Role of Adenosine Deaminase, Ecto-(5'-Nucleotidase) and Ecto-(Non-Specific Phosphatase) in Cyanide-Induced Adenosine Monophosphate Catabolism in Rat Polymorphonuclear Leucocytes

Andrew C. NEWBY*

Department of Clinical Biochemistry, University of Cambridge,
Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QR, U.K.

(Received 12 September 1979)

1. The role of adenosine deaminase (EC 3.5.4.4), ecto-(5'-nucleotidase) (EC 3.1.3.5) and ecto-(non-specific phosphatase) in the CN-induced catabolism of adenine nucleotides in intact rat polymorphonuclear leucocytes was investigated by inhibiting the enzymes *in situ*. 2. KCN (10 mM for 90 min) induced a 20–30% fall in ATP concentration accompanied by an approximately equimolar increase in hypoxanthine. ADP, AMP and adenosine concentrations were unchanged, and IMP and inosine remained undetectable (<0.05 nmol/ 10^7 cells). 3. Cells remained 98% intact, as judged by loss of the cytoplasmic enzyme lactate dehydrogenase (EC 1.1.1.27). 4. Pentostatin (30 μ M), a specific inhibitor of adenosine deaminase, completely inhibited hypoxanthine production from exogenous adenosine (55 μ M), but did not block CN-induced hypoxanthine production or cause adenosine accumulation in intact cells. This implied that IMP rather than adenosine was an intermediate in AMP breakdown in response to cyanide. 5. Antibodies raised against purified plasma-membrane 5'-nucleotidase inhibited the ecto-(5'-nucleotidase) by 95–98%. Non-specific phosphatases were blocked by 10 mM-sodium β -glycerophosphate. 6. These two agents together blocked hypoxanthine production from exogenous AMP and IMP (200 μ M) by more than 90%, but had no effect on production from endogenous substrates. 7. These data suggest that ectophosphatases do not participate in CN-induced catabolism of intracellular AMP in rat polymorphonuclear leucocytes. 8. A minor IMPase, not inhibited by antiserum, was detected in the soluble fraction of disrupted cells.

Most mammalian cells contain enzymes capable of degrading AMP to nucleosides and purine bases (Murray *et al.*, 1970). This process may operate to transfer preformed purines between cells (Murray, 1971) or to prevent a large increase in AMP concentration during a fall in ATP concentration (Atkinson, 1968; Chapman & Atkinson, 1973; Chapman *et al.*, 1976). AMP degradation to adenosine may have a more specific role in feedback regulation of the catabolic stimulus (Berne, 1964; Fain *et al.*, 1972; Berne & Rubio, 1974).

The enzyme activities detected suggest the existence of two alternative pathways of AMP degradation (Scheme 1), which naturally raises the question as to which, if either, pathway predominates under a

given metabolic circumstance. It is also true that the detection of an enzyme in a cell homogenate does not necessarily imply its participation in a given metabolic pathway. This is a particular problem with the enzyme 5'-nucleotidase, which has been rigorously located on the external face of the plasma membrane in several mammalian cells (de Pierre & Karnovsky, 1974*a,b*; Newby *et al.*, 1975; Frick & Lowenstein, 1976), raising doubts as to whether it can participate in internal AMP degradation.

As a first step to understanding the control of the degradation route of AMP and the possible involvement of ecto-(5'-nucleotidase) in this process, AMP degradation has been examined in a pure intact isolated cell preparation under a single catabolic stimulus. Intact cells have been used in order to preserve the compartmentalization and endogenous regulation of the enzymes involved. Polymorphonuclear leucocytes were chosen because they were obtainable pure, in large quantities and were much

Abbreviations used: pentostatin, R-3-(2-deoxy- β -D-erythro-pentofuranosyl)-3,6,7,8-tetrahydro-[4,5-d][1,3]-diazapin-8-ol; h.p.l.c., high-pressure liquid chromatography.

* Beit Memorial Research Fellow.

Scheme 1. Pathways of AMP degradation

more mechanically stable than collagenase-isolated cells (Rodbell, 1966; Newby *et al.*, 1975; Stanley & Luzio, 1979). This meant that ATP catabolism from lysed cells was minimized and was indeed estimated independently under each experimental circumstance. Cyanide poisoning was used as a convenient model of anoxia.

To determine whether AMP catabolism takes place via IMP or via adenosine, adenosine deaminase was blocked *in situ* by a potent specific inhibitor, pentostatin, and the effect of this on degradation of endogenous substrates and on exogenously added adenosine assessed. The involvement of ecto-(5'-nucleotidase) was examined by inhibition of the enzyme with a specific antibody. Ecto-(non-specific phosphatase) was blocked by saturation with exogenous β -glycerophosphate.

Experimental

Preparation and purification of rat polymorphonuclear leucocytes

Male Wistar rats (200–300 g) were obtained from Ralph Tuck, Rayleigh, Essex, U.K. at least 2 days before use, and fed and watered *ad lib.* throughout. About 16–20 h before they were killed, the rats were injected intraperitoneally with approx. 20 ml of sterile 12% (w/v) sodium caseinate (Difco, Detroit, MI, U.S.A.) in iso-osmotic (0.9%) NaCl (de Pierre & Karnovsky, 1974a). Animals were killed by ether asphyxiation, the peritoneal cavity was opened and the peritoneal exudate collected. After being filtered through three layers of surgical gauze, exudates were centrifuged at 200 g for 10 min, and the pellets resuspended for 5 min in 20 ml of iso-osmotic (0.85%) NH_4Cl per rat. This procedure led to lysis of erythrocyte contaminants (Hempling, 1973). The cell suspension was centrifuged as before and the pellet resuspended in the same volume of Krebs–

Ringer bicarbonate buffer (1.3 mM- Ca^{2+} , pH 7.4; Cohen, 1957) containing 0.2% bovine serum albumin (fraction V, batch no. AC2864; Armour, Eastbourne, Sussex, U.K.). A portion (20 ml) of suspension was layered over 5 ml of a sterile solution of 5.7% (w/v) Ficoll 400 and 9% (w/v) sodium diatrizoate (Ficoll-Paque obtained from Pharmacia, Uppsala, Sweden). After centrifugation at 400 g for 45 min, cells that passed through the Ficoll-Paque layer were collected after aspiration of the supernatant liquids (Böyum, 1968). Purified polymorphonuclear leucocytes were washed twice by resuspension in the original volume of Krebs–Ringer bicarbonate buffer and centrifugation at 200 g for 10 min. Cells were finally resuspended in the same buffer or buffer supplemented with 10 mM-KCN and re-adjusted to pH 7.4 with HCl. All operations were carried out at room temperature (20–23°C).

