Preservation Recrystallization and Preliminary Biochemical Characterizaton of Charcot-Leyden Crystals.* (27442)

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Charcot-Leyden (C-L) crystals are found associated with eosinophils in blood and asthmatic secretions. They are also found in stools in diseases where there is eosinophilic infiltration of the intestinal mucosa (trichuriasis and amebic dysentery) and in eosinophilic granulomas. Beaver and Danaraj(1) demonstrated them in the lung in fatal pulmonary ascariasis (Fig. 1). Using wetting agents, Ayres and Starkey(2) produced these crystals from eosinophils of normal blood. Samter(3) was unable to produce C-L crystals from masses of eosinophils in blister fluid but did produce them from eosinophils removed from this same fluid. Using electron microscopy, Welsh(4) demonstrated intracellular formation of these crystals in eosinophils. He also showed that the granules of the eosinophils were incorporated into the crystals. Neither the crystals nor the incorporated granules stained with Sudan black although the remaining granules continued to do so. He concluded that a protein matrix within the granule formed the nidus for the crystal. It is possible that further knowledge of the chemical properties of C-L crystals might contribute to an understanding of the function of eosinophils, which is presently unknown.

Identification of C-L crystals is made solely on the basis of their shape, which, however, is quite characteristic and constant. The crystal has the appearance of 2 hexagonal pyramids placed base to base, each side tapering from the base at an angle of 80° and the sides meeting to form a 20° angle at either apex. The crystals vary in size from those barely visible at 960 × magnification up to those approximately 100 μ in length. The usual length is between 20 and 40 μ (Fig. 2, inset). The chemical composition of the C-L crystal has been variously reported to be spermine phosphate(5), nucleoprotein(6) or a zinc containing protein(7). This wide divergence of opinion reflects the fact that up to now there has been no satisfactory method of separating large numbers of crystals from other heterogeneous elements, concentrating them and preserving them without addition of fixatives.

This paper reports a method of concentrating and preserving C-L crystals from feces. Determinations were made of their solubility, histochemical reactions and behavior in presence of various enzymes. Preliminary efforts to characterize the crystals included estimation of their nitrogen content, ninhydrin reaction, absorption spectrum, color reactions for protein, ultracentrifugation of dissolved crystals, electrophoretic studies, spectrographic analysis and recrystallization. The results suggest that the material composing C-L crystals is a polypeptide of low molecular weight, soluble in weak acid or base, which readily recrystallizes at pH 7.2. It was not possible to demonstrate the presence of zinc or phosphorus by spectrographic analysis.

Materials and methods. Concentration and

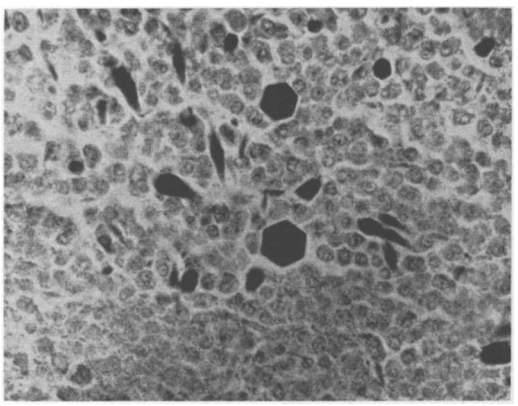

FIG. 1. Charcot-Leyden crystals in section of lung in fatal pulmonary ascariasis. Giemsa stain ×540. (Courtesy of P. C. Beaver.)

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FIG. 2. Charcot-Leyden crystals concentrated from feces. ×300; inset, same ×390.

