locity distributions of heavy ions and the global thermal structure of the torus [for example, see (*32*)]. The relative importance of different heating and cooling effects [that is, fast thermalization of hot ions through collective plasma processes (*33, 34*) versus energy transfer through ion-ion and electron-ion Coulomb collisions] must be evaluated in connection with the analysis of spectral data exemplified in Fig. 1.

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Acetylcholine Synthesis in Synaptosomes: Mode of Transfer of Mitochondrial Acetyl Coenzyme A

Abstract. *Labeled acetylcholine derived from labeled pyruvate in a synaptosomal preparation from rat brain, incubated with nicotinamide adenine dinucleotide as well as coenzyme A, is stimulated by calcium ions in the absence but not in the presence of Triton X-100. Whereas citrate is taken up by cholinergic synaptosomes because it suppresses the formation of acetylcholine from pyruvate, it is not itself converted into acetylcholine. The evidence suggests that there is a calcium-dependent transfer of mitochondrial acetyl coenzyme A into the cholinergic synaptoplasm, which is apparently devoid of the citrate cleavage enzyme, and is there converted into acetylcholine. The permeability of the inner mitochondrial membrane to coenzyme A and acetyl coenzyme A seems to be enhanced by calcium ions, and this effect may be mediated by mitochondrial phospholipase A_2.*

An outstanding question in neurochemistry is how acetyl coenzyme A (CoA) originating in the mitochondria enters the cholinergic cytoplasm, where it is converted into acetylcholine. The inner mitochondrial membrane has been held to be largely impermeable to acetyl CoA (*1*). Various proposals have been made: (i) the conversion of mitochondrial acetyl CoA into citrate that enters the cytoplasm where the citrate cleavage enzyme, adenosine triphosphate (ATP) citrate lyase (E.C. 4.1.3.8), forms acetyl CoA and oxaloacetate (*2*); (ii) the presence in cholinergic nerve terminals of soluble, extramitochondrial pyruvate dehydrogenase (*3*); and (iii) the transfer of sufficient acetyl CoA across the mitochondrial barrier to supply all the brain acetylcholine (*4, 5*). These and other proposals have been reviewed (*5, 6*).

We report that the cholinergic synaptoplasm is apparently almost devoid of the citrate cleavage enzyme. Our evidence also indicates that acetyl CoA emanates as such from the cholinergic mitochondria by a calcium-dependent mechanism and that the outflow is much facilitated by phospholipase A_2. It has been suggested that acetyl CoA may

Table 1. The synthesis of [^{14}C]acetylcholine ([^{14}C]ACh) from [^{14}C]pyruvate and [^{14}C]citrate in crude synaptosomal fractions of rat brain in the presence and absence of Triton X-100. A crude synaptosomal fraction (3 to 5 mg of protein) was incubated aerobically for 1 hour at 37°C in 3 ml of incubation medium of the following composition: KCl, 100 m*M*; $MgSO_4$, 1.3 m*M*; choline, 5 m*M*; eserine, 0.4 m*M*; Na_2HPO_4, *p*H 7.4, 10 m*M*; and sucrose, 53.3 m*M*. Further additions of NAD (1 m*M*); EGTA (3 m*M*); $CaCl_2$ (1 m*M*); CoA (0.1 m*M*); sodium citrate (2 m*M*); sodium succinate (2 m*M*), sodium oxaloacetate (2 m*M*); amobarbital (1 m*M*); and Triton X-100 (0.12 percent) were made. Labeled precursors (Amersham), sodium pyruvate (5 m*M*), or sodium citrate (5 m*M*), had activities of 0.5 μCi per vessel. Each result is the mean of at least four determinations ± 1 standard deviation; N.M., not measurable.

