during incubation with leucocidin for 10 min. is associated with a 20-fold increase in incorporation of ^{32}P into an acidic phospholipid distinct from the inositides. As the radioactivity of the phosphate-containing precursors of the leucocyte lipids has not been measured it is not certain that the increased incorporation represents an increased turnover of the phospholipids. However, as the increased metabolic activity leading to increased labelling of the phospholipids is induced during leucocidin action, and as reconstitution of the granules of the leucocyte does not occur, it is probable that the increased metabolic activity is associated with the actual extrusion of the proteins through the cell membrane.

There have been speculations that leucocytes may have a secretory function (cf. Richter, 1955), but direct demonstration of selective transfer of the proteins of the granules to the exterior of the cell does not appear to have been shown before. It is not at present clear if the release of protein occurs under other conditions or if the two components of leucocidin provide the cell surface with an enzymic equipment it otherwise lacks, permitting the transfer of protein to the exterior of the cell.

SUMMARY

1. The effect of leucocidin on the distribution of protein and nucleic acid in subcellular fractions of the leucocyte has been assessed.

2. Ribonuclease, deoxyribonuclease, β -glucuronidase and peroxidase have been found to be present in the proteins liberated from the leucocyte by leucocidin.

3. It is concluded that the protein released by leucocidin from the leucocyte is derived mainly if not entirely from the cell granules.

4. There is an increased incorporation of ^{32}P into the phospholipids of the leucocyte, which is

largely localized in an acidic lipid distinct from phosphoinositide.

5. There is no increased incorporation of ^{32}P into the ribonucleic acids of the leucocyte during leucocidin action.

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Further Fractionations of Histones from Calf Thymus

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Calf-thymus histones have been separated into fractions relatively rich in lysine or arginine by many methods (Stedman & Stedman, 1951; Davison & Butler, 1954; Grégoire & Limozin, 1954; Crampton, Moore & Stein, 1955; Daly & Mirsky,

1955; Davison & Shooter, 1956; Luck, Rasmussen, Satake & Tsvetkov, 1958). Recently, Johns, Phillips, Simson & Butler (1960) have shown that by combining two previous chromatographic methods (Davison, 1957; Phillips & Johns, 1959)

three main fractions can be obtained, namely: f1, a very lysine-rich histone, f2, a slightly lysine-rich histone, and f3, an arginine-rich histone. It was also found that f3 could be prepared by extracting the whole tissue with 80% ethanol made 0.25N with respect to hydrochloric acid followed by dialysis against 100% ethanol, when f3 was obtained as a precipitate.

It has been shown by starch-gel electrophoresis (Johns, Phillips, Simson & Butler, 1961) that each of these fractions, which yield bands having mobilities in the order $f2 > f1 > f3$, is complex. The fraction f2 gives three main bands and the further fractionation of this group is described in this paper. It was found that after f3 was precipitated as mentioned above, a subfraction of f2, designated f2(a), remained in the supernatant. A further part of f2, designated f2(b), could be obtained by a subsequent extraction of the residue by using a lower ethanol concentration.

A new rapid method for preparing the very lysine-rich histone directly from calf-thymus tissue, without prior isolation of the nuclei or nucleoprotein, is also given. The preparation is based on the solubility of this fraction in 5% perchloric acid. A similar extraction with trichloroacetic acid was mentioned by Bigwood (1959) but no details have been given.

EXPERIMENTAL AND RESULTS

Abbreviation. CMC, Carboxymethylcellulose.

A summary of the extraction methods employed is given in Fig. 1.

Preparation of f2(a). Calf thymus (100 g.) was extracted with ethanol-1.25N-HCl (80:20, v/v) as described for the preparation of the arginine-rich histones (Johns *et al.* 1960). The clarified extract was dialysed three times for 4 hr. at 4° against 600 ml. of absolute ethanol, to precipitate the arginine-rich histones (Plate 1b). The supernatant remaining contained the fraction f2(a), which was recovered by adding 3 vol. of acetone. The white precipitate that formed was washed twice with acetone and dried under vacuum. The yield, which was 823 mg., corresponds approximately to 35% of all the histones that can be extracted from calf thymus. The starch-gel electrophoresis picture of this fraction is given in Plate 1(a) and may be compared with that of the whole group f2, Plate 1(g), and whole histone, Plate 1(c). The total amino acid and *N*-terminal group analyses are given in Table 1, together with the amino acid analysis of this fraction extracted from starch gel as described by Johns *et al.* (1961) after a run with whole histone.

