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TRANSPORT AND METABOLISM OF DOUBLE-LABELLED CDPCHOLINE IN MAMMALIAN TISSUES

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Abstract—Double-labelled [methyl-^{14}C,5-^{3}H]CDPcholine has been synthesized and subjected to a pharmacokinetic analysis in several biological systems.

In transport experiments with intact human erythrocytes no incorporation of radioactivity is observable. On the other hand the results obtained with perfused rat liver suggest a rapid cleavage of the pyrophosphate bridge of the molecule, followed by a rapid uptake of the hydrolytic products.

The plasma half-lives of intravenously injected CDPcholine and of its metabolites have been evaluated within 60 sec range. Renal and fecal excretion of the injected radioactivity is negligible: only 2.5% of administered ^{14}C- and 6.5% of the ^{3}H- is excreted up to 48 hr after administration.

Liver and kidney are the major CDPcholine metabolizing organs, characterized by a fast and extensive uptake of choline metabolites, followed by a slow release; conversely the rate of uptake of both ^{3}H and ^{14}C-labelled moieties by rat brain is significantly slower, reaching a steady-state level after 10 hr.

The characterization of the labelled compounds detectable in the investigated organs provides some insights on the metabolism of the drug: (i) the ^{3}H-cytidine moiety in all the examined organs appears to be incorporated into the nucleic acid fraction via the cytidine nucleotide pool; (ii) the [^{14}C]choline moiety of the molecule is in part converted, at the mitochondrial level, into betaine which accounts for about 60% of the total ^{14}C-radioactivity associated with liver and kidney 30 min after administration; (iii) [^{14}C]betaine in turn acts as methyl donor to homocysteine yielding [^{14}C]methionine subsequently incorporated into proteins; (iv) the time dependent increase in labelled phospholipids is indicative of a recycling of the choline methyl-groups in this lipid fraction via CDPcholine and/or *S*-adenosylmethionine; (v) the rather extensive amount of labelled methionine detectable in brain probably arises from its uptake from the blood stream, since the enzyme catalyzing the conversion of betaine into methionine is lacking in brain.

Cytidine-5′-diphosphocholine (CDPcholine), the "active" form of choline, represents a key intermediate in the biosynthesis of phospholipids [1, 2]; lecithins and, indirectly, sphyngomielins are indeed synthesized by reactions involving this molecule as the phosphorylcholine (Pcholine) donor [3–6]. CDPcholine is in turn synthesized from Pcholine and cytidine triphosphate (CTP) through the action of CTP:choline phosphate cytidylyl-transferase (EC 2.7.7.15). This reaction represents the rate limiting step in the overall metabolic pathway of phosphatidyl-choline biosynthesis [7], which is then regulated by the intracellular levels of this co-substrate.

In the last few years CDPcholine has been extensively employed in therapy, mainly in the treatment of Central Nervous System pathologies such as brain edema [8], head injury [9], cerebral ischemia [10], cronical cerebral vascular diseases [11] and Parkinson's disease [12–15]. The mechanism of the claimed pharmacological action of the molecule may be ascribed to: (i) an increase of the impaired phospholipid biosynthesis [16]; (ii) a restoration of the brain "energy charge" [17, 18]; (iii) a possible interference with neurotransmitters and receptors function [16, 19, 20].

In this respect the exogenously administered CDPcholine was found to interfere with several aspects of the nervous tissue metabolism, not merely related to the restoration of the impaired membrane phospholipids. The molecule is indeed effective in restoring RNA and protein biosynthesis impaired by hypoxic treatment in rabbit brain [21, 22] and in preventing the release of free fatty acids, particularly arachidonic acid, caused by brain ischemia in gerbil [23, 24]. The intravenous injection of CDPcholine induces in rabbit a significant regression of brain edema, provoked by cold injury, restoring the sensitivity of the mitochondrial ATPase towards oligomicyn and the sensitivity of the Na^+,K^+ATPase to the K^+/Na^+ ratio [17]. Moreover intracarotid perfusion with CDPcholine increases the synaptosomal phosphorylation in hypoxic dog brain [18]. Finally, the molecule has been found to inhibit the uptake of norepinephrine, dopamine and serotonin and to increase that of tyrosine and tryptophan when added to striatal synaptosomal homogenates of rat brain [19].

In spite of the above mentioned claimed pharmacological effects of the exogenously administered CDPcholine, the mechanism of the transport of this molecule through biological membranes, as well as

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its metabolic fate, has not been adequately investigated so far.

This problem has been faced in our laboratory by means of double-labelled [methyl-^{14}C,5-^{3}H] CDPcholine, using perfused rat liver and human erythrocytes as model systems. Double-labelled CDPcholine represents, in fact, a useful probe to test the structural integrity of the molecule during transport processes and to discriminate and follow the metabolic fate of cytidine and choline fragments [25].

In vivo experiments carried out with double-labelled CDPcholine allowed us to elucidate the pharmacokinetics as well as the metabolism of the molecule and to draw inferences about its pharmacological mechanism(s).

MATERIALS AND METHODS

Chemicals. [5-^{3}H]CMP and [methyl-^{14}C]Pcholine, ammonium salts and [methyl-^{14}C]CDPcholine were obtained from the Radiochemical Centre, Amersham, Bucks, U.K.; Dicyclohexylcarbodiimide (DCC) was obtained from BDH (Poole, U.K.); heparin was Liquemin, Roche (Welwyn Garden City, U.K.). All other chemicals were of the purest grade available from standard commercial sources.

Preparation of double-labelled CDPcholine. The chemical synthesis of [methyl-^{14}C,5-^{3}H]CDPcholine, was performed according to Khorana [26], condensing [5-^{3}H]CMP free acid (102 Ci/mole), with [methyl-^{14}C]Pcholine (50 Ci/mole) in the presence of DCC as condensing reagent for the pyrophosphate bond formation as described elsewhere [27]. The purity of the compound was checked by HPLC analysis and spectroscopic characterization of the double-labelled molecule diluted with non radioactive CDPcholine, obtained in a 10-times scaled-up parallel experiment.

