J. Pharm. Pharmac., 1970, **22**, 767–773 *Received March* 18, 1970

The effect of magnesium ions and Tris buffer on the uptake of cetyl trimethyl ammonium bromide by *Escherichia coli*

W. G. SALT AND DAVID WISEMAN

School of Studies in Pharmacy, University of Bradford, Bradford 7, Yorks, U.K.

The uptake of CTAB by *E. coli* from a glucose-free mineral salts medium occurs in two distinct phases. Increasing the magnesium ion content of the suspending medium emphasizes the diphasic nature of the isotherm. The primary phase is extended and the height of the "saturation" plateau of the secondary phase, as a function of CTAB uptake, is reduced. The CTAB uptake isotherm for *E. coli* suspended in water is not obviously diphasic and the maximum amount of CTAB taken up by the cells at "saturation" is less than that in medium. The height of this plateau is influenced by Tris buffer, an increase in the Tris concentration increasing the maximum CTAB uptake. A method for the rapid screening of uptake isotherms based on turbidity changes is also discussed.

Isotherms for the uptake of CTAB by *E. coli* have been described by Salton (1951) and by Salt & Wiseman (1968). Salt & Wiseman showed that uptake occurs in two distinct phases and suggested that the first phase was predominantly a surface phenomenon, whilst the second phase resulted from penetration into the cells. These studies were of uptake of CTAB from a buffered medium containing large numbers of inorganic and organic ions. The results reported here describe the effects of changes in the concentration of magnesium ions and the buffer tris (hydroxymethyl)aminomethane on the shape of the uptake isotherm.

The use of changes in turbidity of cell suspensions treated with CTAB for the rapid screening of uptake isotherms is also discussed.

EXPERIMENTAL

NNN-Trimethyl[cetyl-1-^{14}C]ammonium bromide (^{14}C-CTAB) was obtained from the Radiochemical Centre, Amersham, Bucks, England and *NNN*-trimethylcetylammonium bromide (CTAB) was kindly prepared by J. E. Adderson using the method of Adderson & Taylor (1964). Tris (hydroxymethyl)aminomethane (Tris), was obtained from Mann Research Laboratories, New York, U.S.A.

The organism was *Escherichia coli* NCTC 1093; the culture and suspending media, conditions of cultivation and method of measuring uptake of ^{14}C-CTAB were, unless otherwise stated, as described by Salt & Wiseman (1968).

Turbidity changes. Equal volumes of suspensions of *E. coli* and solutions of CTAB in various media were mixed and maintained at 25° for 15 min. The absorbance of these mixtures was measured at 650 nm using a 1 cm path length in a Unicam SP500 series 2 spectrophotometer.

Magnesium release. CTAB-treated suspensions were prepared and maintained as above after which the cells were removed by centrifuging at 5000 rev/min for 10 min.

Samples of the supernatant fluids were assayed for magnesium content using a calibrated Unicam SP900 flame photometer correcting for surface tension effects by the addition of 0·1 ml of 1% CTAB solution to each sample before assay.

Dye solubilization. The solubilization of waxoline yellow IS by solutions of CTAB in either water of glucose free medium (GFM) was studied by equilibrating the solutions with an excess of solid dye for 24 h at 25°, centrifuging the mixtures at 5000 rev/min for 15 min to remove the undissolved dye, and assaying the solubilized waxoline yellow spectrophotometrically.

RESULTS

Cells of *E. coli* suspended in GFM of different magnesium ion concentrations exhibit different isotherms for the uptake of ^{14}C-CTAB (Fig. 1b). As the magnesium ion concentration of the suspending medium is increased, the maximum amount of CTAB taken up by the cells is reduced, by an amount that is a function of the magnesium ion concentration (Fig. 2). In addition to this decrease in the height of the saturation plateau, the equilibrium concentration of CTAB required to complete the primary phase of uptake increases, accentuating the diphasic nature of the isotherm. The second phase of uptake still commences, however, after the same amount of CTAB has been taken up by the cells.

