FORMATION OF CHARCOT-LEYDEN CRYSTALS IN HUMAN EOSINOPHILS AND BASOPHILS AND STUDY OF THE COMPOSITION OF ISOLATED CRYSTALS

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PLATES 17 TO 19

(Received for publication, February 9, 1965)

In 1853 Charcot and Robin (1) observed microscopic crystals in the spleen and blood postmortem in a case of chronic leukaemia. Nineteen years later Leyden (2) described crystals of similar appearance in the sputum of a patient suffering from asthma. The crystals had the characteristic shape of two pyramids joined base to base, and it is now customary to call crystals of this shape Charcot-Leyden crystals. Charcot-Leyden crystals have been found in blister fluid (3) and in eosinophilic granuloma of bone (4) and have been formed *in vitro* from the blood of patients with eosinophilia (5).

We report here certain conditions necessary for the formation of Charcot-Leyden crystals from human eosinophils and basophils. Methods are described for the isolation of these two cell types from donor blood and for the preparation of cell extracts from which Charcot-Leyden crystals were formed. The results are given of some chemical investigations performed on the crystals.

The patient providing the eosinophils was a young man recently returned to Australia from a stay in Rabaul, New Guinea, where he had lately been hospitalized for several weeks with what was diagnosed as hepatitis. Soon after returning he was admitted to the Sydney Hospital where he was found to have a marked anemia, coupled with a great increase in his leukocytes, 75 per cent of which were eosinophils. A biopsy of the liver showed it to be heavily infiltrated with eosinophils along the portal tracts extending into its lobules. The hepatic parenchymal cells were themselves well preserved. Examination of the faeces showed numerous pus cells and a profusion of Charcot-Leyden crystals.

Since the patient's discharge from the hospital, nearly 2 years ago, he has seemed clinically well and has returned to his position as a bank clerk; but he still has about 35,000 white blood cells per mm^3 in his blood, of which approximately 90 per cent are eosinophils.

The case is obviously one of "tropical eosinophilia," an entity of which so little is known that the term is placed between quotation marks in books on tropical diseases.[1]

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* Supported in part by United States Public Health Service, Grant RF 70-01.

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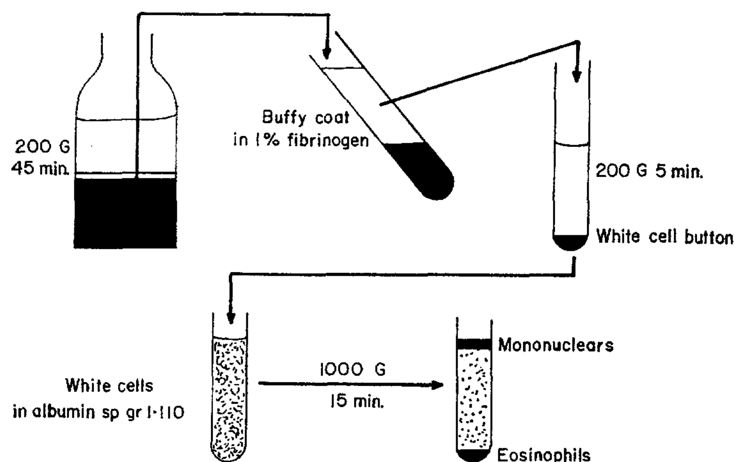
[1] Cheever, G. C., Diseases of the Tropics, New York, Appleton-Century-Crofts, 1951, 518.

Materials and Methods

White cells were obtained from a 500 ml bottle of fresh citrated donor blood by a method shown diagrammatically in Text-fig. 1. The blood was centrifuged at 200 *g* for 45 minutes and the supernatant plasma aspirated into another bottle. The aspirating needle was then inserted further into the blood bottle and the buffy coat layer, together with a small amount of residual plasma and some red cells, was collected. Five volumes of the buffy coat suspension mixed with one volume of 5 per cent human fibrinogen solution in saline were poured into an inclined 6 × 1 inch test tube. Human fibrinogen was obtained as a sterile powder from Commonwealth Serum Laboratories, Melbourne, Australia. The red cells formed rouleaux in the fibrinogen solution and settled to the bottom of the test tube within 15 minutes. The supernatant plasma containing white cells, platelets, and a few red cells was aspirated into two plastic test tubes 3 × ¾ inches and the tubes and contents were centrifuged at 200 *g* for 5 minutes to deposit the white cells.



