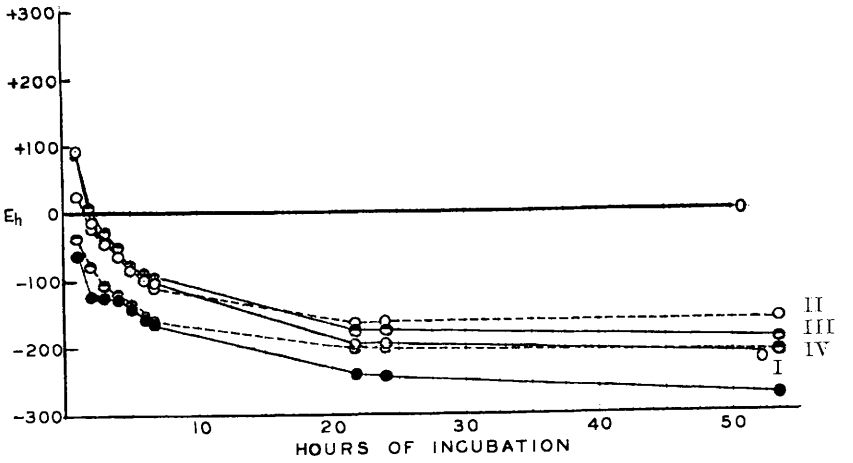

FIG. 4.

Changes in oxidation-reduction potential (E_h) of cultures of *Entamoeba histolytica* (*UC* strain with mixed flora) in different concentrations of prodigiosin, with different amoebic inocula.

● Control.
I—1:200,000 (102,000 amoebae)
II—1:200,000 (391,000 amoebae)
III—1:100,000 (102,000 amoebae)
IV—1:100,000 (391,000 amoebae)

30 hours to reach −200 mv in comparison to 10 hours for the *UC* strain, but in all cases controls and experimentals behaved similarly. The pH of all cultures was held in the range pH 6.8 to 7.2 through the use of well-buffered media.

Summary and conclusions. (1) Prodigiosin was employed in the range 1:20,000,000 to 1:100,000 against two distinct strains of *E. histolytica.*

(2) The monobacterial culture (*NRS* strain grown with *A. aerogenes*) proved more susceptible, failing to survive at 1:400,000. The mixed culture (*UC* strain) failed to survive at 1:100,000, even with relatively huge inocula of amoebae.

(3) Analysis disclosed that the bacterial flora, oxidation-reduction potentials and pH were not adversely affected by the experimental treatment.

(4) The general conclusion is reached that prodigiosin exerts a profound, direct action against *E. histolytica in vitro.* This is apparently the first record of proved direct action of an antibiotic against *E. histolytica.*

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Further Studies on the Thermostable Leukocytosis Factor of Exudates. (18204)

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The rise in the number of circulating leukocytes with inflammation is referable to at least two factors released by cells injured at the site of injury: (1) a thermolabile leukocytosis-promoting factor (the so-called LPF) and (2) a thermostable leukocytosis factor which is closely associated with the

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1. Menkin, V., *Am. J. Path.,* 1940, v16, 13.

pyrogenic factor, pyrexin, recovered particularly from acid exudates(1-3).

An attempt has been made to purify further the thermostable leukocytosis factor. The latter has been recently recovered in a crystalline form. This has been obtained by freeing it from pyrexin, so that these crystals induce only a leukocytosis without any pyrogenic or leukopenic(4) properties. The crystals, the extraction of which is to be presently described, are thermostable in nature. They thus seem to reproduce all the effects of the original and crude thermostable component described(3).

Method. An active fraction of canine pyrexin from exudates is weighed in varying amounts, usually around 20 mg. The powder is suspended in about 20 cc of distilled water. About 20 cc of saturated $(NH_4)_2SO_4$ is added, so that the pyrogenic factor is consequently subjected to the salt at one-half saturation. The entire mixture is then dialyzed in a cellophane tube against tap water. About 24 hours later when the material is free of sulfate ions, the mixture is thoroughly centrifuged. The supernatant phase is a crude material which contains 3 components, namely the pyrogenic, the leukopenic, and the thermostable leukocytosis factors. The precipitate fraction contains considerable pyrogenic activity, when injected intravenously into rabbits. The crude supernatant, containing the 3 components listed, is now diluted with an equal volume of distilled water. About 10 cc portions are poured into a test tube which in turn is sealed. The tube is then refluxed in boiling water for a period ranging from 17 to 20 hours. After that time, the tube is cooled either at room temperature or preferably in a refrigerator. Crystal formations then occur, which by gross examination appear as small needles, but microscopically usually resemble irregularly-shaped crystalline flakes. The contents of the tube are centrifuged, and the crystals are collected as a precipitate. The crystals are injected into the circulation of dogs and periodic white counts are made for about 6 to 24 hours.

