time we added amboceptor and erythrocytes. After 10 minutes on the waterbath we read the reaction and controlled this after 20 minutes at room temperature. The complement-fixation reaction was also done at ice-box temperature. Our impression was that the reaction proceeded more rapidly and in higher titre. Otherwise our results were essentially the same. We compared these results with those obtained with an antigen prepared exactly after Craig's method. The 2 antigens gave in our hands identical results. A total of 121 sera were tested with the 2 antigens. In only 5 of these patients could we demonstrate *E. histolytica* in the feces. The serological reaction was positive in only one of the healthy amebic carriers as well as in one patient who showed no ameba in the feces. This latter patient had an uncertain liver disturbance, but our numerous fecal examinations remained negative for ameba. Our sole purpose for presenting these data is to induce investigators having access to greater clinical material, to repeat the serological reaction in amebic dysentery with the saline suspension antigen of *E. histolytica.*

Summary. A saline suspension of *E. histolytica* was employed as antigen in the complement-fixation test which gave identical results with those obtained with the Craig alcoholic extract antigen of *E. histolytica.*

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Protection of Animals Against *Cl. welchii* (Type A) Toxin by Injection of Certain Purified Lipids.*†

PAUL C. ZAMECNIK, JORDI FOLCH, AND LYDIA BREWSTER.
(Introduced by Joseph C. Aub.)

From the Medical Laboratories, Collis P. Huntington Memorial Hospital of Harvard University, and at the Massachusetts General Hospital, Boston, Mass.

This communication reports evidence that purified total lipid extracts of certain animal organs protect mice and dogs against the lethal action of the toxin of *Cl. welchii* (type A).‡ In the case of hog liver, this protective action has been traced to the acetone insoluble, alcohol soluble (lecithin) fraction. Wüth[1] reported that hemolysis of red cells *in vitro* by *Cl. welchii* could be inhibited by lecithin. Since bacterial toxins have been inhibited *in vitro* in a non-specific way by a number of substances, including soaps,[2] olive oil,[3] lanolin,[4] and cholesterol,[5] this observation received little attention, other than mention by van Heyningen[6] that the effect might

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‡ The authors are indebted to Professor Milan A. Logan for the purified *Cl. welchii* (type A) toxin used. This is a glycerol dialyzed toxin, containing chiefly alpha toxin, a small amount of theta toxin (accounting for less than 5 out of a thousand MLD's), and approximately 2000 mucin clot prevention units of hyaluronidase activity per cc.

1 Wüth, O., *Biochem. Z.*, 1923, **142**, 19.

2 Larson, W. P., Halvorson, H. O., Evans, R. D., and Greene, R. G., *Colloid Symposium Monographs*, 1925, Vol. 3, p. 152, New York Chemical Catalogue Co. (Reinhold Publishing Corp.).

3 Myers, G. N., *J. Hygiene*, 1934, **34**, 250.

4 Weinberg, M., and Guillaumie, M., *C. R. Soc. Biol.*, 1935, **120**, 936.

5 Degkwitz, R., *Erg. d. physiol.*, 1931, Bd. 32, S. 821.

6 van Heyningen, W. E., *Biochem. J.*, 1941, **35**, 1246.

be due to substrate competition. MacFarlane and Knight[7] showed that *Cl. welchii* toxin contained an enzyme which hydrolyzed lecithin into phosphorylcholine and a diglyceride. They considered this lecithinase to be probably identical with the alpha or principal toxin of *Cl. welchii* (type A) culture filtrates. Since MacFarlane and Knight did not determine whether the enzymatic action was specifically restricted to lecithin, the possibility must be considered that phosphatide substrates other than lecithin may be hydrolyzed by the alpha toxin. On this particular point we have found that phosphatidyl serine, sphingomyelin, and soy bean phosphatides[8] are not hydrolyzed by the *Cl. welchii* alpha toxin.

Clostridium welchii alpha toxin hemolyzes erythrocytes and destroys tissue cells,[9,10] an effect presumably related to its action on phosphatides of the cell surface. It occurred to the writers that intravenous injection of phosphatides might divide the action of *Cl. welchii* toxin between hydrolysis of the added phosphatides and of cell phosphatides. Provided a sufficiently high concentration of added phosphatides were present, the hydrolysis of the cell phosphatides might be reduced to a minimal figure, by a *substrate partition effect.* The increasing concentration of split products of the reaction might also be expected eventually to decrease the rate of cytolysis.