TEXT-FIG. 1. Diagrammatic representation of the method used for isolation of human eosinophils from a 500 ml bottle of donor blood.

Isolation of Eosinophils.—The white cell buttons from the two test tubes were mixed with 3.5 ml 60 per cent bovine albumin solution in physiological saline (sp gr 1.110). The bovine albumin was obtained as a sterile powder from the Commonwealth Serum Laboratories. The cells in albumin were centrifuged at 1000 *g* for 15 minutes. Eosinophils are the heaviest white cells and passed to the bottom of the tube while the other white cells remained suspended in the albumin or rose to the top of the albumin column. Approximately 50 per cent of the white cells present in the cell button were eosinophils, the remainder being neutrophils. The cells in the button were washed twice in physiological saline solution.

Isolation of Basophils.—The white cells from one bottle of blood were mixed with 3.5 ml 40 per cent bovine albumin solution (sp gr 1.085) and the suspension centrifuged at 1000 *g* for 15 minutes. Eosinophils and neutrophils settled to the bottom of the tube and monocytes rose to the top of the column. The monocyte layer was removed by aspiration and the albumin solution containing basophils and lymphocytes was transferred to another test tube with 0.1 ml of physiological saline. The tube was centrifuged at 1000 *g* for 10 minutes and the basophils, together with lymphocytes and a small number of neutrophils and monocytes, were deposited

at the bottom of the tube. Basophils constituted approximately 20 per cent of the white cells present in the cell button. The cells were washed twice in physiological saline.

Preparation of Charcot-Leyden Crystals from Eosinophil Extracts.—Contaminating red cells in the eosinophil-rich cell preparation were lysed by the addition of 2 ml 0.15 per cent sodium chloride solution. The cells were suspended in the hypotonic saline for 15 seconds, when the tube was filled with physiological saline and centrifuged. The deposited cells were suspended in a small volume of 0.15 per cent sodium chloride solution and homogenized in a teflon tissue grinder. The homogenate was centrifuged at 1000 *g* for 10 minutes to deposit solid elements and the supernatant solution was left in the refrigerator overnight to allow aggregation of insoluble particles. The particles were removed by centrifugation at 10,000 *g* for 30 minutes and the supernatant solution was ultrafiltered through 8 mm diameter visking casing tubing under reduced pressure.

Preparation of Charcot-Leyden Crystals from Basophil Extracts.—The method used was similar to the one described above for the preparation of crystals from eosinophil extracts.

Preparation of Human Eosinophil Cell Fractions.—500 ml of fresh citrated blood were obtained from the donor who had the white cell count of 42,000 with 95 per cent of them eosinophils. The white cells were obtained from the whole blood by the method outlined above for donor buffy coat. Eosinophils were not separated from other white cell types and the contaminating red cells were lysed by suspension of the cells for 15 seconds in 0.15 per cent sodium chloride solution. The white cells were then suspended in 10 ml 0.06 M sodium citrate and disrupted by suction through a fine mesh screen under negative pressure as previously described (6). The citrate solution containing eosinophil granules and disrupted cells was centrifuged at 100 *g* for 5 minutes to deposit the cell nuclei and at 500 *g* for 10 minutes to deposit eosinophil granules. The "nuclear" fraction and the granule fraction were suspended in 5 ml 0.06 M sodium citrate and were frozen and thawed four times. The supernatant solution obtained after centrifugation of the granule and nuclear fractions was subjected to ultrafiltration as described above.

Column Chromatography of Eosinophil Extracts.—Eosinophils obtained from the donor (40,000 eosinophils/mm^3) were homogenized in 0.15 per cent sodium chloride solution. The homogenate was centrifuged at 1000 *g* for 10 minutes and a clear supernatant solution obtained. The solution (50 ml) was dialysed against 0.01 M sodium barbitone buffer and applied to a DEAE-cellulose column. Elution was performed with a continuous gradient of buffer varying from 0.01 M sodium barbitone to 0.1 M sodium phosphate buffer at pH 6 and 20 ml samples were collected. The original extract was tested for protein, pentose, deoxyribose, phosphorus, and carbohydrate content and eluates were tested for protein, deoxyribose, and pentose content. 2 ml aliquots of the eluates were submitted to ultrafiltration and solid material inside the dialysis tubing was examined microscopically for the presence of Charcot-Leyden crystals.

