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Inflammation. VII. Selective Inhibitory Action of Tuberculo-Carbohydrate and Phosphatide on Cellular Cathepsins from Tuberculous Tissues.

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In an enzymologic investigation designed to throw light on the mechanism of the specific cytotoxic effect of tuberculin *in vitro*, we observed that the purified protein derivative of tuberculin (PPD) inhibits to an equal degree the proteinases (Cathepsin II) from organs of normal animals and those infected with either virulent or nonvirulent tubercle bacilli.[1] Tuberculin (OT), on the other hand, had but slight effect, in tissue culture, on cells from normal animals, but severely injured cells derived from animals infected with either strain of tubercle bacilli.[2] These experiments therefore did not throw any new light upon the mechanism of the tuberculin reaction. In the present communication we report upon experiments with carbohydrate and phosphatide fractions of the tubercle bacillus, *M. tuberculosis*, H-37, in which the results were positive and significant.

Experimental. The cathepsin solutions were prepared as previously described[3] by the methods of Bergmann and Fruton[4] from livers of normal rabbits (Series A), those infected with a strain (R_1) of tubercle bacilli of low virulence (Series B), those infected with a virulent, Ravenel culture (Series C), and those from animals first injected with an R_1 culture and later reinfected with the Ravenel strain (Series D). For purposes of comparison, normal beef spleen cathepsin and papain, purified by the method of Greenberg,[5] were also tested.

The enzyme preparations were dialyzed for 24 hours at 7°C against 1% NaCl and used immediately, or, in some cases, were preserved in a frozen state at −70° for several days. These dialyzed extracts show increased NPN and lower protein nitrogen, but their hydrolytic activity (unit of enzyme per mg of protein N) remains constant up to about one

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1 Weiss, C., and Halliday, N., *Arch. Path.*, 1944, **37**, 272.

2 Moen, J. K., and Swift, H. F., *J. Exp. Med.*, 1936, **64**, 339.

3 Weiss, C., and Halliday, N., *J. Immunol.*, 1944, **49**, 251.

4 Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, 1939, **130**, 19.

5 Winnick, T., Davis, A. R., and Greenberg, D. M., *J. Gen. Physiol.*, 1939, **23**, 301.

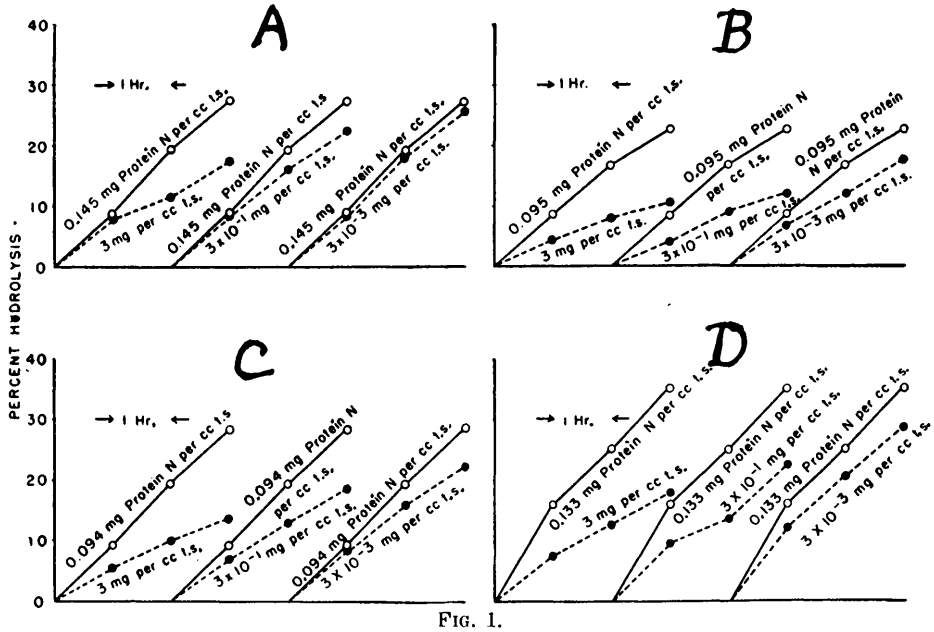

FIG. 1.

