Biochemical Pharmacology, Vol. 23, pp. 2857–2865. Pergamon Press, 1974. Printed in Great Britain.

KINETICS OF CHOLINE UPTAKE IN NEUROBLASTOMA CLONES*

RAFFAELE MASSARELLI, JARKA CIESIELSKI-TRESKA, ALIX EBEL† and PAUL MANDEL

Centre du Neurochimie du CNRS and Institut de Chimie Biologique, Faculté de Médecine, 67085 Strasbourg Cedex, France

(*Received* 21 *December* 1973; *accepted* 6 *March* 1974)

Abstract—The uptake of choline has been investigated in an inactive clone (N_{18}) and in a cholinergic clone (S_{21}) of mouse neuroblastoma C1300. Choline uptake was linear with time and a saturation plateau appeared at about 120 min for clone N_{18} and at 40 min for clone S_{21}. Sodium substitution inhibited the incorporation of choline, sucrose and cesium being more inhibitory than lithium. Potassium ferricyanide and ouabain inhibited the uptake in both clones. Two mechanisms, which differ for their affinity towards choline, have been revealed, with different substrate concentrations. Clone S_{21} showed a high affinity component with an apparent K_m which was much lower ($1{\cdot}1 \times 10^{-7}$ M) than the one presented by clone N_{18} ($2{\cdot}6 \times 10^{-6}$ M).

CHOLINE cannot be synthesized by nervous tissue[1,2] and thus it has to be taken up by the cells of this tissue. The transport of choline across the neuronal membrane is then indispensable for neuronal phospholipid metabolism and for the synthesis of acetylcholine.

Choline uptake has been studied in a number of non-neuronal tissues such as kidney[3] and erythrocytes,[4,5] and in brain slices,[6] nervous tissue cultures[7] and synaptosomes.[8–15] Complex kinetics have been determined for the uptake into synaptosomes and two systems with different affinity for the substrate have been defined. The high affinity system appears to be specifically directed towards acetylcholine synthesis[12,14,15] possibly being rate limiting in the synthesis of the neurotransmitters.

In cultures of the nervous system similar kinetics have been observed and we have detected an active high affinity uptake system which is specifically located in neurons.[16] Studies have also been carried out on neuroblastoma cells with similar results.[17] This paper reports on choline uptake in a cholinergic (S_{21}) and in an inactive (N_{18}) clone of neuroblastoma C1300.[18–23]

MATERIALS AND METHODS

Cell cultures. Two clones (gifts of Dr. M. Nirenberg) were studied. Clone S_{21} was defined cholinergic by the presence of choline acetyltransferase[18] (acetyl CoA: choline *O*-acetyltransferase, EC 2.3.1.5; ChAc). Clone N_{18} was defined as inactive since

* Part of this paper was presented at the IVth Meeting Int. Soc. Neurochem., Tokyo, 1973.

† Chargée de Recherche au CNRS.

Abbreviations used: AChE, acetylcholinesterase; ChAc, choline acetyltransferase; IsoOMPA, tetraethylpyrophosphoramide.

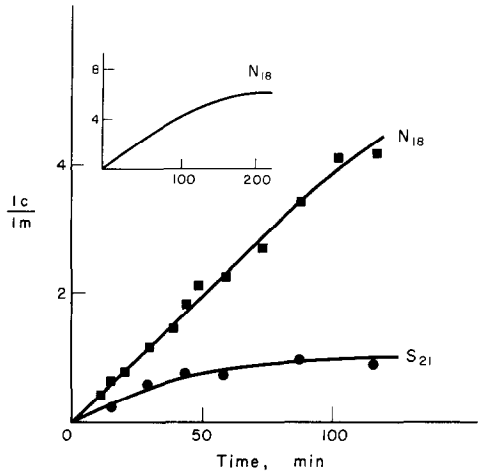

FIG. 1. Cells were incubated with 0·5 μCi/ml of Me-^{14}C choline. The medium choline concentration was lower than 0·5 μM. Each point is the mean of at least five experiments. The data are plotted as ratio of total radioactivity in the cells (Ic)/mg of protein to total radioactivity in the medium (Im) $\times$ 10^{-3}. The non-saturable component has been calculated by experiment performed at 4°.[17]

it lacked ChAc activity,[18] though having a high acetylcholinesterase (acetylcholine hydrolase EC 3.1.1.7; AchE) activity.[23] Both enzyme activities were assayed in our laboratory as explained in the text.