Chemical Estimations.—Protein estimations were performed by the Method of Lowry *et al.* (7). Hydrolytic enzymes were assayed by methods described by Cohn and Hirsch (8). Peroxidase was estimated by the method of Jermyn and Thomas (9) using guaiacol as the chromogenic receptor. Total phosphorus was determined by the method of Fiske and Subba-Row (10). Absorbancy at 260 μ and 280 μ was determined using a Unicam Sp 500 spectrophotometer. Pentose was assayed by the method of Ogur and Rosen (11), deoxyribose by the method of Seibert (12), and the crystals were tested for the presence of carbohydrate by the Molisch test.

RESULTS

Formation of Charcot-Leyden Crystals in Eosinophils.—Charcot-Leyden crystals were seen in the cytoplasm of human eosinophils suspended in physiological

saline for a period of more than 24 hours. A greater percentage of eosinophils was found to contain crystals after 48 hours than at 24 hours and almost all eosinophils contained a Charcot-Leyden crystal in the cytoplasm after 72 hours (Fig. 1.). The cytoplasmic granules appeared normal in many of the cells and the nuclear membrane was intact although frequently the nucleus had become spherical. Charcot-Leyden crystals were found at similar time intervals in eosinophils suspended in serum or in hypotonic saline (0.15 per cent sodium chloride solution).

Formation of Charcot-Leyden Crystals in Basophils.—Charcot-Leyden crystals formed within 2 minutes when basophils were suspended in hypotonic saline. The process of formation of a crystal was observed on many occasions and involved the following changes: There was a gradual swelling of the cell and the cell nucleus; the nuclear lobes fused together until only one large rounded structure remained; the basophil granules became swollen and burst; at one point in the cytoplasm a tiny crystal formed and increased in size until it stretched across the cell (Figs. 3 *a* and 3 *b*). Of interest was the frequent observation of a tiny Charcot-Leyden crystal inside a swollen basophil granule (Fig. 3 *c*). Occasionally a granule containing a crystal was seen to burst, releasing the crystal into the cytoplasm, where it acted as a nidus for the formation of a large crystal.

Formation of Charcot-Leyden Crystals from Extracts of Eosinophils and Basophils.—Crystals were formed inside the dialysis tubing following ultrafiltration of saline extracts of eosinophils. The crystals had the characteristic shape of Charcot-Leyden crystals (see Figs. 2 *a* and 2 *b*) namely that of a hexagonal dipyramid. The crystals did not dissolve in physiological saline solution or in 0.05 M acetate buffer at pH 5.0. The crystals dissolved slowly in acetate buffer at pH 4.5 and reformed when the solution was dialysed against 0.15 per cent sodium chloride and ultrafiltered. The crystals dissolved rapidly in a solution at pH 4.0 or less but were not reformed following dialysis against saline and ultrafiltration. Charcot-Leyden crystals were formed when extracts of human basophils were submitted to the same treatment as eosinophil extracts.

Eosinophil Fractions.—Charcot-Leyden crystals were present in large numbers in the supernatant fraction held at 4°C for 24 hours, but were not present in the citrate suspensions of nuclei or granules. A small number of crystals formed inside the dialysis tubing following ultrafiltration of the solution obtained from the granules suspension but not from the nuclear suspension.

Chemical Findings.—Charcot-Leyden crystals obtained from eosinophil extracts were dissolved in acetate buffer at pH 4.5. None of the following enzymes was detected in the solution, acid phosphatase, β-glucuronidase, aryl sulphatase, ribonuclease, cathepsin, and peroxidase. The saline extract of white cells from the patient with eosinophilia was found to contain 17 μg protein per million cells and following ultrafiltration Charcot-Leyden crystals formed inside the

dialysis tubing. A protein estimation performed on the crystals after washing in physiological saline showed approximately one-half (8.1 μg million cells) of the protein in the original solution was present in the crystals. Microscopic examination of the crystal preparation revealed that there was very little insoluble material present apart from the crystals (see Fig. 4). The saline extract of eosinophils contained 0.33 μg bound phosphorus and 1.5 μg pentose per million cells but no deoxyribose was detected. Phosphorus and pentose were not found present in preparations of washed Charcot-Leyden crystals and no carbohydrate was detected. Table I shows the distribution of protein and

TABLE I

Protein and Pentose Concentrations (μg/ml) in Fractions Obtained Following Gradient Elution of an Eosinophil Extract from a DEAE-Cellulose Column

The pH varied from pH 9 to pH 6 and the ionic strength from 0.01 M (sodium barbitane) to 0.1 M (sodium phosphate).

