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STUDIES ON CHOLINE TRANSPORT AND METABOLISM IN RAT BRAIN SYNAPTOSOMES

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Abstract—Choline uptake into isolated synaptosomes from rat brain cerebra was investigated by incubating the particles in an iso-osmotic medium containing ^{14}C-choline and isolating them from the incubation mixture by means of ion-exchange chromatography. Analysis of their radioactive contents by means of high voltage electrophoresis at pH 2·0 showed the per cent distribution of radioactivity for ^{14}C-choline, ^{14}C-acetylcholine, ^{14}C-phosphorylcholine and ^{14}C-betaine to be 61, 19·8, 14·8 and 4·4 respectively. Incorporation of ^{14}C-choline into phosphatidylcholine was also studied under the same conditions of incubation and appears to take place at the surface of the synaptosomal membrane through a Ca^{2+}-activated base exchange reaction. Intact, but not lysed or sonicated, synaptosomes took up ^{14}C-choline rapidly. While the choline transport system became saturated at 0·4 mM of ^{14}C-choline in the external medium, the enzymes involved in its conversion into phosphorylcholine, acetylcholine and betaine leveled off at substrate concentrations lower than 0·2 mM. This uptake is temperature dependent; it is not stimulated appreciably by mono- or divalent ions; it is not influenced by a source of energy or inhibited by 2,4-dinitrophenol (DNP), iodoacetic acid (IAA), KCN or IAA plus KCN, but it is markedly inhibited by *p*-chloromercuribenzoate (PCMB) or hemicholinium-3. DNP, IAA and KCN inhibited the total uptake of radioactivity into synaptosomes by more than 30 per cent. These metabolic inhibitors were found to exert no effect on the choline pool in the synaptosomes, however, they depressed considerably the radioactivity in the phosphorylcholine and acetylcholine pools. It was concluded that these inhibitors inhibit the uptake of total radioactivity into synaptosomes by inhibiting the formation of labeled phosphorylcholine and acetylcholine, thus preventing the flow of ^{14}C-choline from the choline pool to the phosphorylcholine and acetylcholine pools. Studies on the time dependence of choline extrusion from ^{14}C-choline preloaded synaptosomes into a choline-free medium gave half-time values, the time required for loss of 50 per cent of intracellular choline, ranging from 8 to 10 min.

SINCE the brain appears to depend on exogenous choline for the synthesis of acetylcholine and choline-containing phospholipids,[1–5] an effective choline transport system in the presynaptic membrane would be required to reutilize the liberated choline at the synapse, following synaptic transmission, for acetylcholine synthesis. Carrier-mediated transport of choline has been reported in kidney slices,[6] squid axon,[7] brain slices,[8–10] erythrocytes[11–12] and synaptosomes.[13–17] Uptake of choline into synaptosomes exhibits saturation kinetics, does not require energy or monovalent or divalent cations, and is inhibited competitively by hemicholinium.[13–15] Potter[14] and Marchbanks[13] demonstrated a Na^+-dependent, hemicholinium-inhibited, carrier-mediated transport of choline across the synaptosomal membrane. Schuberth *et al.*,[8,9] working on the uptake of labeled choline by mouse brain slices, interpreted their results in terms of an energy-dependent system, sensitive to ouabain and the concentration of extracellular sodium, in parallel with passive diffusion. In contrast, Cooke

and Robinson[10] showed no effect of Na^+ on choline uptake into rat brain slices, and Diamond and Kennedy[15] showed that choline uptake was inhibited by monovalent cations and that sodium was much less inhibitory than potassium or lithium. The latter authors also reported that choline transport did not require energy.[15]

This paper describes the characteristics of choline uptake and its conversion into phosphorylcholine (*p*-choline), betaine, acetylcholine (ACh) and phosphatidylcholine and the effect of various ions, metabolic inhibitors and some drugs thereon in rat brain synaptosomes.

METHODS

Tissue preparation. Young rats ranging from 12 to 25 days old were stunned, decapitated and the whole brains removed to ice-cold 0·25 M sucrose. The brains were washed twice with sucrose and the blood vessels removed. The cerebra were then homogenized in 10 vol. of sucrose by 12 strokes (up and down) at 800 rev/min, of a loose-fitting (0·26 mm clearance) Teflon pestle-glass homogenizer. The synaptosomes were obtained by subfractionating the crude mitochondrial fraction by means of discontinuous density gradient centrifugation in a sucrose–Ficoll medium as described previously.[18–20]

Conditions of incubation and filtration of the synaptosomes. The stock salt solution used for suspending the synaptosomes consisted of (millimolar): NaCl, 166; KCl, 8·3; Tris buffer, pH 7·4, 83; $MgCl_2$, 4·2; $CaCl_2$, 0·85; physostigmine, 1·65; mercaptoethanol, 8·3; glucose, 16·5 and sucrose, 16·5. The synaptosomes were homogenized carefully in the above salt solution such that 0·3 ml of the homogenate contained 2–2·5 mg protein. To 0·2 ml of a solution containing 0·5 μc (0·25 mM) of ^{14}C-methyl-labeled choline, plus other additions as the experiment called for, was added 0·3 ml of the synaptosome suspension to give a final concentration of 340–380 milliosmolar solution. The final volume of the reaction mixture was 0·5 ml. All incubation reactions were run in triplicate. After incubation at 37° for 15 min, the tubes were chilled in ice and 1 ml of cold 0·25 M sucrose containing 20 mM unlabeled choline was added to each tube. The control in these experiments consisted of mixing the reaction mixture at 0° and immediately passing it over the cation-exchange resin (zero time control) as discussed below. The contents of the tubes were then passed over columns (10·5 × 0·8 cm) filled with Amberlite IRC-50 (Na^+) cation-exchange resin (25–50 mesh) previously conditioned with 0·25 M sucrose. An additional 9 ml of 0·25 M sucrose was passed through the column. The eluate, which contained all of the synaptosomes added to the column, was centrifuged at 80,000 *g* for 30 min; the precipitate was washed with sucrose and the centrifugation repeated. The synaptosomes were washed twice with sucrose in order to exclude contamination from the ^{14}C-choline in the reaction mixture not taken up by the Amberlite column. To extract the contents of the synaptosomes, 1 ml of deionized water adjusted to pH 4·0 with HCl and containing 0·1 mM each of physostigmine, ACh, *p*-choline, betaine and choline was added to each tube and then heated at 100° for 10 min. To each tube, 9 ml water was added; the mixture was then shaken vigorously and centrifuged at 80,000 *g* for 30 min. The lysed supernatant was lyophilized, dissolved in 0·3 ml of deionized water, a portion of it (0·05 ml) was counted and 0·1 ml of the remainder was run in high voltage paper electrophoresis at pH 2·0. The paper was dried, exposed to iodine vapor, and the spots, which correspond to *p*-choline, betaine, ACh and choline, were marked, cut and counted in the liquid