Cells were cultured at 37° in Falcon plastic flasks (75 cm^2) with Dulbecco minimal nutrient medium (Gibco) containing 10% foetal calf serum (FCS) under an atmosphere of 95% air and 5% CO_2 at 100% humidity. When the stationary phase of growth was attained, the cells were transferred to Falcon Petri dishes (diameter 6 cm) and when the stationary phase was reattained, the cells were used for the uptake experiments.

Incubation procedures. The Dulbecco medium was discarded, and 5 ml of Krebs–Ringer phosphate (pH 7·2) containing 0·5 μCi/ml of Me^{14}C-choline (60 mCi/m-mole,

TABLE 1. EFFECT OF SODIUM SUBSTITUTION AND OTHER INHIBITORS ON CHOLINE UPTAKE IN CLONES N_{18} AND S_{21}

	Counts min^{-1} μg protein^{-1}	
	Clone S_{21}	Clone N_{18}
Control	73 ± 5·0	210 ± 14
Sucrose	—	129 ± 7·5‡
Lithium	51 ± 3·9‡	147 ± 12·4†
Cesium	45 ± 3·5†	122 ± 17†
Control	65 ± 2·15	270 ± 6
K-ferricyanide 10^{-3} M	53·7 ± 0·9†	239 ± 9*
Ouabain 10^{-4} M	42 ± 4·0	204 ± 3†

Each value corresponds to the mean ± S.E.M. of five experiments. The incubation was performed for 60 min; preincubation 16 min. Incubation conditions: see legend of Fig. 1.

* $P < 0·02$.

† $P < 0·01$.

‡ $P < 0·001$.

Radiochemical Centre, Amersham) was added to each dish. Cultures from clone N_{18} were incubated for 60 min and cultures from clone S_{21} for 30 min. At these times uptake was still linear. At the end of the incubation, the medium was discarded and the dishes were rapidly washed three times with 0·14 M NaCl. The Petri dishes were then dried at 37° and 2 ml of concentrated formic acid was added. The cells dissolved completely, as shown by microscopic observation; 0·5 ml of the solution was transferred to scintillation vials, together with 10 ml of scintillation fluid (Omnifluor, NEN Chemicals, 4 g, toluene 1 liter, ethanol 400 ml) and counted in a scintillation spectrometer (Intertechnique SL30).

Enzyme assays. ChAc and AchE activity were measured by the radiometric method of McCaman and Hunt,[24] modified by Goldberg *et al.*[25] In clone S_{21} ChAc activity was 13·6 nmoles hr^{-1} mg $protein^{-1}$ and AchE activity 0·41 μmole hr^{-1} mg $protein^{-1}$. In clone N_{18} ChAc activity was not detectable; AChE activity was revealed histochemically.[23]

Drugs. When the effect of drugs was to be tested, the cells were preincubated in the presence of the drug for 15 min prior to addition of the labelled choline.

The following drugs were used: eserine sulphate (Sigma Chemical Co. Ltd.), tetraisopropyl pyrophosphoramide (isoOMPA) (Koch-Light Lab.); ouabain (Sigma Chemical Co. Ltd.).

The K_m and V_{max} values were calculated by the algebraic method of Wilkinson[26] using an Olivetti Programma 602.

Proteins were determined by the method of Lowry *et al.*[27]

RESULTS

Uptake of choline into N_{18} cells was linear for more than 1 hr while S_{21} cells showed a saturation plateau at about 40 min (Fig. 1). The uptake in clone N_{18} was sodium-dependent. Substitution of sodium chloride with sucrose markedly reduced choline uptake, while neither lithium nor cesium ions could replace the sodium ions although lithium was somewhat more active than cesium (Table 1). Similar results were obtained with clone S_{21} (Table 1).

