Effects of serum, lithium, ethacrynic acid, and low external concentration of potassium on specific [^3H]-ouabain binding to human lymphocytes

Rapeport *et al.* (1986) confirmed the increase in specific [^3H]-ouabain binding to normal lymphocytes incubated with *undialysed* fetal calf serum (FCS), which had earlier been shown (Oh *et al.*, 1987). However, their results obtained with dialysed serum are difficult to interpret.

The authors conclude that similar increases in specific [^3H]-ouabain binding occur with *dialysed* FCS in the presence of ethacrynic acid, lithium, and external potassium, at certain concentrations. However, their median values of specific [^3H]-ouabain binding before cell incubation, 27.0–32.0 fmol per 10^6 cells (Table 1), are about half the mean values in the normal subjects studied by Boon *et al.* (1984, 1985) in the same laboratory, and half those in our healthy subjects (Oh & Taylor, 1986; and below).

We have found that the specific *maximum* binding (B_{max}) of [^3H]-ouabain to healthy lymphocytes (the number of sodium pumps) is normally distributed, and not significantly affected by gender, race, or age (Oh & Taylor, 1985). In contrast, specific [^3H]-ouabain binding is significantly reduced both in healthy subjects who have hypertensive first-degree relatives (Oh & Taylor, 1985), and in patients with untreated essential hypertension (Oh & Taylor, 1986). In our study in 182 healthy subjects (aged 19–55 years; 116 men; 24 Europeans, 115 Chinese,

and 43 Indians) the maximum specific binding of [^3H]-ouabain was 68.4 ± 1.7 (mean $\pm$ s.e. mean) fmol per 10^6 cells. In those 124 subjects without familial hypertension, maximum specific binding was 73.2 ± 1.5 (s.e. mean) fmol per 10^6 cells.

Rapeport *et al.* (1986); then, may not in fact have measured the specific *maximum* binding of [^3H]-ouabain to the cells. Their data on [^3H]-ouabain binding should therefore be interpreted with caution.

Rapeport *et al.* (1986) found that specific [^3H]-ouabain binding after cell culture with *dialysed* FCS increased by 30% in 11 subjects but decreased in the others (their Table 1). This variation also hinders interpretation. We have found that specific [^3H]-ouabain binding usually decreases slightly, but does not increase, when normal lymphocytes are incubated for 72 h with serum dialysed as described, except that we use deionised distilled water. These findings apply to both FCS (Gibco and Flow Labs) and human serum (unpublished data). Therefore the authors may sometimes have removed incompletely by dialysis the factor or factors in FCS that enhance specific [^3H]-ouabain binding.

Rapeport *et al.* (1986) state that 1 mmol l^{-1} lithium increased and 2–20 mmol l^{-1} reduced specific [^3H]-ouabain binding over 72 h (data for 2–20 mmol l^{-1} not given). We have studied the

Table 1 Specific maximum binding of [^3H]-ouabain (B_{max}) and ouabain sensitive ^{86}Rb influx in normal human lymphocytes before and after incubation in the absence (control) and presence of ethacrynic acid. The culture medium in 72 h experiments contained dialysed fetal calf serum (10% v/v). Data are given as mean $\pm$ s.d.

		Specific maximum [^3H]-ouabain binding (fmol per 10^6 cells)							
		After incubation for 2 h with ethacrynic acid ($\mu\text{mol l}^{-1}$); (n = 8)							
		0	0.1	1	3	10	30	100	300
		62.3 $\pm$ 5.8	NM	63.0 $\pm$ 4.9	63.7 $\pm$ 4.4	65.2 $\pm$ 4.2*	66.1 $\pm$ 6.0‡	66.9 $\pm$ 6.8†	67.5 $\pm$ 7.5†
Before incubation	After incubation for 72 h with ethacrynic acid ($\mu\text{mol l}^{-1}$); (n = 6)								
	0	0.1	1	3	10	30	100	300	
74.2 $\pm$ 8.7	72.4 $\pm$ 8.6	67.2 $\pm$ 8.4	66.7 $\pm$ 8.7	65.6 $\pm$ 8.2*	38.4 $\pm$ 7.7‡	5.8 $\pm$ 4.4‡	1.3 $\pm$ 2.5‡	NM	
		Ouabain sensitive $^{86}\text{rubidium}$ influx (pmol h^{-1} per 10^6 cells)							
		After incubation for 1 h with ethacrynic acid ($\mu\text{mol l}^{-1}$); (n = 8)							
		0	0.1	1	3	10	30	100	300
		9.5 $\pm$ 2.4	NM	10.9 $\pm$ 2.5	11.0 $\pm$ 1.8**	11.4 $\pm$ 1.2‡	11.1 $\pm$ 2.4*	11.8 $\pm$ 2.0**	13.0 $\pm$ 1.0‡
Before incubation	After incubation for 72 h with ethacrynic acid ($\mu\text{mol l}^{-1}$); (n = 6)								
	0	0.1	1	3	10	30	100	300	
9.2 $\pm$ 2.8	8.9 $\pm$ 2.6	9.1 $\pm$ 1.4	8.2 $\pm$ 1.7	7.6 $\pm$ 2.1†	4.2 $\pm$ 0.6‡	0.3 $\pm$ 0.2‡	0.2 $\pm$ 0.1‡	NM	

