A Study on the Tricarboxylic Acid Cycle and the Synthesis of Acetylcholine in the Lobster Nerve

By S.-C. CHENG AND R. NAKAMURA

New York State Research Institute for Neurochemistry and Drug Addiction, Ward's Island, New York, N.Y. 10035, U.S.A.

(Received 11 August 1969)

1. The pattern of metabolism of ^{14}C -labelled substrates in the lobster nerve suggested a normal tricarboxylic acid cycle with a slow turnover. 2. Acetylcholine was synthesized from $[2\text{-}^{14}\text{C}]\text{acetate}$, $[2\text{-}^{14}\text{C}]\text{pyruvate}$ and $[1,5\text{-}^{14}\text{C}]\text{citrate}$, implying the presence of acetate thiokinase, choline acetylase and citrate-cleavage enzyme. 3. $[2\text{-}^{14}\text{C}]\text{acetate}$ was the best precursor. 4. The formation of acetyl-CoA from citrate was limited, probably by the citrate-cleavage enzyme, although the magnitude of the reversed reactions of the tricarboxylic acid cycle was large when compared with that of the forward reactions. 5. The relative magnitude of the two pathways (acetyl-CoA and carbon dioxide fixation) in pyruvate utilization was nearly equal. 6. The probable presence of metabolic compartments in the lobster nerve is discussed.

In the preceding paper (Nakamura, Cheng & Naruse, 1970), we reinvestigated the possible precursors for the synthesis of the acetyl moiety of ACh* in brain slices. We found that only acetate and pyruvate contributed significant amounts of acetyl group for such a synthesis, and that pyruvate was a much better precursor than was acetate. Glutamate could also contribute but quantitatively lacked significance. A similar approach was applied in the present work to the isolated lobster nerve, which also synthesizes ACh *in vitro* (Dettbarn & Rosenberg, 1966). The general metabolic pattern of carbohydrate utilization in the lobster nerve, particularly the part that involves the tricarboxylic acid cycle (Scheme 1), was also studied in the same experiments. Preliminary reports on this work have appeared (Cheng & Nakamura, 1967; Nakamura & Cheng, 1967; Nakamura, Cheng, Naruse & Waelsch, 1966-67).

MATERIALS AND METHODS

Lobsters (*Homarus americana*) were purchased in local fish markets. The nerves from the first two pairs of walking legs were removed for experiments. Six nerves were incubated in 5 ml of medium prepared as described by Cheng & Waelsch (1962) except that 0.1 mM-physostigmine and 25 μCi of a radioactive substrate were also included. Table 1 shows the concentrations and the specific radioactivities (c.p.m./ μmol) of these substrates. The apparent specific radioactivity of DL-glutamate

should be halved to take account of the L-isomer only. That of citrate was assumed to be divided equally between C-1 and C-5. The incubation was carried out at 15°C for 3 h with an atmosphere of $\text{CO}_2 + \text{O}_2 + \text{N}_2$ (2:50:48) (Cheng & Waelsch, 1962). Other procedures and fraction numbers are described earlier (Cheng & Waelsch, 1963; Naruse, Cheng & Waelsch, 1966a; Nakamura *et al.* 1970).

Each 1 mg of protein was found to correspond, on the average, to 33 mg wet wt. of nerve. This is in general agreement with the value of 27 mg reported by Cheng & Waelsch (1963).

RESULTS

Concentration of metabolites. The concentrations of glutamate, aspartate, malate and citrate in the nerves all decreased during incubation (Table 2).

Table 1. *Concentration and specific radioactivity of added labelled substrates in the medium*

The efficiency of the counting system was approximately 32%.

Substrate	Concn. $10^{-6} \times$ (mM)	Sp. radioactivity (c.p.m./ μmol)
$[2\text{-}^{14}\text{C}]\text{Acetate}$	2.5	1.2
$[^{14}\text{C}]\text{Bicarbonate}$	1.56	2.1
$[1,5\text{-}^{14}\text{C}]\text{Citrate}$	1	3.0
D- $[1\text{-}^{14}\text{C}]\text{Glucose}$	2	1.5
DL- $[1\text{-}^{14}\text{C}]\text{Glutamate}$	1	3.0
DL- $[5\text{-}^{14}\text{C}]\text{Glutamate}$	1	3.0
$[1\text{-}^{14}\text{C}]\text{Pyruvate}$	2	1.5
$[2\text{-}^{14}\text{C}]\text{Pyruvate}$	2	1.5
$[1,4\text{-}^{14}\text{C}]\text{Succinate}$	0.93	3.22
$[2,3\text{-}^{14}\text{C}]\text{Succinate}$	1.14	2.64

* Abbreviation: ACh, acetylcholine.

