one. It is not clearly understood why a glucocorticoid, and particularly a mineralocorticoid, should potentiate the action of a pressor agent in a favorable manner. It has been clearly established in the dog and in man that adrenocortical insufficiency within physiologic limits is not usually present in endotoxin shock(7,8). There is accumulating evidence that normal hemodynamic function in the human may involve the interrelationship of aldosterone, angiotensin II and Na. Under the conditions of severe peripheral vascular collapse it is probable that during some periods pharmacologic amounts of hormone and a pressor agent will augment the physiologic effort of the host.

It would appear that in endotoxin shock in the dog and in the monkey an optimum amount of aldosterone must be used in conjunction with a pressor drug. Excessive amounts of aldosterone can have a deleterious effect. This may be related to the hyperkalemia and oliguria or anuria that occur. There is also an associated acidosis.

Summary. Severe endotoxin shock in monkeys resulted in hypotension, oliguria or anuria, hyperkalemia and death. No characteristic electrocardiographic findings were elicited. Aldosterone and angiotensin II were employed alone and in combination in attempts to reverse the shock. The most favorable results were obtained with a combination of the steroid and pressor drug. As in canine endotoxin shock, aldosterone markedly potentiated the pressor action of angiotensin II. Optimum results were correlated with the amount of aldosterone administered.

1. Spink, W. W., Vick, J., PROC. SOC. EXP. BIOL. AND MED., 1962, v109, 521.

2. Halberg, F., Spink, W. W., *Lab. Invest.,* 1956, v5, 283.

3. Spink, W., in *Shock: Pathogenesis and Therapy,* Bock, K. D., Ed., Springer-Verlag Publishers, Berlin-Gottingen-Heidelberg, 1962, 15.

4. Dexan, W. J., Massey, F. J., Jr., *Introduction to Statistical Analysis,* McGraw-Hill, New York, 1957.

5. Spink, W. W., Vick, J. A., PROC. SOC. EXP. BIOL. AND MED., 1961, v107, 777.

6. ———, *Circulation Res.,* 1961, v9, 184.

7. Melby, J. C., Spink, W. W., *J. Clin. Invest.,* 1958, v137, 1791.

8. Melby, J. C., Egdahl, R. H., Spink, W. W., *J. Lab. & Clin. Med.,* 1960, v56, 50.

Received December 10, 1962. P.S.E.B.M., 1963, v112.

Antigens of *Bordetella pertussis.* III. The Protective Antigen. (28173)

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The most practical prophylactic agent for immunization of children against whooping cough is still a vaccine prepared from killed whole cells of *Bordetella pertussis*(1,2). However, protection of children can be achieved with cell-free extracts of *B. pertussis*(3,4,5). All cell-free extracts so far described have been impure and the exact nature of the protective antigen is unknown. The mouse protective activity of *B. pertussis* cells is not associated with the so-called agglutinogen(6,7, 8), the hemagglutinin(9), the heat labile toxin (10,11,12), the heat stable toxin(13,14) or any substances found in the protoplasm of the cell(10,11). Since mouse protective activity of pertussis vaccines correlates well with their ability to confer immunity to children(15,16), it may be reasonable to assume that the *B. pertussis* protective antigen for children is not found in those substances lacking mouse protective activity. The mouse protective activity is found in cell walls(10,11, 17), and the active substance can be extracted from whole cells by various procedures (5,18-21). Some investigators have observed that the histamine sensitizing activity of pertussis vaccines parallels protective potency (22,23), and others have stated that the histamine sensitizing factor (HSF) is similar, if not identical, to the protective antigen, al-

TABLE I. Protective and Histamine Sensitizing Activities of Various *B. pertussis* Extracts.

	Test for HSF μg/mouse			Test for protection μg/mouse		
	10	30	90	3.2	16	80
Starting cells*	4/20†	8/20	8/17	15/20	8/20	0/20
Acetone dried cells	1/20	9/20	9/20	16/17	11/18	2/20
Saline extracted cells	3/20	3/20	12/20	20/20	17/20	4/17
Saline extracted (SE)	3/20	13/20	15/20	19/19	11/20	2/20
Acid precipitated SE(APSE)	6/20	14/20	14/20	16/20	6/20	4/20
Uninoculated controls		0/13			20/20	

* In this test the starting cells were heated at 56°C for 15 min to destroy heat-labile toxin.
† Deaths/total No. of mice used.

though the evidence on which this statement was based was indirect and not convincing (24). On the other hand, some evidence has indicated that the two substances are different (12,18,20,25). In this paper a method of preparing the protective antigen free of most other cellular components is described and evidence indicating that protective antigen and HSF might be identical is presented.

