BACTERICIDAL ACTIVITY OF RAT LEUCOCYTIC EXTRACTS

II. Characterization of the Bactericidal Substance in Leucocyte Mitochondrial Extracts

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In the preceding paper (1), it was reported that rat leucocytic extracts prepared by ultrasonic disintegration of polymorphonuclear neutrophils (leucocytes) were bactericidal against both Gram-negative and Gram-positive organisms. The bactericidal activity of these extracts was confined primarily to the mitochondria. The purpose of this report is to characterize this bactericidal substance or substances in terms of its physico-chemical properties.

Methods and Results

The preparation of the extracts and the method of bactericidal assay have been previously described (1).

Dialysis.—

Aliquots from a leucocytic and mitochondrial extract were dialyzed against distilled water for 24 hours, passed through a Seitz filter, and tested for their bactericidal activity.

It can be seen from the results given in Fig. 1 that dialysis did not effect the rate or extent of the bactericidal activity. The fact that the bactericidal factor of either extract is non-dialyzable suggests that the active substance or substances has a relatively high molecular weight.

Heat Stability.—The effect of heating on the bactericidal activity of leucocytic and mitochondrial extracts was determined.

Aliquots of the leucocytic extract were exposed to temperatures of 37, 56, 72 and 100°C. for 30 minutes and then assayed for bactericidal activity. Aliquots of the mitochondrial extract were heated at 100°C. for 30 minutes and 121°C. (autoclaved under 15 pounds' pressure) for 15 minutes.

The results of these experiments (Table I) clearly show that the substance responsible for the bactericidal activity of the leucocytic and mitochondrial

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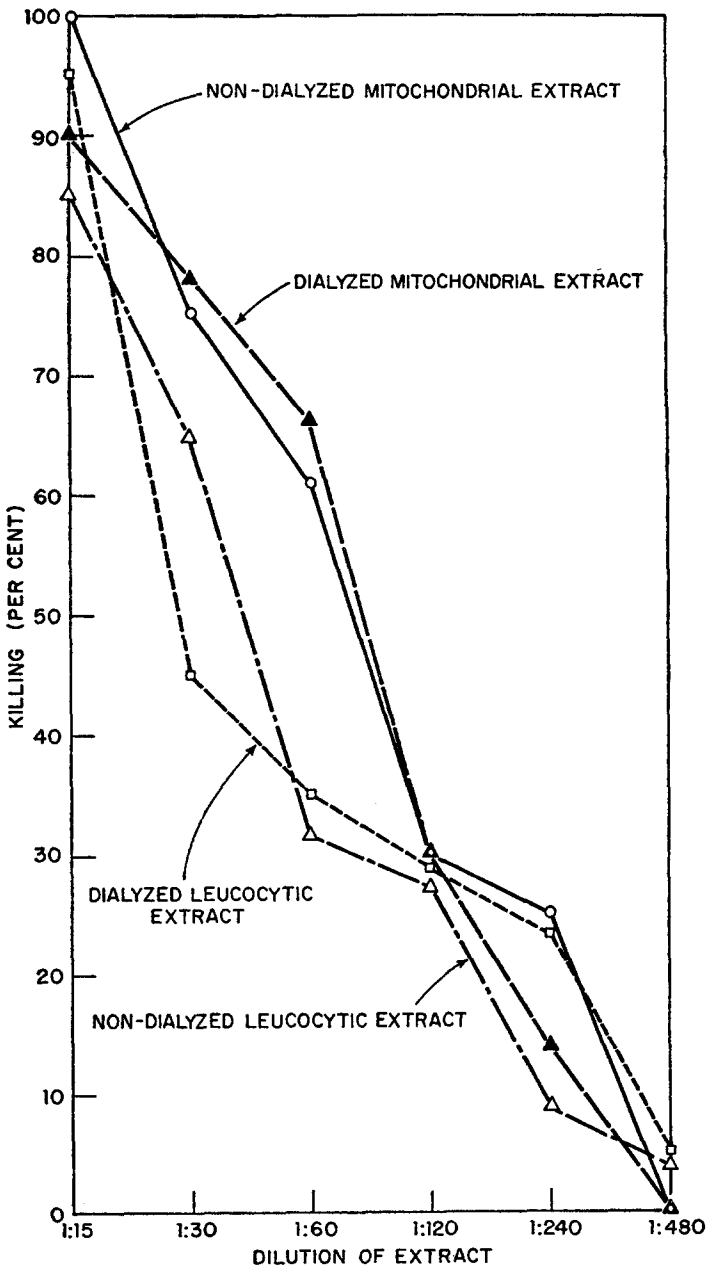