Hydrolysis of benzoyl-*l* arginineamide (BAA) in the presence and absence of tuberculo-phosphatide by cathepsin II prepared from livers of normal and tuberculous rabbits. (Series A, B, C, and D are described in the text under "Experimental.")

week. All hydrolyses were carried out simultaneously in the presence and absence of the phosphatide or carbohydrate, as previously described.[1] The substrate employed was benzoyl-*l*-arginineamide (BAA); the enzymes were activated by cysteine and buffered with 0.05 M citrate, pH 4.9. The enzyme concentrations were selected to give about 20 to 30% substrate hydrolysis in a 3-hour period, using the Grassmann and Heyde titration method,[6] where an increase of 1 cc of 0.01 M alcoholic KOH represents 100% splitting of a peptide bond.

The tuberculo-carbohydrate ("B.M. Supt. II") was prepared by Dr. Sidney Raffel of Stanford University[7] according to the method of Heidelberger and Menzel.[8] The tubercle bacilli were grown on Long's synthetic medium, the defatted organisms pooled, and the carbohydrate extracted with acidified water. This polysaccharide precipitated with homologous immune horse serum in a dilution of one to one million.

The tuberculo-phosphatide (H-37; A-3) was obtained from Dr. R. J. Anderson of Yale University. The concentrations which could be employed were governed by its low solubility in water. The levels selected for this substance as well as for the carbohydrate were approximately: 3, 3×10^{-1}, and 3×10^{-3} mg per cc of test solution. The final mixtures of phosphatide with the two higher levels were opaque, but since the material was readily soluble in alcohol, it was possible to obtain good titration end-points.

In general, phospholipids are relatively unstable. Losses of 20 to 25% were observed by Halliday[9] and Boyd[10] when alcohol-ether extracts of tissues were stored up to 3 months at room temperature even in the dark. It was thought that aqueous suspensions could be preserved in the ice-box up to 2 to 3 weeks, but it was found that after only 1 week there was loss of inhibitory power. In subsequent experiments, therefore, solutions were freshly prepared for each experiment or used after only 24 hours of storage.

Three types of experiments were performed with phosphatide: (1) the substrate, cysteine-

[6] Grassmann, W., and Heyde, W., *Z. physiol. Chem.*, 1929, **183**, 32.

[7] Raffel, S., *Stanford Med. Bull.*, 1943, **1**, 209.

[8] Heidelberger, M., and Menzel, A. E. O., *J. Biol. Chem.*, 1937, **118**, 79.

[9] Halliday, N., *J. Nutr.*, 1938, **16**, 285.

[10] Boyd, E. M., *J. Biol. Chem.*, 1937, **121**, 485.

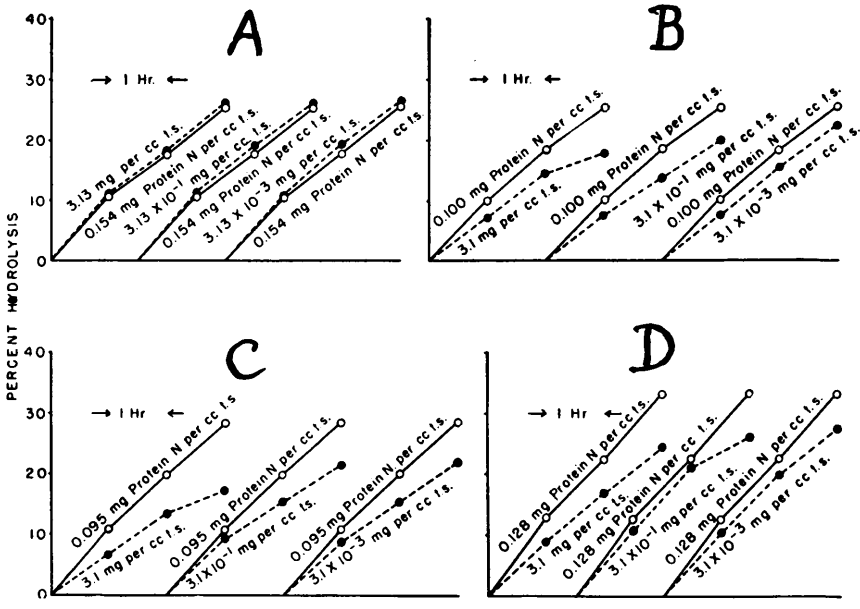

FIG. 2.