Preparation of f2(b). After the removal of the histones, f3 and f2(a), from calf thymus as above, the sediment remaining was extracted with ethanol-water-N-HCl (50:25:25, by vol.) and then with ethanol-water-N-HCl (10:65:25, by vol.) as described by Johns *et al.* 1960. The intermediate extraction serves merely as a washing, and on dialysis against ethanol only 90 mg. of protein is precipitated.

Fig. 1. Summary of methods used for the extraction of histone fractions from calf thymus.

Table 1. *Composition of histone fractions*

The amino acids are expressed as moles/100 moles of all amino acids found and no correction has been made for the hydrolytic losses of amino acids. The proportions of *N*-terminal amino acids are molar percentages of all such groups found.

Amino acid	Extraction methods			Starch gel	
	f2 (a)	f2 (b)	f1	f2 (a)	f2 (b)
Aspartic acid	6.4	{ 16.9	2.7	7.3	7.4
Glutamic acid	9.0		7.0	9.2	5.9
Glycine	11.8	7.8	5.6	10.7	8.9
Alanine	10.3	11.4	22.9	12.1	11.0
Valine	6.5	7.2	4.3	6.9	6.9
Leucine + isoleucine	14.9	10.3	6.4	12.8	10.6
Phenylalanine	1.7	1.8	1.3	1.4	1.9
Tyrosine	3.0	2.7	—	2.0	2.5
Serine*	3.2	7.6	5.5	4.3	7.2
Threonine	5.7	5.9	4.2	4.9	6.6
Proline	3.2	5.2	7.6	4.3	4.2
Histidine	1.9	3.0	0.7	3.0	3.0
Lysine	11.4	15.8	29.7	11.6	14.8
Arginine	10.9	4.5	2.1	10.3	9.2
<i>N</i> -Terminal amino acids					
Alanine	54.0	11.0	9.0	—	—
Proline	12.0	77.0	8.0	—	—
Glycine	11.0	3.0	34.0	—	—
Others	23.0	8.0	49.0	—	—
Wt. (g./mole of <i>N</i> -terminal amino acids)	112 000	17 000	52 000	—	—

* Serine values for eight separate preparations of f2 (a) have varied from 2.9 to 4.7 and from four separate preparations of f2 (b) from 7.6 to 9.8.

The ethanol-water-*N*-HCl (10:65:25, by vol.) extract was clarified by centrifuging at 20 000*g* for 1 hr., and the histones were precipitated by dialysing against ethanol. The precipitate was washed in ethanol and acetone and dried under vacuum. The yield was 454 mg. Plate 1 (e) shows that this precipitate consists of the very lysine-rich histones (f1) plus that part of the slightly lysine-rich histone that is responsible for the two slower electrophoretic bands. These were then separated as follows:

The precipitate (200 mg.) was dissolved in 50 ml. of a buffer, pH 4.2, containing 0.17*M*-acetic acid, 0.51*M*-NaOH and 0.42*M*-NaCl. This was applied to a CMC column (20 cm. × 2.4 cm.) in the H⁺ ion form, which had been equilibrated with this buffer. The same buffer (200 ml.) was then run through the column. The very lysine-rich histone did not adsorb on the CMC under these conditions and came straight through the column. The fraction f2(b) was then eluted from the column by 0.1*N*-HCl. Trichloroacetic acid was added to the solution obtained to a final concentration of 12% and left at 4° for 18 hr. The precipitate that formed was washed in acetone acidified with a few drops of conc. HCl, then three times in acetone and finally dried under vacuum; 64.6 mg. was obtained. The starch-gel electrophoresis picture (Plate 1*d*) shows that the very lysine-rich histone has been completely removed.

The total amino acid and *N*-terminal group analyses are given in Table 1, together with the total amino acid analysis of the two slower bands of f2 extracted from starch gel after a run with whole histone.

A method of preparing the very lysine-rich histone (f1). Minced calf thymus (100 g.) was homogenized with 800 ml. of 5% HClO₄ (0.75*N*) in an M.S.E. Ato-Mix blender

at top speed for 2 min. The homogenate was centrifuged at 1100*g* for 10 min. The sediment was extracted again in the same manner with 200 ml. of 5% HClO₄. The combined supernatants were clarified by filtering through a no. 4 sinter. Trichloroacetic acid was then added to the clear extract, to bring the final concentration up to 12% (0.74*M*).