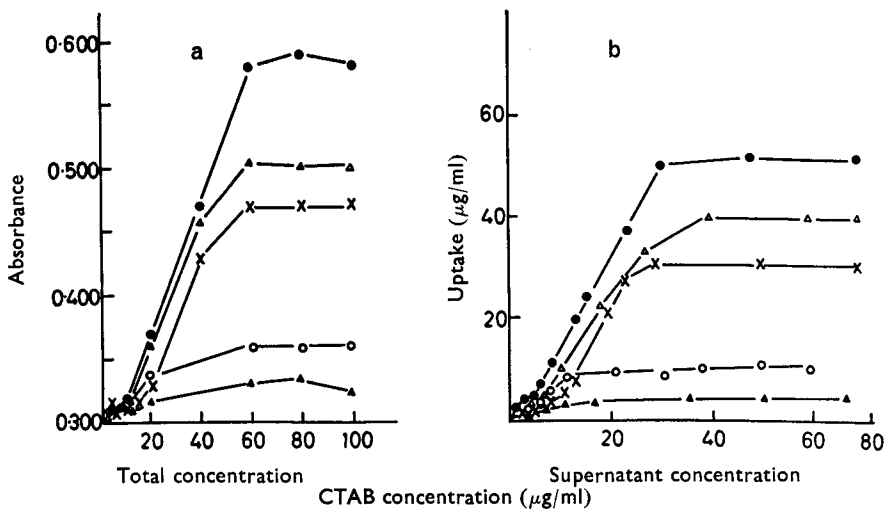

FIG. 1. A. Turbidity changes in CTAB-treated cells of *E. coli* suspended in glucose-free medium (pH 7·7) containing different concentrations of magnesium ions.

B. Uptake of ^{14}C-CTAB by *E. coli* suspended in glucose-free medium (pH 7·7) containing different concentrations of magnesium ions, plotted as a function of the ^{14}C-CTAB concentration in the supernatant fluid. Cell concentration 0·125 mg/ml, 3·2 × 10^8 cells/ml. Temperature 25°. Contact time 15 min. ●—● GFM (M/2000 Mg^{++}), △—△ M/100 Mg^{++}, ×—× M/50 Mg^{++}, ○—○ M/20 Mg^{++}, ▲—▲ M/10 Mg^{++} in GFM.

Cells treated with CTAB in water release magnesium into the suspending medium. Magnesium release occurs even at CTAB concentrations as low as 2 μg/ml and the extent of the release increases with increase in the CTAB concentrations.

The release of magnesium (in μg/ml) from CTAB-treated cells of *E. coli* suspended in water at a cell concentration 3·2 × 10^8 cells/ml (0·125 mg/ml) at 25°; contact time 15 min, is 0·01, 0·04, 0·06, 0·08, 0·10, 0·15 and 0·29 for CTAB concentrations of 1, 2, 3, 4, 6, 10, 20 μg/ml respectively.

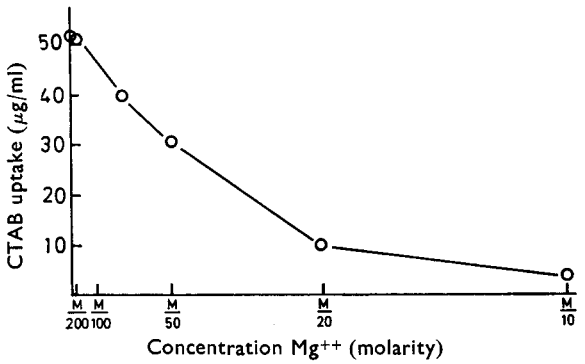

FIG. 2. The effect of increasing magnesium ion concentration on the maximum uptake of ^{14}C-CTAB by cells of *E. coli* suspended in glucose-free medium (pH 7·7). Cell concentration 0·125 mg/ml, 3·2 × 10^8 cells/ml. Temperature 25°. Contact time 15 min.

Fig. 1a shows the effect of different CTAB concentrations on the turbidity of suspensions of *E. coli* in GFM of various magnesium ion concentrations. The curves show two distinct regions. A first region where little change in absorbance occurs, as the CTAB concentration is increased, followed by a second region in which the absorbance increases approximately linearly with CTAB concentration, finally flattening into a plateau where little further change in turbidity occurs. The height of this plateau is decreased by increasing the magnesium ion concentration whilst the length of the first region, in terms of range of CTAB concentration, is increased. In all cases the concentration of CTAB equivalent to the beginning of the plateau in the turbidity curves approximates to the concentration just required to "saturate" the cells with CTAB. In addition, the threshold concentration of CTAB required to cause a marked increase in turbidity corresponds to the concentration at which the secondary phase of uptake commences.