Hydrolysis of benzoyl-*l*-arginineamide (BAA) in the presence and absence of tuberculo-carbohydrate by cathepsin II prepared from livers of normal and tuberculous rabbits. (Series A, B, C, and D are described in the text under "Experimental.")

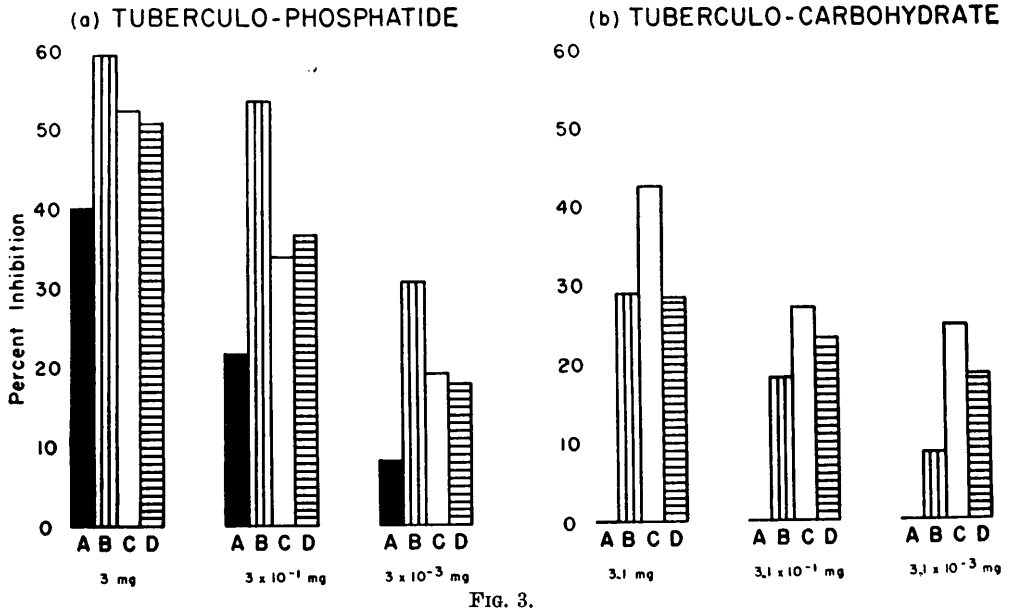

FIG. 3.

Percentages of inhibition of hydrolysis of BAA by (a) tuberculo-phosphatide and (b) tuberculo-carbohydrate. Results are based upon a three-hour interval. Concentrations of inhibitor per cc of test solution are shown under the columns.

buffer solution, and phosphatide were mixed and warmed to 40° just before addition of enzyme and beginning of the 3-hour hydrolysis period; (2) the bath temperature was lowered to 30° and hydrolyses continued for 24 hours, with titrations at zero, 5 and 24 hours; and

TABLE I.

Effect of Tuberculo-phosphatide on the Hydrolysis of Benzoyl-*l*-argininеamide (BAA) by Cathepsin II Prepared from Livers of Normal Rabbits and Those Infected with Virulent and Non-Virulent Tubercle Bacilli.

Conc. of phosphatide mg per cc test sol.	Conc. of enzyme	K × 10⁴		C × 10³		Unit		Unit per mg		
		Without phospha-tide	With phospha-tide	Without phospha-tide	With phospha-tide	Without phospha-tide	With phospha-tide	Without phospha-tide	With phospha-tide	% decrease
				Series A. Normal Rabbits.						
0.0	0.291	15.6		5.35		0.374		2.67		
3.0	0.145	7.76	4.64	5.34	3.19	0.375	0.627	2.67	1.60	40.1
3 × 10-1	0.145		6.09		4.19		0.478		2.09	21.7
3 × 10-3	0.145		7.13		4.91		0.408		2.45	8.24
				Avg: 5.35		Avg: 0.375		Avg: 2.67		
				Series B. Rabbits Infected with a Non-Virulent (R_1) Strain of Tubercle Bacilli.						
0.0	0.189	14.4		7.59		0.263		3.80		
3.0	0.095	6.21	2.71	6.56	2.86	0.305	0.700	3.28	1.43	59.6
3 × 10-1	0.095		3.11		3.29		0.609		1.64	53.7
3 × 10-3	0.095		4.64		4.90		0.408		2.45	30.8
				Avg: 7.08		Avg: 0.284		Avg: 3.54		
				Series C. Rabbits Infected with a Virulent Ravenel Strain of Bovine Tubercle Bacilli.						
0.0	0.188	13.7		7.31		0.274		3.66		
3.0	0.094	8.06	3.56	8.58	3.79	0.233	0.528	4.29	1.89	52.5
3 × 10-1	0.094		4.93		5.25		0.381		2.63	33.9
3 × 10-3	0.094		6.05		6.45		0.310		3.22	19.1
				Avg: 7.95		Avg: 0.254		Avg: 3.98		
				Series D. Rabbits Immunized with an R_1 Strain and Reinfected with a Ravenel Strain.						
0.0	0.265	18.0		6.80		0.294		3.40		
3.0	0.133	10.5	4.79	7.90	3.61	0.253	0.553	3.95	1.81	50.8
3 × 10-1	0.133		6.18		4.67		0.429		2.33	36.7
3 × 10-3	0.133		8.03		6.06		0.330		3.03	17.7
				Avg: 7.35		Avg: 0.274		Avg: 3.68		