scintillation counter. In general, the radioactivity recovered in the four bases after electrophoresis was about 60 per cent of that applied from the lysed supernatant. The 40 per cent loss in radioactivity could not have been due to the formation of other choline metabolites since, when the paper electrophoresis was cut at 1-cm intervals and the paper strips were counted, only the spots corresponding to the four bases were radioactive. The loss in radioactivity could have been due to the fact that only part of the radioactivity on the paper strip was eluted into the liquid scintillation medium. Furthermore, since the bases were localized with iodine vapor, a small amount of the latter could have remained on the paper strips, although every effort was made to evaporate all the iodine. Both of these factors could lower the efficiency of counting.

Isolation of phosphatidylcholine. The precipitate obtained after lysing of the synaptosomes was homogenized in 8 ml of chloroform–methanol (2:1). Phosphatidylcholine was isolated from the lipid extract on Silica gel G by means of two-dimensional thin-layer chromatography and its specific radioactivity (sp. act.) was determined as described previously.[20]

Protein content was measured by the method of Lowry *et al.*[21] using crystalline bovine serum albumin as the standard. Phospholipid phosphorus was determined according to the procedure of Bartlett.[22]

Materials. [Me-^{14}C] choline chloride (15 mc/m-mole) was purchased from New England Nuclear; hemicholinium-3 from Aldrich Chemical Co. Inc. and Gramicidin C from California Corp. for Biochemical Research. Ficoll was purchased from Pharmacia Fine Chemicals, Inc. and purified by dialysis against deionized water.

RESULTS

Effect of ionic change on ^{14}C-choline uptake by synaptosomes. There were no significant changes in choline uptake upon the addition or omission of Na^+, K^+, Ca^{2+}, phosphate, glucose or pyruvate and only Mg^{2+} exerted a slight stimulatory effect (Table 1). In previous studies, Ca^{2+} was found to have no effect on choline uptake

TABLE 1. EFFECT OF ADDITION OF VARIOUS IONS ON CHOLINE UPTAKE INTO RAT BRAIN SYNAPTOSOMES

Exp. No.	Ions added	Conc. of ions added (mM)	Total radioactivity in lysed supernatant (% of control)
1	None*		100
2	1 + Mg^{2+}	5	117
3	2 + Na^+	100	98
4	3 + K^+	5	102
5	4 + Ca^{2+}	1	97
6	5 + K_2HPO_4	5	100
7	6 + Glucose	10	105
8	7 + Na-pyruvate	5	101

* The incubation mixture consisted of 55 mM Tris buffer, pH 7·5, 0·25 mM ^{14}C-methyl-labeled choline synaptosomes, suspended in 0·25 M sucrose and equivalent to 2·1 mg protein, and enough sucrose to maintain the osmolarity of the medium at 320–350 milliosmolar. Other ions were added as indicated and the incubation conditions and assay for choline uptake were as described in the text. The effect of a certain ion on choline uptake was obtained by dividing the activity in the lysed supernatant in the presence of the ion by that in the preceding experiment lacking that particular ion.

into synaptosomes, but it stimulated choline incorporation into phosphatidylcholine by more than three times.[17] In subsequent studies, the synaptosomes were incubated in a physiological uptake medium in order to preserve their structural integrity.

Effect of external concentration of ^{14}C-choline on its uptake by synaptosomes. The uptake of ^{14}C-choline into synaptosomes was linear with added substrate up to 0·4 mM, then leveled off at higher choline concentrations (Fig. 1). Thus, as can be seen from Fig. 1, saturation of choline transport occurred at concentrations greater than 0·4 mM.

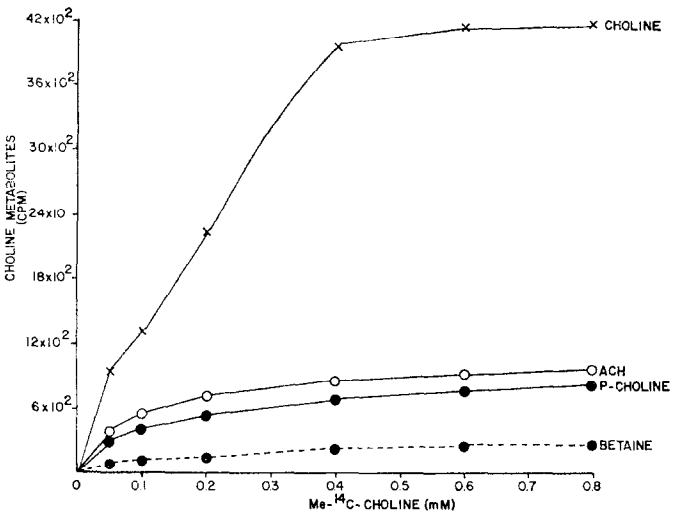

FIG. 1. Effect of ^{14}C-choline concentration on choline uptake and metabolism in synaptosomes. Synaptosomes from rat cerebra equivalent to 2·2 mg protein were incubated under standard uptake conditions for 15 min at 37° (see Methods), then passed over Amberlite IRC-50 cation-exchange resin. The choline and its metabolites in the eluted synaptosomes were then separated by means of high voltage electrophoresis (Fig. 2) and analyzed as described in the text. The concentration of ^{14}C-choline was varied as shown. Each point represents the average of two different experiments and each experiment was run in triplicate.

While the choline uptake system became saturated at 0·4 mM of ^{14}C-choline in the external medium, the enzymes involved in its metabolism into *p*-choline, betaine and ACh leveled off at lower substrate concentrations (< 0·2 mM; Fig. 1). At 0·2 mM, the percentage distribution of radioactivity in choline, ACh, *p*-choline and betaine was found to be 61, 19·8, 14·8 and 4·4 respectively (Fig. 1). The separation of the water-soluble ^{14}C-choline metabolites was accomplished by means of high voltage paper electrophoresis as shown in Fig. 2. At this pH, it was found that physostigmine moves along with betaine. Under the same conditions of incubation, the labeling of phosphatidylcholine continued to increase with the added choline (Fig. 3). It was shown in a previous communication that in synaptosomes ^{14}C-choline can enter phosphatidylcholine by base exchange.[17] The latter reaction appears to take place in the synaptosomal membrane (see below under lysing of synaptosomes).

