An Investigation of Acid-Soluble Nuclear Proteins of Human Leucocytes in Relation to Fraction RP2-L, a Component of Neoplastic Cells

By A. J. MacGILLIVRAY* AND J. P. P. V. MONJARDINO

Division of Chemistry and Biochemistry, Imperial Cancer Research Fund, London, W.C. 2

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1. The claim that tumour cells contain a specific nuclear protein was investigated. The presence of this component was confirmed in Walker tumour cells by the chromatography on CM-cellulose of nuclear proteins labelled with [^{14}C]lysine. This protein was studied further in a number of human leucocyte cells. 2. The labelling of leucocyte nuclear proteins with [^{14}C]lysine was attempted during incubation and culture *in vitro*. Incorporation of the label into acid-soluble nuclear proteins was highest in normal lymphocytes cultured with phytohaemagglutinin, followed by chronic-myeloid-leukaemic leucocytes and mixed samples of normal leucocytes incubated in plasma. Little incorporation was seen in similar extracts of chronic-lymphatic or normal leucocytes. 3. Lymphocytes were the only cells that gave nuclear extracts with amino acid analysis similar to that of unfractionated histones. 4. Little of the [^{14}C]lysine in nuclear extracts of incubated leucocytes proved to be of chromosomal origin. No evidence was found of an RP2-L component in the highly labelled nuclear extracts of phytohaemagglutinin-treated lymphocytes until after 6 days of culture with [^{14}C]lysine. This component was soluble in saline. 5. Evidence is presented that fraction RP2-L is a non-histone protein constituent of cell nuclei whose labelling with [^{14}C]lysine may be dependent on the metabolic state of the cell. Thus this component is not specific to the neoplastic state.

Davis & Busch (1959, 1960) examined the acid-soluble nuclear proteins of a number of animal tissues and reported the presence of a fraction specific to tumour cells. This fraction was demonstrated by means of labelling *in vivo* with [^{14}C]lysine followed by chromatography on CM-cellulose. It was thus designated as fraction RP2-L (i.e. radioactive peak 2 with lysine). No evidence was found for the presence of this RP2-L fraction in normal, embryonic or regenerating tissues. Since the RP2-L fraction was shown to be associated with chromosomal material (Byvoet & Busch, 1961), its origin was thought to be the histones of the tumour cells.

Considerable interest has been shown in the biochemistry of histones since it has been suggested that they play a role in the control of gene expression and DNA replication (Bonner & T'so, 1964; Busch, 1965; De Reuck & Knight, 1966). However, isolation and characterization of the histones of tumour cells have shown their marked similarity to those of normal tissues (Hnilica, Johns & Butler, 1962; Laurence, Simson & Butler, 1963; Laurence, Phillips & Butler, 1966; Hnilica, 1966). Therefore

the work of Davis & Busch (1959, 1960) appeared to reveal a distinction sufficient to warrant further investigation.

The leukaemias are similar to neoplasia in solid tissues since they are associated with a breakdown in the normal control of cell differentiation. Hence in leukaemia the peripheral blood contains many immature forms of leucocytes. Of the mature cell types seen in normal peripheral blood the lymphocytes are capable of undergoing mitosis when cultured *in vitro* with a number of stimulants, e.g. PHA† (Robbins, 1964). The present paper describes an investigation of the acid-soluble nuclear proteins of human leucocytes by procedures based on those of Davis & Busch (1959).

Preliminary reports of this work have been previously published (MacGillivray & Greenwood, 1962; MacGillivray, Monjardino & Moss, 1966).

EXPERIMENTAL

Solutions. In some experiments a solution consisting of 0.8% (w/v) citric acid, 2.2% (w/v) trisodium citrate and

* Present address: Beatson Institute for Cancer Research, Glasgow, C. 3.

† Abbreviations: PHA, phytohaemagglutinin; MEM-S, minimal essential medium modified for suspension culture.

2.45% (w/v) glucose was used as anticoagulant. This is referred to as acid-citrate-dextrose. The balanced salt solution of Earle (1943), modified as described by Eagle (1959), was used as the solvent for dextran. Phosphate-saline solution consisted of 0.14M-NaCl buffered at pH 7.0 with sodium phosphate.