FIG. 1. Effect of dialysis on the bactericidal activity of leucocytic and mitochondrial extracts against *M. Aureus*.

extract is thermostable, withstanding temperatures as high as 100°C. However, complete inactivation of the bactericidal activity occurred when the mitochondrial extract was autoclaved.

TABLE I

Bactericidal Activity of Heated Leucocytic (LE) and Mitochondrial (ME) Extracts from Rats Against M. Aureus

Temperature heated	Dilution	Colonies per 0.1 ml. after incubation for			Per cent killing after incubation for	
		0 min.	30 min.	60 min.	30 min.	60 min.
°C.						
LE						
37	1:15	130	0	0	100	100
56	1:15	140	1	0	99	100
72	1:15	122	0	0	100	100
100	1:15	126	0	0	100	100
ME						
25	1:15	144	2	0	99	100
	1:30	137	60	0	56	100
	1:60	115	82	5	29	96
	1:120	118	120	49	0	59
	1:240	112	113	52	0	52
	1:480	125	121	90	0	28
121	1:15	123	130	127	0	0
(Autoclaved)	1:30	129	125	128	0	0
	1:60	133	129	130	0	0
	1:120	127	132	130	0	0
	1:240	132	142	131	0	0
	1:480	120	117	123	0	0
100	1:15	107	37	0	65	100

Ammonium Sulfate Fractionation.—

Initially $(NH_4)_2SO_4$ fractionation of the leucocytic extract was performed to remove the purine and pyrimidine compounds which appeared to be implicated, in preliminary experiments, as the bactericidal substance. A three-step fractionation of the leucocyte extracts was performed with $(NH_4)_2SO_4$. To 8 ml. of the leucocytic extract was added 3 ml. of the saturated $(NH_4)_2SO_4$. The extract was allowed to stand for 5 minutes at 0°C. and then centrifuged at 10,000 R.P.M. for 5 minutes. The precipitate, redissolved in 5 ml. of the phosphate buffered solution, pH 7.5, constituted a 0 to 30 per cent fractionation step. To the supernatant (8 ml.) was added 2 ml. of the $(NH_4)_2SO_4$. The resulting precipitate represented a 30 to 50 per cent fractionation step. To the remaining supernatant (10 ml.) was added 10 ml. of the $(NH_4)_2SO_4$ solution and the resulting precipitate represented a 50 to 100 per cent fractionation step. All three fractions were dialyzed in the cold against distilled water for 30 hours, at which time a barium chloride test indicated the absence of $(NH_4)_2SO_4$ in the fractions.

The results of the three-step fractionation (Table II) clearly show that the 0 to 30 percent $(NH_4)_2SO_4$ fraction was highly bactericidal whereas the other two fractions, 30 to 50 per cent and 50 to 100 per cent were inactive against *M. aureus*. The ultraviolet absorption spectrum of the 0 to 30 per cent $(NH_4)_2SO_4$ fractions revealed no peak between 220 to 280 mμ thus discounting the purine and pyrimidine compounds as the responsible bactericidal substance.