Effect of time and temperature on choline uptake and metabolism. Both the uptake of ^{14}C-choline into synaptosomes and its subsequent metabolism were fairly rapid with time and did not reach equilibrium values even after 60 min of incubation (Fig. 4).

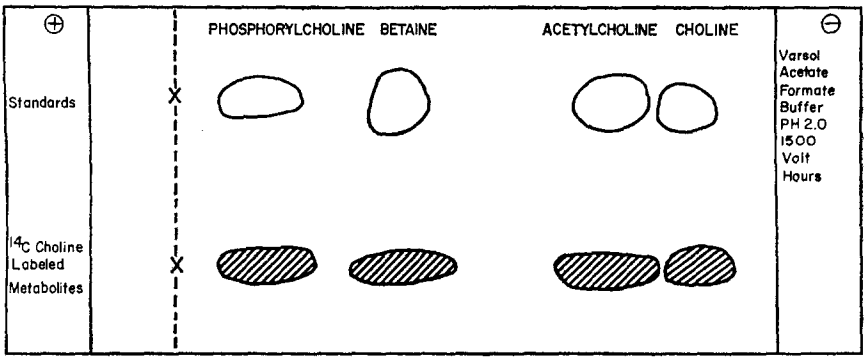

FIG. 2. Relative positions of ^{14}C-labeled choline and its metabolites in paper ionopherograms after uptake into synaptosomes. The length of the spots corresponds, on scale, to the actual spot length. Spots were applied along the dotted line, then electrophoresed at 3000 V for 30 min. After electrophoresis, the paper was exposed to iodine vapor, the spots were cut and counted in the liquid scintallation counter.

While ACh and *p*-choline synthesis continued near linearity after 10 min, betaine plateaued after 15 min of incubation. It is possible that the efflux of betaine from synaptosomes is much higher than that of ACh or *p*-choline. This conclusion is in

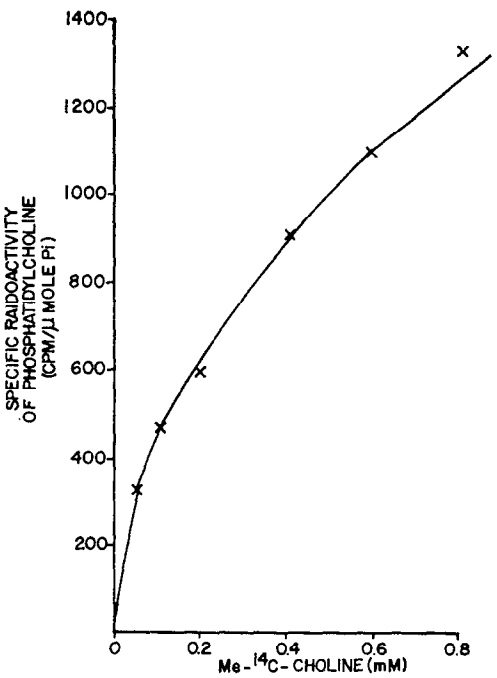

FIG. 3. Effect of ^{14}C-choline concentration on choline incorporation into phosphatidylcholine of synaptosomes. The lipids in the lysed synaptosomes, obtained in the experiment described under Fig. 1, were extracted, phosphatidylcholine was separated and its specific activity determined as described in the text. The concentration of ^{14}C-choline was varied as shown.

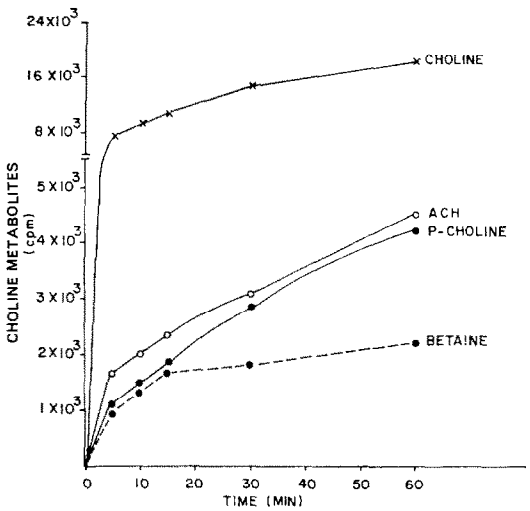

FIG. 4. Variation with time of choline uptake into synaptosomes. Synaptosomes from rat cerebra equivalent to 2·1 mg protein were incubated and assayed as described under Fig. 1. The time of incubation was varied as shown. The reaction was stopped immediately after each time interval by adding 1 ml of 0·25 M sucrose containing 20 mM unlabeled choline (see text), and the mixture was assayed and analyzed for choline and its metabolites as described under Fig. 1.

accord with the results obtained from the preloading experiments (see below). In this connection Sung and Johnstone,[6] working with the uptake of choline into rat kidney cortex slices, found most of the betaine formed in the incubation medium. The incorporation of ^{14}C-choline into phosphatidylcholine was almost linear with time up to 60 min of incubation.

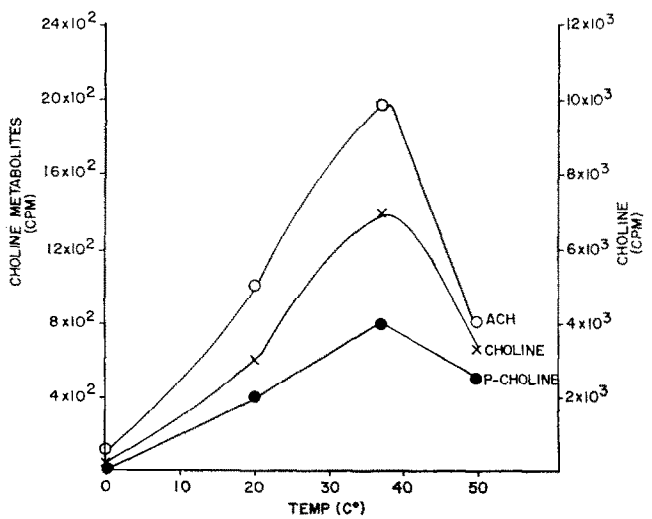

FIG. 5. Effect of temperature on the uptake and metabolism of ^{14}C-choline in synaptosomes. Conditions of incubation and analysis for choline uptake and metabolism were as described in Fig. 1. The temperature of incubation was varied as shown.