HPLC analysis. A model LC-65T Perkin–Elmer Liquid Chromatograph, equipped with a high performance model LC-15 UV detector, operating at the fixed wavelength of 254 nm, was used. The column (25 cm × 4.6 mm i.d.) was pre-packed with Partisil 10 SCX (Whatman). Integration was performed electronically using a Spectra Physics Minigrator. Either sodium phosphate buffer, 250 mM, pH 6.8 or sulphuric acid, 50 mM, pH 1.5 were used as eluents at room temperature, with a flow rate of 1.0 ml/min. When sodium phosphate buffer was used as eluent, the following retention times have been observed for CDPcholine and its metabolites: cytidine nucleotides 4.5 min, methionine 4.5 min, CDPcholine 6 min, betaine 8 min, Pcholine 12 min, choline 19 min. Methionine and cytidine nucleotides could be discriminated by virtue of their different labelling.

Spectroscopic characterizations. Proton and ^{13}C NMR spectra were run on the WH-270 Bruker Spectrospin spectrometer, equipped with a Fourier Transform accessory. The samples (~50 mg) were analyzed using D_2O as a solvent. Ultraviolet measurements were performed in a water solution using a Varian DMS 100 Spectrometer, equipped with a real time interfaced Varian Techtron DS 15 Data System.

Liver perfusion. Male rats, Sprague–Dawley strain, 750 ± 10 g, were used as liver donors. They were kept on a standard laboratory diet and fasted 18 hr before hepatectomy. The liver was perfused by recirculation at 37° according to Miller *et al.* [28], with 100 ml Krebs–Ringer bicarbonate buffer containing 3% bovine serum albumin, 0.2% glucose, 15,000 units heparin and 0.6 μmoles of [methyl-^{14}C,5-^{3}H]CDPcholine (13.6×10^6 dpm ^{14}C; 30.5×10^6 dpm ^{3}H). The pH, adusted to 7.4, was monitored during the perfusion. The flow of the medium, saturated by a gas mixture containing 95% O_2 + 5% CO_2, was set at 5 ml/min/g of the organ. The absence of non-enzymatic alteration of CDPcholine was tested in a preliminary experiment with the perfusate circulating in a closed system without the liver. At the end of the perfusion the liver was washed with 5 × 10 ml of 0.9% (w/v) NaCl solution and rapidly frozen (liver washing out). The radioactivity associated with the washing fractions was measured and the CO_2, released from the liver during the perfusion was trapped in 100 ml of 40% (w/v) KOH. Bile was collected quantitatively and analyzed for radioactivity. The samples of perfusion medium (10 ml), taken at different time intervals, were ultrafiltered on Amicon Ultrafiltration Cell to remove albumin and then analyzed by HPLC using H_2SO_4 50 mM, pH 1.5, as an eluent.

Transport experiments with intact human erythrocytes. Erythrocytes were obtained from EDTA-treated human blood, no later than 30 min after withdrawal. The whole blood was centrifuged for 10 min at 5000 *g*. The plasma and buffy coat were sucked off and the red cell pellet was resuspended in 5 vol. of isotonic glucose-phosphate buffered saline (GPBS). All the previous procedures were carried out at 4°. Aliquots, corresponding to 100 μl erythrocyte suspension, were placed in a 37° Dubnoff incubator and gently shaken. After 5 min of preincubation, 150 μl of GPBS containing separately 100 pmoles of [methyl-^{14}C,5-^{3}H]CDPcholine (10.2 Ci/mmole ^{14}C; 22.5 Ci/mmole ^{3}H) or 318 nmoles of 5′-[methyl-^{14}C]methylthioadenosine (50 Ci/mmole) were added. The uptake was quickly stopped, at the indicated times, by adding 2 ml of ice-cold GPBS, and the samples were immediately centrifuged at 0° for 30 sec at 5000 *g*. After centrifugation the pellet was hemolyzed with 2 ml of distilled water at 0° and the proteins were precipitated with 0.4 ml of 0.3 M TCA. The interval between the addition of GPBS and TCA precipitation was shorter than 200 sec. After the TCA treatment the samples were centrifuged, for 10 min, at 15,000 *g*, and the pellet discarded. One-millilitre aliquots of the incubation medium and of the TCA supernatant were counted for ^{3}H- and ^{14}C-radioactivity and analyzed by HPLC adding CMP and CDPcholine as internal standards.

In vivo *experiments.* Male rats, Sprague–Dawley, 210 ± 10 g body wt, 12 weeks old, were used. The animals were intravenously injected, unless otherwise specified, either with [methyl-^{14}C]CDPcholine or with [methyl-^{14}C,5-^{3}H]CDPcholine, in physiological solution. At different time intervals the animals were killed and the organs promptly frozen in liquid nitrogen.

Treatment of the tissues. Aliquots corresponding to 1–2 g wet tissue were processed according to Wilgram *et al.* [5], with minor modifications. The samples were homogenized in 20 ml ethanol/water (2:1, v/v) for 1 min in a Waring Blender, heated in a boiling water bath for 20 min in closed tubes and centrifuged (5000 *g*, 20 min). The precipitate was washed several times with the same solution and then extracted with chloroform/methanol (1:1 v/v), in order to remove the associated lipids. The pooled aqueous ethanol supernatants were concentrated under reduced pressure, clarified by centrifugation (18,000 *g*, 2 hr) and then extracted three times with 100 ml of ether. The ether excess was removed under vacuum and the water-soluble metabolites were characterized by HPLC.

For the lipid extraction, 1–2 g of tissue were homogenized, lyophilized and then extracted three times with 20 ml of chloroform/methanol (1:1 v/v). The extracted lipids were resolved by analytical silica gel TLC, using chloroform/methanol/water (65:25:4 by vol.) as an eluent. Phospholipids were detected using subliming iodine.

Quantitative evaluation of the radioactivity associated with phospholipid fraction was carried out after purification of crude lipid extract on silica gel column eluted with the above mentioned eluent.