Homogenates were produced at 0°C by sonication at 20 μm tip amplitude for 30 s in a model PG 100 sonicator (MSE, Crawley, Sussex, U.K.).

Incubations and inhibitors

Cell suspensions (0.5 or 1.0 ml containing 1×10^7 – 4×10^7 cells/ml) were incubated in 10 ml-capacity flat-bottomed polystyrene tubes (D10; Sherwood, Crawley, W. Sussex, U.K.) with tight-fitting polythene caps. Incubation was for up to 90 min at 37°C in a shaking incubator (80 rev./min) and under an atmosphere of O_2/CO_2 (19:1). For the determination of intact-cell AMPase activity, to 0.5 ml of cell suspension was added 50 μl containing 154 mM-NaCl, 15 mM-adenosine, 2 mM-AMP and 50 000–100 000 c.p.m. of [^3H]AMP. Incubations were terminated after 5–15 min and processed as described previously (method 3 of Newby *et al.*, 1975).

Anti-(rat erythrocyte) and non-immune rabbit sera were gifts from Dr. J. P. Luzio of this labora-

tory and were prepared as described previously (Luzio *et al.*, 1976). Anti-(5'-nucleotidase) sera, prepared by multiple intradermal injection of 5'-nucleotidase purified as described by Widnell (1975) into rabbits, were given by Dr. K. K. Stanley of this laboratory. Complement was inactivated by heating sera at 56°C for 45 min (Lachmann, 1968). Anti-sera were added to cell suspensions without prior dilution.

Pentostatin (a generous gift from Dr. H. W. Dion, Parke-Davis, Detroit, MI, U.S.A.) in aqueous solution was added in a small volume (1–3% of total incubation volume) to yield the required final concentration. Sodium β -glycerophosphate was added from an iso-osmotic (103 mM) solution to the required final concentration and the resultant dilution of the cell suspension allowed for.

Separation of cells and medium

Centrifugation at 15 000 *g* for 15 s in pointed tubes in an Eppendorf model 5412 centrifuge (Anderman, E. Molesey, Surrey, U.K.) was followed by aspiration of the medium. Alternatively centrifugation at 200 *g* for 10 min was used.

High-pressure liquid chromatography

H.p.l.c was conducted as described by Anderson & Murphy (1976). For the improved estimation of adenosine, the concentration of methanol in the elution buffer was increased to 10% (v/v). Elution was at 2 ml/min and samples (10–200 μ l) were injected after processing as follows. To 1 ml of cell suspension or medium separated from cells as described above, 100 μ l of 50% (w/v) trichloroacetic acid was added, and the mixture immediately cooled on ice for 30 min. The mixture was then centrifuged at 3000 *g* for 5 min at 4°C and the supernatant aspirated. After addition of 100 μ l of 1 M-HCl the solution was extracted at room temperature with 3 $\times$ 10 ml of water-saturated diethyl ether. The solution was then neutralized with 100 μ l of aq. 1 M-NH₃. Traces of ether were removed by evacuation to a pressure of 133 Pa by using a vacuum pump with a liquid-N₂ trap. The samples were then passed through a 0.22 μ m Millipore filter (Millipore, London N.W.10, U.K.) to remove particulate matter.

The amount of compounds present in experimental samples was quantified by comparison of the peak absorption at 254 nm of eluted compounds with the peak absorption of standards (Anderson & Murphy, 1976). Standards were prepared in neutral aqueous solution and themselves standardized by u.v. spectroscopy (Dawson *et al.*, 1969).

Validation

Peak heights of absorption were linearly dependent on the amount of uric acid, ATP, ADP, AMP, IMP, inosine, adenosine and hypoxanthine applied in the range 20–2000 pmol. Peak heights were inde-

pendent of the volume of sample applied up to 50 μ l for uric acid and up to 200 μ l for all other compounds. When standard solutions (10 μ M) each of compound were processed in the same manner as polymorphonuclear leucocyte suspensions, greater than 98% recovery was achieved. The only new peak arising as a result of sample processing was approx. 2% of adenine obtained from depurination of adenine nucleotides and nucleosides. In contrast, IMP gave rise to no detectable hypoxanthine. Indeed, there was no detectable production of any of the compounds under investigation from any other. For example extraction of 10 μ M-ATP led to the production of no detectable ADP, AMP, adenosine, inosine or hypoxanthine.

The identity of the compounds giving rise to the peaks in the elution pattern of extracted polymorphonuclear leucocytes (Fig. 1) was determined by three increasingly stringent criteria: (1) by comparison of retention times with standards; (2) by co-migration with added standard; (3) by removal of the peak after treatment of extracts with a specific enzyme. These experiments are summarized in Table 1.

By these criteria it was possible to conclude that ATP, ADP, AMP, hypoxanthine and adenosine were the major contributors to the appropriately labelled absorption peaks obtained from polymorphonuclear-leucocyte extracts (see Fig. 1). No peaks exactly co-migrating with added uric acid, IMP or inosine were found.

Miscellaneous analytical procedures

Cells were counted by using a haemocytometer under light microscopy. Different cell types were identified and counted in dried smears by the Leishmann procedure (Dacie & Lewis, 1968).

Distribution spaces were determined as described previously (Newby *et al.*, 1975), with the following modifications. Cells (approx. 2×10^7 /ml) and medium were separated by using a 1:1 (w/w) mixture of silicone oils DC550 (ρ 1.07 g/ml) and DC556 (ρ 0.97 g/ml) (Hopkin and Williams, Chadwell Heath, Essex, U.K.). After recovery of the cell pellet, this was dissolved in 1 ml of Soluene-350 followed by 5 ml of Dimilune-30 (Packard, Downers Grove, IL, U.S.A.), and ³H and ¹⁴C were determined in the same sample in a Packard Tri-Carb scintillation spectrometer.

Osmotic pressure was determined by depression of freezing point with a Knauer osmometer (Shandon, London N.W.10, U.K.) as described by Johnson & Hoch (1965).

Sonicated polymorphonuclear leucocytes were assayed for adenosine deaminase by the method of Hopkinson *et al.* (1969), and for nucleoside phosphorylase (EC 2.4.2.1) and xanthine oxidase (EC 1.2.3.2) as described by Trotta *et al.* (1976). Lac-

Table 1. *Identity and characteristics of absorbance peaks obtained by h.p.l.c. of polymorphonuclear leucocytes*

Standards (2nmol) were subjected to h.p.l.c. as described by Anderson & Murphy (1976), the elution buffer being varied as shown. The absorbance of the eluate was monitored at 254 nm (Fig. 1). From these absorbance profiles, retention times and the peak absorbance per μmol of each standard were determined. The limit of detection of each standard compound was estimated as that amount giving rise to a peak A_{254} of 0.0002. Trichloroacetic acid extracts of rat polymorphonuclear leucocytes were similarly chromatographed and the peaks in the absorbance profile obtained (Fig. 1) tentatively assigned on the basis of retention time. Assignments were further tested by co-chromatography of standards with polymorphonuclear leucocyte extract (column 7) and/or by removal of the peak with an enzyme specifically degrading that compound (columns 8 and 9). These were: for ATP, 1 μg of hexokinase + 2 mM-glucose; for ADP, 2 units of pyruvate kinase + 1 mM-phosphoenolpyruvate; for AMP, 1 μg of AMP deaminase; for hypoxanthine, 1 μg of xanthine oxidase; for adenosine, 1 μg of adenosine deaminase.