At 0°, choline uptake and subsequent metabolism were negligible, but increased rapidly with near linearity up to 37°, then dropped by more than 50 per cent at 50° (Fig. 5). The optimum temperature for ^{14}C-choline incorporation into phosphatidylcholine was also at 37° (Fig. 6).

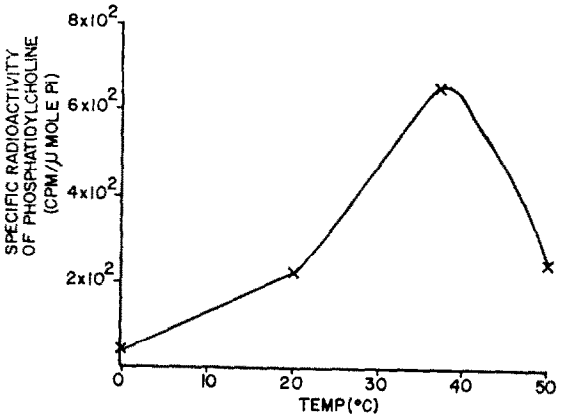

FIG. 6. Effect of temperature on the incorporation of ^{14}C-choline into phosphatidylcholine of synaptosomes. These results were taken from the same experiment described in Fig. 5.

Effect of lysing and sonication on choline uptake into synaptosomes. Upon disruption of the synaptosomal membrane by lysing or sonication the rate of ^{14}C-choline uptake decreased to less than 15 per cent of that of the intact synaptosomes (Table 2). This treatment was found to have a negligible effect on the incorporation of ^{14}C-choline into phosphatidylcholine. These observations suggest that: (1) choline uptake into synaptosomes is not a simple adsorption to the synaptosomal membrane, and (2) labeling of phosphatidylcholine takes place at the membrane. These conclusions were

TABLE 2. EFFECT OF LYSING AND SONICATION ON CHOLINE UPTAKE INTO RAT BRAIN SYNAPTOSOMES

Condition of synaptosomes*	No. of experiments	Total radioactivity in lysed supernantant (counts/min)	Specific radioactvitiy of phosphatidylcholine (counts/min/μmole Pi)
Intact	2	15,500	1200
Lysed†	2	1250	1240
Sonicated‡	2	2230	1150
Synaptosomal membrances§	1	260	1050

* Conditions of incubation and analysis for choline uptake and metabolism were as described in Fig. 1.

† The synaptosomes were homogenized in deionized water and the homogenate was added to the incubation mixture.

‡ Sonication was carried out with a Branson sonifier which was operated in an ice bath at 20 kc/sec and 80 W for 2 min. The sonicated preparation was added to the incubation mixture. The three types of preparations contained approximately the same amount of protein (2 mg) per tube. Each experiment was run in triplicate.

§ Prepared from synaptosomes according to the procedure of Whittaker *et al.*[23]

confirmed by preparing synaptosomal membranes from synaptosomes according to the procedure of Whittaker *et al.*[23] When these membranes were incubated in the standard choline uptake medium, there was very little uptake of ^{14}C-choline; however, the phospholipid labeling proceeded as with intact synaptosomes (Table 2).

Effect of metabolic inhibitors on choline uptake and metabolism. Addition or omission of glucose or pyruvate from the medium did not influence the uptake when compared with control experiments (Table 1). To show further if energy is involved in choline transport, the effects of various inhibitors of metabolism on choline transport and its subsequent metabolism were investigated and the results of these experiments are shown in Fig. 7. *p*-Chloromercuribenzoate (PCMB), which attacks sulfhydryl groups, exerted up to 85 per cent inhibition on choline uptake as well as its incorporation into phosphatidylcholine (Table 3), and virtually abolished the synthesis of ACh and to a lesser extent that of *p*-choline and betaine (Fig. 7). In contrast, KCN,2,4-dinitrophenol

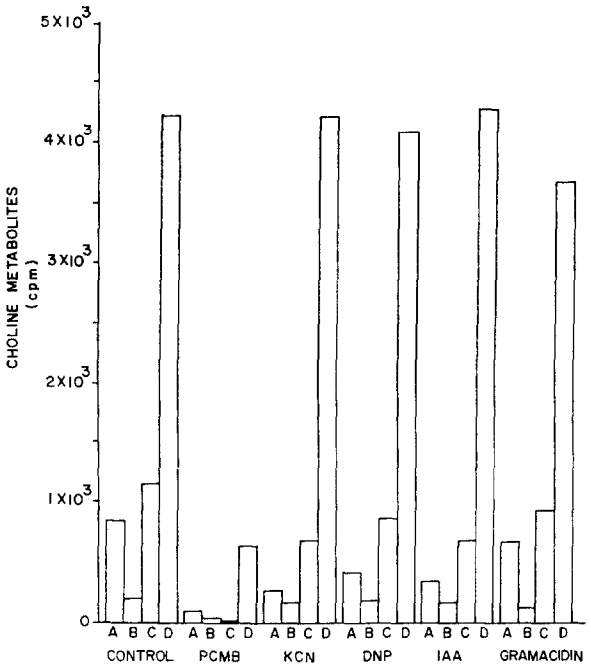

FIG. 7. Effect of metabolic inhibitors on choline uptake and metabolism in synpatosomes. Concentrations of the inhibitors were as follows (mM): *p*-chloromercuribenzoate (PCMB), 1; potassium cyanide (KCN), 1; 2,4-dinitrophenol (DNP), 0·2; iodoacetic acid (IAA), 1; and Gramicidin, 5 μg/0·1 ml containing 17 μmoles ethanol. The final concentration of ethanol was 34 mM and of Gramicidin was 5 μg/0·5 ml of reaction mixture. The letters correspond to: A, *p*-choline; B, betaine; C, ACh; D, choline. Conditions of incubation and analysis for choline uptake and metabolism were as described in Fig. 1.