Preparation of biological samples for radioactive measurement. Aliquots of solid biological samples were previously homogenized in water 1:3 (w/w). 0.6 g of the homogenate were dissolved by digestion at 50° for 24 hr in a shaking water bath, with 2 ml of a mixture of Solulyte (Baker)/isopropyl alcohol (1:1 v/v). The samples were then bleached with a few drops of H_2O_2. The liquid samples were treated in the same way, skipping the homogenization step. The scintillation liquid was Dynagel (Baker)/HCl (10:1, v/v).

Absolute radioactivity was measured in a Beckman LS 7800 scintillation counter, equipped with an automatic quench correction system (AQC). The standardization was performed by using two series of samples containing [5-^{3}H]CDPcholine (2.5×10^5 dpm) or [methyl-^{14}C]CDPcholine (1×10^5 dpm) and increasing aliquots of solubilized liver tissues (0–500 mg) as a quencher.

RESULTS

Transport of CDPcholine through biological membranes

In order to elucidate the mechanism of CDPcholine transmembrane transport, intact human erythrocytes were employed as a first model system. The red blood cells were incubated in the presence of double-labelled CDPcholine as described under Materials and Methods. A control experiment was run using the nucleoside [5'-methyl-^{14}C]methylthioadenosine (MTA), which is known to be actively taken up by these cells [29, 30].

No radioactivity is detectable within the cells incubated with [methyl-^{14}C,5-^{3}H]CDPcholine up to 60 min; furthermore the ^{14}C- and ^{3}H-radioactivity levels in the incubation medium do not significantly change during the same time interval. Conversely, under the same conditions, MTA is actively taken up by the erythrocytes.

The transport of CDPcholine through the hepatocyte membrane was also explored, by means of isolated and perfused rat liver as described under Materials and Methods. The time course of the double-labelled CDPcholine hepatic uptake is shown in Fig. 1: both radioactive isotopes are incorporated

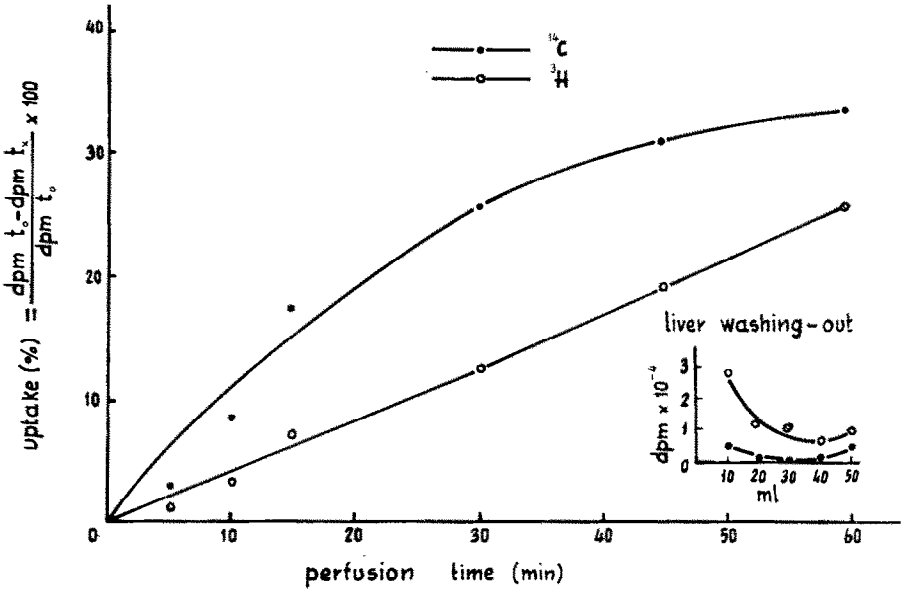


Fig. 1. Time course of double-labelled CDPcholine uptake by isolated and perfused rat liver. The liver was perfused as described under Materials and Methods. The uptake, calculated on the basis of the decrease of ^{3}H- and ^{14}C-radioactivity in the perfusion medium, is reported as a percentage of the two radionuclides taken up by the liver (100 = amount of radioactivity in perfusion medium at $t = 0$). At the end of the perfusion the liver was washed with 5 × 10 ml of saline solution and the radioactivity associated with each fraction is reported in the inset.

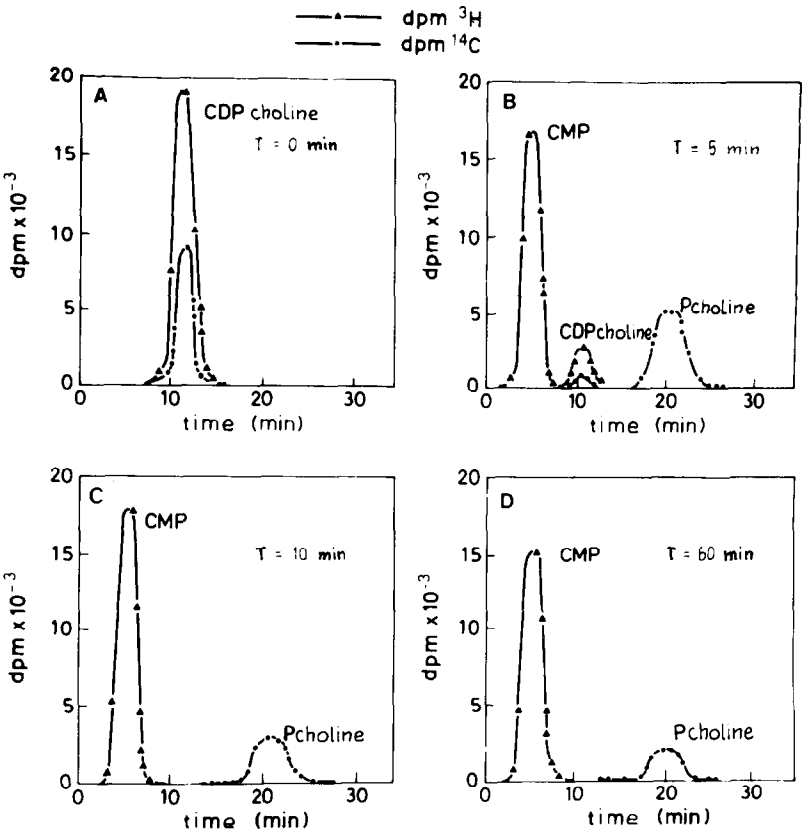


Fig. 2. HPLC analysis of perfusate at different perfusion times. Samples of perfusate were taken at the indicated times, treated and analyzed by HPLC using H_2SO_4 50 mM, pH 1.5 as eluent (see Materials and Methods). Retention times are indicated in the abscissa. The recovery of radioactivity in the eluate range from 80 to 90% of the injected one.