Radioactive compound. [^{14}C]Lysine was obtained from The Radiochemical Centre, Amersham, Berks.

Labelling of rat tissues with [^{14}C]lysine. The procedure described by Davis & Busch (1959) was used. Rats bearing Walker 256 tumours were injected with 0.14M-NaCl (1 ml.) containing $10\text{ }\mu\text{C}$ of [^{14}C]lysine (5–10 c/mole). After 1 hr. the animals were killed, and the tumours and livers removed and placed on solid CO_2 .

Preparation of human leucocytes. All glassware was silicone-treated and each sample of cells was dealt with as soon as possible after withdrawal from the donor. Samples (20–50 ml.) of peripheral blood were collected in heparin from leukaemic patients. Erythrocytes were removed by sedimentation at 37° or by low-speed centrifugation (150g for 10 min. at room temperature). In both methods the leucocytes were removed from the underlying erythrocytes by aspiration in the supernatant plasma. Leucocytes from normal donors were obtained from blood-cell residues from a plasma-fractionation process. Each residue resulted from 500 ml. of peripheral blood being collected in 100 ml. of 3% (w/v) trisodium citrate contained in a blood transfusion bottle, which was then centrifuged at 1000g for 1 hr. On receipt the bottles were recentrifuged at 500g for 10 min. at 5° and the leucocytes removed from the underlying cells by Pasteur pipette.

Incubation with [^{14}C]lysine. Leucocytes from single leukaemic and normal donors were suspended in the plasma obtained during the removal of erythrocytes. Glucose (1 mg./ml.) was added together with 0.25 ml. of 0.14M-NaCl containing 2.5–4 μC of [^{14}C]lysine (5–10 c/mole). In some experiments leucocytes from up to 20 normal donors were pooled and suspended in 100 ml. of mixed plasma to which glucose (1 mg./ml.) and 0.14M-NaCl (1 ml.) containing $10\text{ }\mu\text{C}$ of [^{14}C]lysine were added. Incubations were carried out at 37° for 20 hr. in vessels that were gently agitated.

Tissue culture of normal lymphocytes. All procedures were carried out under aseptic conditions. Samples of whole blood and blood-cell residues were obtained from a plasma-fractionation process. Each sample consisted of 500 ml. of peripheral blood that had been collected in bottles or plastic bags containing 120 ml. and 75 ml. of acid-citrate-dextrose respectively. The cell residues were obtained by centrifugation as described above for blood collected in neutral citrate solution. Each 500 ml. of whole blood was mixed with 20 ml. of balanced salt solution to which had been added 2.5 g. of Dextran 150 (Pharmacia, Uppsala, Sweden). Each cell residue was mixed with 125 ml. of balanced salt solution to which had been added 2.5 g. of Dextran 150 and 750 units of heparin. After thorough mixing sedimentation was allowed to take place by incubation at 37° for 45–60 min. The leucocyte-rich supernatant was transferred to 50 ml. centrifuge tubes, which were centrifuged at 150 g for 10 min. at room temperature. The cell pellets were suspended in a mixture of 15 ml. of pooled human sera and 35 ml. of MEM-S (Eagle, 1959). The medium contained penicillin (100 units/ml.) and streptomycin (100 μg /ml.) and in all experiments lysine was omitted. Before use the serum-MEM-S mixture was warmed to 37° and heparin (6 units/ml.) added.

Lymphocytes were isolated from the leucocyte suspension by a glass-bead-column method based on that of Rabinowitz (1964). The suspension was applied to a column (40 cm. $\times$ 2.5 cm.) of washed silicone-treated glass beads (Glass Beads for gas chromatography, size 80 mesh; British Drug Houses Ltd., Poole, Dorset) maintained at 37° . After 30 min. the unretained cells were recovered by passing MEM-S through the column. Elution of the cells was completed after 100–150 ml. had been collected. The eluted cells consisted of approx. 98% lymphocytes plus a variable amount of erythrocytes. The lymphocyte concentration was adjusted to 1.8×10^6 cells/ml. with MEM-S and serum so that the final MEM-S/serum ratio was 4:1 (v/v). Samples (5 ml.) were placed in 28 ml. (9 cm. $\times$ 2.5 cm.) screw-capped McCartney bottles and incubated for 16–18 hr. at 37° before further additions were made.