(DNP) and iodoacetic acid (IAA) had little influence on choline transport and its oxidation to betaine but, as expected, these agents inhibited considerably (23–64 per cent) choline conversion to ACh and *p*-choline. The antibiotic Gramicidin, which inhibits oxidative phosphorylation and promotes the entry of K^+, Na^+, Li^+, Rb^+ and Cs^+ into mitochondria[24] and of Na^+ and K^+ into synaptosomes,[25] exerted a

slight inhibitory effect on choline transport and metabolism (Fig. 7). A low concentration of ethanol (34 mM) was used in the present work, since it was found that it had no influence on choline uptake and metabolism. However, higher concentrations of the alcohol (e.g. 600 mM) inhibited phosphatidylcholine labeling by almost 60 per cent, but still without much effect on choline transport. Ethanol could compete with choline for the choline in phosphatidylcholine in the base exchange reaction.

TABLE 3. EFFECT OF METABOLIC INHIBITORS ON ^{14}C-CHOLINE INCORPORATION INTO PHOSPHATIDYL CHOLINE*

Exp. No.	Inhibitor	Conc. of metabolic inhibitor (mM)	Specific radioactivity of phosphatidylcholine (counts/min/μmole Pi)
1	None		687
2	*p*-Chloromercuribenzoate	1	105
3	KCN	1	585
4	Iodoacetic acid	1	459
5	2,4-Dinitrophenol	0·2	535
6	Gramicidin	5 μg†	720

* The results on phosphatidylcholine reported in this table were taken from the same experiments described in Fig. 7.

† Five μg/0·5 ml of reaction mixture.

Since KCN and IAA inhibit ATP formation by blocking oxidative phosphorylation and glycolysis, respectively, their effect, when combined at various concentrations, was negligible on choline uptake and its oxidation to betaine. However, a 60 and 90 per cent inhibition on ACh and *p*-choline synthesis, respectively, was observed when both agents were added (Fig. 8).

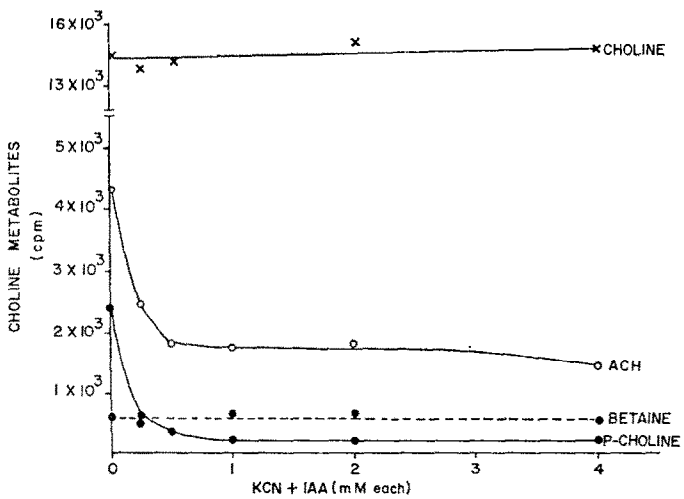

FIG. 8. Effect of KCN plus IAA on choline uptake and metabolism in synaptosomes. Conditions of incubation and analysis for choline uptake and metabolism were as described in Fig. 1. The concentrations of KCN and IAA were varied as shown.

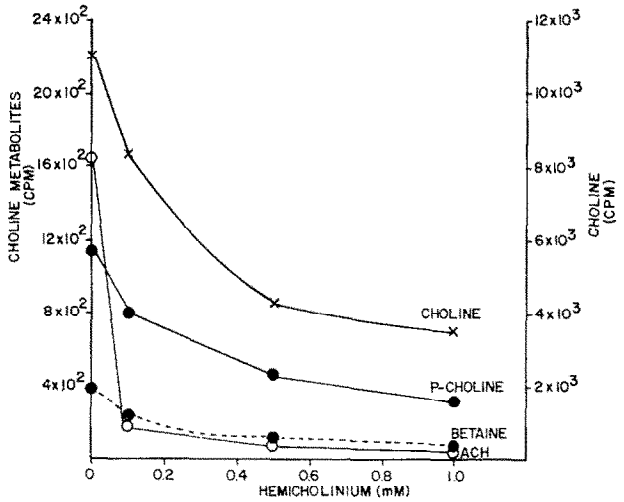

FIG. 9. Effect of hemicholinium-3 on choline uptake and metabolism in synaptosomes. Conditions of incubation and analysis for choline uptake and metabolism were as described in Fig. 1. The concentration of hemicholinium was varied as shown.

Effect of pharmacological agents on choline transport and metabolism. At 0·1 mM concentration, hemicholinium-3, a competitive inhibitor of choline transport,[13–16] inhibited ACh synthesis by more than 90 per cent, choline transport and conversion to *p*-choline by 25–30 per cent and had little influence on phosphatidylcholine labeling (Figs. 9 and 10 respectively). At 1 mM concentration of the drug, a considerable amount of inhibition was observed on the transport and metabolism of choline.

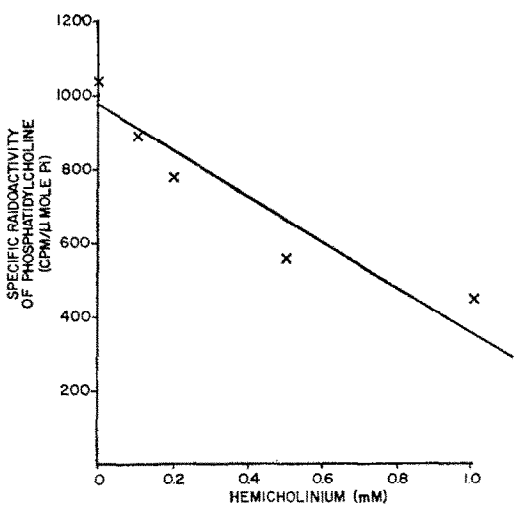

FIG. 10. Effect of various concentrations of hemicholinium-3 on choline incorporation into phosphatidylcholine of synaptosomes. The lipids in the lysed synaptosomes, obtained in the experiment described under Fig. 9, were extracted, phosphatidylcholine was separated and its specific activity determined as described in the text. The concentration of hemicholinium-3 was varied as shown.