Materials and methods. B. pertussis *cells.* These cells were grown in a modified liquid medium of Verwey *et al.*(26) and were kindly supplied by Merck Sharp and Dohme through the courtesy of Mr. L. F. Schuchardt. Living cells in a concentration of 10×10^{12} per ml were kept frozen until needed.

Preparation of cell extracts. Frozen cells were thawed and extracted with acetone at room temperature. To 820 ml of cell suspension, 2460 ml of acetone were added with constant stirring and the cells were then collected by filtration through a Buchner filter fitted with #4 Whatman filter paper. The acetone extraction was repeated once more by resuspending the cells in another 2460 ml of acetone. The cells were again collected by filtration and further washed on the filter with 540 ml of fresh acetone. Vacuum was applied until most of the acetone was removed. The dried cells were spread in a thin layer and placed in a desiccator under continuous vacuum to remove traces of acetone. These acetone-dried cells were stored in a tightly stoppered bottle kept at 2-5°C. From acetone-dried cells, extracts containing the protective activity were prepared as previously described for preparation of HSF(27). The procedure consisted of suspending 15 g of finely powdered acetone-dried cells in 500 ml of cold 0.85% NaCl (saline), homogenizing the suspension, and then rupturing the cells in the pressure cell of Ribi *et al.*(28) at 40,000 pounds per square inch. This last procedure, although not essential, was found to improve extraction of HSF and protective antigen. The ruptured cell suspension was diluted with another 500 ml of cold saline and the pH adjusted to 8.5 with 1 N NaOH. The suspension was kept cold and constantly stirred during the addition of the base and for 1-2 hours thereafter. It was centrifuged at 13,000 rpm (27,000 g) in a Servall refrigerated centrifuge for 40 min. The clear amber-colored supernatant was dialyzed against repeated changes of cold distilled water and lyophilized. This dried saline extracted material (SE) was used as starting material for further fractionation and contained most of the protective and HSF activity of the cells, although considerable activity remained in the extracted sediment (Table I). Reextraction of the sedimented material was not carried out.

The various procedures used to purify the protective antigen further will be described under the corresponding experiment.

Mice. The mice used for HSF activity test were Webster strain Carworth Farms mice raised in a special colony in this laboratory. Mice of both sexes weighing approximately 18-22 g were used. For assay of the protective antigen, approximately 15-18 g mice of the Rocky Mountain Laboratory strain were used because of their availability. These latter mice do not become sensitive to histamine and could not be used for estimating HSF activity. The methods used in testing for HSF and for protective activity were similar to those used previously(10).

Antisera. For most of the gel diffusion

tests a pool of hyperimmune rabbit anti-*B. pertussis* serum was used. This serum was kindly supplied to us by Dr. Margaret Pittman. Other pools used were hyperimmune rabbit sera prepared by inoculating rabbits with whole *B. pertussis* cells over a period of weeks. Gel diffusion tests were performed with minor modifications as described by Ouchterlony(31).

Results. Protective and HSF activity of B. pertussis *cells and SE.* From a single batch of frozen cells, SE was prepared and samples of original cells, extracted cells and SE were dialyzed repeatedly against cold distilled water and lyophilized. A sample of 420 ml of dialyzed but not lyophilized SE was precipitated with 1680 ml of 0.02 M acetate buffer at pH 4.6. The precipitate collected by centrifugation was resuspended in 0.01 M phosphate buffer pH 7, dialyzed against water, lyophilized, and designated acid precipitated SE (APSE).

These materials were tested on a weight basis for their protective and HSF activity (Table I). SE contained most of the protective antigen and HSF originally present in whole cells, although considerable material with activity was not extracted. Protective and HSF material was precipitated at pH 4.6. Notably, the amount of material required to produce appreciable protection was of the same order of magnitude as that required to produce histamine sensitization. It required between 10-30 μg of SE or APSE to sensitize mice to histamine and around 16 μg to protect about 50% of challenged mice.