With this idea in mind, a number of lipid preparations have been tested (a) for inhibitory action of hemolysis by *Cl. welchii* (type A) toxin *in vitro;* (b) for toxicity for mice and dogs on intraperitoneal or intravenous injection; (c) for protective action, on intraperitoneal injection into mice, against *Cl. welchii* toxin injected intraperitoneally by separate injection; and (d) for protective action for mice and dogs when injected intravenously, against *Cl. welchii* toxin administered intravenously from one to 10 minutes after injection of lipids. A number of incidental observations have also been made. The methods used and detailed experimental results are reported below.

Methods. Preparation of purified total lipid extracts: Purified total lipid extracts of erythrocytes have been prepared in slightly different ways: (1) the cells were packed by centrifugation, hemolyzed with an equal volume of distilled water, and the lipid extracted at room temperature by addition of 25 volumes of 1:1 alcohol-ether. After filtration, the extract was concentrated to a small volume in a water suction pump vacuum. The resulting aqueous emulsion was freed of nonlipid impurities by dialysis through Visking cellophane membranes against distilled water at approximately 3° C. After dialysis, the emulsions were lyophilized. (2) The procedure was the same as (1) with the exception that 8 volumes of 1:1 alcohol-ether were used instead of 25 volumes. (3) 100 cc portions of packed erythrocytes were homogenized for 45 seconds in a Waring blendor, 200 cc of alcohol were added, the mixture homogenized for one minute, 200 cc of ether were added, and the homogenization allowed to proceed for another minute. The mixture was filtered through a Buchner funnel and the clear alcohol-ether filtrate handled as described above. The 3 methods yield apparently identical purified total lipid extracts of erythrocytes.[8]

Purified total lipid extracts have been prepared from various other sources by the method described under (3), unless otherwise stated. The best source of protective lipids found so far is liver, which yields approximately 30 g of purified total lipids per kilo of wet tissue, whereas erythrocytes yield only 3-4 g per kilo of packed cells.

The lipid preparations were injected as emulsions (either in 0.9% sodium chloride or distilled water), ranging in concentration from 2 to 10%. The emulsions were made by addition of distilled water to the dried lipid preparations, followed by stirring or rotation until a homogenous emulsion was present. In the case of hog liver lipids so pre-

[7] MacFarlane, M. G., and Knight, B. C. J. G., *Biochem. J.*, 1941, **35**, 884.

[8] Unpublished data.

[9] Bull, C. G., and Pritchett, I. W., *J. Exp. Med.*, 1917, **26**, 119.

[10] Glenny, A. T., Barr, M., Llewellyn-Jones, M., Dalling, T., and Ross, H. E., *J. Path. and Bact.*, 1933, **37**, 53.

pared, the state of the emulsion was improved by adjustment of the pH from 6.2 to 7.5 by careful addition of normal sodium hydroxide. Prior to injection into animals, emulsions were always centrifuged to remove minute amounts of material not well emulsified.

Fractionation of lipid extracts. To a 10% solution of total lipids in chloroform 6 volumes of acetone were added. The mixture was centrifuged and the supernatant collected (acetone soluble fraction). The precipitate was dissolved in half the volume of chloroform used previously, and the solution was treated with a 6-fold excess of alcohol. The mixture was centrifuged and the precipitate (alcohol insoluble or cephalin fraction) collected separately. Aliquots of the 3 fractions thus obtained were combined in such proportions as to reproduce the mother material. The fractions and the recombined mixture were freed of solvents by means of a vacuum water pump, and emulsified as described above.

Technics used in testing for protective effect. As a preliminary screen test, in an effort to sort out ineffective preparations from promising ones, an *in vitro* hemolysis test has been used, as follows: 0.1 cc fresh, washed canine erythrocytes, 1.0 cc 0.1M borate buffer (Sorensen) of pH 7.5, 0.02 cc toxin (containing 36 LD_{50}), 10 cc 0.003 M $CaCl_2$ in 0.9% sodium chloride, 0.2 cc 5% lipid. An Evelyn photoelectric colorimeter, with a 660 $\mu\mu$ filter, was used to measure changes in optical density[12] occurring during the hemolytic reaction. The reaction was carried out at room temperature (24° C) over a one hour period, in a series of colorimeter tubes with included appropriate controls. Colorimeter readings were taken at 10-minute intervals, the tubes being well mixed by inversion just prior to each reading.