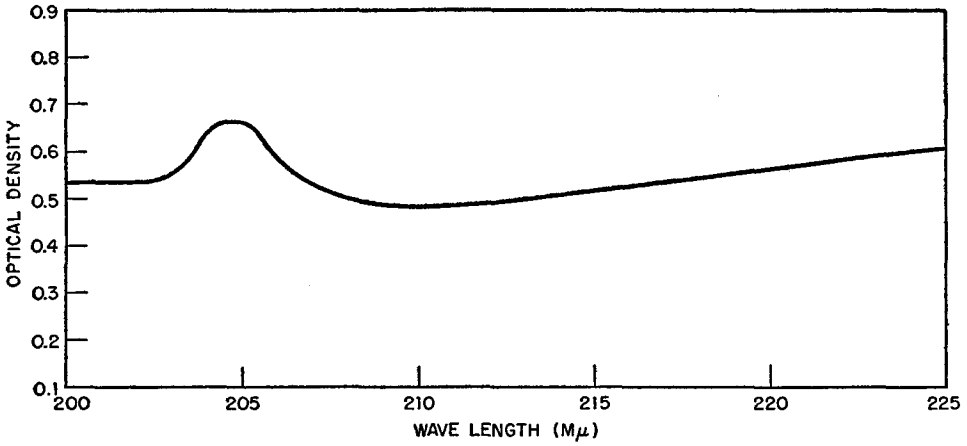

FIG. 2. Ultraviolet spectrum of an active mitochondrial extract

TABLE II

Effect of $(NH_4)_2SO_4$ Fractionation on the Bactericidal Activity of Leucocytic Extracts from Rats Against M. Aureus

Fraction per cent $(NH_4)_2SO_4$	Colonies per 0.1 ml. after incubation for			Per cent killing after incubation for	
	0 min.	30 min.	60 min.	30 min.	60 min.
0	200	4	0	98	100
30	198	0	0	100	100
50	250	248	248	0	0
100	207	208	207	0	0
—	210	213	211	0	0

At this point, a wider absorption range (200 to 700 mμ) obtained by use of the Cary recording spectrophotometer, was used to further characterize the bactericidal substance. The mitochondrial extract gave a definite absorption peak at 203 to 208 mμ (Fig 2) a region reported to be associated with alpha, beta unsaturated fatty acids as well as with some amino acids (2).

Assuming that the ultraviolet peak of 203 to 208 mμ is indicative of an alpha,

beta, unsaturated fatty acid, the bactericidal substance was considered to be, at least in part, lipide in nature. Since the bactericidal activity is non-dialyza-

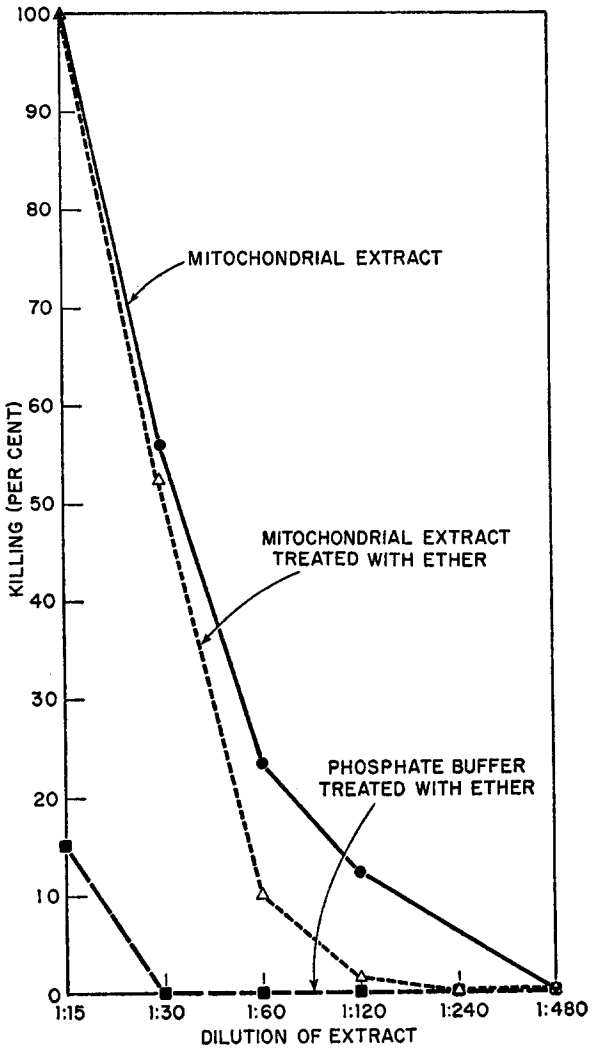

FIG. 3. The effect of ether treatment on the bactericidal activity of mitochondrial extracts against *M. Aureus*.

ble, the substance was considered to be a lipide complex rather than a free lipide. To prove the presence of a lipide, the mitochondrial extract was treated with ethyl ether under conditions which make possible differentiation between free neutral lipide and protein-bound lipide.