Elution buffer	Compound	Retention time (min)	Peak $A_{254}/\mu\text{mol}$	Minimum detectable (approx. pmol)	Amount present (pmol)			
					Control	+50 pmol	+Specific enzyme	+50 pmol + enzyme
Aq. ammonium phosphate	Uric acid	2.7	6.94	30	0	48	—	—
	IMP	3.3	23.8	10	0	46	—	—
	ATP	3.6	18.9	10	322	370	0	0
	ADP	4.4	15.5	10	33	88	8	7
	Hypoxanthine	5.6	21.9	10	134	—	3	—
+5% Methanol	AMP	7.0	14.1	30	36	89	8	0
	Inosine	5.7	22.7	10	0	48	—	—
+10% Methanol	Adenosine	16.2	7.04	50	0	100*	—	—
	Adenosine	7.8	16.9	10	24	71	0	0

* Plus 100 pmol.

tate dehydrogenase (EC 1.1.1.27) was measured as described previously (Luzio *et al.*, 1976).

Adenosine kinase (EC 2.7.1.20) was assayed at 37°C in a 20 μl volume containing 25 mM-sodium phosphate, pH 7.0, 2 mM-ATP, 2 mM-MgSO₄, 5 mM-creatine phosphate, 0.01 mg of creatine kinase (EC 2.7.3.2), 1 μM -erythro-9-(2-hydroxy-nonyl-3-yl)-adenine (a gift from Burroughs Wellcome, Research Triangle, NC, U.S.A.), 10 μM -adenosine, 100 000–200 000 c.p.m. of [2,5',8-³H]adenosine (The Radiochemical Centre, Amersham, Bucks., U.K.) and 10 μl of polymorphonuclear-leucocyte homogenate. The assay was linear for 10 min and with enzyme to 5×10^5 cells per incubation. Incubations were terminated with 20 μl of a solution containing 1 mM-AMP, 1 mM-adenosine and 1% sodium dodecyl sulphate. Then 2 $\times$ 5 μl of reaction mixture was serially applied to prepared ECTEOLA-cellulose t.l.c. plates (Camlab, Cambridge, U.K.). These were developed with glass-distilled water in an ascending direction. After they had been dried, the AMP spot was localized under u.v. light, cut and eluted in 2 ml of 1 M-NaCl/10 mM-Tris/HCl, pH 7.4. Scintillation counting was then performed as described previously (Newby *et al.*, 1975). Recovery of radioactivity was greater than 95%.

AMP deaminase (EC 3.5.4.6) and soluble IMPase were assayed after preparation of a supernatant (100 000g, 60 min) of polymorphonuclear leucocytes. Cells (approx. 10^8) were washed twice with

20 ml of 140 mM-NaCl/20 mM-3,3-dimethylglutarate, pH 6.3, and resuspended in the same buffer to 2×10^7 – 4×10^7 cells/ml. Sonication was performed at 4°C for 5 s as above. AMP deaminase was assayed by the method of Chapman & Atkinson (1973), with 2 mM-AMP as substrate. IMPase was assayed by method 2 of Newby *et al.* (1975), with 2 mM-IMP as substrate.

Results and Discussion

Purity and intactness of rat polymorphonuclear leucocytes

Rat peritoneal exudates elicited by caseinate injection contained 60–70% polymorphonuclear leucocytes, 20–30% macrophages, 2–3% erythrocytes, less than 1% eosinophils and 5–15% erythrocytes. The cell population obtained after the two-stage purification described contained less than 1% of contaminants when 500 cells from three consecutive preparations were examined. One rat yielded 5×10^8 – 8×10^8 total cells and 2×10^8 – 4×10^8 purified polymorphonuclear leucocytes.

Intactness was judged by Trypan Blue exclusion (de Pierre & Karnovsky, 1974a) and by release of the cytosol enzyme lactate dehydrogenase (EC 3.1.1.27) into the medium (Rodbell, 1966; Newby *et al.*, 1975). Since both methods yielded similar results (results not shown), the less-tedious enzymic method was used in each experiment. Only ATP breakdown

or intermediate accumulation in excess of that explicable by cell breakage was considered to be a consequence of intracellular nucleotide catabolism. Cells remained more than 98% intact under all conditions, except in the simultaneous presence of KCN and antisera (see below). Initially, higher degrees of breakage were obtained, but were improved by checking the osmoticity of all solutions used. Cells tolerated slight hyperosmoticity (up to 320 mosM), but were very sensitive to hypo-osmotic buffer (280 mosM).

Partial characterization of the enzymes of AMP degradation in rat polymorphonuclear leucocytes

There are detailed kinetic analyses of the enzymes involved in AMP catabolism (Zielke & Suelter, 1971; Nakatsu & Drummond, 1972; de Pierre & Karnovsky, 1974*a,b*; Van den Berghe *et al.*, 1977*a,b*; Arch & Newsholme, 1978*a*). However, since studies of these enzymes in rat polymorphonuclear leucocytes have not previously been reported, sufficient experiments were performed to compare the enzymes with those from other sources and to establish the inhibitory specificity of pentostatin.

Sonicated preparations of polymorphonuclear leucocytes contained adenosine deaminase (19–32 nmol/min per 10^7 cells; five experiments). The K_m of the enzyme was $26 \pm 3 \mu\text{M}$. When the sonicated material was preincubated at 23°C for 20 min with increasing concentrations of pentostatin and then assayed with 50 μM -adenosine at 37°C, 50% inhibition was caused by 1 nM-pentostatin (Agarwal *et al.*, 1977).

Adenosine kinase (0.17–0.21 nmol/min per 10^7 cells; three experiments) was also present. Because of the relatively low activity of this enzyme, it was necessary to include an inhibitor of adenosine deaminase in the assay to protect the substrate (Carson *et al.*, 1977): 1 μM -erythro-9-(2-hydroxy-

non-3-yl)adenine was routinely used. No further effect was seen when the concentration of this compound was raised to 100 μM . Pentostatin (0.1–100 μM) had no effect on the enzyme activity measured in this way (results not shown). With rat liver homogenate, where adenosine kinase (1400–1600 nmol/min per g wet wt.) and adenosine deaminase (1500–1600 nmol/min per g wet wt.) were comparable, it was not necessary to inhibit adenosine deaminase during the assay. No effect of either adenosine deaminase inhibitor on rat liver adenosine kinase was observed at concentrations of 0.1–100 μM .