The concentrations of glutamate and malate after incubation were not determined in these experiments but were obtained from some other experiments under similar conditions. The decrease was less marked for citrate than for the other intermediates. The mean concentration of ACh in nerves examined mainly in the spring was 2.4 ± 0.1 nmol/mg of protein ($n = 36$); however, there was a seasonal variation of ACh content. The results from 28 experiments collected during 1966 and 1967 could be fitted with a regression line $y = 2.71 - 0.006x$, where y was nmol/mg of protein and x the number of days from 1 March. The overall average ACh content on wet-weight basis (72 nmol/g) was higher than that (39 nmol/g) reported by Dettbarn & Rosenberg (1966). Since they did all their experiments during the summer, their values should be less than ours, which were obtained primarily during the spring.

Total uptake of radioactivity. Results on the total uptake of radioactivity (fraction D; Nakamura *et al.* 1970) from various labelled substrates are summarized in Table 3. The highest radioactivity uptake was found from labelled glucose and this

uptake was several-fold larger than that from pyruvate. If this uptake is assumed to represent the total incorporation of glucose (about 7×10^6 c.p.m./g wet wt.) then there would be an active uptake of glucose from the medium (3×10^6 c.p.m./ml). Radioactivity uptake from glutamate was less than that from pyruvate. This was surprising since glutamate is actively accumulated in the brain (Takagaki, Hirano & Nagata, 1959). The uptake of radioactivity from citrate should be half of the amount listed in Table 3 because of double labelling. The extent of bicarbonate uptake was less than that of citrate but more than those of succinate and acetate. This large variation in total uptake suggested the presence of some sort of barrier towards the metabolic incorporation of these substrates.

Incorporation of radioactivity into different metabolites. Further breakdown of the radioactivity taken up is shown in Table 3. Significant amounts of radioactivity were found in the acetyl moiety of ACh in the [2- 14 C]acetate, [2- 14 C]pyruvate and [1,5- 14 C]citrate experiments. The radioactivity in the ACh fraction in these experiments was essentially all in the acetyl moiety (97–99%), as shown by hydrolysis with cholinesterase (Nakamura *et al.* 1970). With labelled glucose the total radioactivity incorporated into the ACh fraction was low and only 45% was found in the acetyl moiety, giving a final value of only 7 c.p.m./mg of protein. The significance of this value is questionable. ACh isolated from experiments with other added substrates contained practically no radioactivity.

The incorporation of radioactivity into aspartate was best from [14 C]bicarbonate. This agreed with our previous findings of CO₂ fixation at the oxaloacetate level and that the aspartate pool probably functioned like a trap (Cheng & Waelsch, 1962; Naruse, Cheng & Waelsch, 1966b). This incorporation should be compared with that from pyruvate, which amounted to only about one-quarter of that from bicarbonate. If CO₂ and pyruvate are pre-

Table 2. *Concentration of metabolites in the lobster nerve after incubation in vitro*

The nerves were incubated at 15°C with CO₂ + O₂ + N₂ (2:50:48). Values quoted are the means $\pm$ S.E.M. with the numbers of determinations in parentheses. Values for 0.5 h incubation are taken from previous experiments (Cheng & Mela, 1966a; Naruse *et al.* 1966a).

Compounds	Concn. (μ mol/mg of protein)	
	After 0.5 h incubation	After 3 h incubation
ACh		0.0024 $\pm$ 0.0001 (36)
Glutamate	0.33 $\pm$ 0.01 (179)	0.13 $\pm$ 0.01 (18)
Aspartate	3.5 $\pm$ 0.03 (179)	1.2 $\pm$ 0.06 (36)
Malate	0.17 $\pm$ 0.01 (179)	0.06 $\pm$ 0.01 (18)
Citrate	0.012 $\pm$ 0.005 (12)	0.008 $\pm$ 0.001 (36)

Table 3. *Radioactivity uptake by the lobster nerve from various 14 C-labelled substrates*

All values are given in three significant digits.

