fer reaction is ultimately bound to the protein anabolism of immunologically active cells, *i.e.* intact protein synthesis is essential for expression of the reaction. However, it remains to be determined whether this is an increased synthesis of specific protein or antibody in situ ("local secondary specific protein or antibody response") on second contact of immunologically active cells with specific antigen, or the increased synthesis of protein accompanying increased cell size and/or proliferation.

Summary. Incubation of homograft sensitized lymphoid cells with ribonuclease inhibits their ability to produce the "transfer reaction" on intradermal injection into the antigen donor. Ribonuclease in the concentration used does not kill the cells as determined by trypan blue staining and culture in Millipore filter chambers. The data indicate that intact protein synthesis of transferred immunologically active cells is essential for the expression of the "transfer reaction."

1. Brent, L., Brown, J. B., Medawar, P. B., *Lancet*, 1958, v2, 561.
2. ————, in *Biological Problems of Grafting*, 1959, Blackwell Scientific Publ., Oxford, p64.
3. Allfrey, V. G., Daly, M. M., Mirsky, A. E., *J. Gen. Physiol.*, 1953, v37, 157.
4. Brachet, J., *Nature*, 1954, v174, 876.
5. Ledoux, L., Baltus, E., *Experientia*, 1954, v10, 500.
6. Brachet, J., *Nature*, 1960, v186, 194.
7. ————, *Biochemical Cytology*, 1957, Academic Press Inc., New York, p264.

Received August 11, 1961 P.S.E.B.M., 1961, v108

Effect of Solvents on Eosinophile-Stimulating Substance of Erythrocyte Stroma.* (26998)

JOHN S. CHAPMAN (Introduced by D. W. Seldin)
Department of Internal Medicine and William Buchanan Laboratory, University of Texas Southwestern Medical School

It has been shown that blood is capable of exciting in animals a peritoneal reaction which in its later stages is characterized by the appearance of eosinophiles in definitely elevated proportions. It was then established that the component of blood responsible for this reaction was neither plasma nor hemoglobin but the stroma and membranes of erythrocytes (1,2).

The present studies were undertaken as the first steps toward isolation and identification of the eosinophile-stimulating substance (E.S.S.), if such exists.

Materials and Methods. Erythrocyte stroma was prepared as previously described.

Procedure 1. 2.5 ml of stroma was shaken repeatedly in 7.5 ml ether at room temperature, allowed to stand over night, reshaken and allowed to sediment. After filtration the soluble fraction was evaporated in partial vacuum to dryness. The residue was suspended with repeated shaking in sterile 0.7% saline to make 10 ml, and 1 ml of the suspension was injected into the peritoneal cavity of each of 10 male albino mice. The insoluble portion was likewise suspended in 0.7% sterile saline to make 10 ml, and 1 ml was injected intraperitoneally into each of 10 male albino mice.

Procedure 2. 2.5 ml of erythrocyte stroma was suspended in 7.5 ml petroleum ether and was treated in the same manner as described in procedure 1. One ml of the hexane soluble fraction suspended in 0.7% sterile saline was injected intraperitoneally into each of 10 male albino mice, and the insoluble portion was similarly injected.

Procedure 3. This experiment was carried out in the same way as procedure 1, except that chloroform was used as the initial extraction agent. Both soluble and insoluble fractions were resuspended and injected as before.

Procedure 4. Except for the use of acetone as the initial solvent this procedure was iden-

* This study was supported originally by a grant from the Dallas Tuberculosis Assn. and has been extended under grant from Dept. of HEW.

tical with procedure 1. Resuspended fractions were treated and injected into mice in the identical way.

Procedure 5. Alcohol 95% was employed as the initial extraction agent. All other steps were carried out as in procedure 1.

Procedure 6. Equal parts of 95% alcohol and ether were used as the initial extraction agents. All other steps were the same as in procedure 1.

