velope" antigen of Schütze[1] and others. They are probably present on the surface of the bacilli, because antisera prepared against these fractions will agglutinate the strains of plague bacilli thus far tested. Only one avirulent strain, TRU,[1] obtained from Dr. Harry Schütze, Lister Institute, London, England, is not agglutinated by Fraction I sera, although it is slightly agglutinated by sera prepared against whole plague bacilli. This strain appears to be devoid of these 2 fractions. The exact relationship between Fractions IA and IB is not clear. Serologically, they are almost identical. Thus far, the crystalline Fraction IB proved of slightly lower immunogenic value. It might be suggested that in the intact cell the normal antigen is the carbohydrate protein Fraction IA, and that the crystalline protein is an artifact formed by disaggregation of the molecule during the death of the cell and subsequent treatment. All attempts to alter Fraction IA short of denaturation have failed. It may be that plague bacilli contain an enzyme capable of converting Fraction IA to IB. Serologic studies have shown that Fraction I (IA and IB) is formed by the virulent strains tested, and the avirulent strain 1122 which forms a stable suspension in saline; avirulent strains showing salt instability produce only traces. These antigens are formed in quantity at 37°C; extracts of cells grown at room temperature contain relatively small amounts.

In view of the observed differences in response of the various laboratory animals to the different antigens of plague bacilli, it is considered advisable at the present time to use vaccines containing all of the antigens of *P. pestis* for prophylaxis of plague in man, and for the production of antiplague serum for therapeutic use.

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1 Schütze, H., *Brit. J. Exp. Path.*, 1932, **13**, 284; 1939, **20**, 235.

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Liberation of Histamine and Heparin by Peptone from the Isolated Dog's Liver.*

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In previous reports[1-3] one of us has shown that several agents (antigen, *e.g.* horse serum in the sensitized animal, or peptone or Ascaris extracts) which *in vivo* produce a shock-like syndrome in the dog associated with a discharge of histamine and heparin from the liver, are unable to liberate significant amounts of these substances when perfused through the isolated liver, using Tyrode's solution as a vehicle. When citrated blood or heparinized blood is used as the perfusion fluid, detectable quantities of histamine and sometimes of heparin are discharged, but the total amounts that can be found in the perfusates are far smaller than those which are discharged in experiments *in vivo*. These

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1 Rocha e Silva, M., and Grana, A., *Arch. Surg.*, 1946, **52**, 713.

2 Rocha e Silva, M., Porto, A., and Andrade, S. O., *Arch. Surg.*, 1946, **53**, 199.

3 Rocha e Silva, M., and Teixeira, R. M., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 376.

results lead to the conclusion that whole blood is necessary to produce the discharge of histamine and heparin from the hepatic cells in the forms of shock mentioned. In the experiments to be reported here, it has been possible on occasion to obtain the liberation from the liver of amounts of histamine and heparin greater even than *in vivo* and hence it has been possible to suggest the nature of the factors necessary for the liberation of these substances from the liver.

Methods. Ten dogs were used in the present experiments, all of them anaesthetized with sodium pentobarbital ("Ibital," Ingram and Bell). The liver was prepared for perfusion as described previously.[2] To avoid the use of anticoagulants in the blood, glassware treated with the silicone (Dri-Film, No. 9987, General Electric Co.) was used. In vessels so coated, blood will remain unclotted for 2 or 3 hours and the platelets are preserved for at least 30 minutes.[4] It was assumed that, by thus completely excluding anticoagulants, conditions would be obtained more closely resembling those *in vivo*. All glassware entering into contact with the blood was carefully coated with the silicone, as described elsewhere,[4] and contact with rubber tubing was reduced to a minimum. The blood was collected from the carotid through cannulae treated with silicone. Care was exercised to minimize trauma to the artery. Only the first 400 ml of blood were used for the perfusion. As soon as the animal had been exsanguinated, a cannula was inserted into the portal vein and the liver was perfused with warm (38°C) Tyrode's solution. The entire left lobe of the liver was tied off and was excised. The remaining portion of the liver was isolated and was placed in a warm chamber. The perfusion with warm Tyrode's solution was continued for 5 to 10 minutes, until the perfusate was free of blood. Three g of peptone in 10 ml of saline were then added to 250 ml of blood and the mixture was pumped rapidly through the organ. The perfusate was collected in small portions of 20 ml each and was assayed for histamine and heparin. The organ was perfused further with 200 ml of blood and finally was washed out by prolonged perfusion with Tyrode's solution.