Additions	[^{14}C]ACh (nanomoles per 100 mg of protein per hour)	
	No Triton	With Triton
With [2-^{14}C]pyruvate		
NAD and Ca^{2+}	48 ± 5*	737 ± 23†
NAD and CoA	61 ± 10*	2300 ± 100‡
NAD, Ca^{2+}, and CoA	203 ± 8	1988 ± 99*
Ca^{2+}	53 ± 5*	
Ca^{2+} and CoA	85 ± 5	74 ± 14†
Ca^{2+}, CoA, and amobarbital	55 ± 6§	
NAD, Ca^{2+}, CoA, and amobarbital	247 ± 16‖	1961 ± 110
NAD, Ca^{2+}, CoA, and oxaloacetate	31 ± 4*	115 ± 30†
NAD, Ca^{2+}, CoA, and succinate	69 ± 12*	1775 ± 49‡
NAD, Ca^{2+}, CoA, and citrate	121 ± 5*	1780 ± 95‡
EGTA	26 ± 5*	
NAD and EGTA	41 ± 7*	
NAD, EGTA, and CoA	35 ± 3*	2463 ± 82†
NAD, Ca^{2+}, CoA, and EGTA	42 ± 9*	
With [1, 5-^{14}C]citrate		
None	N. M.	332 ± 18†
Oxaloacetate	N. M.	71 ± 20†
ATP	N. M.	394 ± 22
NAD and CoA	N. M.	464 ± 20
NAD, CoA, and Ca^{2+}	N. M.	470 ± 17

* $P < .001$ with respect to the control (NAD, Ca^{2+}, and CoA). † $P < .001$ with respect to controls (NAD, Ca^{2+}, CoA, and Triton). ‡ $P > .02$ with respect to controls (NAD, Ca^{2+}, CoA, and Triton). § $P < .001$ with respect to control (Ca^{2+} and CoA). ‖ $P < .005$ with respect to control (NAD, Ca^{2+}, and CoA).

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Table 2. The synthesis of [^{14}C]acetylcholine from 5 m*M* [2-^{14}C]pyruvate in synaptosomal fractions from rat brain in the presence of various concentrations of sodium citrate and calcium chloride. Conditions are those given in Table 1 except that NAD (1 m*M*) and CoA (0.1 m*M*) were always present. Numbers in parentheses represent the percentage inhibition at the various concentrations.

Citrate added (m*M*)	[^{14}C]ACh (nmole/100 mg of protein/hour) with added $CaCl_2$ (m*M*)			
	0.25	0.5	1.0	2.0
0	107 ± 5	171 ± 6	234 ± 5	252 ± 1
1.0	87 ± 19 (19)	133 ± 8 (22)	187 ± 4 (20)	210 ± 4 (17)
2.0	68 ± 19 (36)	96 ± 5 (44)	143 ± 2 (39)	164 ± 4 (35)

Table 3. Effects of pancreatic phospholipase A_2 (Sigma) on [^{14}C]acetylcholine synthesis from [2-^{14}C]pyruvate (5 m*M*) by a crude synaptosomal fraction of rat brain. Conditions are as described in Table 1.

Additions	[^{14}C]ACh (nmole/100 mg of protein/hour) with added phospholipase A_2		
	0	1.5 μg	15 μg
$CaCl_2$	53 ± 5	16 ± 6	30 ± 10
CoA and $CaCl_2$	81 ± 4	16 ± 2	2 ± 2
NAD and $CaCl_2$	48 ± 3	73 ± 5	271 ± 13
NAD and CoA	61 ± 10	118 ± 8*	159 ± 13*
NAD, CoA, and $CaCl_2$	206 ± 6	382 ± 8*	880 ± 50*

*$P < .001$.

diffuse from isolated sheep and rabbit brain mitochondria, especially those treated with ether or exposed to calcium ions (*4, 5*).

We prepared a suspension from fresh rat brain (without cerebellum), as described in (*7*), that consisted largely of resealed nerve endings and of mitochondria derived from glia and neurons. This preparation was at least as active in forming acetylcholine from pyruvate as rat brain cortex slices, for the same amount of protein, when incubated in a physiological saline-glucose medium (*8*). Acetylcholine was measured by the reineckate method (*8*).

The presence of calcium ions, as well as of nicotinamide adenine dinucleotide (NAD) and CoA, are important for the synaptosomal transformation of labeled pyruvate into labeled acetylcholine (Table 1). The addition of EGTA, a powerful calcium chelator (*9*), abolishes the action of calcium ions, which is largely confined to cell barriers; in the presence of detergent Triton X-100 (0.12 percent) and of CoA and NAD, EGTA no longer suppresses the formation of acetylcholine (Table 1). Moreover, Triton X-100, by destroying permeability barriers, increases the rate of acetylcholine synthesis (Table 1) (*10*). There is an accompanying large (90 percent in 1 hour) suppression of respiration; thus, the acetyl CoA formed from pyruvate by pyruvate dehydrogenase in the presence of CoA and NAD is diverted from oxidation by the citric acid cycle and becomes available for acetylcholine synthesis.