Procedure 7. 2.5 ml of erythrocyte stroma was shaken repeatedly with N/10 HCl. Complete solution occurred. When N/10 NaOH was added a precipitate occurred at pH below 7, presumably the iso-electric point of certain proteins. The pH was brought to 7.0, the material centrifuged and filtered. Soluble and insoluble fractions were reconstituted to 10 ml, and mice were then injected intraperitoneally, 10 with 1 ml of the soluble and 10 with 1 ml of the insoluble portions.

Procedure 8. 2.5 ml erythrocyte stroma was treated with 7.5 ml N/10 NaOH. Complete solution occurred, and adjustment with N/10 HCl to pH 7 produced no precipitation. One ml of the neutralized solution was injected intraperitoneally into each of 10 mice.

Procedure 9. 2.5 ml of stroma was shaken with 7.5 ml 10% urea in distilled water. Complete solution took place. One ml of the urea solution was then injected intraperitoneally into each of 10 male albino mice.

Procedure 10. 2.5 ml of stroma was suspended in 7.5 ml, 0.7% NaCl solution. The mixture was placed for one hour in a water bath held at 50°C. The material was then filtered and soluble and insoluble fractions brought up to 10 ml with 0.7% NaCl solution. Ten mice each received intraperitoneal injections of 1 ml of the insoluble suspension and 10 received each 1 ml of the solution.

Procedure 11. 2.5 ml stroma was boiled for one hour in 7.5 ml of 0.7% NaCl solution. The material was filtered, and the soluble portion brought up to 10 ml with 0.7% NaCl solution. Each of 10 male albino mice received 1 ml of the solution intraperitoneally.

Procedure 12. 2.5 ml stroma was digested at 37° with 7.5 cc 1% trypsin solution. Almost all material was dissolved. The solution was filtered, and the volume of filtrate brought to 10 ml. The undissolved residue was suspended in 10 ml 0.7% NaCl. One ml of filtrate was injected into each of 10 albino mice. One ml of suspension was injected into each of 10 animals.

Procedure 13. 2.5 ml stroma was digested with 7.5 ml 5% pepsin for 18 hours at 37°. The filtrate and residue were treated as in procedure 11.

Procedure 14. This procedure was identical with procedures 12 and 13, except that 1% caroid was employed as digestant. Mice were injected as before.

Procedure 15. Except that papain 0.1% was employed as the enzyme, this procedure was identical with the previous ones.

Procedure 16. Stroma was digested with 1% ptyalin.

Procedure 17. The enzyme employed was 5% hyaluronidase. In all other respects the procedure was carried out as before.

Procedure 18. 5% pepsin in 10 ml 0.7% NaCl was injected into albino mice as a control.

Procedure 19. 1% ptyalin in 10 ml 0.7% NaCl was injected into animals as a control.

Procedure 20. 5% hyaluronidase in 10 ml 0.7% NaCl was injected intraperitoneally.

Procedure 21. Injections of 1% caroid, papain and 1% trypsin, were associated with very high mortality within 48-72 hours.

Procedure 22. 2.5 ml RBC stroma was digested at 37° over night in 7.5 cc 5% pancreatic lipase solution. After repeated shakings the material was filtered and injected into mice as in other procedures.

All mice were sacrificed at 120 hours following injection and smears made from the peritoneal surface as described previously. Slides were stained with Wright-Giemsa and 300-500 white cells counted, except in those instances in which a cellular response was so slight as to limit the observable white cells to 100-200 in 30 minutes of study.