Fraction No. (20 ml volume)	Protein concentration	Pentose concentration	Ability to form Charcot-Leyden crystals
	μg/ml	*μg/ml*	
1	24	1.4	−
2	21	1.5	−
3	6	0.7	−
4	3	0.6	−
5	9	0.5	−
6	21	0.9	−
7	90	3.8	−
8	100	12.3	−
9	294	2.8	+++
10	232	3.0	+++
11	165	2.4	++
12	76	1.6	+
13	48	1.5	−
14	39	1.4	−
15	42	0.9	−

pentose in the eluates following DEAE-cellulose chromatography of the eosinophil extract. It will be seen that maximum pentose activity was detected in the eighth 20 ml sample and maximum protein was present in the ninth sample. Charcot-Leyden crystals were formed only in the fractions containing protein and less Charcot-Leyden crystals formed when an equal volume of fraction 8 containing RNA was added to fraction 9 prior to ultrafiltration.

DISCUSSION

It is known Charcot-Leyden crystals are formed frequently at sites of eosinophil accumulation and Ayres and Starkey have supposed the crystals to origi-

nate from the nucleus of eosinophils (13). This assumption arose from experiments in which eosinophils were treated with various anionic detergents. The detergents caused breakdown of the cell membrane and nuclear membrane with formation of Charcot-Leyden crystals in the solution. Eosinophil granules appeared unaffected by this treatment and the authors concluded the granules played no part in the formation of the crystals. In the present work, Charcot-Leyden crystals were found to consist of protein and to be formed from eosinophil extracts containing protein and RNA but not DNA. Crystals did not form in nuclei-rich fractions of eosinophils. From the results of these experiments it seems unlikely the crystals originate from the nucleus.

Only small numbers of crystals were formed from extracts of eosinophil granules. In contrast large numbers of crystals were obtained from granule-free and nuclear-free extracts of human eosinophils and it can be concluded that the crystals originate from the cell cytoplasm. Of interest was the finding that the RNA-rich fraction inhibited the formation of Charcot-Leyden crystals. The nature of the relationship, if any, between RNA and the cytoplasmic proteins of the eosinophil is not known but it has been suggested by Szafarz (14) that the protein of the cytoplasm of cells possesses special ability to form stable complexes with RNA. It is tempting to suggest an association between the cytoplasmic protein and RNA in eosinophils. Such an hypothesis could explain the time interval (2 to 3 days) necessary for the formation of crystals in eosinophils *in vitro*. Depolymerization of RNA is likely to occur during this time interval and with the loss of RNA crystallization of the protein may no longer be inhibited. RNAase has been shown to be present in eosinophil granules but it is not known whether release of this enzyme occurs into the cytoplasm of degenerating eosinophils.

Bessis (15) has reported previously on a formation of crystals in basophils exposed to a hypotonic saline environment. This finding was confirmed in the present study and the crystals morphologically were found similar to Charcot-Leyden crystals. The contribution of the basophil granules to crystal formation was suggested by the presence of tiny crystals inside swollen granules of basophils exposed to a hypotonic saline environment. Furthermore the formation of Charcot-Leyden crystals in the cytoplasm of basophils was usually associated with a breakdown of the cytoplasmic granules. However, insufficient numbers of basophils were available in the present study to determine the origin of the crystals in cells of this type with certainty.

Charcot-Leyden crystals were not observed in human neutrophils, lymphocytes, or monocytes, and it must be concluded that the specific protein is absent from these cells or is not present in sufficient quantity to crystallize out of solution.