After standing at 4° overnight the trichloroacetic acid precipitate that had formed was removed by centrifuging and dissolved in 100 ml. of ethanol. Conc. HCl (2 ml.) was then added, followed by 100 ml. of acetone. The white precipitate which formed was washed three times with acetone and dried under vacuum. The yield of 563 mg. is about 23% of all the histones that can be extracted from calf thymus. The total amino acid and *N*-terminal group analyses are given in Table 1 and the starch-gel electrophoresis picture in Plate 1 (f).

Amino acid analyses. Total amino acid and *N*-terminal group analyses were determined as previously described (Phillips, 1958; Phillips & Johns, 1959).

Starch-gel electrophoresis. The method used is that described by Johns *et al.* (1961).

DISCUSSION

The slightly lysine-rich histones have been separated into two distinct subfractions, f2 (a) and f2 (b), by the methods described above. The subfraction f2 (a) gives one main band on starch-gel electrophoresis whereas f2 (b) gives two main bands. Significant differences occur in the total

amino acid values for glycine, leucine + isoleucine, and lysine in these subfractions.

The yield of *N*-terminal amino acid groups is markedly different in the two subfractions (Table 1). The apparent mol.wt. of 112 000 based on the total amino acid *N*-terminal groups of f2 (a) is too high to be acceptable as the real average molecular weight. Other experiments in this Laboratory have shown that this fraction also contains a terminal acetyl group amounting to about 1 mole/20 000 g. (D. M. P. Phillips, unpublished work). The amino acid *N*-terminal groups found thus belong to minor constituents of this fraction.

Although f2(a) is the major fraction of calf-thymus histone (approx. 35 %) it is possible that many procedures for the preparation of whole histone, involving the precipitation of the chloride with ethanol, do not in fact isolate it. Its solubility in high concentrations of ethanol is obvious from its method of preparation.

Fraction f2 (b) has proline as its main *N*-terminal amino acid and an average molecular weight of about 17 000. This fraction is thus the main source of *N*-terminal proline found in the histone. It is possible that this subfraction corresponds to the fraction IIb isolated by Satake, Rasmussen & Luck (1960), which has a molecular weight of 18 000 and *N*-terminal proline amounting to 97 % of all end groups. The total amino acid analysis of this fraction was, however, not given.

A fraction (0.8S_y) giving a similar total amino acid analysis to f2 (b) was isolated by Cruft, Hindley, Mauritzen & Stedman (1957) by zone electrophoresis and ultracentrifuging. The small amount obtained by these methods was insufficient for further characterization.

The preparation of the very lysine-rich histone in quantity from calf thymus without prior isolation of nuclei or nucleoprotein is, as far as we are aware, the first method that does not involve the preparation of the whole histone and its subsequent fractionation. The fraction is a good one as judged by the lysine content and the starch-gel electrophoresis picture. The absence of tyrosine and a high proline content, other characteristics of this fraction, are apparent in the analyses. The high average molecular weight based on the *N*-terminal amino acids again indicates that at least one of the subfractions of this group is without an amino acid *N*-terminal group. It has been shown by D. M. P. Phillips (unpublished work) that acetyl groups are present in this fraction.

SUMMARY

1. The slightly lysine-rich histones have been separated into two distinct subfractions by extraction and chromatographic procedures. These

subfractions have been characterized by total and *N*-terminal amino acid analyses and starch-gel electrophoresis.

2. An extraction method for preparing the very lysine-rich histone from calf thymus, by using perchloric acid, is given.

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EXPLANATION OF PLATE 1

Plate 1. Starch-gel electrophoresis patterns under similar conditions of whole histone and histone fractions in 0.01 *N*-HCl. (In each case the origin is the edge of photograph at right.) (a) f2(a), Subfraction of the slightly lysine-rich histones. (b) f3, Arginine-rich histones (dissolved in water and reprecipitated by dialysis against ethanol). (c) Whole histone. (d) f2(b), Subfraction of the slightly lysine-rich histones. (e) f2(b), Plus the very lysine-rich histones (f1), prepared by extraction with ethanol-water-*N*-HCl (10:65:25, by vol.). (f) Very lysine-rich histone extracted with 5 % HClO₄. (g) f2, The slightly lysine-rich group of histones, prepared by chromatography on CMC.