Purification of protective antigen and HSF by starch block electrophoresis. It has been possible to purify the HSF considerably by curtain electrophoresis and by DEAE column chromatography. Difficulty was encountered, however, in obtaining adequate yields(29). Starch block electrophoresis has proved more efficient in this respect. This technic with slight modifications is similar to that previously used(27). A starch block 52 cm long, 6 cm wide and 1 cm deep was prepared with purified potato starch (Baker) suspended in phosphate buffer (pH 6.2 and 0.024 ionic strength). Eighty mg of SE were dissolved in 8 ml of the same buffer, and 7 ml of this

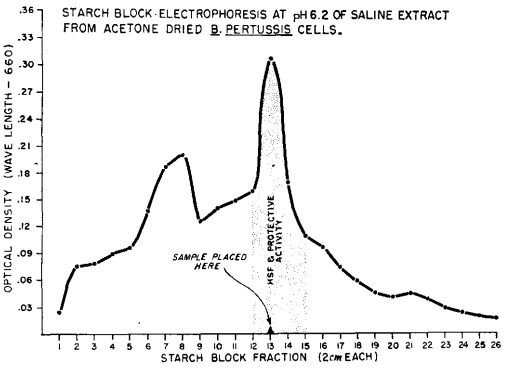

FIG. 1

solution were mixed with 8 g starch and placed in a 1 × 2 × 6 cm well cut out of the starch block. Electrophoresis was carried out for 46 hours at 10 ma and 310 volts. (The voltage varied somewhat during the run but the current was kept constant.) The starch block was then cut into pieces 2 cm long. Each section was extracted with 20 ml of phosphate buffer (pH 7.2 and 0.024 ionic strength). Five-tenths ml of each fraction was analyzed for the presence of tyrosine by the Folin-Ciocalteau reagent method. The color developed was measured in a Coleman Jr. spectrophotometer at a wave length of 660 mμ. This measurement gave a close estimate of the degree of resolution obtained as well as of the complexity of the material. Obviously any material which did not contain aromatic amino acids was not detected. From previous experience(27), however, it was known that HSF activity is associated with one of the tyrosine peaks. At least 7 different tyrosine peaks were obtained (Fig. 1) and some material may have migrated into the buffer at the cathode. From preliminary experiments it was found that the HSF activity did not move appreciably at pH 6.2 and, in order to obtain as much resolution as possible, the period of electrophoresis was 46 hours regardless of loss of some materials with no activity of immediate interest. Selected fractions were tested for HSF and protective activity. The results are given in Table II. Protective and HSF activities were found mainly in fraction 13, although fractions 12, 14, and 15 also had some activity. Fractions 8, 11, and 18 had no protective or HSF activity. In other ex-

TABLE II. Protective and Histamine Sensitizing Activities of Various Starch Block Electrophoresis Fractions.

Fraction No. (see Fig. 1)	Test for HSF dilution of sample 1:1	1:5	Qualitative interpretation	Test for protection dilution of sample 1:1	1:5	Qualitative interpretation
8	0/10*	1/10	—	13/15	13/13	—
11	3/10	3/10	—	15/15	12/14	—
12	6/10	1/10	+	10/14	14/15	±
13	9/10	9/10	+	4/12	6/15	+
14	10/10	2/10	+	1/14	11/14	+
15	ND†	0/10	?	1/10	15/15	+
18	3/10	3/10	—	13/14	13/14	—
Controls	0/10			13/14		

* Deaths/total No. of mice used.
† ND = not done.

periments, protective or HSF activities have not been found in fractions 1-11 or 18-26. In gel diffusion tests, at least 12 antigenic materials were found in these fractions (Table III). Unfractionated SE formed only 3 to 4 detectable bands with the antiserum used (27). Fig. 2 is a photograph of the serum-agar diffusion test in which these fractions and immune rabbit antiserum were used, and a diagrammatic representation of the same test. In Table III the sequence of appearance and disappearance of the various different bands is given, as well as the presence or absence of protective and HSF activities of the different fractions. The only band that can be correlated with protective and HSF activities is a rather diffused band (band "J" in Table III) found close to the antigen wells in fractions 12, 13, 14, 15, and 16. This is the only band common to all the protective or histamine-sensitizing fractions. It is interesting that of 6 pools of rabbit hyperimmune *B. pertussis* sera only 2 showed this band, although