SUMMARY

Methods are described for the isolation of human eosinophils and basophils from donor blood. Using these cell preparations Charcot-Leyden crystals were found to originate from both eosinophils and basophils when the cells were suspended in hypotonic saline solution. The crystals formed also when saline extracts of eosinophils and basophils were concentrated by ultrafiltration through dialysis tubing. Fractions of eosinophils were prepared and the crystals were obtained from the cytoplasmic fraction but not from the nuclear or granular fractions. Chemical studies showed the crystals to be protein in nature and some evidence is presented which suggests that RNA may decrease the tendency for the protein to crystallize out of solution.

We would like to acknowledge the technical assistance of Miss Mary Howard. Microscopic sections of the crystals were prepared by Dr. V. J. McGovern, Royal Prince Alfred Hospital, Sydney. Clinical notes of the patient with eosinophilia were provided by Dr. Susan Gordon, haematologist at the Sydney Hospital.

Addendum.—Zinc estimations have been performed on a sample of Charcot-Leyden crystals by Dr. L. G. Smythe, Australian Atomic Energy Commission. The zinc content of the dried crystals was found to be 188 ± 19 parts per million.

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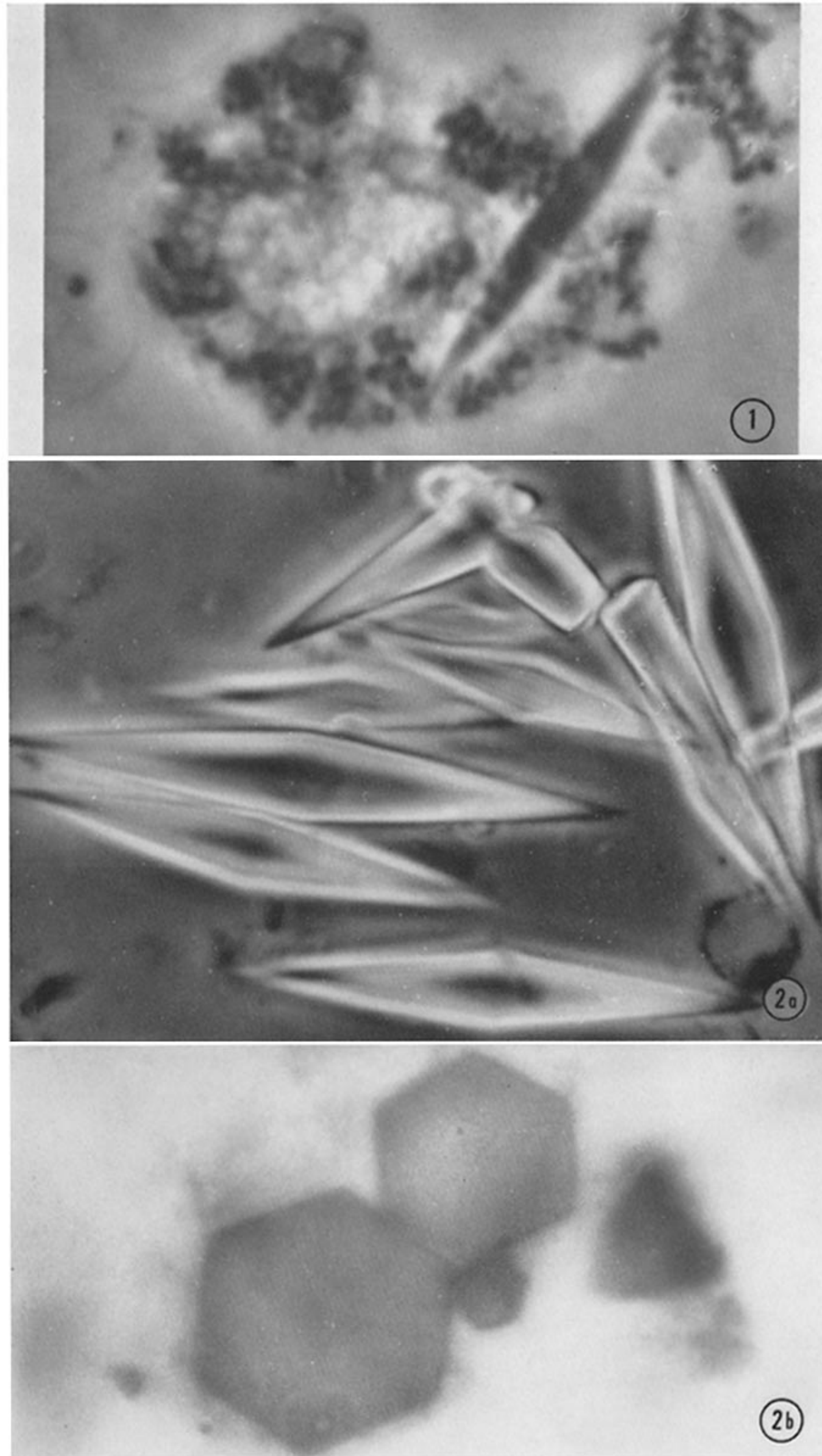
EXPLANATION OF PLATES