For the estimation of the histamine content of the perfusates, a piece of guinea pig ileum was employed. The heparin content was determined by the protamine-titration method of Jaques and Waters.[5] In the experiments in which Tyrode's solution was used as the vehicle, the presence of heparin was investigated by concentrating the perfusates and then testing for metachromasia with the dye, Azure A.[6] By this method, heparin can be estimated quantitatively in concentrations as low as 0.02 mg/ml, while it can be detected qualitatively with concentrations as low as 0.0005 mg/ml. We are indebted to Mr. E. Napke for conducting this test. The figures for total histamine and heparin given in Table I cover the whole discharge, since the perfusates were collected until only traces of histamine were detected by the biological test. In some of the experiments, after the perfusion with blood, a piece of the liver was taken in a 3.8% solution of sodium citrate and smears were prepared according to a technic previously described,[2] to observe the degree of disintegration of the clumps of platelets found within the liver parenchyma after perfusion with peptone. The same technic applied to the normal liver shows only intact, isolated platelets. Red cells, leucocytes and platelets were counted in the perfusing blood in 6 of the experiments and the degree of agglutination is indicated as the percentage of aggregated platelets to the total count.

The peptone was a sample of proteose-peptone (Difco) in 30% solution. n previous experiments we used a peptone which contained histamine as an impurity and it was necessary to rely upon increases in the histamine content of the perfusing blood. In these experiments the histamine was removed from the peptone by treatment with permutit,

[4] Jaques, L. B., Fidlar, E., Feldsted, E. T., and Macdonald, A. G., *Can. Med. Assn. J.*, 1946, **55**, 26.

[5] Jaques, L. B., and Waters, E. T., *J. Physiol.*, 1941, **99**, 454.

[6] Jaques, L. B., Mitford, M. B., and Macdonald, A. G., to be published.

TABLE I.
Total Histamine and Heparin Released from Isolated Dog's Liver Following Addition of Peptone to the Perfusion Fluid.

Dog, No.	Wt, kg	Liver, g	Perfusion fluid	Histamine, μg	Heparin,† mg
11	16.0	170	Tyrode's solution	26.8	None
17	15.5	402	" "	38.0	Traces
"	"	"	Heparinized blood—45 min. contact (8 units/ml)	93.7	1.75
19	17.6	365	Defibrinated blood	1,162.0	Not est'd
18	12.5	365	Heparinized blood—22 min. contact (8 units/ml)	538.0	Doubtful
16	10.1	295	" " 15 " " 5 "	2,511.0	10.5
13	22.5	280	Silicone blood‡	89.0	3.65
15	8.3	140	" "	417.7	5.45
14	29.6	359	" "	607.7	8.15
12	16.2	—	" "	3,313.0	16.5
20*	23.0	470	" "	8,550.0	25.4

* The figures for histamine and heparin in Dog 20 would not cover the whole discharge since after stoppage of the drainage with Tyrode the histamine was still high (2.8 μg per ml) and the heparin was estimated only in the tubes containing enough blood to be used in the protamine assay (350 ml of perfusate).

† The ratio of heparin : protamine under the conditions of this experiment was found to be 1:2.

‡ Blood collected and preserved in silicone-coated vessels.

as described by Gotzl and Dragstedt,[7] thus permitting a much more accurate estimation of the histamine eventually liberated in the perfusion experiments. When the solution was diluted 25 times, as in the perfusion experiments, and was tested upon the guinea pig gut, only traces of histamine were found. It should be noted that a larger quantity of peptone was used in the present experiments than in those referred to above. Although we formerly used 1 or 2 g of peptone for 500 or 600 ml of blood (diluted 20% with Tyrode's solution), in the experiments presented in this paper we have used 3 g of peptone added to 250 ml of undiluted blood. This difference was partly due to the fact that the sample of peptone used appeared to be less active than previous samples. The trypsin used was a crystalline sample containing 50% of $MgSO_4$ and produced by the Lehn and Fink Corporation. The protamine was a sample of salmine hydrochloride supplied by the Connaught Laboratories, University of Toronto.