into the liver, although to a different extent. One-hour perfusion results in the uptake of 4.6×10^6 dpm of ^{14}C-radioactivity (33.6% of total) and 7.8×10^6 dpm of ^{3}H-radioactivity (25.7%), with a parallel change in $^{3}H/^{14}C$ ratio in the perfusate, which increases from 2.2 to 2.4. No radioactivity was detectable in the bile at the end of the perfusion, nor was $^{14}CO_2$ found to diffuse from the perfusate. In addition, 99% of the label lost by the perfusion medium was found in the liver. In fact less than 1% of both ^{14}C and ^{3}H-radioactivity was recovered in the washing out of the organ.

In order to identify the radioactive species present in the perfusate, samples were taken at different perfusion times and analyzed by HPLC (Fig. 2 A–D). As shown in the figure even at 5 min (Fig. 2B), CDPcholine pyrophosphate bridge is already extensively cleaved and 5-^{3}H CMP and methyl-^{14}C Pcholine are the major radioactive products present in the perfusate, where no more double-labelled CDPcholine is detectable at as early as 10 min (Fig. 2C).

These results clearly indicate that CDPcholine is not taken up as such by the liver, although a fast uptake of the CDPcholine metabolites is operative.

The CDPcholine metabolites were also characterized in the liver, after 1 hr perfusion, by processing samples of this organ as described under Materials and Methods. Fifty per cent of ^{3}H-radioactivity associated with liver could be precipitated by ethanol treatment, suggesting the incorporation of the cytidine moiety into the nucleic acid fraction, via cytidine nucleotides. The latter are indeed the major tritiated species detected in the liver water-soluble fraction, when analyzed by HPLC. Conversely the whole ^{14}C-radioactivity associated with the liver is present in the water-soluble fraction of the homogenate and the HPLC analysis shows two major labelled peaks, identified as Pcholine (peak C, Fig. 3) and betaine (peak B, Fig. 3), respectively.

Pharmacokinetics of double-labelled CDPcholine in rat

For a screening of the most suitable administration way of the drug three groups of rats were injected either intramuscularly, or intraperitoneally or intravenously with 200 μl of methyl-^{14}C CDPcholine (0.4 mg; 11×10^6 dpm). Forty-five minutes after the injection, the animals were killed and the ^{14}C-radioactivity associated with kidney, liver, heart and brain was measured. As shown in Fig. 4, although no difference could be detected in the distribution of radioactivity among the organs, the total ^{14}C-incorporation is significantly higher when the drug is injected intravenously. In this case the percentages of injected radioactivity, associated with the various

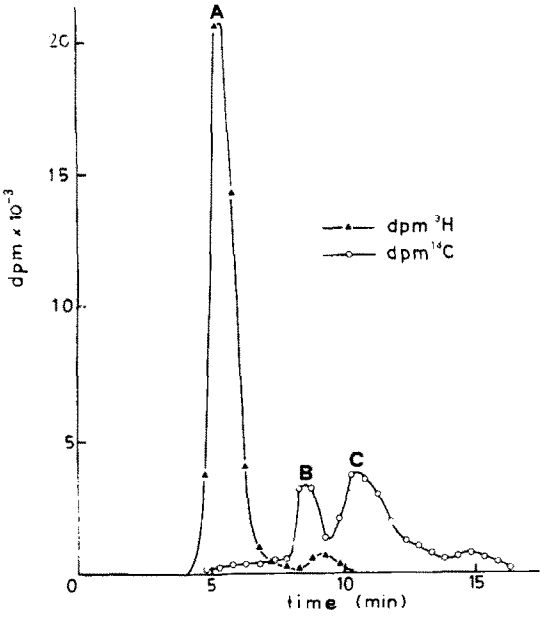


Fig. 3. HPLC analysis of water-soluble labelled metabolites in the liver after 1 hr perfusion with double-labelled CDPcholine. Abscissa reports the retention times. Liver perfusion and tissue extraction are described under Materials and Methods. The HPLC analysis was run using Na-phosphate buffer 250 mM, pH 5.8 as eluent. The recovery of radioactivity eluate range from 80 to 90% of the injected one. Peak A = CMP, peak B = betaine; peak C = Pcholine.

organs are as follows: liver, 31.7%; kidney, 11.8%; heart, 1%; brain, 0.1%.

In order to explore CDPcholine metabolic utilization as a function of the dose two groups of rats were injected intravenously either with 0.04 mg (11×10^6 dpm ^{14}C) or with 2 mg (11×10^6 dpm ^{14}C) of [methyl-^{14}C]-CDPcholine respectively. After 45 min the rats were killed and their kidneys, livers and brains analyzed for radioactivity. As shown in Fig. 5 the extent of radioactivity incorporation in the organs of both groups of animals does not significantly change. However, the amount of CDPcholine taken up by the organs is proportional to the administered dose. This result suggests that, from 0.02 to 1 mg CDPcholine/100 g body wt, the putative recognition sites of the molecule are not yet saturated.