Substrate	Total (c.p.m./mg of protein)	Acetylcholine		Glutamate (c.p.m./mg of protein)	Aspartate		Citrate	
		(c.p.m./mg of protein)	(c.p.m./ μ mol)		(c.p.m./mg of protein)	(c.p.m./ μ mol)	(c.p.m./mg of protein)	(c.p.m./ μ mol)
[2- 14 C]Acetate	7660	226.3	111 000	2620	339	229	1130	160 000
[14 C]Bicarbonate	25 900	0.1	31	1940	14 900	14 700	1100	126 000
[1,5- 14 C]Citrate	80 400	27.0	11 500	435	24	26	—	—
[1- 14 C]Glucose	240 000	7.1	2000	469	490	321	—	—
[1- 14 C]Glutamate	51 200	0.3	60	—	66	67	45	5410
[5- 14 C]Glutamate	49 500	0.3	111	—	350	385	55	8340
[1- 14 C]Pyruvate	87 000	1.8	608	87	3180	2430	299	49 700
[2- 14 C]Pyruvate	84 100	52.8	20 700	1560	3600	3180	892	119 000
[1,4- 14 C]Succinate	10 200	0.3	258	73	272	302	24	4520
[2,3- 14 C]Succinate	14 600	2.9	1230	186	731	592	46	4690

Scheme 1. Diagram of the tricarboxylic acid cycle with the emphasis on the paths of carbon atoms.

cursors for oxaloacetate formation (Scheme 1), the extent of radioactivity incorporation should be comparable with their specific radioactivities in the medium (Table 1). Similar rationale could be

applied in the comparison of the radioactivity incorporation into aspartate from pyruvate and glucose; again a large discrepancy was found. The meaning of these differences is not clear, but it

could be due to metabolic compartmentation. The radioactivity incorporation into aspartate from other labelled substrates was in general low. There should be no radioactivity from [1-¹⁴C]glutamate (Scheme 1), but some was found. This probably arose from the recarboxylation of the decarboxylated C-1 of glutamate.

The incorporation of radioactivity into glutamate followed a different order. [¹⁴C]Acetate was better than [¹⁴C]bicarbonate and [2-¹⁴C]pyruvate, all of which were better than the other substrates. The radioactivity in [1-¹⁴C]pyruvate should not be incorporated into glutamate (Scheme 1); the actual finding of 87 c.p.m./mg of protein suggested the recarboxylation of decarboxylated C-1 of pyruvate or a randomization of C-1 and C-4 of the dicarboxylic acids or both. The radioactivity incorporation into citrate followed the pattern for glutamate and requires no further discussion.

Specific radioactivities of some of the metabolites. The specific radioactivity of ACh, aspartate and citrate are shown in Table 3. The pattern is essentially the same as that for c.p.m./mg of protein (see above). The relative specific radioactivity ratios of citrate to ACh were 1.4 and 5.8 respectively with [2-¹⁴C]-acetate and [2-¹⁴C]pyruvate as the added labelled substrate. Since citrate was doubly labelled when pyruvate was the substrate (Scheme 1) and the relative contribution from both the acetyl-CoA and CO₂-fixation pathways was nearly the same (see below), the latter ratio should be halved to 2.9. The specific radioactivities of acetate and pyruvate in the medium were 1.2×10^6 and 1.5×10^6 respectively, thus further decreasing the latter ratio to 2.3. This is still quite different from 1.4 and indicates that ACh and citrate are derived from two separate metabolic pools of acetyl-CoA (Nakamura & Cheng, 1969).

The relative specific radioactivity ratios of citrate to aspartate derived from labelled succinate and glutamate gave an idea of the extent of the backwards reactions of the tricarboxylic acid cycle. The ratio for [2,3-¹⁴C]succinate was 7.9, which represented only the transfer of radioactivity via the forward reactions, and that for [5-¹⁴C]glutamate was 21.6, which represented the transfer of radioactivity to aspartate in the forward direction and to citrate in both the forward and backward directions. The difference between them, representing the backwards flux, is 13.7, which was larger than 7.9, the forwards flux. In other words, with [5-¹⁴C]-glutamate as the labelled substrate about twice as much glutamate reached citrate via the reversed reactions of the tricarboxylic acid cycle than via the forward reactions. Estimations based on [1,4-¹⁴C]succinate and [1-¹⁴C]glutamate are not valid because of the recarboxylation of ¹⁴CO₂ from labelled carboxylic acid groups.

