Nucleoside transport in human erythrocytes

APPARENT MOLECULAR WEIGHT OF THE NITROBENZYLTHIOINOSINE-BINDING COMPLEX ESTIMATED BY RADIATION-INACTIVATION ANALYSIS

Simon M. JARVIS*, James D. YOUNG* and J. Clive ELLORY†

*ARC Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, and

†Physiological Laboratory, University of Cambridge, Downing Street, Cambridge CB2 3EG, U.K.

(Received 12 December 1979)

Nitrobenzylthioinosine, a potent nucleoside-transport inhibitor, binds specifically to functional nucleoside transport sites. Irradiation of freeze-dried human erythrocyte membranes with high-energy electrons was used to estimate the apparent molecular weight of the nitrobenzylthioinosine-binding complex *in situ*. The nitrobenzylthioinosine-binding complex had an apparent mol.wt. of 122 000.

Radiation inactivation with high-energy electrons is a simple but powerful technique for obtaining information on the molecular size of unpurified proteins (Pollard *et al.*, 1955; Kepner & Macey, 1968; Ellory, 1979; Kempner & Schlegel, 1979). The method is particularly attractive for membrane studies since it allows measurement to be made *in situ* with native membranes. The only requirements are, first, a suitable assay for biological activity (e.g. enzyme activity or ligand binding), and secondly, that activity survives freeze-drying and subsequent reconstitution. By using this technique it has been possible to estimate the molecular weight of a wide range of soluble proteins as well as several membrane-bound enzymes, including acetylcholinesterase and adenyl cyclase (Levinson & Ellory, 1974; Houslay *et al.*, 1977), but a lack of suitable assays in isolated membrane preparations has so far restricted investigations of membrane transport systems to the sodium pump and the sodium channel in nerve membranes (Nakao *et al.*, 1967; Kepner & Macey, 1968; Levinson & Ellory, 1973; Ellory *et al.*, 1979).

In the following paper (Jarvis & Young, 1980) we have shown that nitrobenzylthioinosine [6-(4-nitrobenzyl)thio-9- β -D-ribofuranosylpurine], a potent inhibitor of nucleoside transport, binds to high-affinity sites on the membrane of human and nucleoside-permeable sheep erythrocytes with an apparent K_D of 1 nM and that this binding represents a specific and reversible interaction with functional nucleoside-transport sites. The properties of nitrobenzylthioinosine binding to erythrocyte membranes and the availability of nitro[3 H]benzylthioinosine make this an ideal system for radiation-inactivation analysis. In the present paper we describe the effects of high-energy electrons on the

nitrobenzylthioinosine-binding activity of human erythrocyte membranes. Human cells were chosen because they contain considerably more nitrobenzylthioinosine-binding sites than sheep erythrocytes. Our results show that the nitrobenzylthioinosine-binding complex *in situ* has an apparent mol.wt. of 122 000. This represents the first molecular information on the physical properties of the erythrocyte nucleoside-transport mechanism.

Materials and methods

Blood from healthy volunteers was collected into heparin, and haemoglobin-free erythrocyte 'ghosts' were prepared by hypo-osmotic lysis and centrifugation as described previously (Ellory & Maker, 1977). Portions of membrane suspension (equivalent to 1 ml of packed erythrocytes) were freeze-dried in individual Pyrex tubes and stored dry at -20°C until required. In some experiments dithiothreitol (5 mM) was added before freeze-drying.

Samples were irradiated by using the experimental beam facility of a clinical MEL SL 75/20 20 MeV linear accelerator (Addenbrooke's Hospital, Cambridge) at a dose rate of 2–2.5 Mrad/min, with air/solid CO_2 cooling (Ellory *et al.*, 1979). Tubes were either gassed with dry N_2 or evacuated (<0.1 mmHg) during irradiation to minimize free-radical effects. Machine calibration was by HX-Perspex dosimetry (Berry & Marshall, 1969). Samples were reconstituted by the addition of 1 ml of 5 mM-sodium phosphate buffer, pH 7.2.

Nitrobenzylthioinosine binding was measured at 37°C in the presence and absence of 25 μM -nitrobenzylthioguanosine as described in the following paper (Jarvis & Young, 1980). Initial influx rates for

uridine uptake were determined at 25°C (Jarvis & Young, 1980). Acetylcholinesterase activity was assayed by the method of Chow & Islam (1970).