Intact polymorphonuclear leucocytes hydrolysed AMP (5.5–7.5 nmol/min per 10^7 cells; four experiments). This activity was not affected by 0.1–100 μM -pentostatin. Only 2–3% of the activity was present in the medium from which cells had been removed. As with guinea-pig polymorphonuclear leucocytes (de Pierre & Karnovsky, 1974*a,b*), adding sodium β -glycerophosphate in addition to AMP revealed an inhibitable component ($43 \pm 6\%$), hereafter referred to as non-specific phosphatase (Table 2). No additional inhibition of AMPase was seen when the concentration of β -glycerophosphate was raised from 10 to 30 mM (results not shown). The remaining activity (3.2–5.7 nmol/min per 10^7 cells) was, however, inhibited by 0.5 mM-ATP (66%), -GMP (55%), -IMP (53%), -CMP (21%) and -UMP (36%). It was also inhibited by 67% by 5 mM-EDTA and by 92% by 0.5 mM-adenosine[α,β -methylene]diphosphate. In all respects this was typical of 5'-nucleotidase from other sources (Burger & Lowenstein, 1970; Evans & Gurd, 1973; de Pierre & Karnovsky, 1974*a,b*; Newby *et al.*, 1975). Intact cells exhibited $85 \pm 4\%$ of AMPase activity found in sonicated cells and $89 \pm 3\%$ of 5'-nucleotidase activity found in sonicated cells.

In addition the 5'-nucleotidase was inhibited by

Table 2. *Effects of KCN and antibodies on total AMPase and 5'-nucleotidase in rat polymorphonuclear leucocytes*
Polymorphonuclear leucocytes (2.9×10^7 cells/ml) were incubated for 5 min at 37°C with or without 10 mM-KCN. Antiserum at a dilution of 1 in 100 was present as indicated. Triplicate 500 μl samples of cell suspension were then transferred to tubes to which 50 μl containing 154 mM-NaCl, 15 mM-adenosine, 2 mM-AMP and 50000–100000 c.p.m. of [^3H]AMP had previously been added. Another three 500 μl portions were transferred to tubes in which the above 50 μl had been supplemented with 50 μl of 103 mM-sodium β -glycerophosphate. Incubations proceeded for a further 15 min at 37°C before termination and estimation of [^3H]adenosine formed as described (Newby *et al.*, 1975). 5'-Nucleotidase was taken to be the activity expressed in the presence of β -glycerophosphate, whereas non-specific phosphatase was calculated from the difference between incubation with and without this compound. Values are means $\pm$ S.E.M.

Condition	AMPase (nmol/15 min per 10^7 cells)		
	Total	5'-Nucleotidase	Non-specific
Intact cells	83.0 ± 0.3	48.2 ± 0.3	34.8 ± 0.4
+ antiserum	40.0 ± 0.2	2.0 ± 0.3	38.0 ± 0.4
+ KCN	43.8 ± 0.2	24.2 ± 0.2	19.6 ± 0.3
+ KCN + antiserum	25.4 ± 0.4	0.8 ± 0.5	24.6 ± 0.6

95–98% by a 1:100 dilution of antiserum raised against the purified rat liver plasma-membrane enzyme (Table 2). Non-immune sera were not inhibitory. The non-specific phosphatase was unaffected, confirming it to be a distinct enzyme or enzymes. Antibodies plus 10 mM- β -glycerophosphate inhibited total intact-cell AMPase activity by approx. 98%. Owing to the unexpected 50% inhibition of both 5'-nucleotidase and non-specific phosphatase by 10 mM-KCN (Table 2), the combined effect of KCN, antibodies and β -glycerophosphate was a 99% blockade of intact-cell AMPase.

To eliminate contribution to the AMP deaminase assay of the consecutive actions of 5'-nucleotidase and adenosine deaminase a 100 000 g supernatant was used. The supernatant contained AMP deaminase (23–32 nmol/min per 10^7 cells; three experiments). Deamination was not blocked by 1–100 μ M-erythro-9-(2-hydroxynon-3-yl)adenine, although this compound completely blocked deamina-

tion of adenosine under the same conditions. This demonstrated that the activity observed was indeed due to direct deamination of AMP. The enzyme required K^+ (100 mM) for maximum activity, and under these conditions displayed hyperbolic kinetics with a K_m of 1.2 mM. Activity was inhibited by 60–70% by 10 mM-phosphate, but was insensitive to ATP or ADP when added in the Mg-complexed form at concentrations up to 2 mM. This was equally true when the enzyme was assayed at a physiological AMP concentration of 0.2 mM. It seems therefore to be similar to the enzyme from muscle (Zielke & Suelte, 1971), but quite different from the rat liver enzyme (Van den Berghe *et al.*, 1977a). The enzyme activity was unaffected by pentostatin at concentrations up to 100 μ M.

Nucleoside phosphorylase (13–15 nmol/min per 10^7 cells; two experiments) was present and was not affected by pentostatin at concentrations up to 100 μ M. However, xanthine oxidase, which converts

Fig. 1. Elution profile of standards and polymorphonuclear leucocyte extracts from h.p.l.c.

Trichloroacetic acid extracts of freshly resuspended polymorphonuclear leucocytes and aqueous standard solutions were prepared as described and subjected to reverse-phase chromatography as described by Anderson & Murphy (1976). The eluting buffer was 50 mM-ammonium phosphate, pH 6.0 (a and b), or the same buffer supplemented with 5% (v/v) methanol (c and d) or with 10% (v/v) methanol (e and f). In each case the flow rate was 2 ml/min and 2 nmol of each standard was loaded. Extracts from the following number of leucocytes were loaded in each case; (b) $6.2 \times 10^5 \pm 0.2 \times 10^5$; (d) $6.7 \times 10^6 \pm 0.2 \times 10^6$; (f) $3.3 \times 10^6 \pm 0.1 \times 10^6$. In (b) after 5 min (↓) the sensitivity of the detector was increased 4-fold in order more accurately to measure AMP concentration.

hypoxanthine to uric acid, was not detectable (<0.5 nmol/min per 10^7 cells).

Measurement of CN-induced nucleotide catabolism in intact polymorphonuclear leucocytes

Extracts of polymorphonuclear leucocytes gave rise to the absorption peaks shown in Fig. 1, when fractionated by h.p.l.c. Peaks were assigned and metabolites quantified by comparison with standards, as detailed in the Experimental section. The mean values of adenine nucleotide, adenosine and hypoxanthine contents of control and CN-treated polymorphonuclear leucocytes are given in Table 3. These data were pooled from several experiments as indicated and show the degree of inter-experiment variation.