Red kidney beans (*Phaseolus vulgaris*, var. Canadian Red Wonder Bean) were obtained from Carters Seeds Ltd., Raynes Park, London, S.W. 20. PHA was extracted from these beans by the method of Borjeson, Bouveng, Gardell, Norden & Thunell (1964), taken to step 3. Cell growth and mitoses were stimulated in the lymphocyte cultures by the addition of 0.1 ml. of phosphate-saline containing 125 μg . of PHA to each McCartney bottle.

For the labelling of nuclear proteins of PHA-treated lymphocytes 1–2 μC of [^{14}C]lysine (150 c/mole) in 0.1 ml. of phosphate-saline was added to each of a number of 5 ml. cultures.

Preparation of nuclear extracts. All procedures were carried out at 4° unless otherwise stated.

(1) Rat tissue. The Walker 256 tumours and livers were minced and homogenized in 9 vol. (v/w) of 0.25 M-sucrose in a glass homogenizer fitted with a Teflon plunger giving a clearance of 0.006 in. Five to ten passes of the plunger driven by an electric motor were used. The plunger was replaced by one giving a clearance of 0.003 in. and the homogenization was repeated. The homogenate was filtered through several layers of cheesecloth and was then centrifuged at 600g for 10 min. Acid-soluble nuclear proteins were prepared by extracting the 600g pellet in approx. 5 ml. of 0.25 N-HCl/g. wet wt. of original tissue by suspension in a glass homogenizer with an electrically driven Teflon plunger. The suspension was stirred for at least 30 min. (Davis & Busch, 1959) and was then centrifuged at 41 000 rev./min. for 30 min. in the 50 rotor of the Spinco model L ultracentrifuge.

(2) Total leucocytes. After incubation with [^{14}C]lysine, the leucocytes were recovered and contaminating erythrocytes removed by the density-separator technique of Ballentine & Burford (1960). The leucocyte suspension was placed over one-tenth of its volume of diethyl phthalate-di-n-butyl phthalate (1:1.07, v/v), sp.gr. 1.081. After centrifugation at 2000g for 10 min. at room temperature the leucocytes were removed from the underlying material by aspiration in the supernatant plasma. The leucocytes were recovered by centrifugation at 300g for 10 min.

Remaining erythrocytes were removed by the osmotic-shock technique of Fallon, Frei, Davidson, Trier & Burk (1962). The leucocyte pellet from the density-separator procedure was suspended in 3–5 vol. of 0.14M-NaCl. Distilled water (three times this total volume) was added; after mixing for exactly 30 sec. iso-osmoticity was restored by adding a volume of 3.5% (w/v) NaCl equal to that of the initial leucocyte suspension. The erythrocyte-free leucocytes were recovered by centrifugation at 300g for 10 min.

After one washing in 0.14M-NaCl the cells were either used directly for the preparation of nuclei or stored at -20° .

Nuclear extracts were prepared by either of two methods.

(a) The leucocyte pellets were homogenized in 0.25M-sucrose containing 0.2% (w/v) of citric acid (Meischer, 1957) as described above for rat tissues. Acid-soluble nuclear proteins were prepared by extracting the 600g pellet in approx. 5ml. of 0.25N-HCl/g. wet wt. of original cells by suspension in a glass homogenizer with an electrically driven Teflon plunger. The suspension was stirred for at least 2hr. and was then centrifuged at 1100g for 10min. The pellet was extracted twice more with 2.5ml. of 0.25N-HCl/g. of original cells and stirring for 1hr. The three supernatants were combined and were cleared by centrifugation at 41000 rev./min. in the 50 rotor of the Spinco model L ultracentrifuge. The extract was then precipitated by the slow addition with stirring of 10vol. of acetone. The precipitate was collected by centrifugation, washed three times in acetone and dried under vacuum at room temperature.