Choline uptake was inhibited in both N_{18} and S_{21} clones by potassium ferricyanide (10^{-3} M) and ouabain (10^{-4} M) suggesting active uptake (Table 1).

When the uptake of choline by the N_{18} cells was studied as a function of substrate concentration (1–40 μM) (Fig. 2a, b) two components were detected, which were more clearly seen when $[S]/v$ was plotted as a function of [S] (Fig. 3). The K_m and V_{max} are given in Table 2.

The kinetics of clone S_{21} cells showed only a low affinity component when the data were plotted according to Lineweaver and Burk[28] (Fig. 4a, b) of $[S]/v$ as a function of [S] (Fig. 5).[29,30] The K_m and V_{max} are shown in Table 2. The substrate concentration range was the same as in clone N_{18} (1–40 μM). In order to clarify the role of the active component in the transport of choline in both N_{18} and S_{21} clones, the concentrations of the substrate were reduced (0·1–10 μM). A more clear distinction appeared between the high affinity and the low affinity components (Figs. 6 and 7). K_m and V_{max} are given in Table 3.

When cholinesterase inhibitors were added to the incubation medium of N_{18} cells, contradictory results were obtained. Surprisingly eserine sulphate (10^{-3} M) increased the uptake of choline while isoOMPA (10^{-4} M) inhibited the uptake (Table

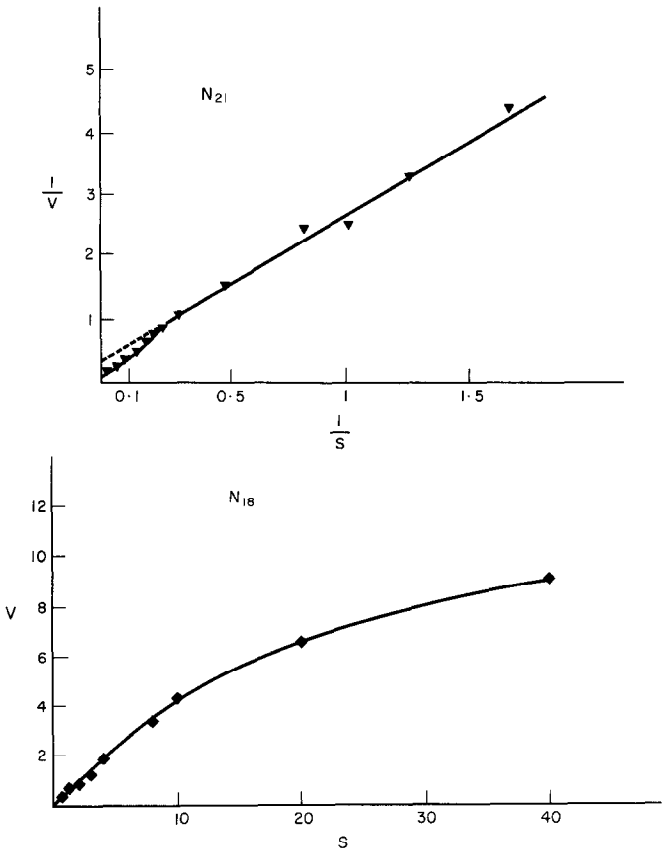

FIG. 2. Lineweaver–Burk plot. Abscissa; substrate concentrations ranging from 1–40 μM: ordinates; nmoles uptake mg protein^{-1} hr^{-1}. Each point is the mean of eleven experiments.

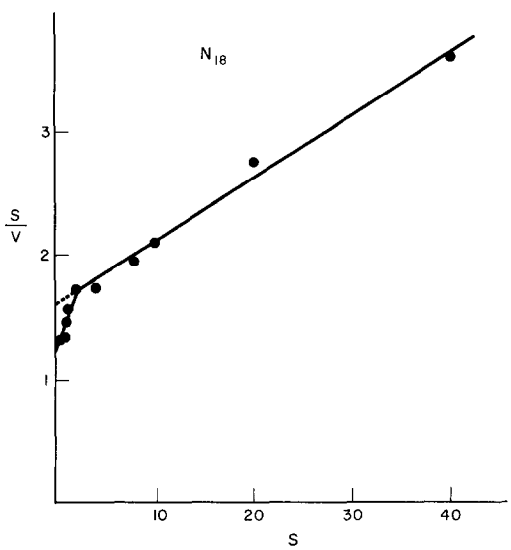

FIG. 3. Eadie's plot of the data presented in Fig. 2.