TABLE II.
Effect of Tuberculo-phosphatide on the Hydrolysis of Benzoyl-*l*-argininеamide (BAA) by Purified Papain and by Cathepsin II Prepared from Normal Beef Spleen.

Conc. of phosphatide mg per cc test sol.	Conc. of enzyme	K × 10⁴		C × 10³		Unit		Unit per mg		% decrease
		Without phosphatide	With phosphatide	Without phosphatide	With phosphatide	Without phosphatide	With phosphatide	Without phosphatide	With phosphatide	
				Purified Papain.						
0.0	0.0155	22.5		14.6		0.0137		72.8		
3.0	0.0077	11.6	12.4	15.0	16.0	0.0133	0.0125	75.0	80.0	0.0
3 × 10⁻³	0.0077		11.5		14.9		0.0134		74.5	0.0
				Avg: 14.8		Avg: 0.0135		Avg: 73.9		
				Beef Spleen Cathepsin.						
0.0	0.136	13.8		10.2		0.197		5.09		
3.0	0.068	7.01	3.87	10.3	5.69	0.194	0.351	5.16	2.85	44.4
3 × 10⁻¹	0.068		5.78		8.51		0.235		4.25	17.2
3 × 10⁻³	0.068		7.46		11.0		0.182		5.49	0.0
				Avg: 10.3		Avg: 0.196		Avg: 5.13		

(3) the cysteine-buffer solution and phosphatide were warmed to 40°, the enzyme then added, and the material held at this temperature for 2 hours before the substrate was added and the titrations begun. The hydrolysis periods were 3 hours. The latter was the only type of experiment employed with the carbohydrate.

In the first method there was little or no effect of phosphatide on the enzyme. If digestion was allowed to proceed at 30° for 24 hours, the rate of enzyme activity was decreased, but only the highest level of phosphatide exerted any inhibitory action. The third method proved of greatest value. Graded differences were observed with graded levels of phosphatide. The results with the carbohydrate were not so closely correlated with the concentration of carbohydrate, but significant inhibition was obtained with enzymes prepared from tuberculous organs, whereas there was no effect whatever on those from normal liver or from beef spleen.

The method of calculating activity constants and units of enzyme was that of Bergmann, Fruton, and coworkers.[11] In the experiments without inhibitor, first-order kinetics were found. In the presence of higher levels of phosphatide or carbohydrate, however, the K values were not constant at the various time intervals.

Results. Results are shown in Figs. 1 to 3 and Tables I to IV. For the sake of comparison with data without inhibitor, the various constants for the time interval of zero to 180 minutes were employed. It is possible that a two-phase system is present. The inhibition is not due to the presence of end-products of the hydrolysis since a pure substrate was employed and the phosphatide yields the following substances[12] after saponification with dilute alcoholic KOH: alcoholic soap solution of the fatty acids, a small amount of free glycerol and phosphoric acid, and an alcohol-insoluble residue consisting of a mixture containing a complex organic phosphoric acid and a neutral carbohydrate (man-

[11] Irving, G. W., Jr., Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, 1941, **138**, 231.

[12] Anderson, R. J., and Roberts, E. G., *J. Am. Chem. Soc.*, 1930, **52**, 5023.

TABLE III.