In some experiments the 600g pellet was purified by the method of Chaveau, Moulé & Rouiller (1956). The pellet was suspended in approx. 20 vol. of 2.2M-sucrose with the aid of a loose homogenizer, the Teflon plunger of which was driven by an electric motor. The suspension was centrifuged at 25000 rev./min. for 1hr. in the 30 rotor of the Spinco model L ultracentrifuge. The supernatants were carefully decanted and the pellets containing the nuclei were retained. Nucleo-protein was obtained from such nuclear preparations by blending in 0.14M-NaCl in 0.01M-trisodium citrate in the MSE homogenizer at full speed for 2min. The homogenate was centrifuged at 1100g for 15min. The extraction was repeated but with blending at half-speed for 30sec. After centrifugation at 1100g for 15min., the pellet was retained. Leucocyte whole histone was prepared by extracting the nucleoprotein with 0.25N-HCl as described above for the extraction of acid-soluble nuclear proteins.

(b) The leucocyte pellets were suspended in 5vol. (v/w) of 4% (v/v) acetic acid (Stedman & Stedman, 1950) in a loose Teflon-glass homogenizer by hand. The suspension was then stirred vigorously for at least 2hr. by using a Teflon-coated metal bar driven by a magnetic stirrer. After filtration through fine nylon or muslin, the suspension was centrifuged at 600g for 10min. The pellet was washed at least twice in 1% (v/v) acetic acid followed by four washes in phosphate-saline containing 1mM-CaCl₂ by suspension and centrifugation at 600g for 10min. Nucleoprotein and whole histone were then prepared as described in (a).

(3) PHA-treated lymphocytes. After exposure to [¹⁴C]lysine, lymphocyte cultures were cooled in ice and the cells recovered by centrifugation at 1000g for 10min. Erythrocytes were removed by the osmotic-shock technique as described in (2). Nuclei were prepared from 3×10^7 – 6×10^7 cells by the acetic acid method described in 2(b). Acid-soluble nuclear proteins, nucleoprotein and whole histone were prepared as described in 2(a). The extracts of nuclear proteins and whole histone were dialysed for 24hr. against 2l. of 0.25N-HCl.

Preparation of other leucocyte proteins. Samples (20g.) of normal leucocytes were homogenized in 0.25M-sucrose containing 0.2% of citric acid as described above. After centrifugation at 600g for 10min. the supernatant was dialysed exhaustively against 0.25N-HCl at 4° and then centrifuged at 30000 rev./min. in the 30 rotor of the Spinco model L ultracentrifuge. Acid-soluble proteins were

precipitated from the supernatant by the addition of 10vol. of acetone. The precipitate was collected by centrifugation, washed three times in acetone and dried under vacuum. The yield of acid-soluble cytoplasmic protein was 510mg. The 600g pellet was treated with 2.2M-sucrose as described above to yield a preparation of purified leucocyte nuclei. Proteins of the nuclear sap, nucleoprotein complex and nuclear residue were then prepared by the method of Steele & Busch (1963).

Fractionation of acid-soluble nuclear proteins. CM-cellulose (0.6–0.7m-equiv. of ionizing groups/g.) was obtained as Whatman CM11 or CM70 and was washed and treated as described by Peterson & Sober (1956). Column chromatography on CM-cellulose was carried out at 4° by a method similar to that of Davis & Busch (1959). Columns (15cm. $\times$ 1cm.) were packed with CM-cellulose equilibrated with a 0.05M-acetic acid-sodium acetate buffer, pH4.0. Before chromatography acid-soluble extracts (5–12ml.) of nuclei and nucleoprotein were dialysed overnight against 1l. of 0.5N-acetic acid. The contents of the bags were then applied to the CM-cellulose columns and followed by two portions (approx. 1ml. each) of water. Gradient elution was then carried out by using a constant-volume mixing chamber containing 170ml. of water. N-Formic acid was introduced into the chamber at a flow rate of 12–15ml./hr. from a reservoir containing 500ml. of the acid solution. After 80 3ml. fractions had been collected the solution in the reservoir was changed to 8N-formic acid. A further 40 fractions were collected. Under those conditions the RP2-L fraction of the Walker 256 tumour was eluted between fractions 45 and 70 at pH2.0–2.1.

Protein concentrations in column fractions were determined by measuring E_{280} in cells of path length 1cm. In experiments with small amounts of protein the absorption was masked by that of the formic acid and hence no u.v. values have been recorded.

Radioactivity was assayed by plating 0.5ml. samples from fractions on aluminium planchets that were then dried under an infrared lamp. The samples were then counted in a gas-flow counter with a thin end window (model C110B or model C115 low-background system; Nuclear-Chicago Corp.). Counting efficiencies of these instruments were approx. 20%.