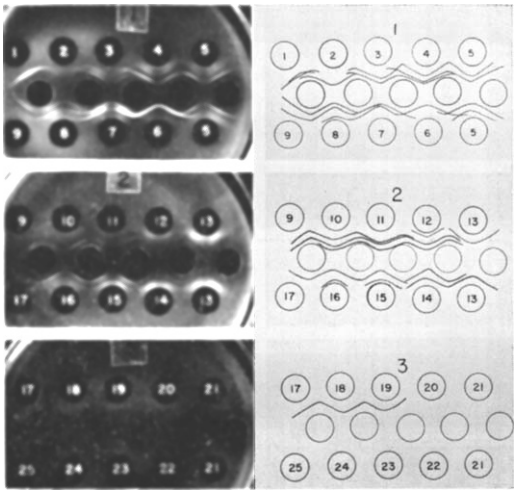

FIG. 2. Ouchterlony type gel diffusion test performed with various starch block electrophoresis fractions of SE. Center wells of each plate contained hyperimmune rabbit serum. Top and bottom rows of wells in each plate contained the various electrophoretic fractions, starting with fraction 1 at negative pole. Left = actual photograph of plates. Right = diagrammatic sketches of same plates.

TABLE III. Antigens in Various Starch Block Electrophoresis Fractions as Shown by Ouchterlony Agar Diffusion Test.

Fraction No. (see Fig. 2)	Bands observed	HSF	Protection
1	a,b,e	—	—
2*	b,e	—	—
3	b, d	—	—
4	b, d,e	—	—
5	b, e,f	—	—
6	e,f	—	—
7	f,g	—	—
8	f,g,h	—	—
9	g,h	—	—
10	g,h,i	—	—
11	g,h,i	—	—
12	g,h,i,j	+	+
13	h,i,j,k	+	+
14	j,k	+	+
15	j, l	+	+
16	j, l	+	ND†
17	l	—	—
18	l	—	—
19	l	—	—
20		—	—
21		—	—
22		—	—
23		—	—
24		—	—
25		—	—

* It was difficult to tell whether the "a" or the "b" band was present in fraction 2. In this table band b was assumed to continue in wells, 3, 4 and 5 as the same antigen found in fraction 1.
† ND = not done.

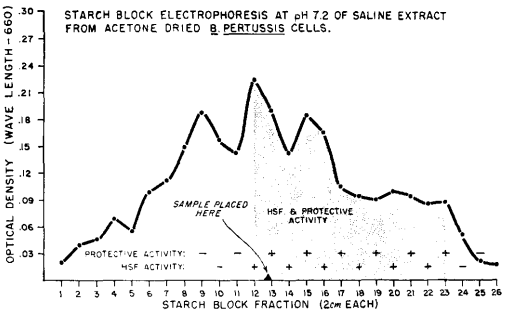

FIG. 3

all the sera had high titer of agglutinins and gave various precipitin bands. It should be noticed that fraction 13 shows one main band as well as traces of 3 more which are visible only at the sides adjacent to wells 12 or 14. At the side where no other fraction was placed, only one main band was formed (Fig. 2, Plate 2, right hand side).

When electrophoresis is performed at a higher pH the HSF moved towards the anode and spread through quite a broad zone. Whether the protective antigen behaved similarly was determined in an experiment in which the conditions were similar to those in the above experiment except that the phosphate buffer was adjusted to pH 7.2 (0.024 ionic strength), the current was 7 ma, the voltage 200 volts and the time of electrophoresis 40 hours and 40 min. The results are recorded in Fig. 3 and Table IV. Due to insufficient material, the same fraction was not tested for the two activities, but it is clear that both HSF and protective antigen spread to the same parts of the starch block.

From this experiment, as well as the appearance of the band in the serum agar test and from unpublished ultracentrifugal studies, it is evident that the protective antigen is quite polydispersed. Studies are now under way to determine the exact chemical composition of this interesting substance.

Discussion. Data are presented which indicate that the protective antigen of *B. pertussis* is closely associated with, and perhaps identical to, the HSF. All efforts to separate the agents causing the two activities by mild procedures have failed. Thus both protective antigen and HSF activities are precipitable by similar concentration of acetone and by pH lower than 4.9, both have similar electrophoretic mobility in starch blocks, both are found in the cell walls(10,11), and the protective potency of vaccines correlates with their ability to sensitize mice to histamine (22,23). Differences in stability of the 2 factors have been reported by various investigators(12,18,20,22), and the conclusion was

TABLE IV. Protective and Histamine Sensitizing Activities of Various Starch Block Electrophoresis Fractions.