* $P < 0.05$, ** $P < 0.01$, † $P < 0.005$, ‡ $P < 0.001$; paired *t*-tests comparing values with control.
NM = not measured.

acute and longer term effects of lithium and ethacrynate on [^3H]-ouabain binding in lymphocytes incubated with 10% v/v dialysed FCS, in controlled experiments using the method described by Boon *et al.* (1984). The cell donors were healthy, non-obese Chinese without familial hypertension. Ouabain sensitive $^{86}\text{rubidium}$ influx (sodium pump activity) was also measured over 1 h using the method described by Oh *et al.* (1987). Trypan blue exclusion assessed the cell viability.

Compared with controls, lithium (1, 3, 10, and 30 mmol l^{-1}) (final concentrations) acutely decreased [^3H]-ouabain binding (2 h) but significantly increased ouabain sensitive $^{86}\text{rubidium}$ influx (1 h) ($n = 6$). Over 72 h lithium (3–30 mmol l^{-1}) significantly enhanced specific ouabain binding, and tended to increase total and ouabain sensitive influx of $^{86}\text{rubidium}$, in a dose related manner. Lithium (1 mmol l^{-1}) did not significantly affect any measurement. The mean ($\pm$ s.d.) control values of specific ouabain binding were 66.9 ± 7.7 and 71.7 ± 11.8 fmol per 10^6 cells ($n = 6$ (1 h); $n = 6$ (72 h)). Ouabain resistant $^{86}\text{rubidium}$ influx was significantly reduced in both 1 and 72 h experiments (data not shown).

Thus lithium (3–30 mmol l^{-1}) increases both the number and activity of sodium pumps in lymphocytes, 95% of which excluded Trypan blue. Over 72 h lithium (100 mmol l^{-1}) greatly reduced ouabain binding, total $^{86}\text{rubidium}$ influx, cell counts, and Trypan blue exclusion. These ouabain binding results are qualitatively similar to those of Boardman *et al.* (1975) in HeLa cells, but opposite to those of Rapeport *et al.* (1986).

Rapeport *et al.* (1986) suggest that the increase in specific [^3H]-ouabain binding produced by incubating lymphocytes for 72 h with ethacrynate is initiated by a rise in intracellular sodium concentration. However, neither they nor Naylor & Smith (1981) tested this inference by measuring the sodium concentration, or the rate of $^{86}\text{rubidium}$ influx or sodium efflux, in lymphocytes.

We found that ethacrynate (10–300 $\mu\text{mol l}^{-1}$)

over 2 h significantly increased specific [^3H]-ouabain binding in a dose related way. In contrast, over 72 h ethacrynate (3–10 $\mu\text{mol l}^{-1}$) significantly *reduced* specific [^3H]-ouabain binding in a dose related way without altering cell viability (Table 1).

Ethacrynate (3–300 $\mu\text{mol l}^{-1}$) also significantly increased total $^{86}\text{rubidium}$ influx and ouabain sensitive influx acutely, while reducing ouabain resistant influx. Over 72 h ethacrynate (3–10 $\mu\text{mol l}^{-1}$) *decreased* total $^{86}\text{rubidium}$ influx, and ouabain sensitive and ouabain resistant influx in a dose related way. Over 72 h ethacrynate (30 and 100 $\mu\text{mol l}^{-1}$) greatly reduced ouabain binding, total $^{86}\text{rubidium}$ influx, cell counts, and Trypan blue exclusion.