Preparation of antisera. Acid-soluble proteins were prepared from the 600g pellet of normal leucocytes homogenized in acid sucrose as described above. A sample (150mg.) of the resulting acetone precipitate was fractionated on a column (20cm. $\times$ 2cm.) of CM-cellulose as described by Johns, Phillips, Simson & Butler (1960). The following yields were obtained: fraction 1, 55mg.; fraction 2, 6mg.; fraction 3, 53mg. Rabbits were immunized at weekly intervals by the subcutaneous injection of 1ml. of a suspension consisting of 0.5ml. of Freund's adjuvant and 0.5ml. of water containing 2mg. of a CM-cellulose fraction. Two weeks after the third immunization the rabbits were injected intravenously with 5ml. of phosphate-saline containing 5mg. of the fraction. Blood was collected 10 days later by ear-vein puncture and was allowed to coagulate at room temperature. Serum was obtained by centrifugation and was incubated at 56° for 30min. After a second centrifugation the sera were stored in samples at -20° .

Detection of antibody activity. This was carried out by double diffusion in agar (Ouchterlony, 1958). Precipitation

lines were obtained with leucocyte extracts at a concentration of approx. 5 mg./ml. in 5 *MN*-acetic acid.

Amino acid analysis. Samples of protein were hydrolysed under vacuum in 6 *N*-HCl for 70 hr. at 110°. Separation and quantitative analysis of amino acids were carried out with an amino acid analyser (Technicon Instruments Co. Ltd.).

Determination of protein. Protein was determined in extracts of lymphocyte nuclei or nucleoprotein by the method of Lowry, Rosebrough, Farr & Randall (1951). Calf thymus whole histone prepared by the method of Johns, Phillips, Simson & Butler (1961) was used as standard.

RESULTS

Labelling of acid-soluble nuclear proteins of human leucocytes with [¹⁴C]lysine. The labelling of nuclear proteins was examined with different types of leucocytes. Table 1 shows the incorporation of [¹⁴C]lysine into extracts of leucocytes after incubation at 37°. Chronic-myeloid-leukaemic cells require up to 20 hr. to achieve satisfactory incorporation of the isotope into the acid nuclear extract. Under similar conditions little radioactivity was found in nuclear extracts of chronic-lymphatic or normal leucocytes. However, mixed normal leucocyte samples gave a result similar to that found with

chronic-myeloid cells. An extract of nucleoprotein prepared by saline treatment of nuclei showed that little of this [¹⁴C]lysine was associated with chromosomal protein. Lymphocytes grown in culture with PHA have been shown to synthesize histones during the DNA synthetic phase (Pogo, Allfrey & Mirsky, 1966). Table 2 shows the results of experiments with [¹⁴C]lysine used to label nuclear proteins of such cells. Acid extracts of nuclei showed very high specific activities after 1 hr. or 20 hr. labelling periods with [¹⁴C]lysine. The high level of incorporation seen after 6 days' labelling was still evident after treatment of the nuclei with saline.

Analysis of acid extracts. Amino acid analysis showed that extracts of nuclei or nucleoprotein from incubated leucocytes were unlike histone preparations from calf thymus or fresh normal leucocytes (Table 3, preparations A, D, E and F respectively). Smaller amounts of lysine, alanine and valine were found together with greater amounts of aspartic acid, cystine, phenylalanine and histidine. An analysis similar to that given by extracts from incubated normal leucocytes was obtained from a nuclear preparation of incubated chronic-myeloid-leukaemic cells (compare preparations B and A of

Table 1. *Incorporation of [U-¹⁴C]lysine into acid-soluble proteins of leucocyte nuclei*

Leucocytes were isolated by low-speed centrifugation. Then 1×10^8 – 3×10^9 leucocytes from normal or from leukaemic donors were incubated for 20 hr. in autologous plasma containing 2.5–4 μ C of [U-¹⁴C]lysine. In experiments with mixed leucocytes 5×10^{10} – 10×10^{10} cells from normal donors were incubated in a similar fashion with 10 μ C of [U-¹⁴C]lysine. The cells were recovered from the incubation medium by the density-separator technique and contaminating erythrocytes removed by osmotic shock. Nuclei were prepared by homogenization in 0.25 *M*-sucrose–0.2% citric acid. In the preparation of nucleoprotein, nuclei were obtained by the acetic acid method and disrupted in 0.14 *M*-NaCl–0.01 *M*-trisodium citrate. Extracts were made with 0.25 *N*-HCl and were precipitated with 10 vol. of acetone.