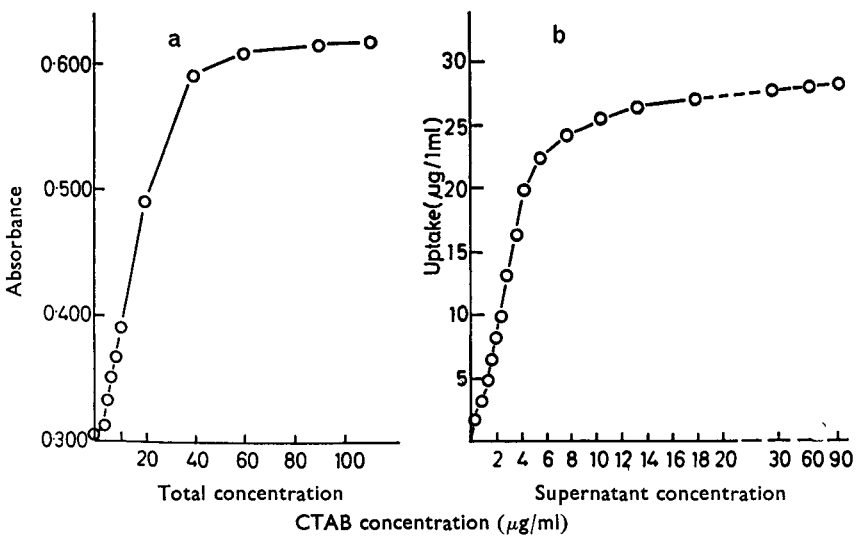

FIG. 3. A. Turbidity changes in CTAB-treated cells of *E. coli* suspended in water. B. Uptake of ^{14}C-CTAB by *E. coli* suspended in water plotted as a function of the ^{14}C-CTAB concentration in the supernatant fluid. Cell concentration 0·125 mg/ml, 3·2 × 10^8 cells/ml. Temperature 25°. Contact time 15 min.

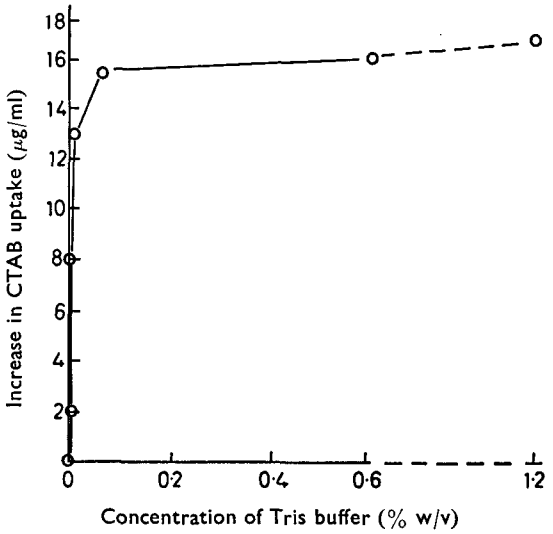

FIG. 4. The effect of Tris buffer concentration (pH 7·7) on the maximum uptake of ^{14}C-CTAB by *E. coli* suspended in water. Cell concentration 0·125 mg/ml, 3·2 × 10^8 cells/ml. Temperature 25°. Contact time 15 min.

Fig. 3a shows the changes in turbidity of suspensions of *E. coli* treated with different concentrations of CTAB in water. Although the curve is not obviously diphasic (cf. Fig. 1a) it is S shaped being initially convex to the CTAB concentration axis, becoming linear and finally flattening off in a plateau at a CTAB concentration of about 60 μg/ml. Fig. 3b is the uptake isotherm from water for CTAB by *E. coli* corresponding to the results shown in Fig. 3a. As in Fig. 3a the curve is not obviously diphasic although there is a change in slope at a CTAB uptake of about