preservation of C-L crystals. The C-L crystals were obtained from diarrheal stools of patients having mixed parasitic infections. In one case of rectal prolapse it was possible to demonstrate an associated intestinal eosinophilia. The feces were emulsified in 50 volumes of distilled water, filtered through 2 layers of surgical gauze and extracted three times with ether. The crystals remained in the sediment. Since the crystals are unaffected by sonication for one hour at maximum breakage, it was possible on occasion further to remove some extraneous material by this procedure. Heavier particles were removed by suspending the washed sediment in 15 volumes of sucrose solution (sp. gr. 1.250) and centrifuging 30 minutes at 34,850 × gravity. Most of the crystals remained in the supernatant fluid which was diluted with distilled water and the crystals recovered by centrifugation at 2000 rpm. After concentration the crystals were suspended in 70% sucrose to which were added 500 units of streptomycin and 500 units of penicillin per ml. They were then stored at 0-4°C. Freezing, with or without sucrose, caused destruction of the crystals on subsequent thawing. Crystal preparations concentrated as described contained several thousand crystals per low power field (Fig. 2). Some yeast-like forms and an occasional tissue cell were also present. When these concentrates were stored in 70% sucrose at 0-4°C there was no change in number or appearance of the crystals over a period of 9 months. It was possible to collect 50 ml of material having the microscopic appearance shown in Fig. 2. These preserved crystals were compared repeatedly with freshly collected crystals and the staining reactions and solubilities of both were the same.

For this purpose, freshly collected stools containing C-L crystals were emulsified with 50 volumes of water, extracted with ether and the sediment containing the crystals washed 5 times with distilled water. Duplicate experiments were run using this type of preparation and crystals preserved as described above.

Dissolved crystals. A concentrated preparation of crystals having the microscopic appearance shown in Fig. 2 was suspended in 250 volumes of .005 N HCl (pH 3.5), allowed to stand 30 minutes and centrifuged. The sediment containing the crystals was then suspended in 50 volumes of .0075 N HCl (pH 2.4). The supernatant fluid containing the dissolved crystals was removed. Fifteen volumes of .0075 N HCl was added to the remaining sediment, centrifuged and the supernatant fluid removed for use as a control. The same procedure was followed with alkaline solutions; washing with .005 N NaOH at pH 10.5 and dissolving in .0075 N NaOH at pH 11.2. A similar control was prepared. These controls were run in all subsequent experiments. Concentration was determined by lyophilizing and weighing a sample of the crystal solution.

Stains. The stains used were Sudan black (saturated solution in 70% alcohol), Sudan III, (sat. sol. in 70% alcohol), phloxine (1% in 1% acetic acid), eosin S (1% in 95% alcohol), eosin Y (1% aqueous) trypan blue (1% aqueous), methylene blue (1% aqueous), neutral red (1% aqueous), Heidenhain's hematoxylin, crystal violet (1% aqueous), methyl green (1% in 1% acetic acid) and the indicator stain bromthymol blue (1%

aqueous). Three staining procedures were used: 1) a small drop of stain was added to a drop of crystal suspension, mixed, placed on a slide with coverslip and examined visually under the microscope for 15 minutes; 2) a drop of crystal suspension was added to 0.5 ml of the stain, allowed to stand 30 minutes with stirring, centrifuged, washed with water and examined microscopically; 3) the same procedure as 2) except that staining time was increased to 16 hours. Hematoxylin staining was done according to Heidenhain's method (8).

Solvents. The solubility of both freshly collected and preserved crystals was determined in hot and cold water, alcohol, ether, xylol, acetone, chloroform, carbon tetrachloride, $NaHCO_3$ (5%), Na_2CO_3 (5%), NH_4OH (1% to 5%), NaOH (.005 N to .01 N), HCl (.005 N to 0.1 N), acetic acid (1% to 5%). One hundred volumes of solvent were added to the crystal concentrate, stirred, allowed to stand for 2 hours, centrifuged and the sediment examined microscopically for crystals. In the case of hot water, the temperature was raised gradually in a water bath, samples removed at various temperatures and examined for the presence of crystals.

Enzymes. Highly active preparations of 1% pepsin, trypsin, papain and ficin were each buffered with phosphate buffer at optimal pH with the exception that pepsin was prepared in .005 N HCl at pH 3.5. A small amount of concentrated crystals was suspended in 100 volumes of each enzyme preparation and duplicates were incubated at room temperature and 37°C for a total period of 4 days. Streptomycin and penicillin (500 units each per ml) were used to inhibit bacterial growth. Samples were examined periodically for change in appearance or number of crystals.

Millon's reaction was carried out according to the procedure outlined in Hawk, Oser and Summerson(9).

Recrystallization. Approximately 0.5% alkaline and acid solutions of crystals were brought to pH 7.2 by addition of .01 N HCl or .01 N NaOH respectively.