The colorimetric test was checked against direct observation of the rate of hemolysis using a microscope counting chamber, and good agreement was obtained. The latter has been found to be a sensitive but tedious method of following the hemolytic reaction.

Animal testing. Strain A mice or market mice weighing 20 to 30 g were injected intravenously by tail vein, or intraperitoneally, with the more promising preparations. The LD_{100} of toxin was determined to be 4.2 LD_{50} when given intravenously as follows: in 3 divided doses of 0.14 cc each of a 10 LD_{50} concentration, injections being given at hourly intervals. This same dose of toxin was also given in later experiments (about half of the total number) as 0.21 cc of a 20 LD_{50} concentration. For the intraperitoneal route, the LD_{100} was 5.0 LD_{50} when given in 3 divided doses. The lipids to be tested were also given either in 3 divided doses intravenously or intraperitoneally, or in a single dose by either route. The precaution of dividing the doses of toxin and of lipids was first adopted in order to lessen the vascular concentrations at a single moment. The survival rate of mice given toxin and lipids was slightly higher where this precaution was taken, but not sufficiently so to justify the extra work involved. In Table III results from both methods of injection have been grouped together. In all cases, the lipid was given one to 10 minutes prior to the toxin, by means of a separate needle and syringe. The dosages of lipids ranged from 0.3 to 1 g per kilo, administered as a 2 to 10% emulsion, and the volume of fluid injected ranged from 0.1 to 0.5 cc per injection.

Results. Inhibitory action of lipid prepara-

TABLE I.

Inhibitory Action of Certain Substances on *in vitro* Hemolysis by *Cl. welchii* Toxin.

	%
Total lipid extract from human erythrocytes	0.1
Egg lecithin*	0.1
Calf brain phosphatidyl serine	0.1
Soy bean phosphatides†	0.1
Oleyl-stearyl-diglyceride‡	0.1
Phosphorylcholine§	0.2
Sodium glycocholate	0.8

* Eastman Kodak Company.

† Obtained through the courtesy of the American Lecithin Company.

‡ Obtained through the courtesy of Lever Brothers.

§ Obtained through the courtesy of Dr. Gerhardt Schmidt.

[11] Folch, J., and Van Slyke, D. D., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 514.

[12] Shohl, A. T., *J. Lab. and Clin. Med.*, 1940, **25**, 1325.

TABLE II.
Experiments on Protection of Mice Against *Cl. welchii* Toxin by Intraperitoneal Injection of Lipid Preparations Administered by Separate Intraperitoneal Injection.

Type of material	Survival rate: Lipids and toxin	Survival rate: Toxin alone
Human erythrocyte total lipids	13 out of 13	1 out of 11
Egg yolk total lipids	7 " " 8	2 " " 6
" " lecithin*	16 " " 16	1 " " 18
Soy bean phosphatides	0 " " 6	0 " " 4
Phosphatidyl serine[13]	0 " " 4	0 " " 2
Human erythrocyte total lipids I-V, and toxin given I-P	0 " " 4	0 " " 2

* Eastman Kodak Company.

tions and other substances on in vitro *hemolysis by* Cl. welchii *(type A) toxin:*

A number of lipid preparations and other compounds were tested for inhibitory action on hemolysis. They all showed inhibitory powers. The substances tested and the respective concentrations at which they prevented hemolysis completely are listed in Table I.

Experiments on mice: Tolerance for intraperitoneal or intravenous injection of lipid preparations:

Preparations listed in Table II were well tolerated on intraperitoneal injection. Preparations listed in Tables III and IV were well tolerated on intravenous injection with the exception of pure total hog brain lipids and of sphingomyelin; the injection of either was followed by death of the mice within a few minutes. Relatively large doses (150 mg) of hog liver, hog erythrocyte, and human erythrocyte lipids were well tolerated by mice intravenously. This quantity of lipid is 15 times that necessary to provide a protective effect.