The mitochondrial extracts were treated by adding an equal volume of doubly distilled ether to the extract and placing the mixture in a dry ice–acetone bath (−70°C.) for 20 minutes. The mixture was then put into an ice bath and allowed to remain there until the extract was

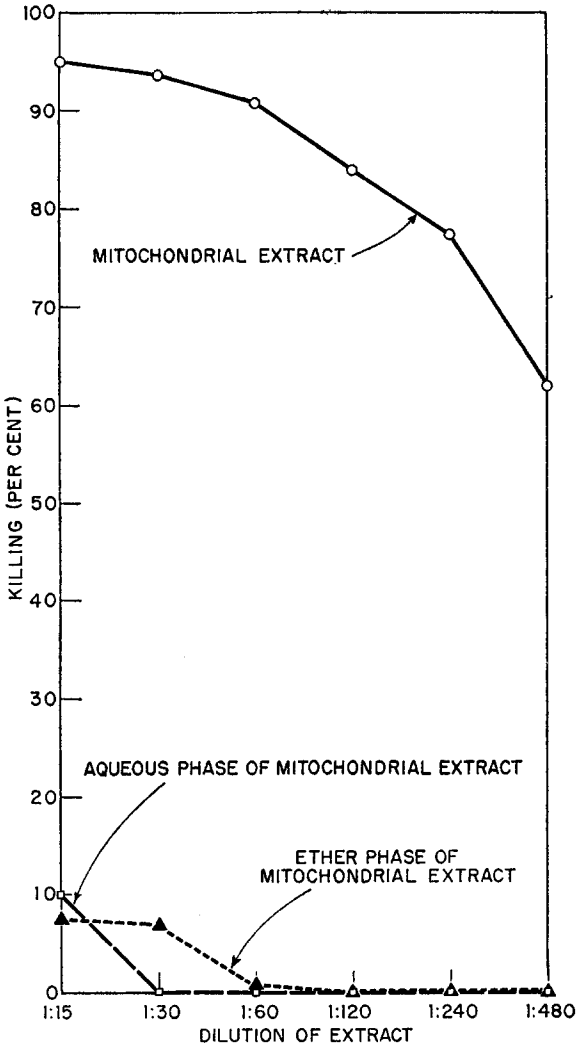

FIG. 4. The effect of "freeze-thaw" method of ether treatment on the bactericidal activity of mitochondrial extracts against *M. Aureus*.

completely thawed out. This "freezing and thawing" procedure (3) was repeated three times. The aqueous phase was removed and washed three times with fresh ether. The ether was removed from both the aqueous and ether phase before testing their bactericidal activity.

Treatment with ether at 5°C. did not affect the bactericidal activity as seen in Fig 3. When, however, the mitochondrial extract was treated by the "freeze

and thaw" method of ether extraction, the bactericidal activity could not be demonstrated in either the aqueous or ether phase (Fig. 4). Furthermore, the UV peak at 203 to 208 mμ was no longer detectable in the aqueous phase, but was still present in the ether phase. The latter observation lends support to the hypothesis that the bactericidal substance is a lipide complex, possibly lipoprotein.

In a further attempt to verify the presence of an active lipide compound, a mitochondrial extract was treated with lipase.

TABLE III

Effect of Lipase on the Bactericidal Activity of Mitochondrial Extracts (ME) from Rat Leucocytes

Dilution	Colonies per 0.1 ml. after incubation for			Per cent killing after incubation for	
	0 min.	30 min.	60 min.	30 min.	60 min.
ME not treated with lipase					
1:15	78	0	0	100	100
1:30	90	69	39	23	56
ME treated with lipase					
1:15	85	90	91	0	0
1:30	81	84	86	0	0
Lipase without *ME*					
Undiluted	77	75	80	0	0

Lipase was extracted from Steapsin with 0.025 M NH_4OH at 0°C. for 30 minutes as described by Korn (4). Two ml. of the enzyme were added to 4 ml. of the mitochondrial extract together with 1 ml. of 0.2 M $CaCl_2$ and 3 ml. of a 0.25 M NH_3-NH_4Cl buffer, pH 8.5 (0.1 M glycine buffer, pH 8.5 was also used). The mixture was incubated at 37°C. for 1 hour. The pH was adjusted to 7.5 and the extract dialyzed against distilled water for 24 hours. The pH of the dialyzed extract was adjusted to 7.5 and then assayed for its bactericidal activity.