Effect of Tuberculo-carbohydrate on the Hydrolysis of Benzoyl-*l*-arginineamide (BAA) by Cathepsin II Prepared from Livers of Normal Rabbits and Those Infected with Virulent and Non-Virulent Tubercle Bacilli.

Conc. of carbohydrate mg per cc test sol.	Conc. of enzyme	K × 10⁴		C × 10³		Unit		Unit per mg		% decrease
		Without carbohydrate	With carbohydrate	Without carbohydrate	With carbohydrate	Without carbohydrate	With carbohydrate	Without carbohydrate	With carbohydrate	
				Series A. Normal Rabbits.						
0.0	0.308	14.4		4.68		0.427		2.34		
3.1	0.154	7.04	7.23	4.57	4.70	0.438	0.426	2.29	2.35	0.0
3.1 × 10-1	0.154		7.20		4.68		0.428		2.34	0.0
3.1 × 10-3	0.154		7.27		4.72		0.424		2.36	0.0
				Avg:	4.63	Avg:	0.433	Avg:	2.32	
				Series B. Rabbits Infected with a Non-Virulent (R_1) Strain of Tubercle Bacilli.						
0.0	0.200	12.2		6.10		0.328		3.05		
3.1	0.100	7.01	4.67	7.00	4.67	0.286	0.429	3.50	2.33	29.0
3.1 × 10-1	0.100		5.36		5.35		0.374		2.68	18.3
3.1 × 10-3	0.100		6.00		5.99		0.334		3.00	8.5
				Avg:	6.55	Avg:	0.307	Avg:	3.28	
				Series C. Rabbits Infected with a Virulent Ravenel Strain of Bovine Tubercle Bacilli.						
0.0	0.190	15.6		8.21		0.244		4.11		
3.1	0.095	8.13	4.58	8.54	4.82	0.234	0.415	4.27	2.41	42.5
3.1 × 10-1	0.095		5.81		6.10		0.328		3.05	27.2
3.1 × 10-3	0.095		6.00		6.30		0.318		3.15	24.8
				Avg:	8.38	Avg:	0.239	Avg:	4.19	
				Series D. Rabbits Immunized with an R_1 Strain and Reinfected with a Ravenel Strain.						
0.0	0.256	18.5		7.21		0.277		3.61		
3.1	0.128	9.73	6.78	7.60	5.30	0.263	0.377	3.80	2.65	28.6
3.1 × 10-1	0.128		7.30		5.70		0.351		2.85	23.2
3.1 × 10-3	0.128		7.73		6.04		0.331		3.02	18.6
				Avg:	7.41	Avg:	0.270	Avg:	3.71	

TABLE IV.
Effect of Tuberculo-carbohydrate on the Hydrolysis of Benzoyl-*l*-argininеamide (BAA) by Cathepsin II Prepared from Normal Beef Spleen.

Conc. of carbohydrate mg per cc test sol.	Conc. of enzyme	K × 10⁴		C × 10³		Unit		Unit per mg		% decrease
		Without carbohydrate	With carbohydrate	Without carbohydrate	With carbohydrate	Without carbohydrate	With carbohydrate	Without carbohydrate	With carbohydrate	
0.0	0.142	13.2		9.26		0.216		4.63		
3.1	0.071	6.43	6.62	**9.04**	9.30	0.221	0.215	4.52	4.65	0.0
3.1 × 10⁻¹	0.071		6.59		9.26		0.216		4.63	0.0
3.1 × 10⁻³	0.071		6.84		9.61		0.208		4.81	0.0
				Avg: 9.15		Avg: 0.219		Avg: 4.58		

ninositose). According to Heidelberger and Menzel[8] the carbohydrate is obtained as a complex mixture of polysaccharides made up of *d*-arabinose and *d*-mannose.

It is evident that tuberculo-phosphatide definitely exerts a much greater inhibitory action on cathepsin derived from tuberculous tissues of rabbits than on similar enzyme solutions prepared from livers of normal animals. Whether or not there is any significance to the observation that the greatest inhibition was observed with cathepsin prepared from the livers of animals infected with the non-virulent R_1 strain remains to be determined.

The results with tuberculo-carbohydrate are even more striking: there was no inhibition whatever exerted upon normal liver cathepsin and very strong inhibition upon the proteinases prepared from tuberculous tissues. The significance of the apparent maximal inhibitory values in the case of tissues derived from animals infected for the first time with virulent, Ravenel tubercle bacilli, will be investigated.