Estimates of molecular weight were obtained by substituting D_{37} (the radiation dose at which 37% of the original activity remains) into the empirical equation:

$$\text{mol.wt.} = 6.4 \times 10^5 / D_{37}$$

where D_{37} is in Mrad (Kepner & Macey, 1968; Ellory, 1979). D_{37} values from simple first-order inactivation plots were obtained by linear regression analysis of $\log[\text{activity (\%)}]$ versus radiation dose (Mrad). A two-component exponential fitting procedure (Maximum Likelihood program MLP, Copyright 1979, Lawes Agricultural Trust, Rothamstead Experimental Station, Harpenden, Herts., U.K.) was used for data not conforming to a single exponential. All data points were equally weighted. D_{37} standard errors were calculated from the residual mean squares about the fitted lines. Additional error in the estimated target size due to the limited precision of the empirical constant used to determine molecular weight is likely to be less than 14% (Kepner & Macey, 1968; Kempner & Schlegel, 1979).

Results and discussion

Fig. 1 shows the results of a typical binding experiment in which the amount of nitro[^3H]benzylthioinosine bound to human erythrocyte 'ghosts' is plotted against the equilibrium free nitrobenzylthioinosine concentration in the medium. Binding was saturable. Pretreatment of membranes with the structurally related nucleoside-transport inhibitor nitrobenzylthioguanosine abolished this high-affinity binding component and revealed a minor nitrobenzylthioguanosine-insensitive non-saturable association (17×10^{-23} mol/cell at 10 nM). Subtraction of this component from the saturable uptake curve gave an apparent K_D of 0.69 nM with maximum binding of 1545×10^{-23} mol/cell (9300 sites/cell) (see the inset curve in Fig. 1). Uridine, a transported nucleoside, was an effective competitive inhibitor of the saturable nitrobenzylthioinosine-binding component giving an apparent K_I value of 1.2 mM at 25°C, a value higher than the apparent K_m for uridine influx in intact cells (0.11 mM at 25°C), but close to the apparent K_m of uridine equilibrium-exchange efflux (1.3 mM) (Cabantchik & Ginsburg, 1977; Shohami & Koren, 1979). The properties of this saturable nitrobenzylthioinosine-binding component are similar to those previously reported for human and nucleoside-permeable sheep erythrocytes, although sheep cells have considerably fewer binding sites (Pickard *et al.*, 1973; Cass *et al.*, 1974; Jarvis & Young, 1980).

Fig. 1. Binding of nitro[^3H]benzylthioinosine to human erythrocyte membranes

Nitro[^3H]benzylthioinosine was incubated at 37°C with membranes in the presence (O) and absence (●) of 25 μM -nitrobenzylthioguanosine. The amount of nitrobenzylthioinosine bound is plotted as a function of the equilibrium free nitrobenzylthioinosine concentration. The inset shows a modified Scatchard plot (analogous to the Hanes plot of enzyme kinetics) of the data (corrected for the nitrobenzylthioguanosine-insensitive component) (Hanes, 1932). Maximum binding (1545×10^{-23} mol/cell) and apparent K_D (0.69 nM) were determined by linear regression analysis.

In subsequent binding assays, differences between the binding of saturating concentrations of nitro[^3H]benzylthioinosine in the presence and absence of excess nitrobenzylthioguanosine were used to quantify the magnitude of the high-affinity nitrobenzylthioinosine-binding component. Initial control experiments established that freeze-drying and reconstitution did not result in significant loss of nitrobenzylthioinosine-binding activity (nitrobenzylthioinosine bound to freeze-dried samples was 97.8% of the value for control membranes).

In the first series of irradiation experiments, freeze-dried samples from five donors were irradiated under N_2 as described in the Materials and methods section. The percentage of saturable nitrobenzylthioinosine-binding activity remaining was plotted on a logarithmic scale against radiation dose (0.1–5.0 Mrad) (Fig. 2). The plot was distinctly biphasic, with 45% of the binding activity being associated with a component of high apparent mol.wt. ($> 10^6$), the remaining 55% of activity being associated with a component of mol.wt. $(128 \pm 25) \times 10^3$ ($n = 5$). Non-saturable nitro-

Fig. 2. Nitro[³H]benzylthioinosine binding and acetylcholinesterase activity as a function of radiation dose. Symbols: ■, acetylcholinesterase activity; ●, nitrobenzylthioinosine-binding activity under N₂; ○, nitrobenzylthioinosine binding activity *in vacuo* and in the presence of 5 mM-dithiothreitol. Error bars denote $\pm$ S.E.M. Data points without S.E.M. are means for two independent estimates.

benzylthioinosine-binding activity also decreased with increasing radiation dose, but the relatively large errors associated with this minor binding component prevented an analysis of its target size. Acetylcholinesterase, used as an internal control, gave an apparent mol.wt. of $(77 \pm 8) \times 10^3$ ($n = 4$), a value close to that obtained for the monomeric unit by conventional methods (Hollunger & Nikalsson, 1973) and previous irradiation studies (Levinson & Ellory, 1974; Folkard *et al.*, 1977). In contrast with saturable nitrobenzylthioinosine binding, there was no evidence of an initial loss of activity at low radiation doses (Fig. 2). The temperature at the end of a 5 Mrad irradiation, measured with a thermocouple, was 35°C. This modest temperature increase had no effect on nitrobenzylthioinosine binding. Heating freeze-dried samples to 50°C for 5 min only decreased binding activity by <2%. Previous studies have shown that acetylcholinesterase activity is also resistant to heating (Levinson & Ellory, 1974).