Fraction	Test for HSF (ml/mouse)			Qualitative interpretation	Test for protective antigen (ml/mouse)			Qualitative interpretation
	.1	.2	.4		.008	0.04	0.2	
9	0/10*	0/10	1/10	—				
10					10/10	10/10	10/10	—
11	0/10	0/10	0/10	—				
12					10/10	8/9	3/9	+
13	6/10	8/10	8/10	+				
14					8/9	6/9	3/9	+
15	3/10	6/10	8/10	+				
16					9/10	5/9	4/8	+
17	3/10	5/10	9/10	+				
18					5/8	10/10	2/9	+
19	3/10	7/10	7/10	+				
20					9/10	6/10	3/9	+
21	3/10	5/10	9/10	+				
22					7/9	8/10	3/7	+
23	3/9	7/10	6/10	+				
24					10/10	9/10	10/10	—
25	2/10	1/10	0/10	—				
26					9/10	9/10	7/8	—
Uninoculated controls		0/10				9/10		

* Deaths/total No. of mice used.

reached by Dolby(20) that, since the 2 factors could be partially separated, the two were found in different parts of the *B. pertussis* cell. Her data, however, showed that the 2 activities could not be completely dissociated from each other. The partial separation obtained might be explained by differences in stability of the two activities in the same complex molecule, or by a dependence of one activity on certain degree of polymerization or rearrangement of the molecular species involved. If either of these hypotheses is true, it can easily be seen that preparations could be obtained which vary in their protective and HSF activities, even though the two may be due to the same chemical entity. It has been our experience, as well as that of others, that the 2 activities are easily lost by a variety of treatments and that some treatments seem to affect one activity more readily than the other (20,22,25). Consequently there seems to be no doubt that the 2 activities can be separated by preferential destruction of one while leaving the other intact or only slightly affected. A well-known analogy can be found in the formation of toxoid from exotoxins. It should be made clear that there still remains a possibility that our observations could be due to 2 different substances with unusually similar properties. We are presently investigating other properties of purified preparations in an effort to settle this question.

It should be emphasized that, as found by Fishel(30), the histamine sensitization phenomenon is not due to production of sensitizing antibodies, but rather to some still not clearly understood physiological effect on the susceptible mouse. Protective activity, on the other hand, is dependent on the stimulation of the immune mechanisms and can be demonstrated in various strains of mice regardless of whether they are susceptible to HSF or not. The strain of mice used for protection experiments in the present work is resistant to sensitization to histamine by previous injection of whole cells or purified fractions.

Regardless of the problem of identity or difference between protective antigen and HSF, the present work gives a method of obtaining highly purified preparations of protective antigen containing mainly one antigenic component as demonstrated by the serum-agar precipitation test of Ouchterlony. Since the tests were performed with a hyperimmune serum which gave about 12 bands with the SE, considerable purification was achieved. This method may prove to be of practical value in preparation of purified protective antigen for immunization of children against whooping cough.

Summary. A method of preparing extracts from *Bordetella pertussis* with high protective and histamine sensitizing activity is described. From these extracts, starch block electrophoresis yielded active purified fractions which not only protected mice against intracerebral challenge with living *B. pertussis* cells but also sensitized mice to histamine. From electrophoretic properties and agar diffusion studies, it is tentatively concluded that the mouse protective antigen from *B. pertussis* is similar, and perhaps identical to the histamine sensitizing substance. The purified preparations were shown to contain mainly one antigenic component. The starting material contained at least 12 antigenic substances, as demonstrated by gel diffusion.

1. Provenzano, W. R., Wetterlow, C. H., Ipsen, J., *New Engl. J. Med.*, 1959, v261, 473.

2. Pittman, M., *Pediatrics*, 1954, v45, 57.

3. Felton, H. M., Verwey, W. F., *ibid.*, 1955, v16, 637.

4. Petrilla, A., Barsy, G., *Acta Microbiol. Acad. Scient. Hungaricae*, 1958, v5, 35.

5. Behrens, O. K., Ensminger, P. W., 1959, U. S. Patent No. 24748.

6. Schuchardt, L. F., Sagin, J. F., Munoz, J., *Fed. Proc.*, 1957, v16, 432.

7. Onoue, K., Kitagawa, M., Yamamura, Y., *J. Bact.*, 1961, v82, 648.

8. Verwey, W. F., Thiele, E. H., *J. Immunol.*, 1949, v61, 27.

9. Thiele, E. H., *ibid.*, 1950, v65, 627.

10. Munoz, J., Ribi, E., Larson, C. L., *ibid.*, 1959, v83, 496.

11. Billaudelle, H., Edebo, L., Hammarsten, E., Hedén, C-G., Malmgren, B., Palmstierna, H., *Acta Path. et Micro. Scand.*, 1960, v50, 208.