Results. The total quantity of histamine and heparin liberated from the isolated liver of the dog, following the addition of peptone to the perfusion fluid, is presented in Table I. In agreement with results reported previously, peptone is capable of liberating only small amounts of histamine and traces of heparin when Tyrode's solution is used as the perfusing fluid. It is possible that even this small amount of histamine is liberated because of the presence of traces of blood retained within the perfused organ, since after the injection of peptone some blood appeared in the perfusate. In one experiment, the effect of crystalline trypsin on the perfused liver was tested. After washing the liver of Dog 11 free from peptone and traces of histamine with Tyrode's solution, 50 mg of crystalline trypsin was added to the Tyrode's solution perfusing the liver; 132 μg of histamine and 1.5 mg of heparin were then released by the liver.

In contrast to the results obtained on adding peptone to Tyrode's solution, when whole blood (especially blood kept unclotted by the use of silicone instead of anticoagulants) was used as a vehicle, enormous amounts of heparin and histamine were liberated. As shown in Fig. 1, most of the histamine and heparin was released at the beginning of the perfusion with blood and peptone. In some of the experiments the amount of histamine in the perfusates went up as high as 40 or 50 μg per ml, in contrast to the 1.5 μg per ml that has so far been the maximal content of histamine estimated in the blood of intact animals submitted to peptone shock.[3] The variability shown in Table I in

[7] Gotzl, F. R., and Dragstedt, C. A., *J. Pharm. and Exp. Ther.*, 1941, **74**, 33.

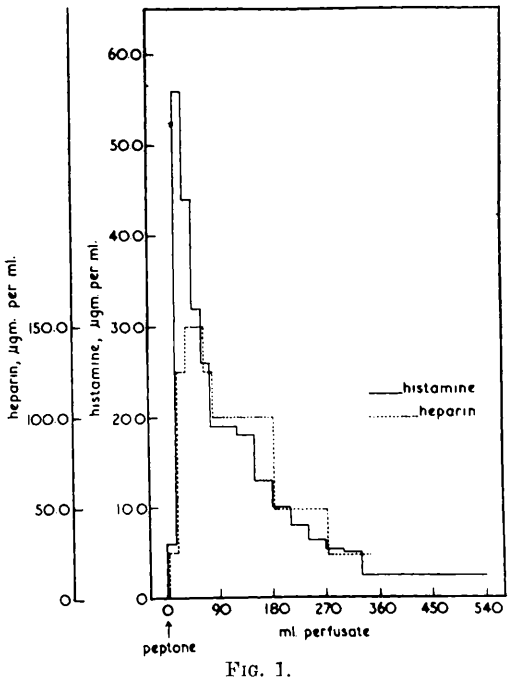

FIG. 1.

Dog No. 20. Liver perfusion with silicone blood + peptone. Discharge of histamine and heparin after passage of the blood through the liver. Note the sharp peak (56 µg histamine per ml of perfusate) occurring before the first 50 ml of blood passed through.

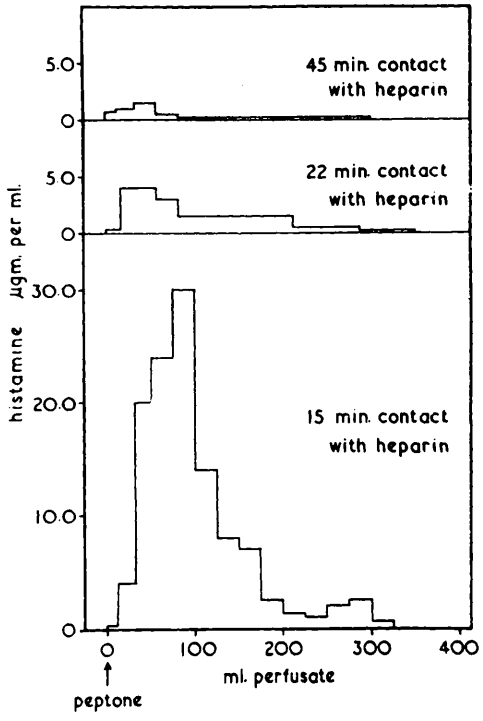

FIG. 2.