	Acid extract	Yield (mg.)	Radioactivity (counts/min./mg.)
Normal leucocytes from one donor	Nuclear	28	41
Normal leucocytes from 20 donors	Nuclear	116	290
Chronic-myeloid leucocytes from one donor	Nuclear	99	309
Chronic-lymphatic leucocytes from one donor	Nuclear	5	64
Normal leucocytes from 20 donors	Nucleoprotein	30	19

Table 2. *Incorporation of [U-¹⁴C]lysine into extracts of nuclei from PHA-treated lymphocytes*

Lymphocytes were isolated and cultured with PHA as described in the text. Then 3×10^7 – 6×10^7 cells were exposed for various intervals to [U-¹⁴C]lysine (4–7 μ C/10⁷ cells). Nuclei were isolated by the acetic acid procedure and nucleoprotein was prepared by disruption of nuclei in saline–citrate. Acid extracts were dialysed against 0.25 *N*-HCl before being analysed for protein and radioactivity.

Period of labelling after addition of PHA (hr.)	Acid extract	Yield (μ g.)	Radioactivity (counts/min./ μ g.)
71–72	Nuclear	333	151
52–72	Nuclear	231	928
0–142	Nuclear	310	490
0–136	Nucleoprotein	408	463

Table 3. *Composition of acid-soluble nuclear extracts*

Acid-soluble extracts of nuclei and nucleoprotein were prepared from fresh and incubated leucocytes as described in Table 1. Calf-thymus whole histone was prepared by the method of Johns *et al.* (1961). The amino acids are expressed as moles/100 moles of all amino acids found. No corrections were made for hydrolytic losses.

Acid-soluble nuclear proteins of incubated leucocytes				Whole histone			
Prep.	...	A	B	C	D	E	F
Amino acid		Normal	Chronic myeloid	Chronic lymphatic	Incubated normal leucocytes	Normal leucocytes	Calf thymus
Asp		8.6	9.4	6.9	7.8	5.4	4.9
Thr		4.9	5.2	5.8	5.0	4.9	5.4
Ser		4.6	4.8	5.8	4.7	4.2	5.6
Glu		9.3	9.0	7.0	10.5	9.9	8.6
Pro		4.9	4.8	5.2	4.2	5.3	5.1
Gly		7.8	7.8	8.5	8.4	8.4	8.7
Ala		10.2	10.8	14.5	9.1	12.8	13.7
Val		1.8	2.0	6.6	6.9	9.2	5.7
Cys		8.1	7.9	0.4	2.2		Trace
Met		1.4	1.2	0.8	1.5	0.8	0.9
Ile		2.9	3.4	4.1	5.0	4.4	4.2
Leu		10.6	9.6	7.9	9.0	7.7	7.9
Tyr		2.7	2.6	2.1	3.0	2.3	2.5
Phe		4.2	4.0	2.2	3.4	2.1	1.7
Lys		8.0	8.1	12.7	7.7	11.9	14.6
His		4.4	3.7	2.0	2.3	1.9	2.0
Arg		5.4	5.6	7.2	9.1	8.5	8.5

Fig. 1. Detection by double diffusion in agar of non-histone contaminants in acid extracts of normal leucocyte nuclei. (a) Centre well, antiserum against CM-cellulose fraction 1; 1, acid-insoluble nuclear proteins; 2, phenol-insoluble nuclear proteins; 3, histones isolated from 2M-NaCl-extracted nucleoprotein; 4, acid extract of whole leucocytes; 5, acid extract of whole nuclei; 6, fraction 1. (b) Centre well, antiserum against CM-cellulose fraction 1; 1, acid-soluble cytoplasmic proteins; 2, saline-soluble nuclear proteins; 3, tris-soluble nuclear proteins; 4, alkali-insoluble nuclear residue; 5, acidic ribonucleoproteins; 6, residual proteins. (c) Centre well, antiserum against CM-cellulose fraction 2; 1, as in (a) 3; 2, histones from leucocyte nucleoprotein; 3 and 6, tris-soluble nuclear proteins; 4 and 5, residual proteins.