12. Kuwajima, Y., Matsui, T., Naka, K., Kanai, Y., *Osaka City Med. J.*, 1958, v4, 177.

13. Munoz, J., Schuchardt, L. F., 1955, unpublished observations.

14. Malmgren, B., 1962, personal communication.

15. Medical Research Council, *Brit. Med. J.*, 1956, v2, 454.

16. Pittman, M., *Revue d'Immunol.*, 1958, v22, 303.

17. Yoshida, N., Tanaka, K., Takaishi, K., Fukuya, I., Nishino, K., Kakutani, I., Inoi, S., Fukui, K., Kono, A., Hashimoto, T., *Tokushima J. Exp. Med.*, 1955, v2, 11.

18. Maitland, H. B., Guerault, A., *J. Path. Bact.*, 1958, v76, 257.

19. Frappier, A., Guerault, A., *Revue Canad. de Biol.*, 1955, v14, 193.

20. Dolby, J. M., *Immunology*, 1958, v1, 328.

21. Pillemer, L., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 704.

22. Pittman, M., *J. Infect. Dis.*, 1951, v89, 300.

23. Maitland, H. B., Kohn, R., MacDonald, A. D., *J. Hyg. Camb.*, 1955, v63, 196.

24. Joó, I., Pusztai, Z., *Nature*, 1960, v188, 331.

25. Pusztai, Z., Joó, I., Juhász, V. P., *Z. für Immunitätsforsch. u. exp. Ther.*, 1961, v122, 278.

26. Verwey, W. F., Thiele, E. H., Sage, D. N., Schuchardt, L. F., *J. Bact.*, 1949, v58, 127.

27. Munoz, J., Hestekin, B. M., *Nature*, 1962, v196, 1192.

28. Ribi, E., Perrine, T., List, R., Brown, W., Goode, G., PROC. SOC. EXP. BIOL. AND MED., 1959, v100, 647.

29. Munoz, J., 1960, unpublished observations.

30. Fishel, C. W., *J. Infect. Dis.*, 1956, v99, 44.

31. Ouchterlony, O., *Progr. Allergy*, 1958, v5, 1.

Received November 27, 1962. P.S.E.B.M., 1963, v112.

Effect of Corticoids on Spleen of the Cichlid Fish, *Tilapia mossambica.** (28174)

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Splenic atrophy is one of the generally observed consequences of glucocorticoid administration to mammals(1). In an experiment designed to determine the effect of short-term treatment with cortisol on interrenal histology of the euryhaline freshwater cichlid teleost, *Tilapia mossambica,* the expected decrease in spleen weight was not observed. Additional experiments were then conducted to examine the response of the spleen in *Tilapia* to the mammalian "glucocorticoid," cortisol, and to the mammalian "mineralocorticoid," deoxycorticosterone.

Materials and methods. Tilapia mossambica of both sexes and weighing less than 30 g, from Oahu, Hawaii, were used. No differences in response relative to sex or size of the animals were noted. In the initial experiment (Table I), 2 groups of 6 fish each were maintained in running tap water. One group received 0.5 mg cortisol acetate (FA) in 0.1 ml aqueous suspension intraperitoneally daily for 7 days; the controls received 0.1 ml distilled water (DW) similarly. Two other groups were injected similarly, but had been transferred to running sea water one month before the beginning of the experiment. When it became apparent that the spleen weight was not decreased by the cortisol treatment, additional groups were set up (Table I), including 3 groups treated as above for 21 days with DW, FA, or deoxycorticosterone acetate (DCA); both steroids were given as a daily intraperitoneal dose of 0.5 mg in 0.1 ml aqueous suspension.

At termination of experiments, spleens were weighed on a 0-500 mg torsion balance, and all spleens except those from the first experimental series were fixed in Bouin's for preparation of hematoxylin and eosin-stained paraffin sections. Final body weights were recorded, and spleen weight/body weight ratios were calculated (Table I). Portions of the hepatopancreas and of the hemopoietic "head kidney" from representatives of Groups III and IV were also fixed and stained.

Results. Short-term treatment with FA had

* Hawaii Marine Laboratory Contribution No. 184. Research conducted during tenure of a National Science Foundation Senior Postdoctoral Fellowship, 1958-9. Author thanks K. B. DeOme for advice on histopathologic findings.