Three experiments with heparinized blood peptone. The maximal discharge of histamine was obtained when the blood was used 15 minutes after addition of heparin and the minimal when the blood was maintained for 45 minutes in contact with the heparin before passing through the liver.

the amounts of histamine and heparin released when "silicone blood" was used in the perfusion suggests that other factors (individual differences in the response of the liver from different dogs, the time of contact of the blood with peptone before coming in contact with the cells of the liver, etc.) are also operative in these experiments. These factors will be the subject of a separate investigation.

In the experiments presented in Table I even heparinized blood with peptone was able to liberate amounts of histamine and heparin far more conspicuous than those detected in previous experiments.[3] This might be due to one or more of the following reasons: (1) the peptone was freed from histamine by treatment with permutit, thus permitting a much more accurate assay of histamine; (2) much higher doses of peptone were used than before; (3) the blood used was undiluted, while in the previous experiments the perfusion solution was blood which contained approximately 20 to 25% Tyrode's solution. Whatever may be the reason for this discrepancy, heparin appeared to afford some protection against the action of peptone when enough time was allowed to elapse between the addition of heparin and the start of the perfusion experiment. As shown in Fig. 2, the maximal discharge of histamine was obtained when the blood remained in contact with heparin only 15 minutes. The minimal discharge resulted when the blood was kept for 45 minutes in contact with heparin. Due to the small number of experiments with heparin, further work is required to establish that heparin inhibits the discharge of histamine from dog's liver. Although defibrinated blood also permits the release of appreciable amounts of histamine and probably also of heparin (not estimated) by peptone, there is no question that the use of silicone-coated vessels for the collec-

TABLE II.
Leucocyte and Platelet Counts per mm³ of Perfusing Blood.*

Dog No.	Normal in silicone		After heparin		After peptone		Perfusate	
	Leucocytes × 100	Platelets × 1000	Leucocytes × 100	Platelets × 1000	Leucocytes × 100	Platelets × 1000	Leucocytes × 100	Platelets × 1000
15	62	411 (6%)†	Heparin not used		135	398	49	50 (87%)
16	77	415	79	368 (16%)	48	151 (67%)	41	70 (79%)
17	45	341	—	—	59	187 (91%)	5	12 (15%)
18	102	291	82	140 (42%)	94	227 (44%)	10	32 (25%)
19	25	363	79	46 (11%)	35	24 (62%)	14	14 (54%)
20	77	171	Heparin not used		74	129 (64%)	32	8.7 (48%)

* The red cells were used as an indicator of accuracy of sampling and of dilution, and in each experiment the leucocyte and platelet counts have been adjusted to the count of red cells in the normal blood.
† The figures in brackets indicate the degree of agglutination.

tion of blood, which thereby may approach its natural condition, permits the discharge of histamine and heparin in far more significant amounts than those obtained with defibrinated or heparinized blood.

Since the liberation of histamine and heparin from the liver by peptone appears to depend on the presence of whole blood, the question arises as to what constituents of the blood are involved in this action. It was previously found[2] with citrated blood as the perfusion fluid and antigen (horse serum) or Ascaris extracts as exciting agents that a sharp decrease in platelet and leucocyte counts occurred on passing the blood through the liver. As shown in Table II (Dogs 15, 17 and 20), this likewise occurred with the blood in silicone. Since the addition of peptone to the blood caused a marked agglutination of the platelets, the decrease in count may be attributed to trapping of these clumps in the hepatic capillaries. Confirming previous reports by Copley,[8,9] heparin itself, when added to blood, caused considerable clumping of platelets and sometimes a sharp decrease in the count. The presence of heparin had little apparent effect on the action of peptone on the platelets and on the further decrease in the count when the blood passed through the liver.