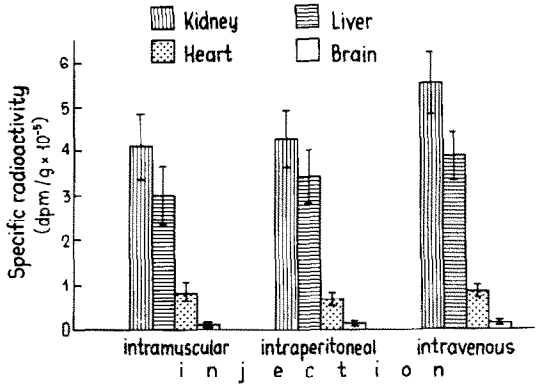
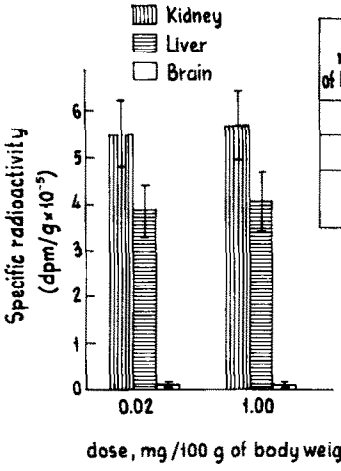


Fig. 4. Radioactivity distribution in organs of rats injected with methyl-labelled CDPcholine, as a function of the route of administration. Three groups of rats were treated with 0.4 mg of [methyl-^{14}C]CDPcholine (11×10^6 dpm) as indicated in the figure. Forty-five minutes after treatment the animals were killed and the organs, processed as reported under Materials and Methods, were analyzed for radioactivity.

To follow up the metabolic fate of CDPcholine in the blood stream, the plasma levels of ^{3}H- and ^{14}C-radioactivity were evaluated at different time intervals after the intravenous injection of 2 mg of double-labelled CDPcholine. As shown in Fig. 6, less than 10% of the administered radioactivity is detectable in the plasma only 2 min after the treatment. From



Dose mg/100 g of body weight	nmoles utilized/g tissue/45 min		
	Liver	Kidney	Brain
0.02	2.8 ± 0.3	4.0 ± 0.5	0.07 ± 0.02
1.00	155 ± 24	218 ± 31	3.8 ± 0.5
Increase factor	55.3	54.5	54.3

Fig. 5. CDPcholine utilization as a function of the dose. Two groups of rats were injected intravenously with 0.04 and 2 mg of [methyl-^{14}C]CDPcholine respectively. The radioactivity administered was in both cases 11×10^6 dpm. The rats were killed 45 min after injection and the organs, processed as described under Materials and Methods, were analyzed for radioactivity. The radioactivity incorporated as dpm × g of tissue is shown on the left; the table on the right reports the absolute amounts of CDPcholine utilized by the organs. The values represent the average of data obtained from four animals. The ratio between the absolute amounts of CDPcholine utilized at higher over lower dose represent the increase factor.

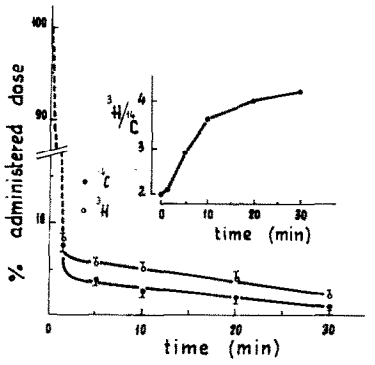


Fig. 6. Kinetic analysis of plasma radioactivity in rats injected with double-labelled CDPcholine. The rats were injected intravenously with 2 mg of [methyl-^{14}C,5-^{3}H]-CDPcholine (20×10^6 dpm ^{14}C; 40×10^6 dpm ^{3}H). The animals were heparin treated, before the drug injection, in order to facilitate the blood drawing. At different time intervals, 1 ml blood samples were drawn, centrifuged (10 min, 3500 *g*) and both the pellet and the supernatant were analyzed for radioactivity. The results are expressed as a percentage of the injected dose in the total plasma volume [34]. The values represent the average of the results obtained from four animals.

this time on a slower decrease of plasma radioactivity is observable. The time-dependent increase in the plasma $^{3}H/^{14}C$ ratio (inset of Fig. 6) is indicative of a preferential uptake of the ^{14}C choline moiety by the tissues. Moreover, as shown in Fig. 7, the total CDPcholine catabolites, excreted with the urine over 48 hr from the injection, represent only 2.5 and 6.5% of the total ^{14}C- and ^{3}H-radioactivity respectively. On the other hand the fecal excretion, in the same time interval, never exceeds 2% of the injected dose. Therefore from the reported results an active utilization of the administered molecule by the peripheral metabolic compartments can be inferred.

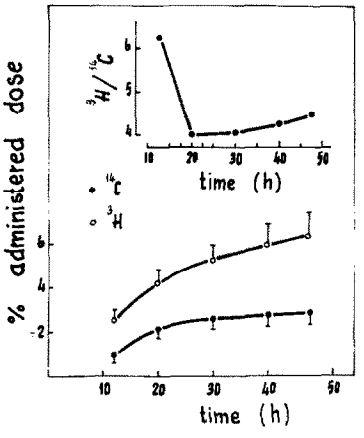


Fig. 7. Renal excretion of labelled catabolites in rats injected with double-labelled CDPcholine. The rats were injected with 2 mg/kg of [methyl-^{14}C,5-^{3}H]CDPcholine (20×10^6 dpm ^{14}C; 40×10^6 dpm ^{3}H) and were kept in metabolic cages. Urine samples were collected under toluene up to the indicated times and analyzed for radioactivity. The results are expressed as percentage of the injected dose. In the inset the $^{3}H/^{14}C$ ratio values are reported. All the values represent the average of 10 different urine samples.

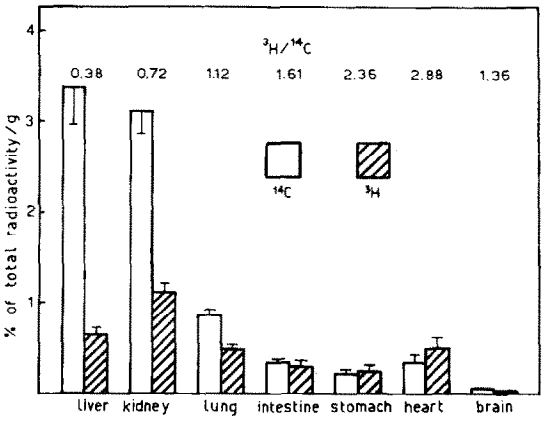


Fig. 8. Radioactive distribution in the organs of rats after injection of double-labelled CDPcholine. The animals were treated as in Fig. 7 and killed 30 min after the injection and the organs were processed as described under Materials and Methods. The radioactivity is reported as percentage of injected ^{3}H- and ^{14}C-radioactivity per g of wet tissue (100 = total injected radioactivity). The values represent the average of data obtained from four animals.