Molecular weight. Using a Schlieren optical system, approximately a 1% solution of crystals in HCl at pH 2.4 was subjected to ultracentrifugation at 59,000 rpm for 4 hours and photographed at 16-minute intervals. It was visually observed for the first 30 minutes.

Electrophoresis. Paper electrophoresis was employed, using paper strips 3 $\times$ 30.6 cm in Durrum cells. Duplicate runs were made at 2.5 ma (70 volts $\pm$ 1) per cell and 10 ma (265 volts $\pm$ 1) per cell for 4, 8 and 16 hours. The strips were dried, fixed with methanol, stained with bromphenol blue, washed with 5% acetic acid, dried and the color developed by exposure to NH_4OH vapors. Buffer for the acid-dissolved crystals was 0.2 M potassium acid phthalate at pH 2.4. The alkaline-dissolved crystals were run in 0.2 M carbonate buffer at pH 10.8. In addition, 2 other samples were prepared in which the crystals were dissolved at pH 2.4 and pH 11.2 respectively and the solutions brought to pH 7.2, with the concentration of crystals kept sufficiently low to prevent recrystallization. These solutions were run in sodium barbital buffer at pH 8.5. In such instances, controls of normal serum were run when the current was 2.5 ma (70 volts $\pm$ 1) per cell, and of hemoglobin when the current was 10 ma (265 volts $\pm$ 1) per cell, in order to show that the system was operating satisfactorily.

Nitrogen determination. The micro-Kjeldahl determination was made on dissolved crystals according to the method of Folin and Wu(10).

Ninhydrin test. A drop of dissolved crystals was placed on Whatman filter paper No. 1 (chromatographic), allowed to dry, sprayed with ninhydrin and heated at 80°C for 30 minutes to develop the color.

Ultraviolet absorption. A 0.3% crystal solution at pH 2.4 was examined in a Beckman automatic recording spectrophotometer.

Spectrographic analysis. A solution of C-L crystals (pH 2.4) and an equal amount of the acidic control were lyophilized and weighed. The C-L crystal sample weighed 6 mg. Since the material in the control was almost imperceptible, 1 ml of hot water was added to the control tube and both samples were absorbed onto magnesium oxide, ashed and examined in a Beckman emission spectrometer for the presence of minerals.

TABLE I. Staining Reactions of Charcot-Leyden Crystals.

Stain	Reaction
Sudan black	Unstained
" III	"
Trypan blue	"
Pheoxine	"
Eosine S	"
Eosin Y	"
Methylene blue	Blue
Methyl green (pH 4.5)	Green
Neutral red	Red
Hematoxylin	Black
Crystal violet	Purple
Toluidine blue	Blue
Bromthymol blue	"

Results. The C-L crystals stained well with nuclear and basic stains, but not with fat stains; nor were they eosinophilic (Table I). They were unaffected by treatment with the proteolytic enzymes tested. They were insoluble in organic solvents but did dissolve in water at 68°C, weak acid and weak base (Table II), and gave a positive Millon's reaction. Freshly collected and preserved crystals gave the same reactions in all cases.

When a 0.5% solution of crystals, either acidic or basic, was brought to pH 7.2, a precipitate formed immediately consisting of hexagonal blocks or plates (Fig. 3). These crystals had the same staining reactions as the C-L crystals and gave a positive Millon's reaction. No precipitate formed in the controls. No boundary line formed in the ultracentrifuge, so it was concluded that the molecular weight was below 1000. In all of the electrophoretic experiments a single band of bromphenol blue binding material was found at the point of application. The serum and hemoglobin controls showed characteristic movement and the acidic and basic solution controls contained no bromphenol blue binding material. It was concluded that the crystal material bound bromphenol blue. The paper electrophoresis system used evidently was not satisfactory for determining the mobility of the crystalline material. There was apparently no ordinary serum protein present however since the serum controls separated satisfactorily. The micro-Kjeldahl analysis gave a value of 14% nitrogen. The ninhydrin test was positive. The U-V absorption curve (Fig. 4) showed a single peak with a maximum at 276 mμ. The emission spectra (Fig. 5) for crystals and controls showed that both contained traces of zinc, aluminum, boron, copper, iron, manganese and phosphorus. These traces (less than 1 part per million) were too small to be measured quantitatively and can be considered as impurities commonly found in biological materials.