TABLE 2. K_m AND V_{max} VALUES FOR CLONES N_{18} AND S_{21} (HIGH SUBSTRATE CONCENTRATION)

	N_{18}	S_{21}
$K_{m\,H}$	$2{\cdot}6 \times 10^{-6}$ M	—
$V_{max}H$	$2{\cdot}5 \times 10^{-6}$ M	—
$K_{m\,L}$	$3{\cdot}6 \times 10^{-5}$ M	$1{\cdot}4 \times 10^{-5}$ M
$V_{max}L$	$2{\cdot}1 \times 10^{-5}$ M	$1{\cdot}1 \times 10^{-5}$ M

The subscripts H and L refer to high and low affinity respectively.

4). Choline uptake in S_{21} cells was not influenced by eserine but markedly reduced by isoOMPA (Table 4).

Low cencentrations of the organophosphate isoOMPA can specifically inhibit pseudo-cholinesterase activities (acetylcholine acylhydrolase, EC 3.1.1.8), without inhibiting true cholinesterase or AChE. At 10^{-6} M this inhibitor still reduced the uptake of choline in both clones (Table 4).

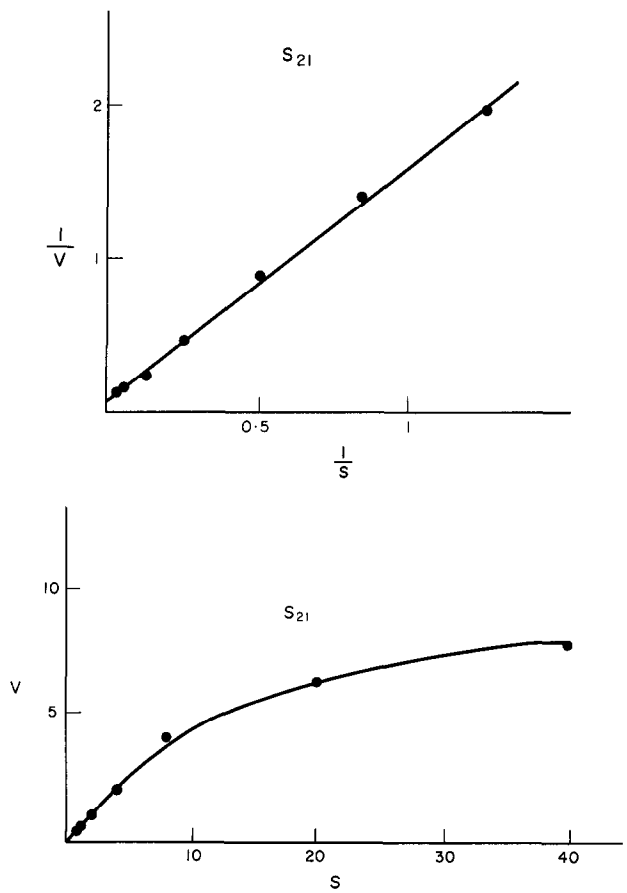

FIG. 4. Lineweaver–Burk plot of S_{21} kinetics. Abscissa; substrate concentration (1–40 μM): ordinates; nmoles uptake mg protein^{-1} hr^{-1}. Each point is the mean of six experiments.

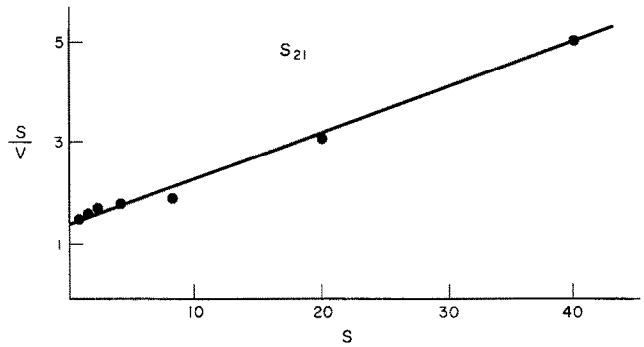

FIG. 5. Eadie's plot of the data presented in Fig. 4.