The distribution of the radioactivity within the various rat organs 30 min after the treatment is reported in Fig. 8. The highest ^{3}H- and ^{14}C-radioactivity levels were found to be associated with liver and kidney while the brain is, among the investigated organs, the less active in CDPcholine utilization. The values of $^{3}H/^{14}C$ ratio are significantly different in the examined tissues indicating that only kidney, liver and lung show a preferential uptake of the ^{14}C-labelled moiety while both radioactive fragments of CDPcholine are equally utilized by the other organs. These results suggest an organ-dependent differential utilization of both choline and cytidine moieties.

In order to gain further information on the metabolism of the exogenously administered CDPcholine, the radioactivity levels in several rat organs were evaluated at different time intervals after the drug injection and the labelled metabolites were determined and characterized. Hereinafter the results obtained in the liver, brain and kidney are reported (Fig. 9A–C).

Figure 9A shows the radioactivity levels, detected in the liver at different times after the injection of double-labelled CDPcholine, as a percentage of the administered dose. A ^{14}C-labelled peak (35% of injected ^{14}C-radioactivity) is detectable 60 min after the treatment; 48 hr after the injection 10% of ^{14}C-radioactivity is still associated with the organ, indicating a slow release of ^{14}C-metabolites by the hepatocytes. The ^{3}H-uptake is strikingly lower and the radioactivity reached a saturation plateau 3 hr after the injection, suggesting an even slower release of the ^{3}H-labelled metabolites by this organ. The preferential uptake of choline moiety by the liver can also be inferred by evaluating the $^{3}H/^{14}C$ ratio in liver samples, which stays always lower than that of the injected molecule. However, a slow time-dependent increase in $^{3}H/^{14}C$ ratio is observable, as the ^{14}C-labelled catabolites are released to a higher extent.

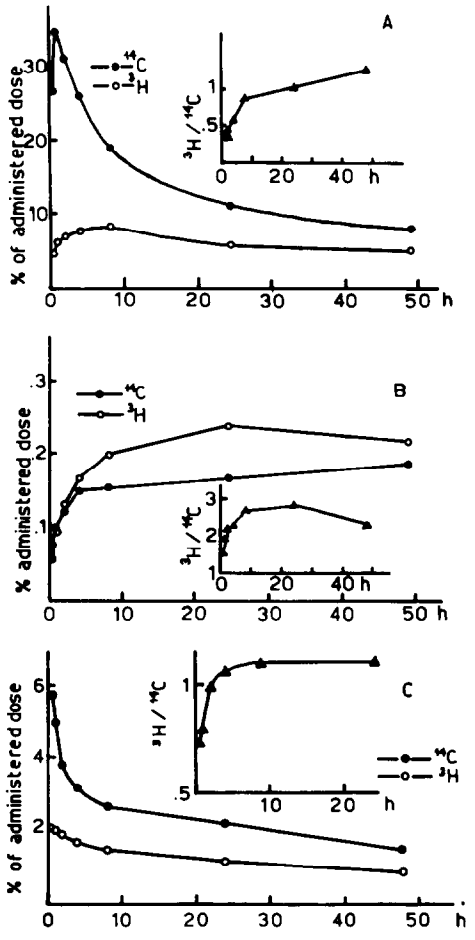


Fig. 9. Radioactivity levels in rat liver (A), brain (B) and kidney (C), at different times from the injection with double-labelled CDPCholine. The rats were treated as reported in Fig. 7. At the indicated times the animals were killed and the radioactivity levels in the organs were evaluated as described under Materials and Methods. The results are expressed as a percentage of the injected dose. The $^{3}H/^{14}C$ ratio values are reported in the inset. All the values represent the average obtained from 10 animals.

Table 1 reports the labelled CDPcholine metabolites detected in this organ at different time intervals. Most of the ^{3}H-radioactivity is initially associated with the cytidine nucleotides and then with nucleic acids; this fraction indeed accounts for the 74% of total liver ^{3}H-radioactivity, 48 hr after the drug injection. Up to 1 hr after the treatment the ^{14}C-labelled metabolites were mostly found in the water-soluble fraction, with [^{14}C]betaine and [^{14}C] Pcholine as the major radioactivity species. It is worth noting, in this respect, the absence of any detectable amounts of [^{14}C]choline within this organ. When the same analysis is carried out at 48 hr the ^{14}C-labelled betaine decreases (5%) while a new water-soluble labelled species, identified as methionine, accounts for 6.2% of the total ^{14}C-radioactivity detected in this organ. Furthermore, at this time, 22% of liver ^{14}C-labelling is associated with proteins. The incorporation of the choline moiety into phospholipids shows a time-dependent increase with 53% of total liver ^{14}C-radioactivity at 48 hr. On

Table 1. Labelled metabolites present in rat organs after intravenous injection of double-labelled CDPcholine. The animals were treated as reported in Fig. 9. At the indicated times they were killed and the organs were processed as described under Materials and Methods. The values represent the average of data obtained from 10 animals, except the brain values which were obtained from a pooled homogenate of the ten brains