Effect of mixing toxin and lecithin in advance and injecting the mixture: When a 10% emulsion of lecithin was mixed with toxin in a test tube for a few minutes at room temperature, and the mixture, containing 4.2 LD_{50} of toxin and 75 mg of lecithin, was given by 3 closely spaced intravenous injections, 5 out of 5 mice survived, in contrast to none out of 3 controls. An effect similar to this was described by Myers[3] as a result of mixing olive oil and gum acacia with *Cl. welchii* toxin prior to administration of the mixture subcutaneously into guinea pigs.

Protection of mice, against Cl. welchii *(type A) toxin injected intraperitoneally, by separate injection of certain lipid preparations:*

Results are summarized in Table II. The minimal protective dose of lipids given by this route was approximately 7 mg.

Protection of mice, against Cl. welchii *(type A) toxin injected intravenously, by separate intravenous injection of certain lipid preparations:*

Results are reported in Table III. The difference in protective power between rat brain lipids and hog brain lipids is most likely due to the toxicity of the latter as reported above. Human erythrocyte total lipids given intravenously have not protected against the effect of toxin given intraperitoneally (Table II). When the toxin was given first intravenously and the lipids were given intravenously 15 minutes thereafter, the survival rate of the mice was cut down greatly. The minimal protective dose of total lipids (erythrocyte or liver) administered intravenously was approximately 10 mg, an order of magnitude 5 times that of the total lipids present in the red cells of a 20 g mouse.

Death of mice as a result of the toxin was associated with intravascular hemolysis, congestion and hemorrhage of the lungs, congestion of the viscera, and the presence of hemolyzed fluid in the pleural and peritoneal cavities. In agreement with the findings of Cooke, Frazer, *et al.*[16,17] fat stains showed a

[13] Folch, J., *J. Biol. Chem.*, 1941, **139**, 973.

[16] Cooke, W. T., Frazer, A. C., Peeney, A. L. P., Govan, A. D. T., Barling, S. G., Thomas, G., Leather, J. B., Elkes, J. J., and Scott Mason, R. P., *Lancet*, 1945, **1**, 487.

[17] Frazer, A. C., Elkes, J. J., Sammons, H. G., Govan, A. D. T., and Cooke, W. T., *Lancet*, 1945, **1**, 457.

TABLE III.
Experiments on Protection of Mice, Against *Cl. welchii* Toxin Injection, by Lipid Preparations Administered by Separate Intravenous Injection.

Preparation used	Survival rate: Lipid and toxin	Survival rate: Toxin alone
A. Exhibiting protective effect:		
Human erythrocyte total lipids	44 out of 53	0 out of 28
Hog erythrocyte total lipids	9 " " 13	0 " " 5
Hog liver total lipids	29 " " 34	1 " " 25
Rat liver total lipids	3 " " 3	0 " " 4
Human plasma total lipids	10 " " 14	0 " " 8
B. Failing to protect, or exhibiting questionable protection:		
Rat brain total lipids	4 " " 12	0 " " 6
Hog brain total lipids without phosphatidyl serine*	0 " " 6	0 " " 5
Hog brain phosphatides:		
Lecithin fraction	1 " " 3	0 " " 4
Cephalin fraction	4 " " 14	0 " " 6
Sphingomyelin fraction	0 " " 2	0 " " 1
Egg yolk lecithin†	0 " " 8	1 " " 13
" " total lipids	0 " " 6	0 " " 5
" " phosphatides:		
Lecithin fraction	0 " " 5	0 " " 2
Cephalin fraction	0 " " 2	
Soy bean phosphatides‡	0 " " 4	0 " " 2
Phosphatidyl serine	0 " " 6	0 " " 2
Cocoanut oil in soy bean phosphatide emulsion	0 " " 4	0 " " 2
Diglycerides in soy bean phosphatide emulsion§	1 " " 7	0 " " 4
Phosphorylcholine¶	0 " " 6	0 " " 10
Cholesterol-cephalin emulsion¶	0 " " 3	0 " " 2
Cholesterol-monoglyceride emulsion	0 " " 3	0 " " 2

* Total lipid extract prepared by the use of $Fe(OH)_3$. This method yields an extract that contains all brain lipids free of impurities and of phosphatidyl serine.[14]

† Eastman Kodak Company.

‡ Purified oil-free phosphatides supplied through the courtesy of the American Lecithin Company.

§ Diglycerides and monoglycerides supplied through the courtesy of the Lever Brothers Company. The emulsions were made by means of a Junior 125 Viscolizer through the courtesy of Dr. J. McKibbon.