TABLE I. Effect of Crude Material on Leukocyte Count in the Blood.

Dog No.	Basal white count, per cubic mm	White count about 1 hr after inj. of material, per cubic mm	Highest white count within about 1 day, per cubic mm	Max. rise in temp., °F
54-T	11150	4000	16350	1.2
156-T	4675	1500	9200	2.7
63-T	9550	3950	19950	2.7
98-T	15500	12700	23800	1.0
63-T*	10950	4750	20100	1.6
157-T	11550	3150	20500	1.9
Avg	10563	5008	18317	1.9

* 5-year-old pyrexin utilized.

TABLE II. Effect of Crystals of Thermostable Leukocytosis Factor on Leukocytes of the Blood.

Dog No.	Basal white blood cell count, per cubic mm	White blood cell level about 1 hr after inj. of crystals, per cubic mm	Highest count within about 24 hr after inj. of crystals, per cubic mm	Highest change in temp., °F
129-T	14800	15300	25400	—0.4
156-T	3100	5450	7550	+1.6
54-T	13550	13400	19450	+0.2
63-T	9875	13300	16425	+0.2
156-T	8875	13750	26800	+1.2
129-T*	18650	15500	23850	+0.7
98-T	13850	13750	19250	+0.4
97-T†	9200	9150	18950	0
Avg	11488	12450	19709	0.5

* 5-year-old pyrexin utilized to oztain the crystals of the thermostable leukocytosis factor.

† Filtered the crystals, and then took the wet filter paper and washed off the crystals in distilled water.

Results. When pyrexin is treated at half saturation with ammonium sulfate and dialyzed, as indicated in the scheme of extraction, a so-called crude material is obtained in which some amorphous material is seen to be suspended. This fraction is thoroughly centrifuged. The precipitate or sediment appears to contain considerable pyrogenic activity. When suspended in a minimal amount of aqueous fluid and injected intravenously into rabbits, the latter develop a rapid rise

2. Menkin, V., *Arch. Path.*, 1940, v30, 363.

3. Menkin, V., *Blood, The Jour. of Hematol.*, 1949, v4, 1323.

4. Menkin, V., *Arch. Path.*, 1946, v41, 50.

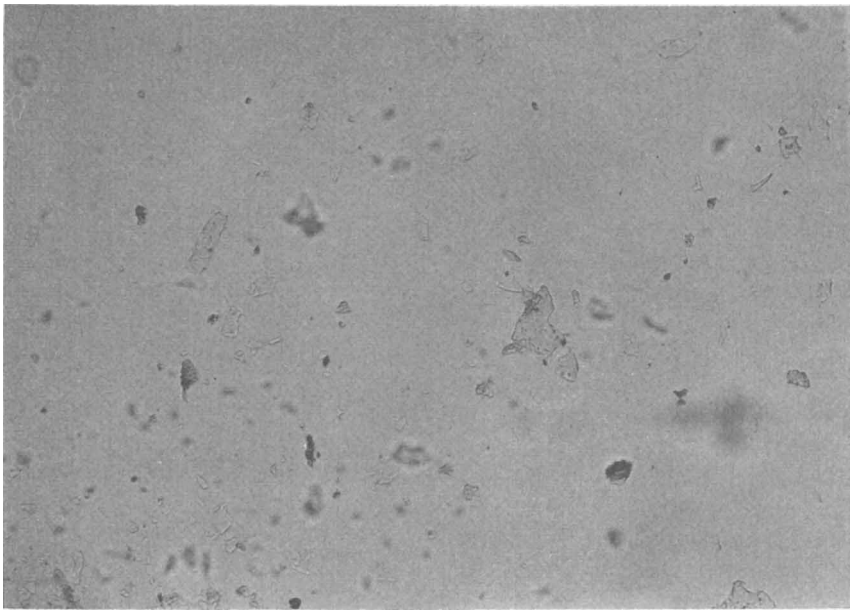

FIG. 1.
One-day-old crystals of the thermostable leukocytosis factor. These crystals were derived from a sample of pyrexin which had been isolated 5 years previously. 400 gamma of these crystals induced a rise of 77.5% in the l-lobe form of granulocytes. ×134.

in rectal temperature. The average rise is 2.7°F. This effect in rabbits has already been described(5).

The supernatant phase after centrifugation of the precipitate, as described above, upon injection intravascularly into dogs yields at first a leukopenia subsequently followed by a leukocytosis. Furthermore, there is a rise in rectal temperature which averages 1.9°F (Table I). These results are similar to the ones described with the thermostable component of the leukocytosis factor(3). The activity of such pyrexin preparations may be found even 5 years after extracting the pyrogenic factor from inflammatory exudates (dog No. 63-T, Table I).