Results. Table I shows that no eosinophile-stimulating substance was dissolved in any lipid solvent. Most of these solvents seemed to destroy or damage the substance. Hot water failed to extract the substance, and boiling for one hour did not add additional amounts. Heat apparently destroyed in large part the eosinophile-stimulating property of stroma. Treatment of red blood cell stroma with alcohol, acetone and alcohol-ether failed to extract

TABLE I. Effects of Solvents, Heat and Enzymes on the Eosinophile Stimulating Substance of Erythrocyte Stroma

Solvents	Animals	% Eosinophiles (mean)	Range
Ether			
Soluble	10	2.6	0.5- 6.3
Insoluble	10	3.52	0 - 8
Petroleum Ether			
Soluble	10	2.23	0 -10.2
Insoluble	7	2.9	0 - 5.6
Chloroform			
Soluble	9	.67	0 - 1.7(13)*
Insoluble	10	2.04	0 - 6.7
Acetone			
Soluble	8	2.31	0 -11
Insoluble	10	10.31	4.7-18.3
Alcohol 95%			
Soluble	9	0	0 - .5
Insoluble	10	7.5	3.7-17
Alcohol—Ether			
Soluble	10	3.32	.4- 6.6
Insoluble	10	10.66	3.6-25.2
N/10 HCl			
Soluble	9	2.3	0 - 7.3(22.3)
Insoluble	10	3.23	.7- 5.7
N/10 NaOH			
Soluble	10	3.87	.7-10.7
Urea 7.5%			
Soluble	10	1.72	.7- 4.5(12)
Hot Water Extraction			
Soluble	9	.9	0 - 2 (12.3)
Insoluble	6	2.08	0 - 5.5(30.6)
Boiling			
Soluble	7	.7	0 - 1 (15.6)
Trypsin 1%			
Soluble	10	0.2	0 - 1.5
Insoluble	10	2.7	0 - 7.3
Pepsin 5%			
Soluble	16	2.8	0 -6.3(16-17)
Insoluble	18	3.9	0 - 9
Caroid 1%			
Soluble	8	2.1	0 - 5.5(15)
Insoluble	12	5.7	0 -12.4
Papain 0.1%			
Soluble	12	5.3	3.6- 8.3
Insoluble	16	5.1	0 -13
Ptyalin 1%			
Soluble	9	5.7	3 - 8.3
Insoluble	9	3.9	1.7- 8.7
Hyaluronidase 5%			
Soluble	9	8.8	0.5-20.3
Insoluble	9	2.2	0 - 5.3
1% Ptyalin	16	11.1	2.3-24
5% Pepsin	5	3	1.3- 6.0
1% Hyaluronidase	10	8.5	2.6-16.6
5% Hyaluronidase	10	9.6	2.0-27.5
Lipase			
Soluble	4	0	0 - 0
Insoluble	10	5.7	2 -11

*Parenthetic figures represent an exceptionally high deviant count found in a single animal and not included in the mean.

a soluble eosinophile-stimulating substance, but did not damage it in the residue. N/10 NaOH caused total solution and partially damaged the substance. N/10 HCl solution resulted in a precipitable fraction at the presumed isoelectric point: both soluble and insoluble fractions showed reduction in activity, the activity being slightly greater in the precipitated portion.

Enzymatic digestion was little more successful than the solvents in producing solution of the E.S.S. of RBC stroma. Trypsin produced nearly complete solution of the entire stroma, but in doing so altered the material so that no further eosinophile stimulus occurred. There was 5% pepsin soluble response, but procedure 17 revealed that pepsin itself produces an eosinophilic reaction. Hyaluronidase digestion produced a solution that provoked eosinophilic response, but control procedure indicates an even more striking response when hyaluronidase alone is used. Papain solution of stroma resulted in a response, but in view of the inability to control this procedure by reason of the early deaths of mice injected with papain alone this observation is not useful.

Some degree of eosinophile stimulating activity was preserved in the insoluble portion after digestion with all protein-splitting enzymes. It was least after tryptic and hyaluronidase and greatest after caroid digestion.

The range of eosinophiles in some of the experiments was rather wide. It was not uncommon to encounter 20-30% eosinophiles in one of 10 animals, when all the others presented 1-5% in their exudate. The significance of this observation will be discussed below.