¶ Supplied through the kindness of Dr. Gerhardt Schmidt. Administered 0.25 cc of a 2% solution.

¶¶ Prepared according to the technic of Hanger.[15]

minor degree of lipid embolism of the lungs and lipid staining material in the spleen. Sections taken from mice, protected for 24 hours against the toxin by injection of liver lipids, were fixed in 10% formalin, and frozen sections therefrom were stained with Sudan IV. No evidence of lipid embolism was found in the lungs, but the livers showed a slight increase in the amount of fat staining material.

Effect of fractionation on the protective action of total lipid extracts of hog liver: During the fractionation, it has been found that the lipid preparation must not be exposed to air or light at room temperature for more than the shortest possible length of time. In two preliminary experiments done on a small scale, in which such precautions were not strictly adhered to, the result was the disappearance of the protective effect of the lipid preparations involved. The purified total lipids appear to be less sensitive to such changes than the fractions obtained therefrom; the protective effect of aqueous emulsions of purified total lipid preparations remained present for a number of days when they were simply kept at ice-box temperatures.

In a third and fourth fractionation experiment, each performed on 15 g of purified total hog lipids the fractions were tested with re-

[14] Folch, J., *J. Biol. Chem.*, 1942, **146**, 35.

[15] Hanger, F. M., *J. Clin. Invest.*, 1939, **18**, 261.

TABLE IV.
Fractionation of Total Hog Liver Lipids. Protective Effect of Fractions Against *Cl. welchii* Toxin in Mice. Route of Injection Intravenous.

Type of material injected	Survival rate
Toxin alone	0 out of 22
Total liver lipids and toxin	18 " " 21
Fractions and toxin:	
Acetone soluble (fat and cholesterol)	2 " " 13
Alcohol soluble (lecithin)	10 " " 16
Alcohol insoluble (cephalin)	0 " " 13
Recombined fractions	11 " " 13

sults summarized in Table IV. The protective action appears to reside with the acetone-insoluble, alcohol-soluble fraction, the fraction presumably richest in lecithin. The slight protective action exhibited by the acetone-soluble fraction is possibly due to contaminating lecithin.

Effect of lipids on development of immunity to Cl. welchii *toxin:* Of a group of 19 mice surviving a lethal dosage of *Cl. welchii* toxin as a result of lipid injection, 10 mice survived a repeat lethal dose (4.2 LD_{50}) 10 days later. Two weeks after survival, 2 dogs listed in Table V were tested and found to have a good serum antitoxin titre against *Cl. welchii.*

Experiments on dogs: Two dogs were given 220 LD_{50} per kilo of *Cl. welchii* toxin by slow intravenous drip into the right femoral vein over a 3 hour period. Over a 4 hour period, beginning one hour prior to the administration of the toxin, a 10% aqueous emulsion of human erythrocyte total lipids was administered by slow intravenous drip into the left femoral vein. (1.6 g per kilo for one dog and 1.1 g per kilo for the other dog). These dogs were under sodium pentobarbital anesthesia (30 mg per kilo), with carotid and tracheal cannulation for measurement of blood pressure and cardiac output. Two control dogs given this dosage of toxin alone under the same experimental conditions died with extensive intravascular hemolysis within 8 hours after the beginning of toxin administration. Autopsies revealed the presence of hemolyzed fluid in the pleural and peritoneal cavities, extensive pulmonary edema and hemorrhage, and congestion of the viscera. At the end of the same length of time, the 2 dogs given erythrocyte lipids in addition to toxin were in good condition. Throughout the course of the experiment, their plasmas showed minimal hemolysis (under 0.1%) with unchanged hematocrits, and their cardiac outputs remained within normal limits. These 2 dogs were sacrificed after 8-10 hours, and the autopsies were negative except for mild pulmonary congestion in one animal and moderate congestion of the liver in the other. One of the dogs showed a temporary marked fall in blood pressure when the rate of administration of lipids exceeded 10 cc per minute.

The plasma concentration of total lipids in the dog given 1.6 g per kilo rose from a control level of 0.6 g % to 2.0 g % at the end of the lipid injection, then declined during the subsequent 5 hours to 1.3 g %. In the dog given 1.1 g total lipids per kilo, the plasma lipid level rose from a control level of 0.6 g % to 1.5 g % at the end of the injection, then declined over the subsequent 4 hours to 1.1 g %.