In order to convert these values to metabolite concentrations, the intracellular water space of rat polymorphonuclear leucocytes was also determined by simultaneously measuring the distribution of $^3\text{H}_2\text{O}$ and inulin- ^{14}C carboxylic acid (Newby *et al.*, 1975). Values for the extracellular water space (inulin space) of $0.64 \pm 0.02 \mu\text{l}$ 10^7 cells and intracellular water space (water-inulin space) of $2.68 \pm 0.07 \mu\text{l}/10^7$ cells were obtained from four experiments. Coupled with the values in Table 3, these yield values for the intracellular concentration of ATP in control polymorphonuclear leucocytes of 2.9 ± 0.1 mM, for ADP of 0.30 ± 0.03 mM, and for AMP of 0.07 ± 0.01 mM. These values are in broad agreement with those determined for other cells (Woods *et al.*, 1970; Van den Berghe *et al.*, 1977a). Adenosine and hypoxanthine were found principally in the incubation medium, where they achieved concentrations of 0.02 – $0.1 \mu\text{M}$ and 0.1 – $5 \mu\text{M}$ respectively. This is consistent with their permeability through the plasma membrane. It is noteworthy that the amount of adenosine present initially in the medium (0.6% of the ATP content) is similar to the

value of 0.7% obtained for cell lysis (Table 3).

Incubation of polymorphonuclear leucocytes for 90 min in Krebs–Ringer bicarbonate buffer led to an increase of approx. 1% in the number of broken cells. Apart from a small increase in ADP content, only the adenosine concentration changed. This decreased to an undetectable value, presumably representing uptake of the nucleoside faster than its formation.

Fig. 2. Time course of CN-induced ATP breakdown and hypoxanthine accumulation in rat polymorphonuclear leucocytes

Cells ($1.63 \times 10^7 \pm 0.03 \times 10^7/\text{ml}$) were incubated at 37°C in Krebs–Ringer bicarbonate buffer supplemented with 10 mM-KCN and readjusted to pH 7.4 with HCl. Samples (1 ml) were withdrawn at intervals and extracted with trichloroacetic acid as described. ATP ($\square$) and hypoxanthine ($\triangle$) contents of duplicate samples were determined simultaneously by h.p.l.c. The sum of ATP and hypoxanthine concentrations ($\circ$) was computed for individual samples. Values shown are means $\pm$ difference/2 (where this was larger than the symbols).

Table 3. Metabolite concentrations in control and CN-treated polymorphonuclear leucocytes

Purified polymorphonuclear leucocytes were incubated in duplicate for the stated times at 37°C before precipitation of protein with trichloroacetic acid. Metabolites were subsequently measured by h.p.l.c. Values are means $\pm$ s.e.m. for the numbers of experiments in parentheses. Homogenate lactate dehydrogenase activity was $1.8 \pm 0.2 \mu\text{mol}/\text{min}$ per 10^7 cells in 15 experiments.

Condition	Incubation time (min)	Metabolite concentration (nmol/ 10^7 cells)					Percentage of homogenate lactate dehydrogenase in the medium
		ATP	ADP	AMP	Adenosine	Hypoxanthine	
Control	0	7.7	0.81	0.16	0.05	0.17	0.7
		± 0.5	± 0.10	± 0.03	± 0.02	± 0.05	± 0.2
		(4)	(4)	(4)	(3)	(4)	(4)
Control	90	7.8	1.1	0.18	± 0.015	0.17	1.5
		± 0.3	± 0.1	± 0.02	± 0.001	± 0.03	± 0.2
		(11)	(11)	(11)	(3)	(11)	(11)
+ 10 mM-KCN	90	5.7	1.1	0.26	0.021	1.9	1.9
		± 0.5	± 0.1	± 0.06	± 0.003	± 0.3	± 0.2
		(11)	(11)	(9)	(3)	(11)	(11)

Incubation in buffer supplemented with KCN led, after a short lag, to an approximately linear fall in ATP content (Fig. 2); 10 mM/KCN was maximally effective. After 90 min the fall in ATP concentration amounted to $27 \pm 3\%$, whereas cell breakage increased at most by 1.5% (Table 3). Of the metabolites measured, only hypoxanthine was increased significantly at any time point. This increase was linear (Fig. 2), and accounted for $80 \pm 10\%$ of the fall in ATP content. It thus appears that, in the presence of KCN, the rate of accumulation of hypoxanthine is a valid estimate of the rate of nucleotide catabolism. This contention was strengthened by the observation that there was no detectable metabolism of exogenously added $50 \mu\text{M}$ -hypoxanthine by CN-poisoned polymorphonuclear leucocytes (results not shown). Hypoxanthine was not further metabolized to uric acid, which is consistent with the absence of xanthine oxidase from these cells. The failure to detect increases in intermediates presumably reflects the low rate of nucleotide catabolism (approx. $0.02 \text{ nmol/min per } 10^7 \text{ cells}$) relative to the activity of the degradative enzymes.

Effects of pentostatin on nucleotide catabolism in polymorphonuclear leucocytes

To determine the contribution of adenosine deaminase to the overall rate of nucleotide catabolism, the enzyme was inhibited *in situ* by pentostatin. No effect on the concentration of any metabolite in either the presence or absence of KCN was observed (Table 4). In particular, the CN-induced rise in hypoxanthine concentration was not affected, nor was there significant accumulation of adenosine.

The degree of inhibition of intracellular adenosine deaminase was judged in the same experiment by observing the breakdown of exogenously added

adenosine ($55 \mu\text{M}$). As indicated in Table 4, adenosine was rapidly metabolized by control and CN-treated polymorphonuclear leucocytes. In control cells, only a minor portion was degraded to hypoxanthine, the rest being presumably incorporated into phosphorylated products. In CN-treated cells adenosine was converted almost quantitatively to hypoxanthine, which is consistent with the blockade of the phosphorylation reactions at lower ATP concentrations. Pentostatin ($30 \mu\text{M}$) completely blocked production of hypoxanthine from adenosine in control and CN-treated polymorphonuclear leucocytes (Table 4). The dose-dependence of the effect is shown in Fig. 3, which also shows that the metabolism of exogenous inosine ($50 \mu\text{M}$) by control or CN-poisoned polymorphonuclear leucocytes was unaffected by pentostatin. Hence an effect on nucleoside phosphorylase or on nucleoside transport (Fox & Kelley, 1978) was ruled out. It is particularly impressive that pentostatin decreased the hypoxanthine concentration in CN-treated cells incubated with exogenous adenosine to precisely that in cells incubated with KCN alone (Fig. 3, Table 4). This suggests a complete blockade of hypoxanthine production from exogenous substrates without an effect on metabolism from endogenous substrates.