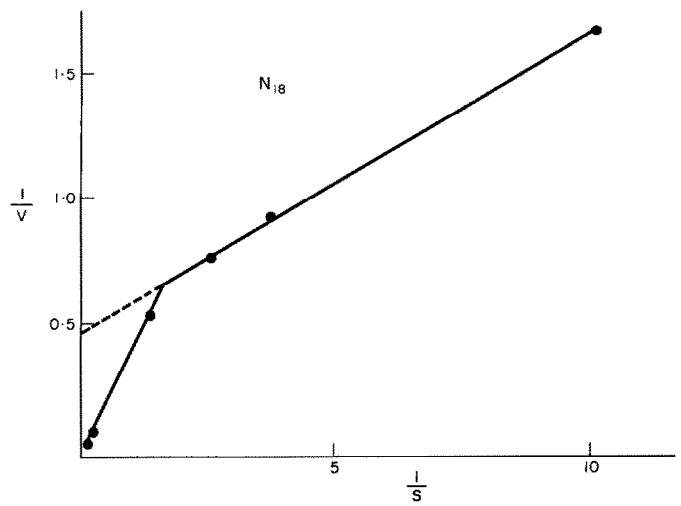

FIG. 6. Lineweaver–Burk plot of clone N_{18} kinetics. Abscissa; substrate concentration (0·1–10 μM): ordinates; nmoles uptake mg protein^{-1} hr^{-1}. Each point is the mean of three experiments.

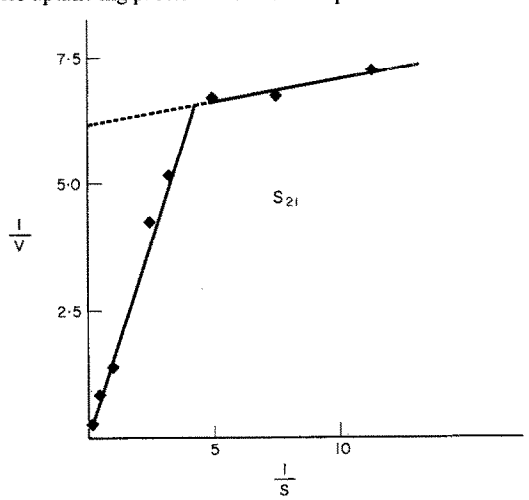

FIG. 7. Lineweaver–Burk plot of clone S_{21} kinetics. Abscissa; substrate concentration (0·1–10 μM): ordinates nmoles uptake mg protein^{-1} hr^{-1}. Each point is the mean of three experiments.

TABLE 3. K_m AND V_{max} VALUES FOR CLONES N_{18} AND S_{21} (LOW SUBSTRATE CONCENTRATION)

	N_{18}	S_{21}
$K_{m\,H}$	$2{\cdot}6 \times 10^{-6}$ M	$1{\cdot}1 \times 10^{-7}$ M
$V_{max}H$	$2{\cdot}1 \times 10^{-6}$ M	$1{\cdot}6 \times 10^{-6}$ M
$K_{m\,L}$	$2{\cdot}8 \times 10^{-5}$ M	$8{\cdot}1 \times 10^{-5}$ M
$V_{max}L$	$6{\cdot}7 \times 10^{-5}$ M	$1{\cdot}3 \times 10^{-5}$ M

The subscripts H and L refer to high and low affinity respectively.