TABLE 4. EFFECT OF ACTYLCHOLINE AND PHYSOSTIGMINE ON CHOLINE UPTAKE AND METABOLISM IN RAT BRAIN SYNAPTOSOMES*

Addition	Conc. (mM)	Distribution of radioactivity (counts/min)					
		Lysed supernatant	Phosphatidylcholine (sp. act.)	Choline	Acetylcholine	Betaine	Phosphorylcholine
None		7590	1780	2270	964	488	820
Acetylcholine	1	3890	513	1480	362	320	344
Physostigmine	1	6380	1390	1910	824	296	732
Acetylcholine + physostigmine	1 (each)	6090	1100	1810	780	232	648

* Conditions of incubation and analysis for choline uptake and metabolism were as described in Fig. 1.

Results of studies on the effect of ACh. Effects of ACh and physostigmine, which were reported previously[15] to act as competitive and noncompetitive inhibitors of choline transport respectively in synaptosomes, are shown in Table 4. Acetylcholine inhibited choline transport and metabolism, and this effect was relieved by adding physostigmine to the incubation medium. This finding suggests to us that at least part of the inhibitory effect of ACh observed by some investigators on choline transport[14–15] and phosphatidylcholine metabolism[26] could be due to the hydrolysis of ACh by AChase, which results in diluting the radioactivity of ^{14}C-choline in the reaction mixture.

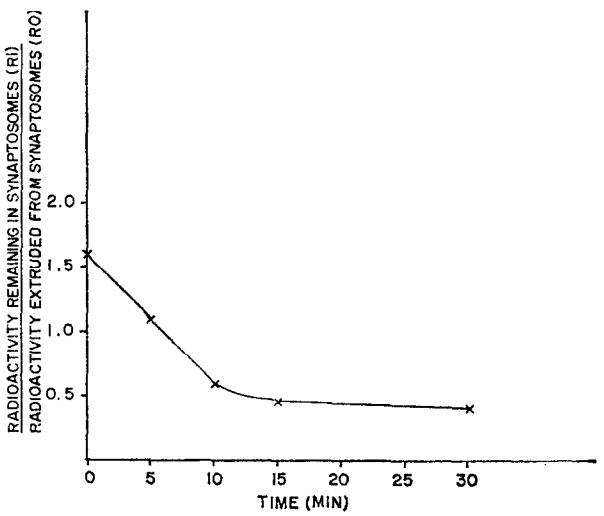

FIG. 11. Effect of preloading synaptosomes with ^{14}C-choline (0·25 mM) on the efflux of ^{14}C-labeled material with time. Synaptosomes were prepared from 10 g cerebra, then incubated in 3 ml of the choline standard salt mixture containing 10 μc ^{14}C-choline (0·25 mM) for 20 min at 37°. At the end of the incubation period, 7 ml of the salt mixture was added, then centrifuged at 30,000 *g* for 15 min. Portions of the precipitate (preloaded synaptosomes) equivalent to 2·2 mg protein were incubated in 0·5 ml of choline-free uptake medium with variation of time of incubation as shown. At the end of incubation, 1 ml of 0·25 M sucrose containing 20 mM choline was added, followed by addition of 9 ml sucrose. The tubes were centrifuged at 30,000 *g* for 15 min and the radioactivity was determined in the supernatant (RO) and the supernatant of the lysed precipitate (RI). In the zero time experiments, the tubes were kept at 0°. The points on the curve correspond to two different experiments; each experiment was run in triplicate. The average variation from the mean was about 5 per cent within the same experiment and about 10 per cent between the two different experiments.

Observations on the efflux of ^{14}C-choline. To study the characteristics of choline efflux from synaptosomes, the latter were first preincubated with the isotope for 20 min and then reincubated in a choline-free salt solution at various time intervals (Fig. 11). At zero time and 0°, the RI/RO ratio was 1·6. Upon incubation at 37°, the rate of efflux increased rapidly during the first 10 min of incubation and the RI/RO ratio dropped to about 50 per cent of that of the zero time value, then became relatively slow and finally leveled off at around 15 min of incubation (Fig. 11). Since 20 mM choline was added to terminate the efflux of ^{14}C-choline from the synaptosomes, the fast drop in the RI/RO ratio could be due to the exchange diffusion. We also observed that when 0·25 mM hemicholinium-3 was added to incubation mixtures containing the preloaded synaptosomes, the rate of efflux was decreased by only 12–17 per cent of

that of the control. In contrast, at the same concentration, this drug inhibited choline uptake into synaptosomes by more than 50 per cent of that of the control (Fig. 9). This is to be expected, since both the drug and the added choline compete for the choline transport system on the outer surface of the synaptosomal membrane. When the contents (RI) of the preloaded synaptosomes were analyzed at zero time and at 15 min after incubation at 37° by means of paper electrophoresis, the efflux of choline was found to be > ACh > *p*-choline (Table 5). The amount of betaine was almost negligible in the preloaded synaptosomes, thus confirming our above conclusions and those of others,[6] namely that after choline is oxidized, most of the betaine is extruded to the medium. This mechanism could be important in exchanging betaine for choline, thus raising the level of intracellular choline concentration over that of the extracellular level.

TABLE 5. DISTRIBUTION OF RADIOACTIVITY IN ^{14}C-CHOLINE PRELOADED SYNAPTOSOMES AFTER INCUBATION IN CHOLINE-FREE MEDIUM*

Time of incubation (min)	Distribution of radioactivity (counts/min)			
	p-choline	Betaine	ACh	Choline
0	1410 (10·8)†	72 (0·5)	702 (5·3)	10900 (83·6)
15	1070 (17·3)	30 (0·49)	408 (6·6)	4670 (75·5)

* In this experiment, the supernatant (RI in Fig. 11) obtained upon lysing the precipitate was electrophoresed at pH 2·0 and the radioactivity analyzed as described in Figs. 1 and 2.

† Distribution of radioactivity as a percentage is given in parentheses.

DISCUSSION

The findings on choline transport and metabolism, reported in this paper give further support to the evidence presented by other workers on choline,[13–16] noradrenaline,[27–31] sodium,[32] potassium,[33] tryptophan[34] and thiamine[35] transport in synaptosomes, that these particulates are enclosed within intact and functional membranes which, like other cell membranes, serve efficiently as a permeability barrier to the influx and outflux of various ions. The rate of entry of the ^{14}C-choline into synaptosomes reaches maximal velocity as the concentration of added choline is increased. Thus in accord with the work of others[6–15] working with synaptosomes and preparations from various tissues, choline transport into synaptosomes involves a saturable system as well as a passive diffusion process and the K_t for choline uptake can be calculated by using the Michaelis–Menten kinetics. After entry into the synaptosome, the ^{14}C-choline is metabolized through various metabolic pathways into *p*-choline, betaine, ACh and phosphatidylcholine (Fig. 12). The formation of the latter appears to take place through a base exchange reaction.[17] The latter conclusion was based on the following findings:[17] (1) Calcium ions exerted a considerable stimulatory effect on the incorporation of labeled choline into phosphatidylcholine. (2) This incorporation was not dependent on cofactors, including CoA, ATP, CTP and Mg^{2+}, which are required for *de novo* synthesis of phospholipids in microsomes.