Plate 17

Fig. 1. A degenerating eosinophil at 72 hours after suspension in physiological saline solution. Some of the granules have been extruded from the cell and surround the tip of a Charcot-Leyden crystal. Phase contrast. Approximately × 1600.

Fig. 2 *a*. Charcot-Leyden crystals formed following ultrafiltration of a saline extract of human eosinophils. Phase contrast. × 400.

Fig. 2 *b*. Eosin-stained section showing the hexagonal appearance of three Charcot-Leyden crystals in cross section. Phase contrast. Approximately × 1600.



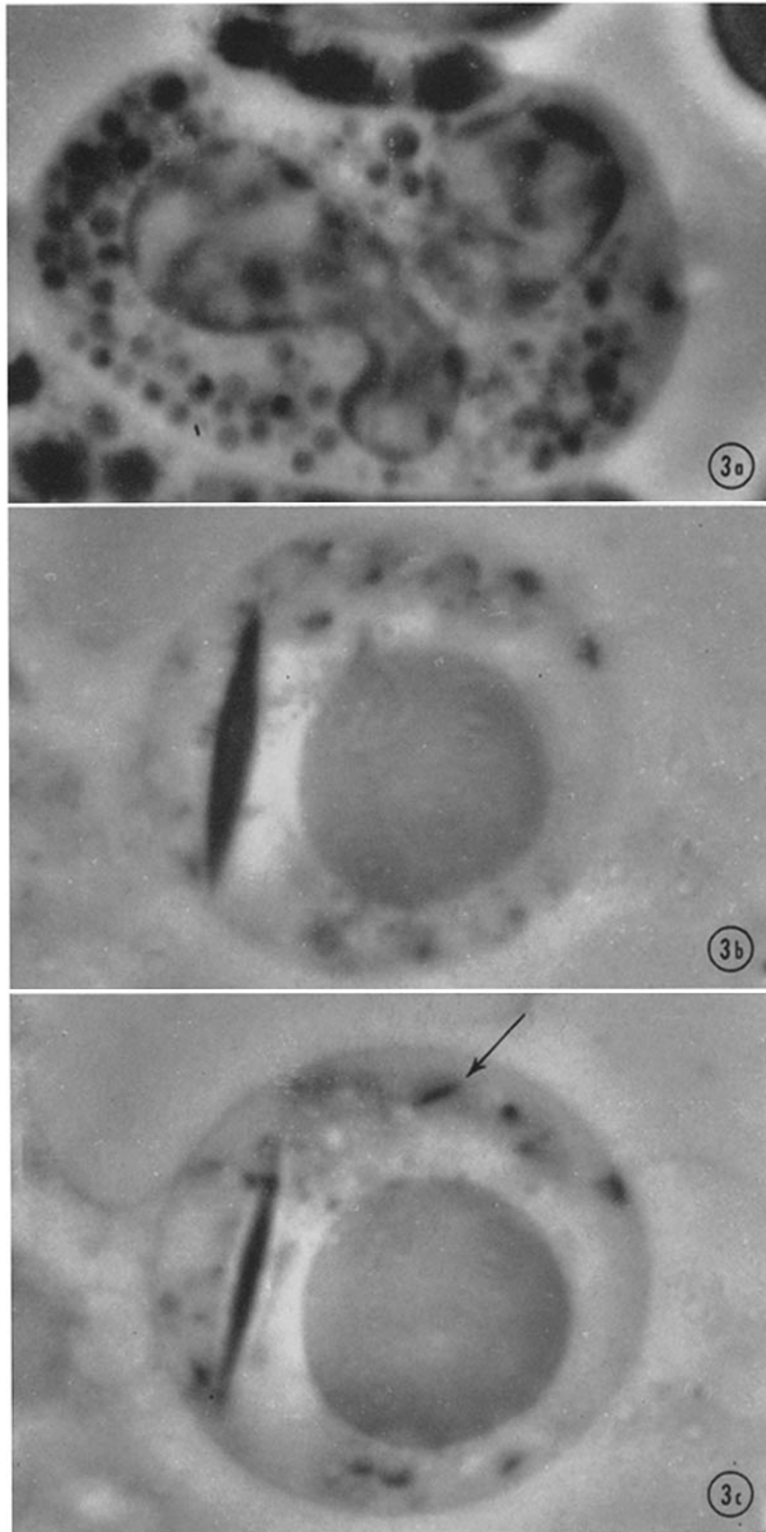
(Archer and Blackwood: Crystals in human eosinophils and basophils)