Thus the sodium pumps are damaged by exposure for 72 h to both lithium (100 mmol l^{-1}) and ethacrynate ($\geq 30 \mu\text{mol l}^{-1}$). Slight injury to the plasma membrane cannot, however, be assessed by dye exclusion.

We conclude that the increase in the number (over 72 h) and activity (over 1 and 72 h) of sodium pumps due to lithium (3–30 mmol l^{-1}) may be achieved by the production of new pumps sites, like in HeLa cells (Boardman *et al.*, 1975). Alternatively, the increase may compensate for the blockade by lithium of the ouabain resistant, frusemide sensitive cotransport; or both mechanisms may apply. Ethacrynate *acutely* enhances both the number and activity of sodium pumps, perhaps in a similar way to lithium. However, the dose dependent reduction in pump number and activity by ethacrynate over 3 days suggests a toxic effect on the lymphocytic plasma membrane which might swamp any continuing stimulatory effect of ethacrynate.

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Effects of serum, lithium, ethacrynic acid, and a low external concentration of potassium on specific [^3H] ouabain binding to human lymphocytes—a reply

Thank you for the opportunity to comment on the letter from Oh & Taylor (1987).

They correctly point out that the mean values given in Rapeport *et al.* (1986) for the B_{max} of specific [^3H] ouabain binding to normal human lymphocytes are lower than those previously quoted from this laboratory. However, there is known to be wide variation in specific [^3H] ouabain binding to fresh lymphocytes from time to time, partly because of interindividual variation, and partly for other reasons which are not clear. For example, in their communal work with us Oh *et al.* (1983) quoted mean values as low as 41.6 fmol per 10^6 cells (with s.d. of 9.7) and as high as 69.6. After incubation in dialysed human serum (10%) the value was 42.5 (s.d. 16.9) fmol per 10^6 cells (Oh *et al.*, 1983, 1987). Furthermore, while Oh & Taylor (1987) accept that the data given in Rapeport *et al.* (1986) confirm our previous communal findings in regard to the effect of undialysed serum in increasing specific [^3H] ouabain binding to human lymphocytes (Oh *et al.*, 1983, 1987), they do not accept the other data given in Rapeport *et al.* (1986), data which were collected using exactly the same techniques in the same laboratory.

Oh & Taylor (1987) imply that data in Rapeport *et al.* (1986) showing small increases and decreases in specific [^3H] ouabain binding to normal lymphocytes after incubation for 72 h in dialysed fetal calf serum were significant, whereas that was not the case, and these are changes which we attribute to the inherent variability of the technique.

Oh & Taylor (1987) report that specific [^3H] ouabain binding to normal lymphocytes does not

increase when the cells are incubated for 72 h with dialysed serum. However, they have themselves been associated with a report of such an increase in a small number of experiments in one of our communal publications, based on work carried out in this laboratory (Oh *et al.*, 1983). In those experiments specific [^3H] ouabain binding to lymphocytes incubated in dialysed fetal calf serum for 72 h was 48.2 fmol per 10^6 cells, compared with a pre-incubation value of 41.6. Furthermore, in the above letter they report control values for specific [^3H] ouabain binding of 66.9 fmol per 10^6 cells after a 1 h incubation (presumably in dialysed serum) and 71.7 after 72 h.

Although it is true that, in general, specific [^3H] ouabain binding to lymphocytes decreases with increasing duration of incubation, the inherent variability in its measurement means that sometimes the alternative result will occur merely by chance. For that reason, in Rapeport *et al.* (1986) we have made statistical comparisons of B_{max} data from comparable 72 h incubations, with and without the manipulations in which we were interested (e.g. the removal of external potassium), and not by comparing pre-incubation values with post-incubation values. Oh & Taylor (1987) do not present any of the results of their measurements of specific [^3H] ouabain binding to lymphocytes which have been incubated with lithium. However, their results, judged from their allusions to them and taken in conjunction with their results with ethacrynic acid, lead to inconsistencies. They report qualitatively similar results after incubation for 72 h with lithium to those found in HeLa cells by Lamb and his