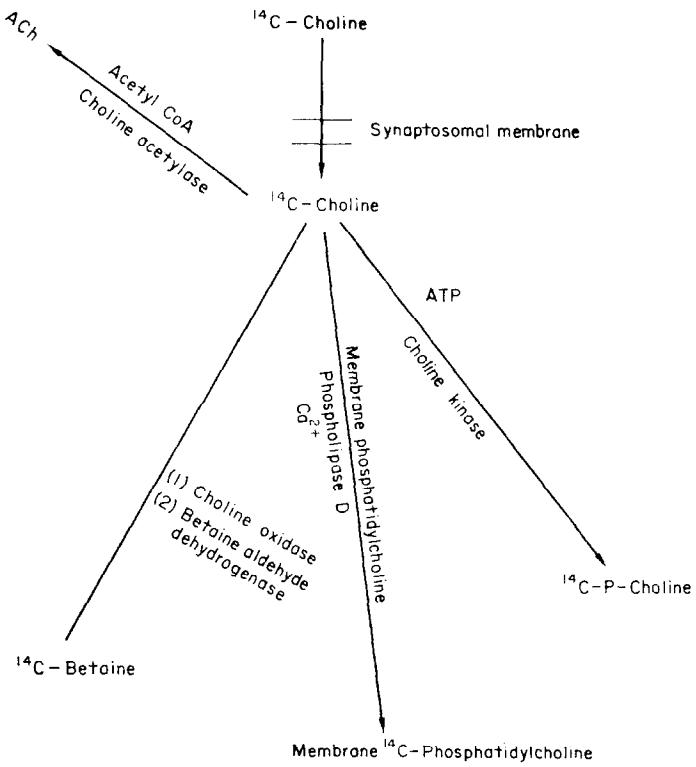

FIG. 12. Metabolism of ^{14}C-choline in rat brain synaptosomes.

(3) No increase in the level of phosphatidylcholine was observed in the reaction mixtures after 2 hr. (4) The pH optimum for this incorporation was about 9, while that for *de novo* synthesis of phospholipids is 7·4–7·8. (5) Incorporation of labeled choline into phosphatidylcholine was inhibited by either L- or D-serine. This conclusion is supported by the findings, first, that lysing had little effect on this reaction, but it reduced the transport of choline markedly, and second, that isolated synaptosomal membranes can incorporate ^{14}C-choline into phosphatidylcholine. The higher value for ACh, 15–20 per cent of that of the total radioactivity obtained in the present work compared to less than 5 per cent reported by Diamond and Kennedy,[15] could be due to the addition of physostigmine at the end of our incubations followed by boiling at 100° for 10 min and the fast electrophoretic method used for isolating the neurotransmitter. More than 60 per cent of the ^{14}C-choline taken up by the synaptosomes was recovered as unmetabolized choline.

Under our experimental conditions, intact synaptosomes are required for choline uptake; the choline transport system is temperature dependent and is not stimulated by mono-or divalent ions, with the exception of Mg^{2+} which exerted a slight stimulatory effect; and it is not influenced by KCN, DNP, IAA or KCN plus IAA.

The present studies demonstrated that the level of a choline metabolite in synaptosomes is closely related to that of the choline uptake system. Thus, as expected, the concentration and time-course studies revealed that the levels of choline metabolites

in synaptosomes vary with time of incubation and external concentration of choline (Figs. 1 and 4 respectively). Furthermore, PCMB exerted more than 85 per cent inhibition on the choline transport system, and this in turn decreased the synthesis of *p*-choline and ACh to less than 15 per cent of that of the control. Similarly the inhibition of choline transport by hemicholinium-3 reduced the synthesis of *p*-choline and ACh considerably. Hemicholinium-3 is known to inhibit ACh synthesis by competing with choline for the choline transport system.[36] The finding that KCN, DNP and IAA had no effect on choline uptake (Fig. 7) is in agreement with the conclusion that the transport of choline into synaptosomes is not energy dependent.[15] However, these metabolic inhibitors exerted up to 40 per cent inhibition on the conversion of choline into *p*-choline and ACh, which require energy for their synthesis (Fig. 12). Since there was little change in betaine formation and choline accumulation in synaptosomes in the presence of these inhibitors (Fig. 7), the marked decrease in the total radioactivity of the lysed synaptosomal supernatant (Table 6) could mostly be accounted for by the inhibition of *p*-choline and ACh synthesis. Thus the findings of Schuberth *et al.*[8,9] on the involvement of energy and Na^+ in choline transport into brain slices could be explained by assuming that there are three pools in the nerve terminal, a choline pool, which does not require energy, and two separate *p*-choline and ACh pools, which require energy and are in equilibrium with the choline pool. In the presence of energy and at maximal velocity for choline uptake into the nerve terminal, the three pools are saturated and the flow of choline from the choline pool into the other two pools is at maximal rate. When energy is interrupted by adding a metabolic inhibitor to the nerve terminal, the flow of choline to the other two pools will stop and only the choline pool will be saturated with the newly added choline (radioactive). The addition of PCMB acts by inhibiting the choline transport system and subsequently the uptake of the newly added choline by the three pools.

TABLE 6. EFFECT OF METABOLIC INHIBITORS ON CHOLINE UPTAKE INTO SYNAPTOSOMES*

Exp. No.	Inhibitor	Conc. (mM)	Total radioactivity in the lysed supernatant (% of control)
1			100
2	PCBM	1	16·3
3	KCN	1	57·5
4	DNP	1	60·5
5	IAA	1	61·7
6	Gramicidin	5 μg†	96·6

* These results are taken from the supernatant obtained upon lysing the synaptosomes in the experiment described under Fig. 7.

† Five μg/0·5 ml of reaction mixture.