The results shown in Table III indicate a complete inactivation of the bactericidal activity by lipase. The mitochondrial extract that was not treated with lipase gave 100 per cent killing after 60 minutes of incubation, whereas the treated extract showed no bactericidal activity.

A mitochondrial extract was treated with trypsin to determine the presence of any protein moiety in the bactericidal substance.

0.1 ml. of a salt free crystalline trypsin preparation, obtained from Armour Company was added to 10 ml. of a M/15 phosphate buffer solution, pH 7.5. 2 ml. of this trypsin solution were added to 5 ml. of a mitochondrial extract from rat leucocytes. The mixture was incubated at 37°C. for 60 minutes. 2 ml. of the incubated mixture were then removed and assayed for

bactericidal activity. A higher concentration of trypsin (0.5 mg./10 ml. phosphate buffer) and a longer incubation period (37°C./2 hrs.) were also used.

It can be noted from Table IV that after 1 hour incubation of the extract with 20 μg. of trypsin per ml., a 62 per cent inactivation of the bactericidal activity occurred. The per cent inactivation could not be increased by either a greater concentration of trypsin (100 μg.) or a longer incubation period (2 hours). These results would tentatively indicate the presence of a *lipoprotein* as a bactericidal substance in rat leucocyte mitochondrial extracts.

TABLE IV

Effect of Trypsin on the Bactericidal Activity of Mitochondrial Extracts (ME) from Rat Leucocytes

Amount of trypsin	Amount of ME	Time of incubation	Amount inactivation in bactericidal activity
mg./ml.	*ml.*	*hrs.*	*per cent*
0.02	5.0	1	62
0.10	5.0	1	58
0.10	5.0	2	61
0.10	0.0	0	0
0.00	5.0	2	0

DISCUSSION

From the present experiments, it appears that the bactericidal substance obtained from the mitochondria of rat leucocytes, is a lipoprotein. The lipoprotein nature, although not definite, is based on heat stability (inactivated by autoclaving), UV spectrum, inactivation with ether at −70°C., and inactivation by the enzymes lipase and trypsin. These properties by themselves would preclude this substance from being lysozyme (5). In addition, this bactericidal substance also differs from lysozyme in that it has a wider antibacterial spectrum of activity (1).

The bactericidal substance, phagocytin, reported by Hirsch (6, 7) does not appear to be an enzyme and is not bacteriolytic. It is also relatively thermostable and inactivated by trypsin. In these respects, phagocytin may be similar to the substance isolated in our studies.

However, Hirsch did not demonstrate any lipide moiety associated with phagocytin. Other differences between phagocytin and the bactericidal substance from rat leucocytes are that the latter substance was found to withstand heating at 100°C. for 30 minutes and to be fractionated at 30 per cent $(NH_4)_2SO_4$ saturation. The pH optimum of both substances cannot be compared since our assay system was sensitive to low pH. In a narrow range, one can estimate that the optimum pH for the *in vitro* assay was 7.2 while that of phagocytin was found to be 4.5.

SUMMARY

The bactericidal substance present in mitochondrial extracts from rat leucocytes was found to be non-dialyzable, fractionated at 30 per cent $(NH_4)_2SO_4$ saturation and was able to withstand temperatures up to 100°C.

The tentative identification of a lipoprotein as the responsible bactericidal substance is based on various observations; an ultraviolet absorption at a peak associated with alpha, beta unsaturated fatty acids, 205 to 208 mμ, inactivation of the bactericidal substance by ether treatment at −70°C. but not at 0°C. and inhibition of the bactericidal activity with lipase treatment as well as with trypsin.

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