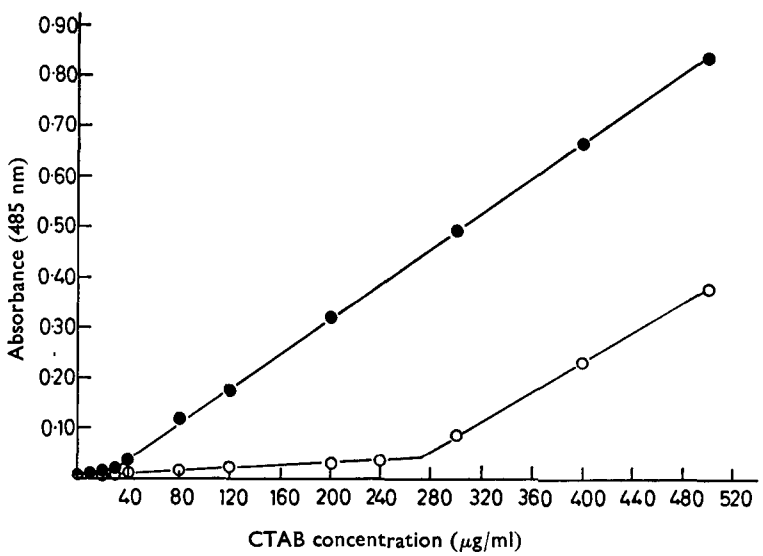

FIG. 5. Solubilization of waxoline yellow IS by CTAB at 25° in ●—● glucose free medium and ○—○ water.

3 μg/ml. Above this uptake the curve is approximately linear flattening into a plateau at an uptake of about 30 μg/ml from an original concentration of 60 μg/ml.

Cells of *E. coli* suspended in GFM have a greater saturation capacity for CTAB than cells suspended in water. Fig. 4 shows the effect of changes in the concentration of Tris (pH 7·7) in water on the height of the uptake saturation plateau. Increasing the concentration from zero to 0·06% w/v causes a 50% increase in the amount of CTAB taken up by the cells at "saturation" but further increases in Tris concentration has little or no effect on uptake. The Tris concentration in GFM is 1·2% w/v.

Fig. 5 shows the solubilization of waxoline yellow IS by solutions of CTAB in water and GFM. The results suggest critical micelle concentrations for CTAB of 280 μg/ml and 30 μg/ml in water and GFM respectively.

DISCUSSION

The diphasic uptake of CTAB by cells of *E. coli* was first reported by Salt & Wiseman (1968). They suggested that CTAB uptake occurred initially as a surface phenomenon, individual cells building up a critical amount of CTAB before the second phase of uptake, penetration into the cells, commences.

Cells suspended in a medium containing an excess of a magnesium salt have a high concentration of magnesium ions loosely bound at their surface (Strange & Shon, 1964; Tempest, Dicks & Meers, 1967). Our results suggest that the appearance and shape of the first phase of CTAB uptake by bacterial cells is due to competition with such ions for sites at the cell surface and that CTAB uptake is primarily an ion exchange process. This is analogous to the uptake of basic stains by bacteria reported by McCalla (1941) who studied the release of hydrogen ions and magnesium ions from bacterial cells treated with crystal violet and methylene blue. The release of magnesium from CTAB-treated cells further supports this analogy, increases in the magnesium content of the suspending medium being detected even in low CTAB concentrations (less than 4 μg/ml). Cations, such as magnesium ions, play a major role in the stabilization of cell membranes. and their removal and replacement by CTAB could well cause phase changes in the cell phospholipids, such as the change from one micellar form to another as suggested by Seufert (1965) who studied the effect of surfactants on spread lipid bilayers. The presence of excess magnesium ions would oppose such an exchange by simple competition.

The diphasic nature of the CTAB uptake isotherm is further emphasized in this paper, and the significance in growth and viability studies of the point of inflection, corresponding to the start of the second phase of uptake, has been demonstrated by Salt & Wiseman (1970).

Tris buffer has a marked effect on the uptake of CTAB by bacteria. The shape of the second region of the uptake isotherm, similar to the C type of Giles, MacEwan & others (1960), together with the increase in the height of the saturation plateau, suggests that Tris may be acting as an indirect carrier for CTAB, penetrating into the cell revealing more sites for uptake than are readily available in its absence. Tris has been shown to alter cellular permeability barriers (Voss, 1967; Mclean, Poland & others, 1967) and its ability to penetrate into intracellular membraned compartments has been reported (Humphreys & Garrard, 1969) tempting the suggestion that the increase in CTAB uptake in its presence is due to binding to the proteins and nucleic acids in normally inaccessible regions of the cell.