	Liver			Kidney			Brain			
Time (hr)	0.5	1	48	0.5	1	48	0.5	1	4	48
Labelled molecular species	Radioactivity (%)*									
^{3}H-cytidine & cytidine nucleotides	74.1 ± 11.8	65.8 ± 10.2	14.2 ± 2.0	73.2 ± 10.3	68.3 ± 11.6	19.4 ± 5.4	60.1	53.2	38.8	28.0
^{3}H-nucleic acids	11.3 ± 1.6	19.2 ± 2.7	73.6 ± 11.3	4.2 ± 1.3	13.3 ± 3.2	66.3 ± 9.7	24.2	33.3	43.2	60.8
^{14}C-betaine	63.2 ± 2.6	47.3 ± 1.8	5.0 ± 0.4	64.1 ± 3.9	27.1 ± 6.1	6.8 ± 0.7	13.8	5.9	2.4	0.3
^{14}C-Pcholine	14.4 ± 1.6	18.1 ± 0.6	3.2 ± 0.5	n.d.	2.8 ± 0.3	1.2 ± 0.2	27.6	17.8	4.8	1.4
^{14}C-choline	n.d.	n.d.	n.d.	3.4 ± 0.8	7.8 ± 2.0	7.1 ± 1.9	25.5	21.1	6.4	2.5
^{14}C-methionine	n.d.	3.4 ± 0.1	6.3 ± 0.5	n.d.	n.d.	3.6 ± 0.9	2.8	9.4	26.4	6.7
^{14}C-phospholipids	9.8 ± 0.4	24.8 ± 0.9	52.9 ± 2.2	16.1 ± 3.1	44.1 ± 5.9	58.3 ± 7.8	7.8	13.1	24.2	47.3
^{14}C-proteins	n.d.	n.d.	22.1 ± 0.9	n.d.	0.8 ± 0.2	6.3 ± 2.1	2.1	18.3	22.1	27.1

* 100 = total radioactivity present in the organ; n.d. = not detectable.

the other hand the absolute amount of radioactivity incorporated in liver phospholipids is higher at 1 hr than at 48 hr, indicating that this pathway is mainly operative in the early times.

Figure 9B illustrates the uptake of ^{3}H- and ^{14}C-labelled species in rat brain. Although the radioactivity incorporation in this organ is significantly lower than in the liver, it slowly increases up to 10 hr from the injection; the ^{3}H-cytidine moiety is preferentially taken up, as can also be inferred from the $^{3}H/^{14}C$ ratio values. Furthermore the brain is characterized by a particularly slow release of CDPcholine labelled catabolites, in that no decrease in both ^{3}H- and ^{14}C-radioactivity levels is observable up to 48 hr.

As reported in Table 1, the cerebral ^{3}H-radioactivity is initially mainly associated with cytidine nucleotides and subsequently with the nucleic acid fraction. Betaine, Pcholine and choline represent the major ^{14}C-labelled species detectable at 30 min, while at 4 hr ^{14}C-methionine and ^{14}C-phospholipids represent 26.4 and 24.2% of total brain ^{14}C-radioactivity, respectively. At 48 hr the brain ^{14}C-labelling was mainly incorporated into choline-phospholipids as well as into proteins. The pool of labelled phospholipids continuously increases up to 48 hr. Figure 10 shows the rate of ^{14}C-incorporation into the brain phospholipids pool as a percentage of total ^{14}C-radioactivity associated with the organ. A fast phase of incorporation is observable in the first 5 hr followed by a 10-fold slower phase, detectable up to 48 hr.

The time course of ^{3}H- and ^{14}C-radioactivity incorporation into kidney is illustrated in Fig. 9C: a rapid uptake of ^{14}C- and ^{3}H-labelled metabolites is observable, both peaks of radioactivity occurring earlier than at 30 min. However, the ^{14}C-labelled molecular species are rapidly released by this organ and 5 hr after the treatment the radioactivity level is reduced to a half of the value detected at 30 min. Since the renal excretion of ^{14}C-labelled catabolites over 10 hr from the injection represents only 1% of the administered dose (Fig. 7), the observed rapid decrease of ^{14}C-radioactivity can be ascribed to an uptake of labelled metabolites from the filtrate for re-use within the organism.

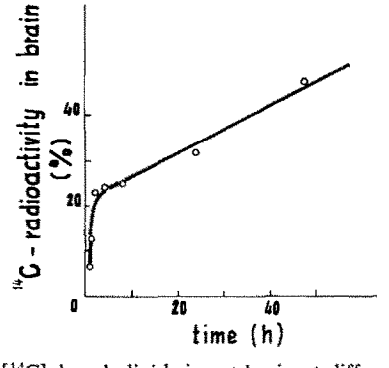


Fig. 10. [^{14}C]phospholipids in rat brain at different times from the injection of double-labelled CDPcholine. The phospholipids were extracted from brain samples, purified as described under Materials and Methods and counted for radioactivity. The values are expressed as percentage of total ^{14}C-radioactivity detected in the brain at the same time intervals and represent the average obtained from three rats.

The labelled molecular species detectable in the kidney are reported in Table 1. As observed in the other examined organs a gradual incorporation of ^{3}H-radioactivity into nucleic acids, via cytidine nucleotides, is observable. The ^{14}C-radioactivity is initially mainly associated with betaine, while the major ^{14}C-labelled species at 48 hr are the choline-phospholipids. The amounts of [^{14}C]choline detectable in this organ keep constantly higher than those of [^{14}C]Pcholine.

DISCUSSION

The study on the transport mechanism of CDPcholine through biological membranes has been carried out using intact human erythrocytes and isolated and perfused rat liver as model systems. Isolated and perfused liver, which duplicates many of the roles played by this organ *in vivo*, was selected because of its key function in lipid metabolism. On the other hand erythrocytes represent a useful tool to investigate CDPcholine transport, in that no significant lipid turnover is operative in these cells.

The reported results suggest that, while the cytidine nucleotide coenzyme is not taken up by the red blood cells, the uptake of the molecule by perfused rat liver is associated with its enzymatic cleavage, probably carried out by a membrane-bound pyrophosphatase activity. The only molecular species present in the perfusate even after 10 min perfusion are [5-^{3}H]CMP and methyl-[^{14}C]Pcholine. Furthermore the analysis of the labelled molecular species in the liver after 1 hr perfusion with double-labelled CDPcholine indicates that only single-labelled metabolites are present while no traces of double-labelled molecules are detectable.