DISCUSSION

Concentration of metabolites. After 3 h of incubation, considerable amounts of intermediates disappeared from the nerves (Table 2). About one-third of citrate was lost and two-thirds of glutamate, aspartate and malate were lost. To gain newly synthesized ACh (Dettbarn & Rosenberg, 1966), we had no recourse but to accept the loss of these acids. The low metabolic rate of the lobster nerve (Naruse *et al.* 1966b), which was confirmed in this study, precludes the possibility that these acids were metabolized. It seemed more reasonable to assume that they diffused out of the nerves. Their amounts in the media were not determined owing to technical difficulties. The concentrations of the acids in the nerves remained essentially the same with or without any substrate added to the medium in addition to glucose. This suggested that the added substrates did not alter the metabolic pattern of the isolated lobster nerve.

Uptake of substrates. The specific radioactivities of various labelled substrates in the medium were all comparable (Table 1). Should diffusion account for all the radioactivity that could enter the nerves, all experiments should have comparable total radioactivity. This was not the case (Table 3). Glucose was probably actively accumulated and thus accounted for the high incorporation. If the total incorporation in the pyruvate experiments represented maximal free diffusion into the nerves, then all other substrates encountered some sort of diffusion barrier. In other words, the permeability of the lobster nerve is selective towards these substrates.

Carbon dioxide fixation. Previous reports (Waelsch, Cheng, Côté & Naruse, 1965; Naruse, Cheng & Waelsch, 1966b,c) of CO₂ fixation in nervous tissue at both the oxaloacetate and oxalosuccinate levels was confirmed. In addition, results presented here also indicated the recarboxylation of decarboxylated C-1 of [1-¹⁴C]glutamate and [1-¹⁴C]pyruvate. Thus an important metabolic recycling of CO₂ was demonstrated. The extremely large amount of CO₂ fixed (Table 3) might be a reflexion of an attempt by the lobster nerve to replenish the lost carbon skeletons (in the form of intermediates) of the tricarboxylic acid cycle (see above) or it might play an essential role in the maintenance of nerve function (Cheng & Waelsch, 1963; Cheng, Naruse, Mela & Waelsch, 1965; Waelsch *et al.* 1965; Côté, Cheng & Waelsch, 1966; Cheng & Mela, 1966a,b; Naruse *et al.* 1966b,c).

On comparison of the incorporation of radioactivity from pyruvate, aspartate derived from both [1-¹⁴C] and [2-¹⁴C]pyruvate was similar quantitatively, but citrate from these sources was not, being approx. 2.4:1 in favour of [2-¹⁴C]-

pyruvate. Clearly decarboxylation of [1^{14}C]-pyruvate yielded non-radioactive acetyl-CoA in contrast with the case with [2^{14}C]-pyruvate (Scheme 1). This could account for the difference. Therefore, the specific radioactivity of citrate from [1^{14}C]-pyruvate was essentially all transmitted through oxaloacetate. If the same amount came through via oxaloacetate in the [2^{14}C]-pyruvate experiments, then the amount of radioactivity from the acetyl-CoA pathway should be the difference between citrate specific radioactivities from [2^{14}C] and [1^{14}C]-pyruvate or $117400 - 49000 = 68400 \text{ c.p.m./}\mu\text{mol}$, or 1.4 times that via oxaloacetate. In other words, the relative contributions of the two pathways (acetyl-CoA and CO_2 fixation) from pyruvate to citrate were nearly equal (Cheng, Nakamura & Waelsch, 1967).

The contribution of radioactivity from C-1 of pyruvate to citrate (Table 3) could also be used to correct the value for citrate in the [^{14}C]bicarbonate experiment. The latter needed correction because of the difference in initial specific radioactivities in the medium (Table 1). Thus $126700 \times (1.5/2.1) - 49000 = 41400 \text{ c.p.m./}\mu\text{mol}$. This difference could be assigned to the CO_2 -fixation mechanism via the reversed reactions of the tricarboxylic acid (see above). This mechanism was approximately the same in magnitude as that of CO_2 fixation at the oxaloacetate level, confirming our earlier estimation (Naruse *et al.* 1966b). In conjunction with the relative formation of citrate from glutamate via the forward and backwards reactions of the tricarboxylic acid cycle (see above), the relative magnitude of the backward reactions was large.