TABLE 4. EFFECT OF AChE INHIBITORS ON CHOLINE UPTAKE IN CLONES N_{18} AND S_{21}

	N_{18}	S_{21}
Control	0·651 ± 0·03	0·095 ± 0·0085
Eserine 10^{-3} M	0·993 ± 0·05*	0·0925 ± 0·0118
Control	0·615 ± 0·05	0·095 ± 0·0085
isoOMPA 10^{-4} M	0·411 ± 0·02*	0·0572 ± 0·0029†
Control	0·580 ± 0·03	0·095 ± 0·0085
isoOMPA 10^{-6} M	0·490 ± 0·03†	0·072 ± 0·0041‡

Values are expressed as nmoles uptake hr^{-1} .mg $protein^{-1}$ ± S.E.M. Each value is the mean of at least three experiments. The drugs were preincubated for 15 min. The incubation lasted 60 min. Incubation conditions: see legend of Fig. 1.

* $P < 0{\cdot}001$.

† $P < 0{\cdot}05$.

‡ $P < 0{\cdot}02$.

DISCUSSION

The finding that choline uptake might be rate limiting in the synthesis of acetylcholine[12,14] has brought into light the necessity of a deeper knowledge of the mechanisms which lie behind such phenomenon.

Since transport mechanisms are generally dependent on the ionic composition of the incubation medium,[31] we started our experiments with a study on the influence of ionic environment on choline uptake. Several reports have shown the importance of sodium ions for the uptake of choline in nervous tissue.[8,12,13] The present experiments confirm that total substitution of sodium ions with cesium and lithium ions or sucrose can produce a sensible inhibition of choline uptake.

Such inhibition, though, might be only partially caused by lack of sodium ions since a direct inhibitory action of cesium and lithium ions on the Na^+-K^+ pump has to be taken into consideration.[32,33]

Furthermore, sucrose has been shown to be an inhibitor of Na^+, K^+-dependent ATPase (adenosine triphosphate pyrophosphohydrolase, EC 3.6.1.8) (M. Ledig, personal communication). The presence of an active component has been suggested by the inhibitions produced by potassium ferricyanide and ouabain; but the data which have clearly shown that choline uptake depends on more than one mechanism are the ones obtained from incubations with different substrate concentrations.

Both mechanisms are present in N_{18} as well as S_{21} clones. The high affinity component, though, seems to have a higher affinity for choline in clone S_{21} than in clone N_{18} (Table 3), a finding which is confirmed by the experiments at high substrate concentrations where clone S_{21} showed only the low affinity component, while clone N_{18} presented both components (Table 2).

It is possible to correlate this difference towards choline affinity between the two clones to the presence of a cholinergic compartment in clone S_{21}. However, data obtained with neuronal-glial cultures of dissociated chick embryo cerebral hemispheres showed that the high affinity K_m was absent in pure glial populations.[16] This implies that the high affinity carrier for choline uptake is present only in neurons irrespective of the fact that a cholinergic compartment is present or not, as our present data on clone N_{18} show.

In other words, the specificity of the high affinity carrier seems to be related to the neuronal entity and partly to its cholinergic capability.

The values of K_m and V_{max} are difficult to compare with those of Richelson and Thompson[7] since these authors did not analyse an inactive neuroblastoma clone and the substrate concentration ranges they used were different.

We could observe a striking behaviour in clone S_{21} which exhibits, at the lowest concentration ranges used ($0{\cdot}1–10 \times 10^{-6}$ M), a transport with a very high affinity for choline, compared to the N_{18} one. Such difference in transport affinity is not detectable at higher concentrations (Tables 2 and 3). It has to be pointed out, though, that the K_m and V_{max} values obtained from the Lineweaver–Burke or Eadie's plots are only an approximation of the real values since they represent the tangents to a curve which might be the result of more than two components; the presence of more than two components might be inferred from data obtained on the action of cholinesterase inhibitors on the kinetics of choline uptake.[34]

Acknowledgements—We thank Miss A. L. Riehl and Miss M. Ostertag for their valuable technical assistance.