Highly reactive membrane thiol groups are essential for normal nitrobenzylthioinosine-binding activity (Dahlig *et al.*, 1979; S. M. Jarvis & J. D. Young, unpublished work). We therefore considered it likely that nitrobenzylthioinosine binding might be particularly susceptible to active radiolytic and free-radical products formed from residual molecular oxygen during irradiation, and that such effects contributed to the substantial loss of saturable nitrobenzylthioinosine-binding activity at low radiation doses. Subsequent irradiation experiments were therefore carried out *in vacuo* and in the presence of the thiol reducing agent and free-radical scavenger dithiothreitol. The results of three such experiments are also shown in Fig. 2. Under these conditions the nitrobenzylthioinosine-binding activity was associated with a single low-molecular-weight component parallel to that observed under N₂. The target size was mol.wt. $(122 \pm 9) \times 10^3$ ($n = 3$). Control experiments confirmed that dithiothreitol (5 mM) had no significant effect on either zero-*trans* uridine influx into intact cells (apparent K_m 0.12 and 0.11 mM in the presence and absence of dithiothreitol respectively, V_{max} 86 and 104 mmol/litre of cells per h respectively at 25°C) or high-affinity nitrobenzylthioinosine binding to isolated cell membranes (apparent K_D 1.01 and 0.86 nM respectively, maximum binding 1030×10^{-23} mol/cell for both). Similarly, dithiothreitol did not interfere with the ability of nitrobenzylthioinosine to inhibit uridine influx [inhibition of zero-*trans* uridine influx (extracellular concentration 3.75 mM) of 49 and 39% in the presence and absence of dithiothreitol respectively, at an initial extracellular nitrobenzylthioinosine concentration of 0.1 μ M and a haematocrit of 15%].

Radiation-inactivation analysis therefore estimates that the nitrobenzylthioinosine-binding complex *in situ* has an apparent mol.wt. of 122000. Thus, assuming a protein density of 1.3 g/ml, we calculate that only 0.1% of the erythrocyte membrane volume is occupied by the nitrobenzylthioinosine-binding complex. For comparison, the human erythrocyte (Na⁺ + K⁺)-dependent ATPase has a target size of mol.wt. 330000 (Ellory *et al.*, 1979). Ouabain-binding activity and K⁺-dependent *p*-nitrophenyl phosphatase activity have a lower apparent mol.wt. of 180000, indicating that the Na⁺-pump is composed of two dissimilar subunits (Ellory *et al.*, 1979; see also Jorgenson, 1975; Kyte, 1972). Since we could not measure nucleoside-transport activity directly, it is necessary to distinguish between the terms 'nitrobenzylthioinosine-binding complex' and 'nucleoside-transport system' in the present work. This is particularly important in view of the possibility that nitrobenzylthioinosine may bind to the nucleoside-transport system at a modifier site distinct from the actual nucleoside-

translocation site (Cass *et al.*, 1974; Cass & Paterson, 1976; Eilam & Cabantchik, 1977; Shohami & Koren, 1979; Turnheim *et al.*, 1978).

A value of 122 000 for the molecular weight of the nitrobenzylthioinosine-binding site *in situ* compares favourably with molecular weights for other erythrocyte-transport systems estimated by different methods (e.g. sodium dodecyl sulphate/gel-electrophoresis). For example, the anion-transport system of human erythrocytes (band 3) has a monomer mol.wt. of 95 000 (Cabantchik *et al.*, 1978), but probably exists in the membrane as a dimer or tetramer (Peters & Richards, 1977; Nigg & Cherry, 1979). The glucose-transport system has been estimated to have a monomer mol.wt. of 45 000–65 000 (band 4.5), but is also likely to be present in the membrane as a dimer with an aggregate molecule weight almost identical with that of the nitrobenzylthioinosine-binding complex (Goldin & Rhoden, 1978; Kasahara & Hinkle, 1977; Baldwin *et al.*, 1979; Hinkle *et al.*, 1979).

We thank Dr. J. Green, Department of Medical Physics, Addenbrooke's Hospital, Cambridge, for access to the linear accelerator. Nitrobenzylthioinosine and nitrobenzylthioguanosine were generous gifts from Professor A. R. P. Paterson, University of Alberta Cancer Research Unit, Edmonton, Canada. We are also grateful to Dr. J. Y. F. Paterson of the ARC Institute of Animal Physiology for assistance with the computer analysis of inactivation data. S. M. J. is in receipt of an M.R.C. postgraduate studentship.