The addition of 1 m*M* amobarbital, an inhibitor of mitochondrial oxidations, inhibits the formation of acetylcholine from pyruvate in the presence of CoA and Ca^{2+} and in the absence of added NAD (Table 1). Amobarbital increases acetylcholine formation, however, in the presence of excess NAD, presumably because, by blocking the oxidation of reduced NAD, it diminishes use of acetyl CoA in the citric acid cycle. In the presence of Triton X-100, when respiration has almost ceased, the addition of amobarbital has no effect on the formation of acetylcholine (Table 1). This evidence of involvement with mitochondria (*11*) is in accord with other observations (*12*). It thus appears unnecessary to postulate the existence of a synaptoplasmic (soluble) pyruvate dehydrogenase as solely responsible for the formation of brain acetylcholine (*3*), and there is evidence (*13*) against the existence of the enzyme in the synaptoplasm.

Citrate, a possible precursor of acetylcholine, enters synaptosomes (*14*), but there is as yet no direct indication that it enters cholinergic synaptosomes. We now show, by methods already described (*7*), that there is a synaptosomal uptake of citrate, an exogenous concentration of 5 m*M* ^{14}C-labeled citrate giving rise, in 30 minutes, to an uptake of 1.25 μmole of [^{14}C]citrate per 100 mg of protein (1.25 m*M*). This is much in excess of the uptake by a hypo-osmotically shocked synaptosomal preparation (*15*) which amounts to 30 percent of the observed uptake. In the presence of 1 m*M* calcium chloride, the corresponding uptake of [^{14}C]citrate by an untreated synaptosomal preparation is 1.65 μmole. It appears that citrate enters the cholinergic synaptosomes because exogenous citrate (2 m*M*) reduces by 40 percent the rate of formation of pyruvate-derived acetylcholine (Table 1). This reduction is almost abolished in the presence of Triton X-100 (Table 1). It is not due to chelation of exogenous calcium ions (Table 2).

These results show that citrate (1 m*M*), although a well known chelator of calcium (*9*), exerts only a relatively small (20 percent) inhibiting effect on the rate of acetylcholine synthesis even in presence of calcium chloride at a quarter of its concentration (Table 2). The percentage inhibition is almost independent of the concentration of calcium chloride (0.25 to 2 m*M*). Doubling the concentration of citrate doubles the percentage inhibition at all concentrations of calcium ions tested. Such results become explicable if the citrate-chelated calcium is able to enter the synaptosome and there dissociates to release free calcium and citrate. The percentage acceleration of the rate of [^{14}C]acetylcholine synthesis from [^{14}C]pyruvate, brought about by 2 m*M* calcium chloride over that produced by 0.25 m*M* calcium chloride, is almost independent of the citrate concentration: 136 percent in the absence of citrate, 141 percent in presence of 1 m*M* citrate, and 141 percent in the presence of 2 m*M* citrate. The apparent constancy of the effect of calcium, within a range of citrate concentrations (0 to 2 m*M*), together with our results on the uptake of citrate, indicate that citrate (1 to 2 m*M*) can enter the cholinergic synaptosomes even in the presence of exogenous calcium at relatively high concentrations (2 m*M*).

Although labeled citrate enters cholinergic synaptosomes, it gives rise to no measurable amount of labeled acetylcholine (Table 1), even in presence of ATP or of NAD, CoA, and Ca^{2+} (Table 1). There is an ample rate of formation of labeled acetylcholine from labeled pyruvate, which indicates that the citrate cleavage enzyme is either absent from or not detectable in the cholinergic synaptosomes. The Michaelis constant of citrate for the cleavage enzyme is 0.15 to 0.2 m*M* (*16*), a value below the concentration in the synaptosomal preparation incubated with 5 m*M* citrate. The virtual absence of the citrate cleavage enzyme in cholinergic synaptosomes has been suggested (*17*), but the evidence is not conclusive. In the presence of Triton X-100, the addition of 5 m*M* of labeled citrate gives a relatively large rate of labeled acetylcholine formation (Table 1); this may be explained by the liberation into the incubation medium of the citrate cleavage enzyme from sources

other than cholinergic nerve terminals.