PLATE 18

Fig. 3 *a*. Normal human basophil suspended in physiological saline solution. Phase contrast. × 1600.

Fig. 3 *b*. Human basophil suspended in hypotonic (0.15) saline solution showing a Charcot-Leyden crystal in the cytoplasm. Many of the basophil granules have burst and the nucleus is rounded up. Phase contrast. × 1600.

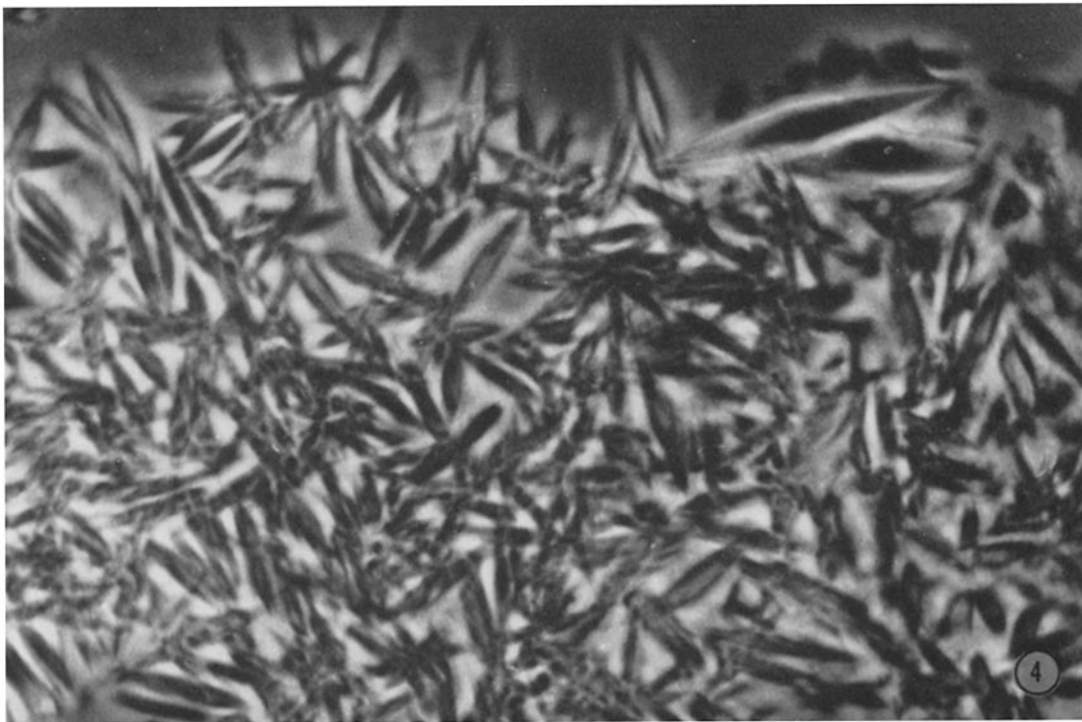
Fig. 3 *c*. The same cell as in Fig. 3 *a* in a different plane of focus showing a tiny Charcot-Leyden crystal inside a swollen granule (arrowed). Phase contrast. × 1600.



(Archer and Blackwood: Crystals in human eosinophils and basophils)

PLATE 19

FIG. 4. Charcot-Leyden crystals prepared from a saline extract of white cells from a patient with eosinophilia. Phase contrast. $\times$ 100.



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