ACh synthesis. In contrast with the results with brain slices (Nakamura *et al.* 1970), acetate was found to be the best substrate for ACh synthesis by the lobster nerve. This implies the presence of active acetate thiokinase. The presence of citrate-cleavage enzyme was deduced from the transfer of radioactivity from [$1,5^{14}\text{C}$]citrate to ACh; this is also different from the results with brain slices. The syntheses of both labelled citrate and ACh from [2^{14}C]-pyruvate were less than those from [2^{14}C]-acetate, implying low activity of the pyruvate dehydrogenase complex. The magnitude of incorporation of radioactivity into ACh from [1^{14}C]-glucose was almost an order lower than that from [2^{14}C]-pyruvate. This suggests that the activity of the pyruvate dehydrogenase complex was not limiting but the glycolytic rate was extremely low. No significant incorporation of radioactivity into ACh was obtained from [5^{14}C]glutamate, in contrast with the 0.06% conversion in rat brain slices (calculated from the average specific radioactivity of isolated ACh and one-half of that of the medium glutamate given by Nakamura *et al.* 1970). This failure to demonstrate the glutamate to citrate to

ACh pathway (Waelsch *et al.* 1965) in lobster nerve became evident when the total radioactivity conversions from glutamate into citrate and from citrate into ACh were calculated. The estimated amount was too small to be determined by our method.

Metabolic compartmentation in the lobster nerve. Certain findings in this study support this idea of different metabolic pools for each metabolite. One is the partition of radioactivity between ACh and citrate. Another concerns the radioactivity in aspartate in the bicarbonate and [1^{14}C]-pyruvate experiments. A third one is based on the comparison of the radioactivity in aspartate in the glucose and [2^{14}C]-pyruvate experiments. In view of an earlier observation of the unusually high γ -aminobutyrate concentration in the inhibitory fibres in the lobster nerve (Kravitz, Molinoff & Hall, 1965), metabolic compartmentation probably exists in these nerves.

The authors thank the U.S. Public Health Service for financial support (Grant NB-05869), Mr B. Arenberg for his technical assistance and Dr A. Lajtha for his continued interest in and discussion on this work. R. N. was a Fulbright Scholar from the University of Tokyo, Japan.

REFERENCES

- Cheng, S.-C. & Mela, P. (1966a). *J. Neurochem.* **13**, 281.
- Cheng, S.-C. & Mela, P. (1966b). *J. Neurochem.* **13**, 289.
- Cheng, S.-C. & Nakamura, R. (1967). *Fedn Proc. Fedn Am. Soc. exp. Biol.* **26**, 403.
- Cheng, S.-C., Nakamura, R. & Waelsch, H. (1967). *Nature, Lond.*, **216**, 928.
- Cheng, S.-C., Naruse, H., Mela, P. & Waelsch, H. (1965). *Biochem. J.* **96**, 67p.
- Cheng, S.-C. & Waelsch, H. (1962). *Science, N. Y.*, **136**, 782.
- Cheng, S.-C. & Waelsch, H. (1963). *Biochem. Z.* **338**, 643.
- Côté, L., Cheng, S.-C. & Waelsch, H. (1966). *J. Neurochem.* **13**, 271.
- Dettbarn, W. D. & Rosenberg, P. (1966). *J. gen. Physiol.* **50**, 447.
- Kravitz, E. A., Molinoff, P. B. & Hall, Z. W. (1965). *Proc. natn. Acad. Sci. U.S.A.* **54**, 778.
- Nakamura, R. & Cheng, S.-C. (1967). *J. gen. Physiol.* **50**, 1086.
- Nakamura, R. & Cheng, S.-C. (1969). *Life Sci.* **8**, 657.
- Nakamura, R., Cheng, S.-C. & Naruse, H. (1970). *Biochem. J.* **118**, 443.
- Nakamura, R., Cheng, S.-C., Naruse, H. & Waelsch, H. (1966-67). *Brain Res.* **3**, 384.
- Naruse, H., Cheng, S.-C. & Waelsch, H. (1966a). *Exp Brain Res.* **1**, 40.
- Naruse, H., Cheng, S.-C. & Waelsch, H. (1966b). *Exp Brain Res.* **1**, 284.
- Naruse, H., Cheng, S.-C. & Waelsch, H. (1966c). *Exp Brain Res.* **1**, 291.
- Takagaki, G., Hirano, S. & Nagata, Y. (1959). *J. Neurochem.* **4**, 124.
- Waelsch, H., Cheng, S.-C., Côté, L. & Naruse, H. (1965). *Proc. natn. Acad. Sci. U.S.A.* **54**, 1249.