This experiment is doubly important, since it shows that no other pathway for the conversion of adenosine to hypoxanthine proceeds at a measurable rate in the presence of pentostatin. Therefore it is not possible to argue that flux to hypoxanthine takes place through adenosine in the absence of pentostatin, but in its presence is simply diverted by an alternative pathway (for instance, rephosphorylation) and therefore the rate of hypoxanthine formation is undiminished. The conclusion is thus

Table 4. *Effect of pentostatin on metabolite concentrations in polymorphonuclear leucocytes*

Polymorphonuclear leucocytes were suspended in Krebs-Ringer bicarbonate buffer or the same buffer supplemented with 10 mM-KCN and readjusted to pH 7.4. Pentostatin was immediately added as indicated to a final concentration of $30 \mu\text{M}$. After 60 min, adenosine was added as indicated to a final concentration of $55 \mu\text{M}$. All incubations were terminated after 90 min, and metabolites determined by h.p.l.c. Values are the means of duplicate incubations. Combined average errors for all estimations of the same metabolite were for ATP 0.2, for ADP 0.1, for AMP 0.04, for adenosine 0.005 and for hypoxanthine 0.1. The percentage of lactate dehydrogenase released into the medium was between 1.5 and 2.2% under all conditions.

Condition	Metabolite concentration (nmol/ 10^7 cells)				
	ATP	ADP	AMP	Adenosine	Hypoxanthine
Control	6.8	0.76	0.18	0.015	0.12
+ Pentostatin	6.8	0.84	0.19	0.014	0.16
+ Adenosine	—	—	—	$8.7 \mu\text{M}$	5.1 ($8.2 \mu\text{M}$)
+ Adenosine + pentostatin	—	—	—	$57 \mu\text{M}$	0.09 ($0.15 \mu\text{M}$)
+ KCN	4.7	0.64	0.19	0.009	3.0
+ KCN + pentostatin	5.3	0.82	0.17	0.014	2.8
+ KCN + adenosine	—	—	—	$12.1 \mu\text{M}$	22.9 ($37.8 \mu\text{M}$)
+ KCN + adenosine + pentostatin	—	—	—	$54 \mu\text{M}$	2.7 ($4.4 \mu\text{M}$)

unequivocal that AMP breakdown to adenosine does not contribute significantly to the overall rate of hypoxanthine production.

The simplest explanation for the failure of pentostatin to increase adenosine concentration is that none was being formed. However, these experiments were not designed to detect cycling between

adenosine and AMP (Arch & Newsholme, 1978b). Both the latter authors and earlier observations (Schrader *et al.*, 1972; Perret & Dean, 1977) suggest that adenosine deaminase only removes adenosine when its concentration is elevated. Hence the role of adenosine deaminase needs to be further evaluated in a situation where AMP degradation is principally via adenosine.

Effect of antibodies and β-glycerophosphate on polymorphonuclear-leucocyte metabolite concentrations

Anti-(5'-nucleotidase) sera decreased ATP concentrations in both control and CN-treated polymorphonuclear leucocytes (Table 5) and caused concomitantly increased hypoxanthine concentrations. When values were pooled from four similar experiments both effects were shown to be significant at the *P* < 0.01 level (Student's *t* test, 14 degrees of freedom). However, these effects were probably unrelated to inhibition of 5'-nucleotidase for the following reasons. ATP concentrations were also reduced by anti-(rat erythrocyte) sera, which bind to many rat cell types (Hales, 1972; Luzio *et al.*, 1976; Luzio, 1977), but do not inhibit 5'-nucleotidase (Newby *et al.*, 1975; Luzio *et al.*, 1976). Secondly, metabolite concentrations were restored to control values in the simultaneous presence of sodium β-glycerophosphate (Table 5), which would have been expected to potentiate an effect specifically on ecto-phosphatases. The combination of KCN and antiserum also caused a variable amount of non-specific cell damage, as judged by lactate dehydrogenase release. This varied between 2.3 and 6% depending on the cell preparation. This value is still much less than the amount of CN-induced ATP breakdown.

Anti-(5'-nucleotidase) antibodies did not prevent the rise in hypoxanthine concentration caused by

Fig. 3. *Effect of pentostatin on hypoxanthine production from polymorphonuclear leucocytes incubated with adenosine or inosine*

Polymorphonuclear leucocytes ($1.65 \times 10^7 \pm 0.03 \times 10^7$ cells/ml) were incubated in duplicate at 37°C in the presence (open symbols) or absence (closed symbols) of 10mM-KCN. Pentostatin at the concentrations showed was simultaneously present. After 60 min the 50μM-adenosine (circles) or 50μM-inosine (squares) was added and the incubation continued for a further 30 min. Trichloroacetic acid extracts were then prepared for h.p.l.c. as described. The concentrations of hypoxanthine in cells incubated without nucleoside are indicated: Δ, +KCN; ▲, -KCN. Values shown are means. The combined variance of all observations was ±5%.

Table 5. *Effect of antiserum and β-glycerophosphate on metabolite concentrations and cell breakage in rat polymorphonuclear leucocytes*

Polymorphonuclear leucocytes (0.67×10^7 cells/ml) were incubated for 90 min at 37°C either with or without 10mM-KCN. Anti-(5'-nucleotidase) serum at a dilution of 1 in 100 or 10mM-sodium β-glycerophosphate was present as indicated. Values are means of duplicate incubations. Combined average errors for all estimates of the same metabolite were: for ATP, 0.2; for ADP, 0.1; for AMP, 0.04; for hypoxanthine, 0.1. Combined average errors for lactate dehydrogenase measurements were ±0.1%.

Conditions	Metabolite concentration (nmol/10 ⁷ cells)				Percentage of homogenate lactate dehydrogenase in the medium
	ATP	ADP	AMP	Hypoxanthine	
Control	7.2	0.84	0.13	0.06	1.4
+Antiserum	6.6	0.71	0.16	0.16	1.7
+β-Glycerophosphate	7.3	0.62	0.12	0.06	1.5
+Antiserum + β-glycerophosphate	6.8	0.73	0.16	0.06	1.5
+KCN	5.3	0.85	0.25	3.4	1.7
+KCN + antiserum	4.2	0.86	0.58	4.0	5.6
+KCN + β-glycerophosphate	5.4	0.85	0.12	3.8	1.8
+KCN + antiserum + β-glycerophosphate	5.2	0.72	0.58	3.6	6.0

KCN in either the presence or absence of sodium β -glycerophosphate (Table 5). 5'-Nucleotidase was inhibited by 98% in the presence of KCN and antibodies, and total ecto-AMPAse by 99% in the presence of KCN, antibodies and β -glycerophosphate (Table 2). Despite this, it was reasonable to ask whether such a blockade of AMP breakdown would influence the rate of hypoxanthine production or merely cause accumulation of some intermediate. This possibility was tested in the same experiment by observing the effect of antiserum and β -glycerophosphate on the metabolism of exogenously added AMP or IMP (200 μ M). Table 6 shows that hypoxanthine production from AMP was blocked by 91–92% by antiserum and β -glycerophosphate, whereas hypoxanthine production from IMP was blocked by 96–99%. In the presence of KCN (results not shown), the amounts of nucleotide degraded and hypoxanthine produced were equimolar.

