrated.

It was found that the treatment of tyrothricin with formaldehyde resulted in a loss of 80 to 90 per cent. of the original hemolytic activity and a loss of up to 50 per cent. of the antibiotic activity. The action of formaldehyde on gramicidin gave a similarly reduced hemolytic effect but the antibiotic activity, estimated with *Staphylococcus aureus,* was found to be unchanged. Preliminary tests with rats (intraperitoneal injections) indicated that the modified gramicidin was considerably less toxic than untreated gramicidin (Table 1). This was particularly striking inasmuch as the derivative was more water-soluble than gramicidin. Because of the possible usefulness of the derivative as a chemotherapeutic agent, this preliminary announcement is being made while further, more detailed studies of its chemical and biological properties are in progress.

TABLE 1

EFFECT OF FORMALDEHYDE ON THE PROPERTIES OF GRAMICIDIN

	Toxicity[a] mg/kg	Hemolytic activity[b] ppm	Antibacterial activity[c] ppm
Formaldehyde-treated gramicidin	>450	0.6	0.003
Gramicidin	20	0.05	0.003

[a] Approximate lethal dose. The products were dissolved in 80 per cent. propylene glycol, 20 per cent. alcohol solution and injected intraperitoneally into a limited number of rats weighing 150–200 gm.

[b] Ppm to achieve 50 per cent. hemolysis of rat erythrocytes in 40 min. at room temperature.

[c] Ppm to achieve 50 per cent. inhibition of *Staphylococcus aureus* growth in 4 hours at 37° C.

The reaction of gramicidin with formaldehyde was usually accomplished in the following manner: To 10 parts of a 5 per cent. solution of gramicidin in 95 per cent. alcohol, 1 part of 1 *N* aqueous sodium hydroxide and 5 parts of commercial 40 per cent. formaldehyde solution were added. The mixture was kept at 53° C. for 2 days; it was then at approximately pH 8. These conditions were not critical. Preparations with similar biological activities were obtained with 4 times as much alkali, or with one tenth of the amount of formaldehyde, at room temperature, or at 70° C., and for various time periods. A marked loss in antibacterial as well as hemolytic activity occurred when the reaction was performed at pH 3.0–3.5. The modified gramicidin was isolated by precipitation with about 5 volumes of 0.1 *N* sodium chloride, washed with water of decreasing salt content, and dried from the frozen state. The average yield was 106 per cent. by weight of the starting material.

The method used for the hemolytic assay of gramicidin was a modification of that published for tyrothricin.[5] The rate of hemolysis of a suspension of rat erythrocytes was measured over a period of up to 90 minutes after the addition of 0.025 to 0.2 ppm of gramicidin.

The antibacterial potency against *Staphylococcus aureus* was estimated by means of serial dilution tests in "medium II" of Schmidt and Moyer.[6] Practical sterilization of dilute solutions of the test substances in 70 per cent. ethanol was achieved by permitting them to stand for several days. Dilution tubes were incubated at 37° C. for 4 hours, at which time they were sterilized by autoclaving and the turbidities due to growth of the organism measured photometrically.

[1] R. D. Hotchkiss, *Adv. in Enzym.*, 4: 153, 1944.

[2] J. C. Lewis, K. P. Dimick and I. C. Feustel, *Ind. Eng. Chem.*, in press, 1945.

[3] H. Fraenkel-Conrat, M. Cooper and H. S. Olcott, *Jour. Am. Chem. Soc.*, 67: 950, 1945.

[4] W. E. Herrell and D. Heilman, *Jour. Am. Med. Asn.*, 118: 1401, 1942.

[5] K. P. Dimick, *Jour. Biol. Chem.*, 149: 387, 1943.

[6] W. H. Schmidt and A. J. Moyer, *Jour. Bact.*, 47: 199, 1944.

The estimates of activities were made by comparison of the 50 per cent. inhibition concentrations for treated and untreated preparations assayed simultaneously. Under these conditions, 50 per cent. inhibition was achieved by approximately 0.003 ppm of gramicidin or modified gramicidin, a concentration considerably lower than the solubility of these substances. The modified gramicidin gave a steeper dosage response curve than did gramicidin.