The critical micelle concentration (CMC) of CTAB in GFM is reduced to one tenth of its normal value in water (Fig. 5). The saturation plateaus reported for the GFM systems (Fig. 1b) may not therefore relate directly to cellular saturation but to competition between the micelles and the cells for the CTAB still in solution. This also suggests that CTAB uptake does not occur as the uptake of aggregates, since uptake might otherwise be expected to continue after the equilibrium concentration had exceeded the CMC. The decrease in the total amount of CTAB taken up by the cells as the magnesium ion concentration is increased, may be a reflection of this CMC effect on plateau height. Alternatively it may be explained on a competition basis, though whether the magnesium ions are competing with the Tris or the CTAB cannot be directly interpreted.

Turbidity changes of cell suspensions treated with quaternary ammonium compounds have been observed by other workers (Avi-Dor, Kuczynski & others, 1956; Gilby & Few, 1960; Hugo & Frier, 1969) but no real attempt has been made to put this phenomenon to use by relating it to the uptake isotherm of the antibacterial agent in question. Figs 1 and 3 show that the CTAB-induced turbidity changes in cells of *E. coli* are qualitatively similar to the CTAB uptake isotherms for that system and the effect of varying the ionic content of the suspending medium produces turbidity changes that can be related to the shape of the later determined isotherms.

It has been suggested that such changes in turbidity may result from shrinkage of the cells due to osmotic effects (Mager, Kuczysnki & others, 1956; Bernheim, 1963) or to changes in the reflective characteristics or refractive index of the cells due to the action of the quaternary salt (Hugo & Frier, 1969), though the latter two possibilities could be a consequence of the former. It can be shown that the large change in turbidity of the cells could be produced by a very small change, about 2%, in their refractive index. Such a small change could be expected in cells taking up large amounts of CTAB, as for example occurs during the second phase of CTAB uptake in GFM (Fig. 1b), especially if such uptake is taking place by penetration into the cells and by combination with their constituents.

Thus the effects of various ions on the uptake of CTAB by *E. coli* can be qualitatively predetermined. The technique is relatively quick, involving none of the centrifuging and sampling of the normal methods available for studying uptake and the effects of several different ions or concentrations of ions on CTAB uptake can be indicated in the time normally required for one experiment.

Acknowledgement

We would like to thank Dr. R. M. Rye for valuable advice, and one of us (W.G.S.) is indebted to the Science Research Council for a Research Studentship.

REFERENCES

ADDERSON, J. E. & TAYLOR, H. (1964). 4th International Congress of Surface Activity, 613–620.

AVI-DOR, Y., KUCZYNSKI, M., SCHATZBERG, F. & MAGER, J. (1956). *J. gen. Microbiol.*, **14**, 76–83.

BERNHEIM, F. (1963). *Ibid.*, **30**, 53–58.

GILBY, A. R. & FEW, A. V. (1960). *Ibid.*, **23**, 19–26.

GILES, C. H., MACEWAN, T. H., NAKHWA, S. N. & SMITH, D. (1960). *J. chem. Soc.*, 3973–3993.

HUGO, W. B. & FRIER, M. (1969). *Appl. Microbiol.*, **17**, 118–127.

HUMPHREYS, T. E. & GARRARD, L. A. (1969). *Phytochemistry*, **8**, 1055–1064.

MAGER, J., KUCZYNSKI, M., SCHATSBERG, F. & AVI-DOR, Y. (1956). *J. gen. Microbiol.*, **14**, 69–75.

MCCALLA, T. M. (1941). *Stain Tech.*, **16**, 27–32.

MCLEAN, W. M., POLAND, D. M., COHON, M. S., PENZOTTE, S. C. & MATTOCK, A. M. (1967). *J. pharm. Sci.*, **56**, 1614–1626.

SALT, W. G. & WISEMAN, D. (1968). *J. Pharm. Pharmac.*, **20**, *Suppl.* 145S–175S.

SALT, W. G. & WISEMAN, D. (1970). *Ibid.*, **22**, 261–264.

SALTON, M. R. J. (1951). *J. gen. Microbiol.*, **5**, 391–404.

SEUFERT, W. D. (1965). *Nature, Lond.*, **207**, 174–176.

STRANGE, R. E. & SHON, M. (1964). *J. gen. Microbiol.*, **34**, 99–114.

TEMPEST, D. W., DICKS, J. W. & MEERS, J. C. (1967). *Ibid.*, **49**, 139–147.

VOSS, J. G. (1967). *Ibid.*, **48**, 391–400.