Discussion. Several investigators, including Moen,[2] have come to the conclusion that the specific toxic action of tuberculin upon hypersensitive tissues is probably not the result of an antigen-antibody reaction. The discovery, in the present communication, of a selective inhibitory action of tuberculo-carbohydrate and tuberculo-phosphatide upon endocellular enzymes (Cathepsin II) derived from tissues of animals infected with tubercle bacilli throws new light upon this mechanism and suggests an enzymological explanation, as follows: Since cathepsin is the enzyme which is concerned with the processes of cellular growth and repair, inhibition thereof leads to injury of the cells. Tuberculin is more toxic for tuberculous than for normal cells in tissue culture because two of its important constituents (phosphatide and carbohydrate) exert a selective inhibitory action upon the proteinases of the former.

Our observations may also throw light on the mechanism of caseation and softening. As pointed out by Jobling and Petersen,[13] "Caseation in tuberculosis is a form of coagu-

[13] Jobling, J. W., and Petersen, W., *J. Exp. Med.*, 1914, **19**, 383.

lation necrosis in which the dead tissues rarely undergo autolysis, except as a result of secondary infection." It is quite likely, therefore, that inhibition of autolysis is accomplished by the carbohydrate and phosphatide fractions of the tubercle bacilli.

It is also possible that the development of an increased susceptibility to the inhibitory action of the products of the tubercle bacillus which comes about as a result of infection is a defense mechanism since, as shown by Lurie,[14] tubercle bacilli usually die in caseous but grow in the softened areas. Furthermore, the former may undergo calcification and healing, whereas in the latter, the bacilli grow and from there disseminate to other sites.

Conclusions. The carbohydrate and phosphatide fractions of the tubercle bacillus (*M. tuberculosis*, H-37) exert a selective inhibitory action upon the endocellular enzyme, Cathepsin II, of tuberculous tissue. This phenomenon may help to explain the cytotoxic action of tuberculin *in vitro* and the failure of autolysis of caseous, tuberculous tissue.

[14] Lurie, M. B., *J. Exp. Med.*, 1933, **57**, 181.

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The Endotoxin of the Cholera Vibrio: Isolation and Properties*

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Though it is generally agreed that the cholera vibrio contains a potent endotoxin, the nature of the toxin is by no means clear. The isolation of a toxic protein has been reported by some workers such as Galeotti,[1] Sanarelli,[2] and Hahn and Hirsch.[3] More recently the trichloracetic acid extraction method of Boivin[4] has been applied to the cholera vibrio by Checcacci,[5] Raynal, Lieou, and Feissole,[6] Damboviceanu and Barber[7] and Gallut,[8] all of whom have reported successful extraction of a toxic fraction. It has been assumed by these workers that the active material is a polysaccharide-lipid complex, though in no instance is this conclusion supported by unequivocal evidence.

In the course of a study of active immunity to infection with *Vibrio choleræ*, we have had occasion to isolate the toxic principle from both Inaba and Ogawa types and partially purify it. Toxic solutions of the vibrio cell substance have been prepared in the following ways: (a) The cells are readily disintegrated in 4-5 hours by high speed grinding with sand, and the cellular debris is spun off, leaving a toxic opalescent supernate. (b) The cells may be dissolved in 6 M urea to give a similar toxic solution. (c) The cells may be digested with pepsin 3-5 days without inactivation of the toxicity, and the insoluble material removed by centrifugation to leave a toxic supernate. (d) The intact cells may be extracted in the cold with M/2 trichloracetic acid, all the toxicity going into solution. (e) Lyophilized cells may be extracted with methyl alcohol, ethyl alcohol, chloroform, or ethyl ether in a Soxhlet apparatus to give toxic

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago.

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[5] Checcacci, L., *Boll. Instituto Sieroterap. Milanese*, 1939, **18**, 391.

[6] Raynal, J., Lieou, Y. C., and Feissole, L., *Rev. Immunol.*, 1939, **5**, 317; *ibid.*, 1940, **6**, 132.

[7] Damboviceanu, A., and Barber, G., *C. R. Soc. Biol.*, 1940, **133**, 501.

[8] Gallut, J., *Ann. Inst. Pasteur*, 1943, **69**, 123.