Table 3). A nuclear extract from chronic-lymphatic-leukaemic cells had an amino acid analysis not unlike that of calf thymus whole histone (Table 3, preparations C and F).

An attempt was made to identify the proteins in an acid extract of nuclei from a mixed leucocyte incubation. The extract was fractionated on CM-

cellulose (Johns *et al.* 1960) and antisera were raised against the three resulting fractions. These antisera were characterized by the Ouchterlony technique and were found to react with proteins of both leucocyte nuclei and cytoplasm (Fig. 1). The nuclear proteins involved appeared to be the tris-soluble proteins of the nuclear sap (Fig. 1b). There was no

Fig. 2. Chromatography on CM-cellulose of ^{14}C -labelled acid-soluble nuclear proteins from tissue of rats bearing Walker 256 tumours. The animals were killed 1 hr. after the injection of $10\text{ }\mu\text{C}$ of $[^{14}\text{C}]\text{lysine}$. Then 12 ml. of each acid-soluble nuclear extract was applied to columns (15 cm. $\times$ 1 cm.) of CM-cellulose equilibrated with 0.05 M sodium acetate buffer, pH 4.0. (a) Tumour extract; (b) liver extract. (1) indicates start of gradient towards 1 N-formic acid through 170 ml. of water; (2) indicates change of limiting concentration of formic acid to 8 N. —, E_{280} ; ----, radioactivity.

cross-reaction with histones prepared by acid extraction of nucleoprotein from fresh normal leucocytes (Fig. 1c).

Chromatography on CM-cellulose. The chromatographic properties of the animal-tumour RP2-L fraction were established by preparing acid extracts of nuclei from tissues of tumour-bearing rats 1 hr. after the injection of $[^{14}\text{C}]\text{lysine}$. Fig. 2 shows the

Fig. 3. Chromatography on CM-cellulose of ^{14}C -labelled acid-soluble proteins of nuclei of lymphocytes cultured with PHA. The extracts were prepared as described in Table 2. Chromatographic conditions were similar to those described in Fig. 2. (a) 71–72 hr. labelling period; (b) 52–72 hr. labelling period. ----, Radioactivity.

elution pattern of protein and ^{14}C radioactivity after chromatography on CM-cellulose under the conditions described by Davis & Busch (1959). The Walker-tumour extract was found to contain a ^{14}C -labelled component that was eluted between fractions 45 and 70 and that was absent from the liver extract. This would appear to be the RP2-L component described by Davis & Busch (1959).

Since the nuclear extracts from PHA-treated lymphocytes showed high specific activities (Table 2) these were fractionated on CM-cellulose under identical conditions. Acid-soluble extracts from nuclei of cells labelled for 1 hr. or 20 hr. periods showed two peaks of $[^{14}\text{C}]\text{lysine}$ activity (Figs. 3a and 3b), whereas a similar extract from cells labelled for 6 days showed three peaks of ^{14}C radioactivity (Fig. 4a). The additional component had an elution volume similar to that of the animal-tumour RP2-L fraction. However, this lymphocyte com-

Fig. 4. Chromatography on CM-cellulose of ^{14}C -labelled nuclear extracts of lymphocytes cultured with PHA and ^{14}C lysine for 6 days. The extracts were prepared as described in Table 2. Chromatographic conditions were similar to those described in Fig. 2. (a) Acid extract of nuclei; (b) acid extract of nucleoprotein. ----, Radioactivity.

ponent could be removed if the nuclei were treated with saline (Fig. 4b).