REFERENCES

1. R. I. BIRKS and F. C. MACINTOSH, *Can. J. Biochem. Physiol.* **39,** 787 (1961).
2. B. T. BROWNING and M. P. SCHULMAN, *J. Neurochem.* **15,** 1391 (1968).
3. C. P. SUNG and R. M. JOHNSTON, *Can. J. Biochem. Physiol.* **43,** 1111 (1965).
4. K. MARTIN, *J. Gen. Physiol.* **51,** 497 (1968).
5. K. MARTIN, *Br. J. Pharmac.* **36,** 458 (1969).
6. J. SCHUBERTH, A. SUNDWALL, B. SÖRBO and J. O. LINDELL, *J. Neurochem.* **13,** 347 (1965).
7. E. RICHELSON and E. J. THOMPSON, *Nature, Lond.* **241,** 201 (1973).
8. I. DIAMOND and E. T. KENNEDY, *J. biol. Chem.* **244,** 3258 (1969).
9. R. M. MARCHBANKS, *Biochem. Pharmac.* **18,** 1763 (1969).
10. B. A. HEMSWORTH, K. I. DARMER and H. B. BOSMANN, *Neuropharmacology* **10,** 109 (1971).
11. V. P. WHITTAKER, *Biochem. J.* **127,** 5 (1972).
12. H. I. YAMAMURA and S. H. SNYDER, *Science, N.Y.* **178,** 626 (1972).
13. T. P. HAGA and H. NODA, *Biochim. biophys. Acta* **291,** 504 (1973).
14. M. J. DOWDALL and E. J. SIMON, *J. Neurochem.* **21,** 969 (1973).
15. P. GUYENET, P. LEFRESNE, J. ROSSIER, J. C. BEAUJOUAN and J. GLOWINSKI, *Molec. Pharmac.* **9,** 630 (1973).
16. R. MASSARELLI, M. SENSENBRENNER, A. EBEL and P. MANDEL, *J. Physiol., France* **67,** 292A (1973).
17. R. MASSARELLI, J. CIESIELSKI-TRESKA, A. EBEL and P. MANDEL, *Pharmac. Res. Commun.* **5,** 397 (1973).
18. A. BLUME, F. GILBERT, S. WILSON, J. FARBER, R. ROSENBERG and M. W. NIRENBERG, *Proc. natn. Acad. Sci. U.S.A.* **67,** 786 (1970).

19. G. AUGUSTI-TOCCO and G. SATO, *Proc. natn. Acad. Sci. U.S.A.* **64,** 311 (1969).
20. M. W. SEEDS, A. C. GILMAN, T. AMANO and M. W. NIRENBERG, *Proc. natn. Acad. Sci. U.S.A.* **66,** 160 (1970).
21. J. C. HERMETET, J. CIESIELSKI-TRESKA and P. MANDEL, *J. Histochem. Cytochem.* **20,** 137 (1972).
22. C. NISSEN, J. CIESIELSKI-TRESKA, L. HERTZ and P. MANDEL, *Brain Res.* **39,** 265 (1972).
23. J. C. HERMETET, J. CIESIELSKI-TRESKA, N. CHAMPY and P. MANDEL, *J. Physiol., France* **65,** 247 (1972).
24. R. E. MCCAMAN and J. M. HUNT, *J. Neurochem.* **12,** 253 (1965).
25. A. M. GOLDBERG, A. A. KAITA and R. E. MCCAMAN, *J. Neurochem.* **16,** 823 (1969).
26. G. M. WILKINSON, *Biochem. J.* **80,** 324 (1961).
27. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 165 (1951).
28. H. LINEWEAVER and D. BURK, *J. Am. chem. Soc.* **56,** 658 (1934).
29. K. D. NEAME and T. G. RICHARDS, *Elementary Kinetics of Membrane Carrier Transport*, pp. 120. Blackwells, Oxford (1972).
30. G. S. EADIE, *J. biol. Chem.* **146,** 85 (1942).
31. H. M. CHRISTENSEN, *Fedn Proc.* **32,** 19 (1973).
32. B. WHITTAM and M. E. AGER, *Biochem. J.* **93,** 337 (1964).
33. L. A. BEAUGE, A. MEDICI and R. A. SJODIN, *J. Physiol., Lond.* **228,** 1 (1973).
34. R. MASSARELLI, M. SENSENBRENNER and A. EBEL, *J. Physiol., France* **67,** 292A (1973).