One unanesthetized dog was first given 1.3 g per kilo of a 3.8% (in 0.9% sodium chlor-

TABLE V.
Dog Protection Experiments.
(Route of injections intravenous. All dogs received 220 LD_{50} *Cl. welchii* toxin per kilo body weight.)

Total lipid dose (g per kilo)	Result
1.6*	Survived
1.3	"
1.1	"
1.1*	"
0.4	"
0.4	Died
0	"
0	"
0	"
0	"

* Human erythrocyte total lipids. The others were hog liver total lipids.

ide) emulsion of hog liver lipids, and then was given 220 LD_{50} per kilo of toxin, and survived. Two unanesthetized dogs were given 0.4 g per kilo of a 2% emulsion of hog liver total lipids. Half of the dose was administered by syringe prior to intravenous injection of toxin (220 LD_{50} per kilo) and the other half after the toxin. One dog survived, and the other died. The results of the dog experiments are summarized in Table V. In these unanesthetized dogs, the rather large volumes of lipid (100-200 cc) injected were administered rapidly in 2 or 3 50-75 cc doses, and were well tolerated. The precaution of adjusting the pH of the emulsion above 7.0 prior to injection was observed in these experiments.

Discussion. A number of substances (Table I) have been found by the authors to inhibit the hemolysis of erythrocytes *in vitro* by *Cl. welchii* toxin. Only lipid preparations hydrolyzable by *Cl. welchii* toxin, however, have been able to protect mice and dogs against *Cl. welchii* toxin given by separate injection. Cholesterol has for a long time been known to exert a protective action against red cell hemolysis *in vitro*;[18,5] but as indicated in Tables III and IV, it has little protective effect against *Cl. welchii in vivo*. It is possible that the presence of cholesterol enhances the protective effect of lecithin as it exists in the total lipid preparations.

The fact that the split products of the hydrolysis of lecithin by the *Cl. welchii* alpha toxin are relatively non-toxic is an important consideration. In contrast to this situation is the production of the highly toxic, hemolytic lysolecithin by cobra venom lecithinase in its action on lecithin.[19] Injection of lipids containing lecithin did not protect mice against the effects of the latter toxin, but rather appeared to aggravate the effect of the toxin.§

There is no explanation of the inability of fresh egg yolk total lipids to protect mice on intravenous injection. As stated previously, purified preparations of lecithin quickly lose their protective effect on exposure to light and air, whereas, purified total lipids retain their protective effect for a number of days if they are simply kept as aqueous emulsions at 3° C.

It should be mentioned that *Cl. welchii* (type A) toxin as it occurs in war wounds and as prepared by Dr. Milan A. Logan, contains small amounts of theta toxin and a considerable amount of hyaluronidase, against which the lipids presumably have no protective effect.

The usefulness of this approach to the problem of *Cl. welchii* infection is limited by the lack of ability of the lipids to reverse damage to cells which has already occurred, as a result of toxin, prior to their administration. It is worth noting, however, that in experiments herein reported the concentrations of toxin introduced into the blood stream at a single instant far exceed those which might be expected in clinical *Cl. welchii* toxemia.

When knowledge of the substrate specificity of other bacterial toxins becomes available, it should be possible to apply a similar chemotherapeutic approach to other cases. This type of approach can theoretically be best used where the enzyme can be "saturated" with substrate (*i.e.* where $V=K_2E$, according to Van Slyke[20]).

Summary. Purified total lipids obtained from erythrocytes, plasma, and liver have been found to have a protective effect against *Cl. welchii* (type A) toxin in mice and dogs. The protection appears to be due to a substrate partition effect in which the lecithinase of the *Cl. welchii* toxin hydrolyzes the relatively large added source of lecithin and in part spares the lecithin of the animal cells from destruction.

[18] Theorell, H., *Biochem. Z.*, 1930, **223**, 1.

[19] Levene, P. A., Rolf, I. P., and Simms, H. S., *J. Biol. Chem.*, 1924, **58**, 859.

[20] Van Slyke, D. D., in Nord, F. F., and Werkman, C. H., *Advances in Enzymology and Related Subjects*, New York, 1942, **2**, 33.

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