Since mitochondria are involved in pyruvate-derived acetylcholine formation and since there is little detectable citrate cleavage enzyme in the cholinergic synaptoplasm, it follows that, in the absence of other known precursors of cerebral acetylcholine (*18–22*), acetyl CoA itself must be able, in presence of Ca^{2+}, to leave the mitochondria. The amount involved (*4, 5*) may be only a small percentage of that formed in the mitochondria during respiration. Oxaloacetate, a potent inhibitor of pyruvate-derived acetylcholine formation, both in the absence or presence of Triton X-100 (Table 1), must combine with acetyl CoA, since citrate synthase (E.C. 4.1.3.7) is present both in the mitochondria of the cholinergic synaptosome and in the Triton-treated preparation. Succinate (2 m*M*) suppresses the formation of pyruvate-derived acetylcholine (Table 1), part of it being converted to oxaloacetate in the cholinergic mitochondria. Succinate has little or no effect in the Triton-treated preparation (Table 1) when the mitochondrial membranes undergo rupture and succinate dehydrogenase activity is suppressed.

It was shown many years ago (*23*) that phospholipase A from venoms stimulates glucose-derived acetylcholine formation. We find that mammalian phospholipase A_2 from the pancreas stimulates the formation of labeled acetylcholine from labeled pyruvate in a preparation from rat brain synaptosomes in the presence of CoA and NAD (Table 3). This stimulation is enhanced by Ca^{2+} (Table 3), which stimulates the uptake of NAD into rat liver mitochondria (*24*). Since phospholipase A_2 is present in brain (*25*) and other mitochondria (*26*) and is stimulated by Ca^{2+} (*25–27*), it seems likely that Ca^{2+} alters the permeability of the mitochondrial membrane to CoA and acetyl CoA and that this effect may be mediated by the activity of endogenous mitochondrial phospholipase A_2. These results may be correlated with findings that indicate a role for intramitochondrial phospholipase A_2 in the processes that establish the permeability properties of the inner mitochondrial membrane (*28*) and with our finding (*29*) that phospholipase A_2 causes an efflux of CoA and acetyl CoA from the synaptosomal fraction into the incubation medium.

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Primary Structure of a Large Aminoacyl-tRNA Synthetase

Abstract. *The complete primary structure of* Escherichia coli *alanyl-tRNA synthetase, a 95,000-dalton polypeptide, was established by sequencing the gene encoding the enzyme and by sequencing oligopeptides in hydrolyzates of the protein by gas chromatography–mass spectrometry. Contrary to expectation, this long polypeptide contains no lengthy duplications. One 13-residue peptide is homologous with* Escherichia coli *tyrosyl-tRNA synthetase, and clusters of charged amino acids occur in several sections of the structure.*

Aminoacyl-tRNA sythetases (tRNA, transfer RNA) play a crucial role in protein biosynthesis by attaching amino acids to their corresponding tRNA's (*1*). We have sequenced the gene that encodes *Escherichia coli* alanyl-tRNA synthetase (*2*) and, in addition, have identified short randomly located oligopeptides of the protein by gas chromatography–mass spectrometry (GC-MS) (*3, 4*). Reports of repeating sequences in tRNA synthetases of large molecular weight (M_r > 60,000) have led to the hypothesis that these enzymes evolved by gene duplication (*1, 5–9*). Alanyl-tRNA synthetase provides the first complete test of this hypothesis because it is the only large (875 residues) synthetase to be sequenced in its entirety. The sequence of the alanine enzyme also allows for a more complete examination of sequence homologies among this class of enzymes, because primary structure data are available only on two small (M_r < 50,000) aminoacyl-tRNA synthetases (*10–12*). Finally, alanyl-tRNA synthetase binds to a specific DNA sequence as part of a transcriptional control mechanism (*13*), and extensive data are available on the general location of catalytic sites (*2*). Therefore, knowledge of the primary structure is essential for understanding the relation between structure and function of this enzyme.

Figure 1 shows the restriction map of the gene for alanyl-tRNA synthetase. The arrows above and below these strands indicate the length and direction

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