Following further the scheme of extraction of the thermostable leukocytosis factor as outlined above, a crystalline fraction can be obtained which has neither any initial leukopenic effect nor has it any pyrogenic capacity. These crystals, however, induce in several to 24 hours an appreciable state of leukocytosis. One hour after administration of the crude supernatant material there is a fall in the leukocyte level amounting to 53% (Table I). On the contrary when the crystalline material is injected there is no initial leukopenia, but there is even a slight rise in the count averaging 8% (Table II). Nevertheless, these crystals induce a leukocytosis similar to the crude material averaging 72% (Table II). The leukocytosis induced by the crude supernatant fraction is of the same order, namely 73% (Table I). Thus, crystals of the thermostable leukocytosis factor which are heat stable and which have neither any initial leukopenic or pyrogenic effects (0.5°F, Table II) have been obtained by the above scheme of extraction.

An illustration of these crystals appears in Fig. 1. The crystals which in the gross resemble needle-like projections, microscopically appear as irregularly-shaped, doubly refractile crystals (Fig. 1). The crystals in contrast to the thermolabile LPF(6) induce no change in the amino nitrogen after hydrolysis. This would suggest that the mater-

5. Menkin, V., *Arch. Path.*, 1945, v39, 28.

6. Menkin, V., *Blood, J. Hematol.*, 1948, v3, 939.

ial is not polypeptide in character. The total nitrogen also yields a figure, namely 2.1%, which is lower than the active polypeptide of the thermolabile LPF. The latter has been found to have 12.3% nitrogen.

The Molisch test for carbohydrates is on some samples of the crystals very strongly positive; whereas in other samples especially if the crystals are dried previously, it is quite weak or absent. The correlation of biological activity and the Molisch reaction has as yet not been studied. The variability in the intensity of this test merely suggests that perhaps the active non-desiccated crystals of the thermostable leukocytosis factor may have a carbohydrate linked to the structure of the crystals. Further studies will establish this point on a firmer basis.

A study was undertaken to determine whether the crystals of the thermostable leukocytosis factor induce a discharge into the circulation of immature or 1-lobe form of granulocytes. At the time that the crude thermostable factor was identified as associated with pyrexin, it was also established that this material causes a discharge of immature granulocytes into the blood stream(3). A similar study was carried out with the crystals of the thermostable leukocytosis factor. The data indicate clearly that the percentage of 1-lobe form of granulocytes augments as early as one hour after administration of either the crude material or the crystals of the thermostable leukocytosis factor. Within about one hour, there is an average increase which almost is double that of the basal level, namely 18 to 35%.

When the crystals are either washed repeatedly with distilled water or when they are desiccated, they lose some of their activity. The absolute white count is barely increased. Nevertheless, the differential microscopic picture indicates a rise in the percentage of 1-lobe form of granulocytes. There is also a marked increase in the absolute number of 1-lobe granulocytes. As pointed out previously, it seems as if a study of the differential leukocytic formula is a more delicate test than the absolute white count in determining the effectiveness of a given material(3,7). On the other hand, when either the distilled water utilized to wash the crystals, or the supernatant fluid alone but freed of suspended thermostable crystals are injected in dogs, there is no appreciable change in either the absolute white count level or in the differential formula. These facts point further to the capacity of the crystals in increasing the number of leukocytes in the blood stream.†

Discussion and conclusions. The significance of the thermostable leukocytosis factor as a contributing factor in the mechanism of leukocytosis with inflammation has already been pointed out(3). At the time no conclusion could be reached as to whether the thermolabile LPF and the thermostable leukocytosis factor are one and the same substance, or whether the two factors are distinct entities. The present studies bearing on the nitrogen content of the crystals of the thermostable leukocytosis factor support the view that the two factors are two distinct substances.

In conclusion, the thermostable leukocytosis factor, recovered especially from acid exudates, and which is found in association with the pyrogenic factor, pyrexin, has been crystallized, and the crystals as such have essentially neither pyrogenic nor leukopenic properties. The crystals of the thermostable leukocytosis factor induce a leukocytosis characterized by a discharge into the circulation of immature 1-lobe form of granulocytes.

7. Menkin, V., PROC. SOC. EXP. BIOL. AND MED., 1947, v64, 448.

† As a further control, several dogs received an intravascular injection of a similar crystalline material derived from a toxic euglobulin derived from exudates and termed necrosin(5). These crystals were in all cases incapable of inducing a leukocytosis in the animals (unpublished studies). These latter studies point to the specificity of the thermostable crystals described in the present communication in inducing a state of leukocytosis.

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