From the studies on the time dependence of chloine exit from synaptosomes which were preloaded with ^{14}C-choline into a choline-free medium (Fig. 11), the time required for loss of 50 per cent of intracellular choline (half-time) was about 8–10 min. Lacko and Burger,[37] working with glucose preloaded erythrocytes, obtained a half-

time of 1 min upon incubation of these cells at 0° in a sugar-free medium. Hemicholinium-3 at 0·25 mM concentration exerted a slight inhibitory effect, in contrast to its marked effect on the entry, on the exit of choline into the medium. It is possible that this drug competes for the choline transport system at the outer surface of the synaptosomal membrane. The finding that the percentage of *p*-choline, as compared to the other metabolites, increased after incubation suggests (as expected) that *p*-choline crosses the synaptosomal membrane less readily than choline. This is in line with the findings of Itokawa and Cooper[35] on the efflux of thiamine and its metabolites from ^{35}S-thiamine preloaded mitochondrial fractions, which were isolated from rat brain after administration of the radioisotope. These authors observed that the bulk of the released material was primarily thiamine monophosphate and free thiamine, whereas the thiamine esters remaining in the particulate fraction were thiamine pyrophosphate and thiamine monophosphate.[35] They also showed that ACh or tetrodotoxin can cause release of thiamine from this particulate fraction.[35]

Since the neurotransmitter, ACh, is hydrolyzed at the synapse by acetylcholinesterase during nerve activity, the present studies could shed some light on the uptake of the liberated free choline into the nerve terminals, its subsequent reutilization in the synthesis of ACh and phospholipids, and the effect of various ions, metabolic poisons and drugs thereon.

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REFERENCES

1. D. P. GROTH, J. A. BAIN and C. C. PFEIFFER, *J. Pharmac. exp. Ther.* **124,** 290 (1958).
2. J. SCHUBERTH, B. SPARF and A. SUNDWALL, *J, Neurochem.* **16,** 695 (1969).
3. G. B. ANSELL and S. SPANNER, *Biochem. J.* **122,** 741 (1971).
4. I. DIAMOND, *Archs. Neurol., Chicago* **24,** 333 (1971).
5. J. R. COOPER, F. E. BLOOM and R. H. ROTH, *The Biochemical Basis of Neuropharmacology* p. 60. Oxford University Press, New York (1970).
6. C.-P. SUNG and R. M. JOHNSTONE, *Can. J. Biochem. Physiol.* **43,** 1111 (1965).
7. A. L. HODGKIN and K. MARTIN, *J. Physiol., Lond.* **179,** 26P (1965).
8. J. SCHUBERTH, A. SUNDWALL, B. SORBO and J-O LINDELL, *J. Neurochem.* **13,** 347 (1966).
9. J. SCHUBERTH, A. SUNDWALL and B. SORBO, *Life Sci.* **6,** 293 (1967).
10. W. J. COOKE and J. D. ROBINSON, *Biochem. Pharmac.* **20,** 2355 (1971).
11. A. ASKARI, *J. gen. Phsyiol.* **49,** 1147 (1966).
12. K. MARTIN, *J. gen. Physiol.* **51,** 497 (1968).
13. R. M. MARCHBANKS, *Biochem. J.* **110,** 533 (1968).
14. L. T. POTTER, in *The Interaction of Drugs and Subcellular Components in Animal Cells* (Ed. P. N. CAMPBELL), p. 293. Little, Brown, Boston (1968).
15. I. DIAMOND and E. P. KENNEDY, *J. biol. Chem.* **244,** 3258 (1969).
16. A. A. ABDEL-LATIF and J. P. SMITH, *Brain Res., Osaka* **31,** 224 (1971).
17. A. A. ABDEL-LATIF and J. P. SMITH, *Biochem. Pharmac.* **21,** 436 (1972).
18. A. A. ABDEL-LATIF, *Biochim. biophys. Acta* **121,** 403 (1966).
19. A. A. ABDEL-LATIF, T. YAMAGUCHI, M. YAMAGUCHI and F. CHANG, *Brain Res., Osaka* **10,** 307 (1968).
20. A. A. ABDEL-LATIF and J. P. SMITH, *Biochim. biophys. Acta* **218,** 134 (1970).
21. O. H. LOWRY, N. J. ROSENBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
22. G. R. BARTLETT, *J. biol. Chem.* **234,** 466 (1959).
23. V. P. WHITTAKER, I. A. MICHAELSON and R. J. A. KIRKLAND, *Biochem. J.* **90,** 293 (1964).
24. B. C. PRESSMAN, E. J. HARRIS, W. S. JAGGER and J. H. JOHNSON, *Proc. natn. Acad. Sci. U.S.A.* **58,** 1949 (1967).
25. P. KEEN and T. D. WHITE, *J. Neurochem.* **18,** 1097 (1971).
26. J. DURELL and J. T. GARLAND, *Ann. N.Y. Acad. Sci.* **165,** 743 (1969).

27. R. W. COLBURN, F. K. GOODWIN, D. L. MURPHY, W. E. BUNNEY and J. M. DAVIS, *Biochem. Pharmac.* **17,** 957 (1968).
28. D. F. BOGDANSKI, A. TISSARI and B. B. BRODIE, *Life Sci.* **7,** 419 (1968).
29. T. D. WHITE and P. KEEN, *Biochim. Biophys. Acta* **196,** 285 (1970).
30. A. S. HORN, J. T. COYIE and S. H. SNYDER, *Molec. Pharmac.* **7,** 66 (1971).
31. D. S. JANOWSKY, J. M. DAVIS, W. E. FANN, J. FREEMAN, R. NIXON and A. A. MICHELAKIS, *Life Sci.* **11,** 1 (1972).
32. C.-M. LING and A. A. ABDEL-LATIF, *J. Neurochem.* **15,** 721 (1968).
33. A. V. ESCUETA and S. H. APPEL, *Biochemistry, N.Y.* **8,** 725 (1969).
34. D. A. GRAHAME-SMITH and A. G. PARFITT, *J. Neurochem.* **17,** 1339 (1970).
35. Y. ITOKAWA and J. R. COOPER, *Biochim. biophys. Acta* **196,** 274 (1970).
36. R. BIRKS and F. C. MACINTOSH, *Can. J. Biochem. Physiol.* **39,** 787 (1961).
37. L. LACKO and M. BURGER, *Biochem. J.* **83,** 622 (1962).