Discussion. The procedures with solvents indicate that the E.S.S. is not a lipid. Damage to the E.S.S. in the residue by ether, petroleum ether and chloroform suggests that the material may be a protein or a protein-linked carbohydrate. The protection of the substance in the residue by alcohol, by alcohol in combination with ether and by acetone may be expected of either protein or protein-carbohydrate compound.

Destruction of E.S.S. by heat, urea solution, N/10 NaOH, N/10 HCl, and tryptic digestion indicates that at least a part of the substance is protein. The simpler protein fragments of tryptic digestion, as shown in procedure 12, do not evoke an eosinophilic response. Nor, as was reported previously(2), do large protein molecules such as the proteins of plasma

or of hemoglobin produce eosinophile migration.

Since weaker proteolytic enzymes of the order of pepsin, caroid and papain fail to eliminate the eosinophilic response, it may be suspected that protein linkage with another type of compound is involved. The procedures reported with fat solvents suggest the linkage is not with a lipid, a point further substantiated by the failure of pancreatic lipase to affect the E.S.S.

There remains the probability that the E.S.S. is a protein-carbohydrate complex. Favoring such a hypothesis is the demonstration that pepsin, ptyalin and hyaluronidase when injected alone produce an eosinophilic reaction. Both ptyalin and pepsin are prepared from biological materials and both are associated with blood group substances that are known to be mucopolysaccharides. Alternately these enzymes and hyaluronidase may release some kind of E.S.S. from the peritoneal surfaces.

It is evident from these procedures that further efforts to fractionate RBC stroma will be unsuccessful since all chemical separations will depend upon obtaining a solution of undamaged E.S.S.

Further work is under way to approach the problem through successive injections of increasingly complex proteins and of increasingly complex carbohydrates as well as to determine if the physical state of material, gel or insoluble suspensions, may determine an eosinophilic response.

The occurrence of an occasionally high eosinophile count in one of 10-20 animals may be the result of an unknown biological difference. In the course of many injections it is almost certain that trauma and peritoneal bleeding will occur from time to time. It is also possible that in spite of efforts to secure a complete mixture of solvents with the gelatinous and gummy stroma, there may not be complete contact between some portions of the mass and the solvent.

Summary. 1. A series of experiments with variously treated RBC stroma injected intraperitoneally into mice clearly demonstrates that an eosinophile-stimulating substance previously noted is not a lipid. 2. The experiments further establish that the substance is either a protein or a protein-carbohydrate compound. 3. The possibility is raised that the physical state of material (gel or insoluble suspension) may determine the eosinophilic response.

The author expresses his gratitude to Maro Speight and Jean Clark, for invaluable assistance in this project.

1. Chapman, J. S., *J. Lab & Clin. Med.*, 1955, v46, 48.

2. ————, Reynolds, R. C., *ibid.*, 1958, v51, 516.

Received March 17, 1961 P.S.E.B.M., 1961, v108

Serum Enzyme Alterations in Cancer. (26999)

H. ALBAUM, W. ANTOPOL, B. KABAKOW, S. SLAPIKOFF, G. BLINICK, L. SUSSMAN AND L. GINZBURG

Levy Laboratories, Beth Israel Hospital, New York City, and Biology Research Laboratory, Brooklyn College, New York

Alterations of serum enzyme levels have been observed in numerous maladies(1,2,3). The fact that similar alterations in level of a particular enzyme such as lactic dehydrogenase occur in many diverse pathological lesions limits the use of a single enzyme as a specific diagnostic tool for cancer detection. The present study was undertaken to determine whether patients with various malignant tumors would reveal different patterns of multiple serum enzyme changes indicative of individual tumor types.

Materials and Methods. The serum enzymes assayed included: lactic dehydrogenase (LDH), glutamic pyruvic transaminase (SGPT), glutamic oxaloacetic transaminase (SGOT), malic dehydrogenase (MDH), aldolase (ALD), enolase (ENOL), pyruvic kinase (PK), myokinase