TABLE II. Solubility of Charcot-Leyden Crystals.*

Solvent	Soluble†
Cold water (20° C)	—
Hot " (68° C)	+
$NaHCO_3$ 5%	—
Na_2CO_3 5%	+
HCl .005 N	—
" .0075 N	+
NaOH .005 N	—
" .0075 N	+
Acetic acid 1.25%	—
" " 2.5%	+
NH_4OH 2%	—
" " 5%	+

* The crystals were insoluble in alcohol, acetone, ether, chloroform and carbon tetrachloride. They dissolved slowly in equeous solvents (16-48 hr in .005 N HCl or .005 N NaOH; several days in distilled water at 4°-8° C).

† Dissolved immediately.

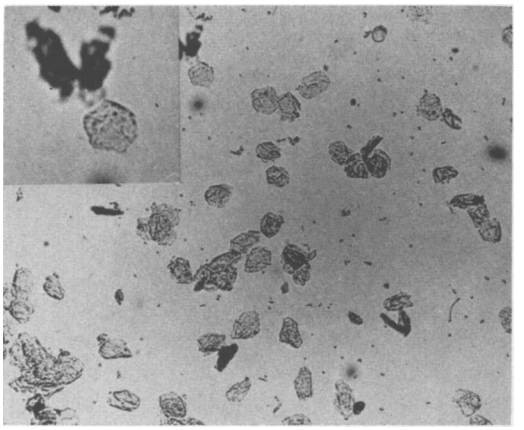

FIG. 3. Charcot-Leyden crystals recrystallized at pH 7.2.

Discussion. Repeated comparisons between freshly collected and preserved crystals seem to indicate that this method of concentration and preservation of C-L crystals causes no change in their histochemical reactions or solubility and is a useful means of accumulating a quantity of crystals suffi-

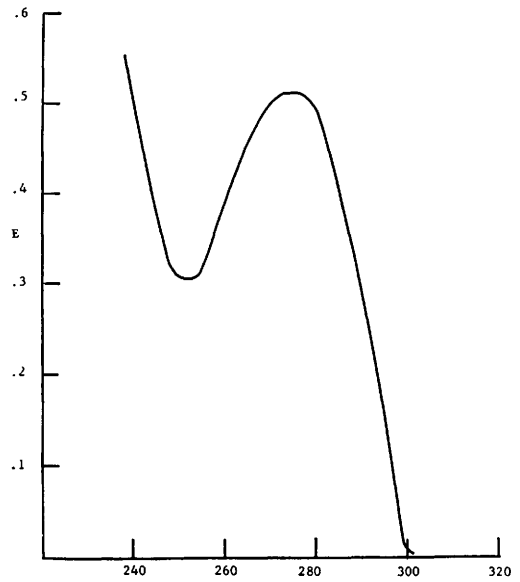

FIG. 4. Absorption spectrum of dissolved Charcot-Leyden crystals (0.3% solution, pH 2).

ciently large for biochemical or other studies. The finding that the crystals do not stain like eosinophil granules agrees with that of Welsh (4) who, nevertheless, demonstrated their formation from these granules. Considering the low solubility of the crystalline material at neutrality, it is possible that the internal medium of the granule is acid and the pH change which occurs when the granule disrupts is responsible for both the tendency to form crystals and the difference in staining reactions between the crystal and the parent granule. With respect to the chemical nature of the crystal, the nitrogen content, positive ninhydrin reaction, positive Millon's reaction and staining reactions argue in favor of a protein type substance. Opposed to these are the low molecular weight (at pH 2.4) and the fact that the crystals were not visibly affected by proteolytic enzymes. Close packing of the molecules in the crystal may have caused the enzyme sites to be inaccessible. Enzyme studies on dissolved crystals would be possible only in the case of pepsin, since the material is relatively insoluble at pH values optimal for the other enzymes studied. However, some low molecular weight polypeptides (*e.g.*, tyrocidin, bacitracin) are not attacked by proteolytic enzymes. The spectrographic analysis indicated that the crystals did not contain significant amounts of either zinc or phosphorous.

