the normal renal function in the first case justifies the inference that structural kidney changes rather than a loss of trophic influence is responsible for the depression of renal function in the second case.

In cases exhibiting Cushing's syndrome, whether associated with an hypophysial adenoma, an adrenal tumor, or with neither, an invariable finding has been hyalinization of the basophil cells of the hypophysis.[10,11] We regard such a change as one which could only lead to a depression of secretory function of these cells. The finding of renal blood flow and renal tubular function within normal limits in our first case indicates that there was no lack of the humoral agent which normally acts to maintain a normal kidney function.

Again, therefore, the evidence seems to indicate that this humoral agent affecting kidney function is secreted by the eosinophil cells, which are histologically normal in all cases exhibiting Cushing's syndrome, rather than by the basophil cells. It is realized that more cases should be studied before final conclusions are drawn. It is hoped that this report will serve as a stimulus to others to obtain data on this subject.

10 Crooke, A. C., *J. Path. and Bact.*, 1935, **41**, 339.

11 Rasmussen, A. T., *Endocrin.*, 1936, **20**, 673.

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Action of Bacterial Toxins on Tumors. III. Some Biological Properties of Purified *Salmonella typhimurium* Endotoxin.

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We have postulated on the basis of a survey of tumor hemorrhage induction by 28 genera of bacteria,[1] and on a more recent extension of this survey,* that aside from immunological specificity the purified O antigens of gram-negative bacteria are probably similar both in toxic action and chemical type. This is consistent with the view, developed by Boivin and his school,[2] based on immuno-chemical investigation.

Morgan and Partridge,[3] working with *Shigella dysenteriæ* and *Salmonella typhi,* have demonstrated that antigenicity is conferred on the O antigen by the protein component (earlier designated by them as a "polypeptide"), but their most recent publication[4] leaves undetermined whether the toxicity of the antigen resides in the protein, the polysaccharide, or in a combination of the two.

The object of the present study was to determine whether the O antigen, following purification, retains activity in respect to: (1) lethality, (2) hemorrhage production in implanted tumors, (3) production of protective antibodies in mice. *Salmonella typhimurium* was chosen as a representative gram-negative possessor of the O antigen. The toxic material was tested for activity at 3 stages of the purification: (1) after acetone precipitation from concentrated culture, (2) after warm phenol extraction of the initial acetone precipitate, and (3) after various formamide treatments of the phenol extract.

Acetone Fraction (A). The toxic material used in this study was prepared from cultures

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* Abstracted in *J. Bacteriol.*, Jan., 1943.

2 Boivin, A., and Mesrobeanu, L., *Ann. Inst. Pasteur*, 1938, **61**, 426.

3 Morgan, W. T. J., and Partridge, S. M., *Biochem. J.*, 1940, **34**, 169.

4 Morgan, W. T. J., and Partridge, S. M., *Brit. J. Exp. Path.*, 1942, **23**, 151.

TABLE I.

Yields and Biological Activity of Various Fractions in the Purification of *Salmonella typhimurium* endotoxin.

Fraction	Dry wt of total material extracted	Bioassay: M.L.D. μg	No. of animals	M.H.D. μg	No. of animals	Range of M.L.D. survived by immunized mice	No. of animals†	Approx. dosage ratio M.H.D.:M.L.D.
Acetone (A-1)	124.2 g	1300	48	6	21	3—9	20	1:200
" (A-2)	239.9 "							
Phenol (B-1)	617 mg	450	40	.1	45	3—9	20	1:4500
" (B-2)	920 "	500	38	.1	38	3—9	10	1:5000
Formamide (C)	126* "	400	42	.1	60	3—9	20	1:4000
" (D)	172* "	350	48	.1	54			1:3500
" (E)	165* "	350	31	.05	48	3—9	20	1:7000
" (F)	103* "	1700	40	.5	34			1:3400

*Yield from 200 mg of phenol fraction B-2.

†Each group of animals in this column was immunized with the same fraction against which it was later tested.

of *S. typhimurium* grown in a synthetic liquid medium.† The cultures were grown in 40 liter batches distributed in 2 or 4 liter flasks. The profuse growth was killed with 2% phenol plus a small amount of toluene and chloroform, and the suspension transferred to cellophane tubing. The contents of the tubes were simultaneously dialyzed against water and reduced to a small volume by pervaporation in a current of warm air. The phenol-free toxic material was precipitated in 80% acetone, the precipitate washed with acetone-ether mixture, and dried as far as possible under reduced pressure. Samples of this product (A-1) were tested for biological activity, as shown in Table I. A duplicate batch designated in the Table as A-2 was not assayed but used as a source of B-2.

Phenol Fraction (B). The acetone precipitates (A-1 and A-2) were separately extracted 3 times with 150 ml 90-95% phenol at 45-55°. The supernatant (450 ml) of each was transferred to cellulose tubing and dialyzed in repeated changes of water at 50°. When all traces of phenol were gone, the volume was reduced to 200 ml by pervaporation. The phenol-free toxic material was precipitated in 80% acetone, and the precipitate dried after washing with acetone-ether mixture, to give fractions B-1 and B-2.