DISCUSSION

During this investigation we have made a number of basic assumptions. The first concerns the possibility that tumour cells contain a specific nuclear protein. The present results confirm those of Davis & Busch (1959, 1960) and Lettré (1965). Davis & Busch (1959, 1960) found that their RP2-L component was eluted from CM-cellulose at pH 2.25. In our experiments the RP2-L fraction appeared at pH 2.0–2.1, a difference probably caused by the

different grade of cellulose used. The second assumption is that the RP2-L fraction is a histone. Initially Byvoet & Busch (1961) showed that the RP2-L fraction was associated with chromosomal material. Since the RP2-L fraction was acid-soluble and was retained on CM-cellulose at pH 4.0, Davis & Busch (1959) assumed it was a histone, but the present results show that this is not necessarily correct. Thirdly, it has been assumed that labelling *in vitro* will yield results similar to those found *in vivo*. This aspect appears valid since RP2-L components have been detected in a human melanoma and in bone-marrow cells after labelling *in vitro* with ^{14}C lysine (Davis & Busch, 1960; Hidvegi, Arky, Antoni & Varteresz, 1963). Other investigations have shown that human leucocytes in short-term culture actively synthesize nucleic acids and proteins in a manner comparable with that found in animal tissues *in vivo* (Rubin & Cooper, 1965; Pogo *et al.* 1966).

Human peripheral leucocytes appear to be relatively inactive in the incorporation of ^{14}C lysine into nuclear proteins. This parallels the low rate of DNA synthesis found in normal leucocytes (Bond, Cronkite, Flidner & Schork, 1958) and the evidence that even leukaemic cells are not highly proliferating systems (Astaldi, Airo & Sauli, 1965; Schmidt, Oechsli, Frick & Moeschlin, 1967). The increased activity seen in the experiments with mixed leucocytes may represent some aspect of the increased cell growth and DNA synthesis seen during the culture together of leucocytes from unrelated donors (Bain, Vas & Lowenstein, 1964). The experiments with mixed leucocytes showed that little of the ^{14}C lysine incorporated into leucocyte nuclei was associated with chromosomal protein. Immunological analysis suggested that acid extracts of leucocyte nuclei contained nuclear-sap proteins and contaminants from the cytoplasm. Nevertheless, an acid extract of nucleoprotein from incubated leucocytes still did not show an amino acid analysis similar to that of unfractionated histones. The presence of considerable amounts of proteolytic activity in leucocytes (Cline, 1965; Haschen & Krug, 1966) may have affected the composition of these extracts. In contrast, acid extracts of lymphocyte nuclei gave an amino acid analysis resembling that of calf thymus whole histone. Moreover, normal lymphocytes when cultured with PHA gave nuclear extracts showing considerable incorporation of ^{14}C lysine. It was therefore decided to fractionate these extracts on CM-cellulose under conditions that showed an RP2-L fraction to be present in tumour nuclei. The results showed that no RP2-L component could be detected in PHA-treated lymphocyte nuclei until after 6 days of exposure to ^{14}C lysine.

These results support those of other investigations

with non-tumour cells. Hidvegi *et al.* (1963) showed that a component similar to the RP2-L fraction is present in normal rabbit bone-marrow cells. Lettré & Otto (1966) demonstrated that an RP2-L protein could be detected in normal rat liver if the time of labelling was increased to 6 hr. Hence the rate of labelling of this fraction with [^{14}C]lysine appears to differ in the tissues examined, indicating that its appearance may be linked to the metabolic state of the cell. Thus the RP2-L component is not specific to neoplasia, since it can be found in normal cells provided that conditions are suitable for it to be labelled with [^{14}C]lysine.

This raises the question of the nature of the RP2-L component. Busch, Byvoet & Adams (1963) showed that, when acid extracts of nucleoprotein were fractionated on CM-cellulose, similar elution patterns were obtained from both tumour and normal tissues. As a result Busch *et al.* (1963) suggested that their RP2-L fraction consisted of a mixture of histones and other nuclear proteins. This proposal was supported by the evidence that the RP2-L fraction of Walker tumour cells was heterogeneous and probably contained non-histone protein (Busch, Hnilica, Chien, Davis & Taylor, 1962). Hence, when nucleoprotein was prepared from normal and tumour cells, only histones were extracted in acid and these gave similar elution patterns on CM-cellulose. The results of the present investigation add confirmation to these suggestions since the RP2-L fraction of PHA-treated lymphocytes was soluble in saline and hence was not a histone. A number of non-histone proteins of the nucleus and nucleolus have been found to show heavy labelling with [^{14}C]lysine (Steele & Busch, 1963; Grogan, Desjardins & Busch, 1966). Since some of these are basic and soluble in saline, it is possible that the RP2-L component could contain a protein of this type.

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