Serum plus KCN did cause a small (relative to total ATP decrease) accumulation of AMP, but this amount was variable between experiments and directly related to the amount of cell breakage (Table 5). In a separate experiment, where lactate dehydrogenase release in the presence of KCN and antiserum was 2.3%, the AMP concentration under

these conditions was 0.27 nmol/ 10^7 cells compared with a control value of 0.20. The accumulation of AMP was therefore interpreted as being the result of prevention of AMP degradation from externally located nucleotides arising from non-specific cell damage.

These data indicate that ecto-phosphatases do not participate in the hydrolysis of intracellular IMP produced during CN-induced AMP catabolism in polymorphonuclear leucocytes. They suggest the existence of an intracellular IMPase capable of this function. Such an enzyme has been described in the liver by Van den Berghe *et al.* (1977b), and hence was sought in polymorphonuclear leucocytes. A 100000g supernatant of cells was produced in order to eliminate the plasma-membrane enzyme. The intact cells hydrolysed IMP, and this hydrolysis was completely blocked by anti-(5'-nucleotidase) serum (Table 7). However, the soluble fraction contained an IMPase that was insensitive to inhibition by antiserum. This enzyme displayed 5–7% (three experiments) of the intact-cell activity under the assay conditions used. Although a more detailed kinetic analysis would require prior purification of the enzyme, it is clear that this activity is sufficient to explain the rate of hypoxanthine formation observed.

Table 6. *Effect of antiserum and β -glycerophosphate on hypoxanthine production from exogenous AMP and IMP* Polymorphonuclear leucocytes (0.67×10^7 cells/ml) from the same preparation described in Table 5 were incubated in duplicate for 90 min at 37°C in the presence or absence of 10 mM-KCN. Anti-(5'-nucleotidase) serum at 1 in 100 dilution and 10 mM-sodium β -glycerophosphate were also present as indicated. After 30 min, 200 μ M-AMP or -IMP was added and incubation continued for a further 60 min. Hypoxanthine was estimated by h.p.l.c. Values are means. Average errors for all estimations were $\pm 5\%$.

Incubation conditions	Hypoxanthine concentration (nmol/ 10^7 cells)		
	+AMP	+IMP	Control
Control	48.6	28.4	0.06
+ Antiserum + β -glycerophosphate	4.5	1.08	0.06
+KCN	67.5	37.8	3.4
+KCN + antiserum + β -glycerophosphate	8.4	4.1	3.6

Table 7. *IMPase and β -glycerophosphatase in intact polymorphonuclear leucocytes and in the soluble fraction of the same cells*

Intact cells (1.1×10^7 cells/ml) or 100000g supernatant from sonicated cells (4.4×10^7 cells/ml) were incubated in duplicate at 37°C in 2 ml containing 140 mM-NaCl, 20 mM-3,3-dimethylglutarate, pH 6.3, and 10 mM- β -glycerophosphate with or without 2 mM-IMP. Samples (300 μ l) were withdrawn at various time intervals up to 1 h, and the amount of P_i released was determined by method 2 of Newby *et al.* (1975). The linear rates reported are the mean $\pm$ difference/2. IMPase was calculated as the difference in the rate of P_i production in the presence and absence of the nucleotide. No increase in P_i production was observed when the β -glycerophosphate concentration was increased to 20 mM.

Preparation	Conditions	β -Glycerophosphatase (nmol/min per 10^7 cells)	IMPase (nmol/min per 10^7 cells)
Intact cells	Control	0.32 ± 0.04	2.9 ± 0.2
	+Antiserum	0.40 ± 0.01	0.0 ± 0.1
Soluble fraction	Control	0.26 ± 0.01	0.21 ± 0.01
	+Antiserum	0.24 ± 0.02	0.17 ± 0.02

Role of 5'-nucleotidase and adenosine deaminase

The action of pentostatin indicates that adenosine deaminase does not participate in CN-induced catabolism of intracellular AMP in polymorphonuclear leucocytes and implies that IMP rather than adenosine is an intermediate in this pathway. A similar conclusion was drawn by Lomax & Henderson (1973) for 2-deoxyglucose poisoning of Ehrlich ascites cells and by Van den Berghe *et al.* (1977a) for AMP catabolism in response to a fructose load on the liver. These observations support the view that adenosine is not a major metabolic intermediate in these tissues, but might have a control function (Arch & Newsholme, 1978b). The situation in the anoxic heart appears to be more complex, with both pathways apparently being active (Richman & Wyborny, 1964; Kaufman *et al.*, 1977). The basis of these tissue differences is clearly worthy of further study.

The observation that ecto-phosphatases do not participate in the catabolism of intracellular IMP is further support for the idea that they function physiologically on external nucleotides. Adenosine deaminase might have a specific role in degrading adenosine from this source. It is interesting that the maximal adenosine deaminase activity of polymorphonuclear leucocytes was 3 times that of rat liver. Moreover, the ratio of maximal adenosine deaminase to adenosine kinase was approx. 1 in liver but 100 in polymorphonuclear leucocytes. The latter cells appear to have a 'degradative bias'. In a series of mouse tissues examined by Arch & Newsholme (1978a), only the jejunum contained adenosine deaminase activity or the ratio of deaminase to kinase activity approaching those in polymorphonuclear leucocytes. Since jejunum is a digestive tissue and polymorphonuclear leucocytes are phagocytes, both cell types are involved in the absorption of exogenous macromolecules.

In view of the wide distribution of 5'-nucleotidase in mammalian tissues (Arch & Newsholme, 1978a), it is unlikely that it is solely a 'digestive' enzyme. Indeed, the maximal activity in polymorphonuclear leucocytes is less than that reported for rat liver, brain or heart (Arch & Newsholme, 1978a). The question remains as to the source of external nucleotides on the surface of these cells. It is clear that adenine nucleotides can arise in the extracellular space as a result of co-export with neurotransmitters (Hillarp, 1958; Burnstock, 1972; Dowdall *et al.*, 1974). Whether or not other cells can also release nucleotides remains to be determined.