References

- Baldwin, S. A., Baldwin, J. M., Gorga, F. R. & Lienhard, G. E. (1979) *Biochim. Biophys. Acta* **552**, 183–188
- Berry, R. J. & Marshall, C. M. (1969) *Phys. Med. Biol.* **14**, 585–596
- Cabantchik, Z. I. & Ginsburg, H. (1977) *J. Gen. Phys.* **69**, 75–96
- Cabantchik, Z. I., Knauf, P. A. & Rothstein, A. (1978) *Biochim. Biophys. Acta* **515**, 239–302
- Cass, C. E. & Paterson, A. R. P. (1976) *Biochim. Biophys. Acta* **419**, 285–294
- Cass, C. E., Gaudette, L. A. & Patterson, A. R. P. (1974) *Biochim. Biophys. Acta* **345**, 1–10
- Chow, C. M. & Islam, M. F. (1970) *Clin. Biochem.* **3**, 295–306
- Dahlig, E., Cass, C. E. & Paterson, A. R. P. (1979) *Abstr. Int. Congr. Biochem.* **XI**, 467
- Eilam, Y. & Cabantchik, Z. I. (1977) *J. Cell Physiol.* **92**, 185–202
- Ellory, J. C. (1979) *Trends Biochem. Sci.* **4**, 99–100
- Ellory, J. C. & Maker, P. (1977) *Biochim. Biophys. Acta* **471**, 111–117
- Ellory, J. C., Green, J. R., Jarvis, S. M. & Young, J. D. (1979) *J. Physiol. (London)* **295**, 10P–11P
- Folkard, E. J., Ellory, J. C. & Hughes-Jones, N. C. (1977) *Immunochemistry* **14**, 529–531
- Goldin, S. M. & Rhoden, V. (1978) *J. Biol. Chem.* **253**, 2575–2583
- Hanes, C. S. (1932) *Biochem. J.* **26**, 1406–1421
- Hinkle, P. C., Sogin, D. C., Wheeler, T. J. & Telford, J. N. (1979) in *Function and Molecular Aspects of Biomembrane Transport (Developments in Bioenergetics and Biomembranes)* (Quagliariello, E., Palmieri, F., Papa, S. & Klingenberg, M., eds.), vol. 3, pp. 487–494, Elsevier/North-Holland Biomedical Press, Amsterdam
- Hollunger, E. G. & Nikalsson, B. H. (1973) *J. Neurochem.* **20**, 821–836
- Houslay, M. D., Ellory, J. C., Smith, G. A., Hesketh, T. R., Stein, J. M., Warren, G. B. & Metcalfe, J. C. (1977) *Biochim. Biophys. Acta* **467**, 208–219
- Jarvis, S. M. & Young, J. D. (1980) *Biochem. J.* **190**, 377–383
- Jorgensen, P. L. (1975) *Quart. Rev. Biophys.* **7**, 239–274
- Kasahara, M. & Hinkle, P. C. (1977) *J. Biol. Chem.* **252**, 7384–7390
- Kempner, E. S. & Schlegel, W. (1979) *Anal. Biochem.* **92**, 2–10
- Kepner, G. R. & Macey, R. I. (1968) *Biochim. Biophys. Acta* **163**, 188–203
- Kyte, J. (1972) *J. Biol. Chem.* **247**, 7642–7649
- Levinson, S. R. & Ellory, J. C. (1973) *Nature (London)* **245**, 122–123
- Levinson, S. R. & Ellory, J. C. (1974) *Biochem. J.* **137**, 123–125
- Nakao, M., Nagano, K., Nakao, T., Mizuno, N., Tashima, Y., Fujita, M., Maeda, H. & Matsudaira, H. (1967) *Biochem. Biophys. Res. Commun.* **29**, 588–592
- Nigg, E. & Cherry, R. J. (1979) *Nature (London)* **277**, 493–494
- Peters, K. & Richards, F. M. (1977) *Annu. Rev. Biochem.* **46**, 523–551
- Pickard, M. R., Brown, R. R., Paul, B. & Paterson, A. R. P. (1973) *Can. J. Biochem.* **51**, 666–672
- Pollard, E. C., Guild, W. R., Hutchinson, F. & Setlow, R. B. (1955) *Prog. Biophys. Biophys. Chem.* **5**, 72–108
- Shohami, E. & Koren, R. (1979) *Biochem. J.* **178**, 271–277
- Turnheim, K., Plank, B. & Kolassa, N. (1978) *Biochem. Pharmacol.* **27**, 2191–2197