In earlier tests with an 18-hour incubation period, the formaldehyde-treated gramicidin was 200 to 400 per cent. more active than gramicidin itself. Subsequently, it was found that this increase was apparently a reflection of the increased solubility of the derivative, since the 50 per cent. inhibition point of the unmodified gramicidin under these conditions was approximately 1 ppm and thus above the solubility range (see below).

The solubilities were estimated as follows: Suspensions of excess gramicidin and its derivative in "medium II" were shaken for two days and permitted to stand for an additional five days. The solutions were filtered and compared in antibacterial activity with solutions of known concentration. By this technique, gramicidin and its derivative were found to be soluble to the extent of approximately 0.4 and 1.4 ppm, respectively.

This increase in water solubility may, in itself, be an advantageous modification, inasmuch as the slow rate of diffusion of gramicidin has appeared to limit its usefulness. For example, Hotchkiss reports that experimental intraperitoneal pneumococci infections could be satisfactorily controlled by gramicidin injected intraperitoneally but not through other routes of administration.[1]

Products of decreased hemolytic activity were obtained from several lots of pure gramicidin and from various crude preparations. The latter were (1) the material that could be extracted from tyrothricin[7] with 50 per cent. acetone-ether, (2) once-crystallized gramicidin prepared from this preparation, and (3) a product from which tyrocidine and other basic substances had been removed by precipitation with phosphotungstic acid. Gramicidin contaminated with tyrocidine, as obtained by the acetone-ether extraction, was not reduced in hemolytic activity to the same extent by formaldehyde as were the other preparations. This reduction could be achieved by a repetition of the formaldehyde treatment.

Gramicidin is distinguished among proteins and polypeptides by containing approximately 40 per cent. tryptophane, but no polar groups known to react reversibly with formaldehyde, such as basic or amide groups.[3] Ross and Stanley[8] and Kassanis and Kleczkowski[9] have shown that the chromogenic property of tryptophane in the Folin reaction is decreased during formaldehyde treatment. Baudouy[10] suggested that proteins containing tryptophane and histidine form complexes with formaldehyde from which the latter can not be recovered. The mode of reaction of formaldehyde with the indole residues of gramicidin and proteins will be described in another place.

The observation that the hemolytic and toxic activity of gramicidin can be lowered without decrease of its antibacterial properties demonstrates that these functions are not necessarily dependent upon the same molecular configuration. In this respect, the formaldehyde reaction with gramicidin is analogous to that with bacterial toxins, in which case the toxicity but not the antigenic activity is reduced.

J. C. LEWIS
K. P. DIMICK
I. C. FEUSTEL
H. L. FEVOLD
H. S. OLCOTT
H. FRAENKEL-CONRAT

WESTERN REGIONAL RESEARCH LABORATORY,
BUREAU OF AGRICULTURAL AND
INDUSTRIAL CHEMISTRY,
U. S. DEPARTMENT OF AGRICULTURE,
ALBANY, CALIF.

THE FREE TRYPTOPHANE CONTENT OF HUMAN URINE*

DURING studies on the metabolism of tryptophane by the rat, samples of human urine were used in standardizing the microbiological method which was being used for the estimation of tryptophane. It was immediately evident that tryptophane figures much lower than the average figure of 281 mg per day reported by Albanese and Frankston[1] were obtained. The results of a more extensive study on the tryptophane content of human urine are reported in this paper.

EXPERIMENTAL

Twenty-four-hour collections of urine were made from nine subjects who consumed average diets. The total urine volume was noted, toluene added as a preservative, and the samples stored in a refrigerator until the following day.

[7] We are indebted to Wallerstein Laboratories for a generous supply of tyrothricin and gramicidin.

[8] A. F. Ross and W. M. Stanley, *Jour. Gen. Physiol.*, 22: 165, 1938.

[9] B. Kassanis and A. Kleczkowski, *Biochem. Jour.*, 38: 20, 1944.

[10] Baudouy, *Compt. rend.*, 214: 692, 1942.

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[1] A. A. Albanese and J. E. Frankston, *Jour. Biol. Chem.*, 157: 59, 1945.