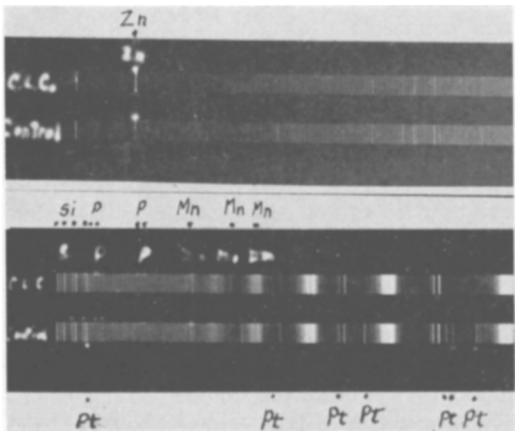

FIG. 5. Emission spectrum of Charcot-Leyden crystals.

The evidence presented thus far suggests that C-L crystals are composed of a low molecular weight polypeptide which is relatively insoluble at neutrality but readily soluble in weak acid or base. The strongly positive Millon reaction and the degree of U-V absorption at pH 2 suggest the possibility that tyrosine may be one of its major components. Further studies are required to determine whether or not it has any physiological activity.

During the course of the work a second type of crystal (Fig. 6) was frequently observed which differed from the typical C-L crystal in shape and which was not soluble in 0.1 N HCl or 0.1 N NaOH. Usually, it was found in small numbers associated with C-L crystals but in one case of dysentery it was the only crystal present. The amount of material available did not permit a further study of its characteristics.

Summary. C-L crystals concentrated and preserved as described did not differ from freshly collected crystals in histochemical reactions or solubility when kept over a period of 9 months. Thus it was feasible to accumulate a quantity of crystals sufficient for biochemical determinations.

Advantage was taken of the solubility of the crystals at pH 2.4 to separate them from residual extraneous material. Histochemical reactions, absorption spectrum, biochemical

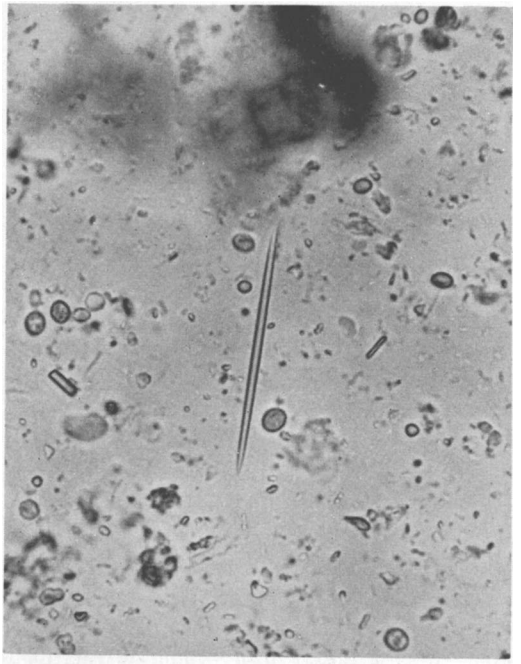

FIG. 6. Crystal frequently found in association with Charcot-Leyden crystals in feces. ×390.

tests and molecular weight studies suggest that the material composing C-L crystals is a polypeptide of low molecular weight containing tyrosine or tryptophane or both. It is soluble at pH 2 and pH 11.2. It was possible to recrystallize a dilute (0.5%) solution of C-L crystals by adjusting the solution to neutrality. No measurable amounts of phosphorus or zinc could be detected by spectrographic analysis.

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Effect of Thiabendazole Upon Experimental Trichinosis in Swine. (27443)

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The anthelmintic property of thiabendazole has been reported recently by Brown *et al.* (1), and Cuckler(2), and the activity of this compound against trichinosis in rats and mice has been reported by Campbell(3). Since subsequent experiments have revealed that large, well-tolerated doses of thiabendazole kill *Trichinella* larvae in the muscle of mice, experiments were carried out to determine the effect of this compound on trichinosis in swine.

Materials and methods. Pigs were exposed to *Trichinella spiralis* larvae recovered by digestion from the ground carcasses of experimentally infected mice and rats. The number of larvae recovered was estimated by a sampling method. In Experiment 1 the larvae were given to the pigs with little or no removal of the digestive solution. In Experiment 2 the larvae were washed by sedimentation in water, before being given to the pigs. The larvae were administered through rubber tubing inserted into the stomach or esophagus. The suspension of larvae was stirred continuously while the inocula were being withdrawn.

In both experiments muscle samples taken