Counts of leucocytes and of platelets cannot afford any direct evidence concerning the disintegration of these cells, since they might be segregated in the network of capillaries and small vessels of the liver after forming clumps on the addition of peptone, as shown in Table II. Information regarding the destruction of these clumps was afforded by the observation of smears taken from pieces of the organ after the perfusion. In the experiments in which an excess of heparin had been used, the platelet clumps could be seen in great numbers throughout the slides, while in those experiments in which there was a considerable discharge of histamine and heparin the clumps were either absent or heavily damaged. The remains of platelets were seen but only very seldom were there any well stained clumps. This suggests that a disintegration of the platelets is associated with the liberation of histamine and heparin, and that while heparin will clump platelets, it will prevent their later disintegration.

The present report is intended to describe conditions under which it is possible to obtain regularly the liberation of histamine and heparin from the dog's liver with peptone. Using this technic we hope to elucidate the mechanism for this liberation. Rocha e Silva and Teixeira have postulated the activation of plasma trypsin as an intermediary in the liberation of histamine and heparin. The finding that the mechanism with peptone depends on the presence of blood, whereas crystalline trypsin by itself was observed to cause some release of histamine and heparin, is suggestive in this regard.

Conclusion. Experiments on the perfusion of dog's liver show that the presence of blood

[8] Copley, A. L., *Am. J. Physiol.*, 1942, **133**, 248.

[9] Copley, A. L., and Robb, T. P., *Am. J. Clin. Path.*, 1942, **12**, 416.

in the perfusing fluid is important for the production of an appreciable discharge of histamine and heparin by peptone. The best results were obtained when anticoagulants were excluded and the blood was preserved from clotting in silicone-treated receptacles. When Tyrode's solution was used as a vehicle for the perfusion, only small amounts of histamine and traces of heparin appeared in the perfusates. The experiments presented in this paper do not afford any direct proof but are suggestive of the participation of platelets and possibly also of leucocytes in the mechanism of the discharge of histamine and heparin from the liver.

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Effect of Reticulo-Endothelial Blockade on Immunity to the Shwartzman Phenomenon.*

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Experiments in this laboratory have shown that repeated intravenous injections of typhoid vaccine cause rabbits to develop a tolerance to the pyrogenic effect of the vaccine, and, furthermore, that this tolerance can be abolished by reticulo-endothelial (R-E) blockade.[1] Additional studies have shown that the development of tolerance to typhoid vaccine carries with it a similar tolerance to the pyrogenic effects of certain other Gram-negative bacilli, not serologically related to the typhoid bacillus.

In view of the fact that the pyrogenic activity of several species of Gram-negative bacilli has been shown to be the property of a carbohydrate component[2,3] and that, at least in the case of *B. prodigiosus*, the same carbohydrate has been shown to be capable of inducing the Shwartzman phenomenon,[4] it was thought that a study of immunity to that form of injury might throw some light on the general problem of immunity to pyrogens. As a tool for study of this problem the Shwartzman phenomenon is advantageous because it provides an observable tissue injury in an area containing few R-E elements.

Materials and Methods. The source of bacterial toxin was the "Mitchell" strain of *E. typhosa*.† An "agar washings" filtrate was prepared by Shwartzman's method.[5a] The potency of the filtrate was determined by titrating the minimum intravenous reacting dose against a constant skin preparatory dose.[5b] Two different lots were prepared. Titration of the first showed 1 reactive unit in 1 ml of a 1:300 dilution, per kg of body weight; while in the second, 1 reactive unit was contained in 1 ml of a 1:400 dilution, per kg of body weight. The skin preparatory dose used in titrations and in all experiments was 0.25 ml of undiluted filtrate.

The rabbits were males, weighing 2 to 3

* Aided by a grant from the United States Public Health Service.

1 Beeson, P. B., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 248.

2 Robinson, C. S., and Flusser, B. A., *J. Biol. Chem.*, 1944, **153**, 529.

3 Hartwell, J. L., Shear, M. J., and Adams, J. R., Jr., *J. Nat. Cancer Inst.*, 1943, **4**, 107.

4 Shwartzman, G., *Cancer Res.*, 1944, **4**, 191.

† Obtained from the Laboratories of the Georgia Department of Public Health.

5 Shwartzman, G., *Phenomenon of Local Tissue Reactivity and Its Immunological, Pathological, and Clinical Significance*, New York, Hoeber, 1937, (a) p. 35, (b) p. 26.