Formamide Fractions (C, D, E, and F). Employing the suggestion made by Morgan and Partridge that the highly associated solvent, formamide, would operate to disassociate the polysaccharide from the protein of the antigen,‡ the phenol product (B-2) was treated in several ways with formamide: (C)—a portion was dissolved at room temperature in formamide containing 1.4% glacial acetic, and then precipitated with acetone. The precipitate was dried and redissolved in formamide, and re-precipitated with acetone. The solution precipitation procedure was repeated a total of 6 times. The final product was washed and dried with acetone and ether; (D)—a portion was dissolved in acid-formamide and allowed to stand in solution for 14

† $(NH_4)_2SO_4$ 0.1 %
K_2HPO_4 0.1 %
$MgSO_4 \cdot 7H_2O$ 0.02%
Na_3 citrate $\cdot 2H_2O$ 0.4 %
$Na_2HPO_4 \cdot 12H_2O$ 0.5 %
Fe 2.0 mg/L.
Mn 0.5 mg/L.
Dextrose 1.0% (added aseptically)
pH 7.9 ± 0.1.

‡ Quantitative determinations by the orcinol or tryptophane procedures, of the polysaccharide content of the formamide-treated products listed in the table, indicated a carbohydrate content of at least 20%, as read against a standard dextrose solution. Unfortunately the sensitivity of these tests, in our hands, is not sufficiently high to give a more precise estimate. Studies are continuing in an effort to establish more precise protein and polysaccharide values following drastic disassociating procedures.

hours at 37°, then precipitated with acetone, washed and dried; (E)—a portion was dissolved in acid-formamide and left in solution for 14 hours at 60°, precipitated with acetone, washed and dried; (F)—a portion was dissolved in acid-formamide and left for 5 hours at 95°, precipitated with acetone, washed and dried. The activities of these various products are shown in Table I.

Bioassay: (1) *Lethality* was measured by injecting the toxic material in aqueous solution intraperitoneally into white male mice weighing 18-20 g. The amount required to kill within 24 hours 50% of the mice so injected was designated as one minimum lethal dose (1 M.L.D.).

(2) *Hemorrhage production* was assayed by injecting the toxic material in aqueous solution intraperitoneally into male mice bearing 7-day-old implanted tumor (mouse sarcoma 180). The amount necessary to produce slight (*) to marked (***) tumor hemorrhage in 50% of the animals tested was designated as one minimum hemorrhage dose (1 M.H.D)

(3) *Antigenicity.* To determine whether the purified products retained their capacity to cause the production of protective antibodies, mice were injected intraperitoneally with 1/20, 1/10, 1/5, 1/2, 1/2, and 1 M.L.D. successively for 6 days. On the 10th day following the first injection, multiple lethal doses of the purified products were injected. Survival was taken to indicate that an immunity had been established and that the fractions tested had retained antigenicity. The results of these tests are shown in Table I.

Discussion. It may be seen in the Table that while phenol extraction furnishes a potent toxin, the yield is relatively low, representing only about 0.4% of the weight of the acetone precipitate. Boivin and others report that as much as 10-12% of the cell body may be extracted as O antigen. The relative smallness of our yield may be due to the fact that we did not facilitate the release of the antigen from the cell bodies by acid or enzymatic digestion, but depended entirely on autolysis occurring during the 2-week growth period of the culture. Palmer and Gerlough[5] in their note on the non-extraction of the O antigen of *S. typhi* by concentrated phenol state that yields on subsequent aqueous extractions were improved by preliminary digestion of the bacteria with trypsin, a finding in harmony with the results of others, notably Raistrick and Topley.[6] The high yields obtained by Boivin with his trichloracetic acid procedure may be referable to a simultaneous hydrolysis and extraction. Our use of warm rather than cold phenol may account for the greater solubility achieved by us, and we are carrying out a further analysis of the extraction procedure with a view to increasing the yield.

From the Table it is seen that the various formamide products are not markedly more toxic or hemorrhagic than are the phenol products. The product of greatest titer is (E) the antigen subjected to formamide solution for 14 hours at 60°. Higher temperature (F) definitely decreased the activity, presumably by inactivating the antigen.

It is noteworthy that the more potent fractions present a wider spread between the M.L.D. and M.H.D. than are found with the crude material (A-1). Shear[7] obtained ratios of about 1:5000 with his most purified preparation. An explanation of this change in the ratio with purification must take into account many undetermined factors such as rates of detoxication, relative induction periods for capillary hemorrhage and lethality, the locus of action of the toxic material in producing death, etc.

All fractions of the antigen tested by us were found to retain the property of inducing the production of antibodies which protected mice against the toxic material. The specificity of this induced protection, as well as its degree, is under study by us.

Conclusion. The acetone, phenol, and various formamide fractions in the extraction of *Salmonella typhimurium* endotoxin when tested on mice were active in respect to (1) lethality, (2) the induction of hemorrhage in implanted tumors, (3) the production of antibodies which protected mice against the toxic material.

[5] Palmer, J. W., and Gerlough, T. D., *Science*, 1940, **92**, 155.

[6] Raistrick, H., and Topley, W. W. C., *Brit. J. Exp. Path.*, 1934, **15**, 113.

[7] Shear, H. J., *Cancer Research*, 1941, **1**, 731.