A second question is whether adenosine, which has been shown to arise in the extracellular fluid of anoxic and ischaemic tissues (Berne & Rubio, 1974), might be a product of external nucleotide metabolism. Many tissues are capable of maintaining low intracellular concentrations of adenosine in the pres-

ence of high external concentrations (Schrader *et al.*, 1972; Berne & Rubio, 1974). This implies that adenosine transport is slow relative to cytoplasmic adenosine metabolism, and poses a problem as to how intracellularly produced adenosine could escape from the cell. This problem would be obviated by producing the adenosine externally.

I acknowledge many helpful discussions with Dr. Blanca Fernandez, and the help and encouragement of Professor C. N. Hales. I thank Dr. M. Allwood and Mr. L. Sizemore of the Addenbrooke's Hospital Pharmacy for use of h.p.l.c. equipment. This work was supported by grants to Professor Hales from the Medical Research Council and the British Diabetic Association.

References

- Agarwal, R. P., Spector, T. & Parks, R. E. (1977) *Biochem. Pharmacol.* **26**, 359–367
- Anderson, F. S. & Murphy, R. C. (1976) *J. Chromatogr.* **121**, 251–262
- Arch, J. R. S. & Newsholme, E. A. (1978a) *Biochem. J.* **174**, 965–977
- Arch, J. R. S. & Newsholme, E. A. (1978b) *Essays Biochem.* **14**, 82–123
- Atkinson, D. E. (1968) *Biochemistry* **7**, 4030–4034
- Berne, R. M. (1964) *Physiol. Rev.* **44**, 1–29
- Berne, R. M. & Rubio, R. (1974) *Circ. Res.* **34** and **35**, Suppl. 3, 109–118
- Böyum, A. (1968) *Scand. J. Clin. Lab. Invest.* **21**, Suppl. **97**, 77–90
- Burger, R. M. & Lowenstein, J. M. (1970) *J. Biol. Chem.* **245**, 6274–6280
- Burnstock, G. (1972) *Pharmacol. Rev.* **24**, 509–581
- Carson, D. A., Kaye, J. & Seegmiller, J. E. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5677–5681
- Chapman, A. G. & Atkinson, D. E. (1973) *J. Biol. Chem.* **248**, 8309–8312
- Chapman, A. G., Miller, A. L. & Atkinson, D. E. (1976) *Cancer Res.* **36**, 1144–1150
- Cohen, P. P. (1957) in *Manometric Techniques* (Umbreit, W. W., Burris, R. H. & Stauffer, J. F., eds.), 3rd edn., pp. 147–150, Burgess Publishing Co., Minneapolis
- Dacie, J. V. & Lewis, S. M. (1968) *Practical Haematology*, 4th edn., p. 59, Churchill, London
- Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. (1969) *Data for Biochemical Research*, 2nd edn., Oxford University Press, Oxford
- de Pierre, J. W. & Karnovsky, M. L. (1974a) *J. Biol. Chem.* **249**, 7111–7120
- de Pierre, J. W. & Karnovsky, M. L. (1974b) *J. Biol. Chem.* **249**, 7121–7129
- Dowdall, M. J., Boyne, A. F. & Whittaker, V. P. (1974) *Biochem. J.* **140**, 1–12
- Evans, W. H. & Gurd, J. W. (1973) *Biochem. J.* **133**, 189–199
- Fain, J. N., Pointer, R. H. & Ward, W. F. (1972) *J. Biol. Chem.* **247**, 6866–6872
- Fox, I. H. & Kelley, W. N. (1978) *Annu. Rev. Biochem.* **47**, 655–686

- Frick, G. P. & Lowenstein, J. M. (1976) *J. Biol. Chem.* **251**, 6372–6278
- Hales, C. N. (1972) *Diabetologia* **8**, 229–235
- Hempling, H. G. (1973) *J. Cell. Physiol.* **81**, 1–10
- Hillarp, N. A. (1958) *Acta Physiol. Scand.* **42**, 321–322
- Hopkinson, D. A., Cook, P. J. L. & Harris, H. (1969) *Ann. Human Genet.* **32**, 361–367
- Johnson, R. B. & Hoch, H. (1965) in *Standard Methods in Clinical Biochemistry* (Meites, S., ed.), vol. 5, p. 159, Academic Press, New York
- Kaufman, I. A., Hall, N. F., De Luca, M. A., Ingwall, J. S. & Mayer, S. E. (1977) *Am. J. Physiol.* **233**, H282–H288
- Lachmann, P. J. (1968) in *Clinical Aspects of Immunology* (Gell, P. G. H. & Coombs, R. A., eds.), 2nd edn., p. 385, Blackwell Scientific Publications, Oxford
- Lomax, C. A. & Henderson, J. F. (1973) *Cancer Res.* **33**, 2825–2829
- Luzio, J. P. (1977) in *Membranous Elements and Movement of Molecules* (Reid, E., ed.), pp. 131–142, Ellis Horwood, Chichester
- Luzio, J. P., Newby, A. C. & Hales, C. N. (1976) *Biochem. J.* **154**, 11–21
- Murray, A. W. (1971) *Annu. Rev. Biochem.* **40**, 811–826
- Murray, A. W., Elliott, D. C. & Atkinson, M. R. (1970) *Prog. Nucleic Acid Res. Mol. Biol.* **10**, 87–119
- Nakatsu, K. & Drummond, G. I. (1972) *Am. J. Physiol.* **223**, 1119–1127
- Newby, A. C., Luzio, J. P. & Hales, C. N. (1975) *Biochem. J.* **146**, 625–633
- Perrett, D. & Dean, B. (1977) *Biochem. Biophys. Res. Commun.* **77**, 374–378
- Richman, H. G. & Wyborny, L. (1964) *Am. J. Physiol.* **207**, 1139–1145
- Rodbell, M. (1966) *J. Biol. Chem.* **241**, 3909–3917
- Schrader, J., Berne, R. M. & Rubio, R. (1972) *Am. J. Physiol.* **223**, 159–166
- Stanley, K. K. & Luzio, J. P. (1979) *Biochem. Soc. Trans.* **7**, 361–362
- Trotta, P. P., Smithwick, E. M. & Balis, M. E. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 104–108
- Van den Berghe, G., Bronfman, M., Vanneste, R. & Hers, H.-G. (1977a) *Biochem. J.* **162**, 601–610
- Van den Berghe, G., Van Pottlesberghe, C. & Hers, H.-G. (1977b) *Biochem. J.* **162**, 611–616
- Widnell, C. C. (1975) *Methods Enzymol.* **32**, 368–374
- Woods, H. F., Eggleston, L. V. & Krebs, H. A. (1970) *Biochem. J.* **119**, 501–510
- Zielke, C. L. & Suelter, C. H. (1971) *Enzymes 3rd Ed.* **4**, 47–78