The reported experiments *in vivo* support the view that an enzymatic cleavage of the molecule occurs before its uptake and utilization by the various organs: only CDPcholine metabolic products are detectable in the examined tissues even at very short times after the administration.

Both cytidine and choline moieties of exogenously administered CDPcholine undergo an active metabolic utilization, as suggested by the slow rate of excretion of labelled catabolites. In this respect, a preferential uptake of the choline moiety by the liver and kidney is detectable, while the brain preferentially utilizes the cytidine fragment. The observed dose dependence of CDPcholine utilization suggests that the putative receptorial biological systems are probably not saturated up to 10 mg/kg body wt, which represents the commonly employed therapeutic dose.

It is worth noting that less than 10% of injected radioactivity is detectable in the blood stream 2 min after the administration. On the other hand the blood is not endowed *per se* with any significant hydrolytic activity towards CDPcholine (data not shown), and the erythrocyte membrane is not permeable to the molecule, therefore an interaction of CDPcholine with endothelial and/or reticulum endothelial systems is predictable.

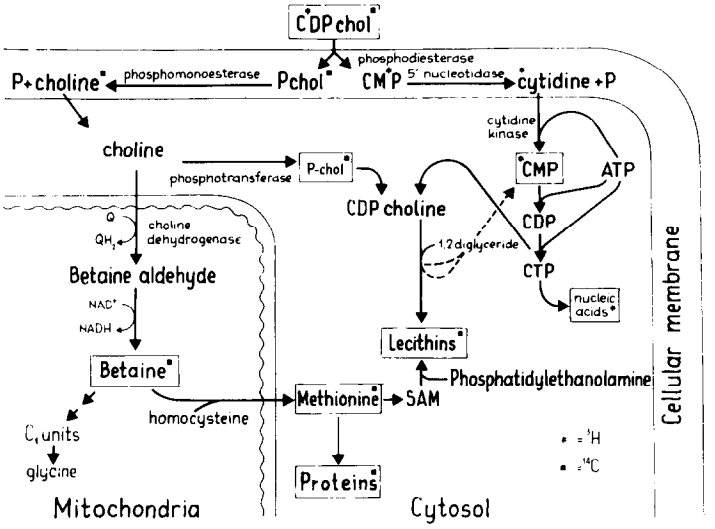


Fig. 11. Metabolic fate of exogenously administered CDPcholine in hepatocytes.

Among the examined organs, liver appears to be the most active in CDPcholine utilization; the hepatic ^{14}C-radioactivity increases rapidly up to 1 hr after the treatment and then slowly decreases. Therefore the liver can be envisioned as the major CDPcholine metabolizing compartment, releasing choline metabolites into the bloodstream. The pattern of the radioactive compounds, detectable in the liver 48 hr after the injection of double-labelled CDPcholine (Table 1), can be explained in terms of the metabolic pathway proposed in Fig. 11. The cleavage of the molecule at membrane level, yielding Pcholine and CMP, is followed by the uptake of choline and cytidine fragments. The cytidine, salvaged in the pool of cytidylic nucleotides, can be subsequently converted into nucleic acids. Moreover the choline moiety is in part rephosphorylated to Pcholine, which through the Kennedy's pathway, is converted into cholinephospholipids, via CDPcholine. The absence of measurable labelled newly synthesized CDPcholine is in agreement with the reported data on CTP:choline phosphate cytidylyl-transferase as the limiting step for the choline-phospholipids biosynthesis [7, 31, 32]. An alternative pathway for choline moiety is operative at mitochondrial level. The choline alcoholic-group is oxidized to betaine aldehyde, in turn dehydrogenated to betaine, which acts as methyl donor to homocysteine yielding methionine, the latter can either be incorporated into proteins or act as *S*-adenosylmethionine (AdoMet) precursor. The remaining dimethylglycine is converted to glycine releasing two molecules of formaldehyde by oxidation of the two methyl-groups. Therefore one out of the three methyl-groups of choline is directly recovered as methionine, while the other two methyls re-enter the one-carbon pool as 5–10-methylene-4H folate via formaldehyde. The time-dependent increase in the ^{14}C-labelling of phospholipids is indicative of a salvage of choline methyl-groups, via CDPcholine and/or AdoMet. In conclusion this metabolic pathway accounts for the ^{14}C-radioactivity associated with both proteins and phospholipids, as well as for the ^{3}H-labelling of nucleic acids.

The different pattern of radioactivity associated with [^{14}C]choline and [^{14}C]Pcholine in liver and kidney suggests that in the latter the rate of choline uptake is higher than that of the phosphorylation process, while in the liver the uptake represents probably the rate-limiting step. The faster ^{14}C-incorporation into phospholipids in kidney, when compared to the liver, could be ascribed either to a higher uptake rate of choline by this organ or to smaller endogenous pool of choline compounds in the kidney.

The pattern of labelled metabolites present in brain is indicative of a rapid salvage of choline moiety into phospholipid fraction and of the cytosine fragment in the cytidine nucleotides pool and nucleic acids. Furthermore it is worth noting the finding of methionine as the major ^{14}C-labelled water-soluble metabolite detectable at 48 hr. This rather extensive amount of radioactive methionine can only be explained by the uptake, from the bloodstream, of methionine as such and/or of methionine precursors like folate derivatives. The brain in fact lacks betaine-homocysteine methyl-transferase [33], which transfers a methyl-group from betaine to homocysteine, yielding methionine and dimethylglycine.

The incorporation in the brain of choline moiety in phospholipids is consistent with the suggested role of the molecule in restoring the structural integrity of membranes impaired by several pathologies of the Central Nervous System. However, on the basis of the wide metabolic utilization of the exogenously administered CDPcholine, a pharmacological action of the molecule not merely related to the phospholipids biosynthesis cannot be excluded.

REFERENCES

1. W. G. Mc Murray and W. L. Magee, *Ann. Rev. Biochem.* **41**, 129 (1972).
2. E. P. Kennedy, *Fed. Proc.* **16**, 847 (1957).
3. E. P. Kennedy and S. B. Weiss, *J. biol. Chem.* **222**, 193 (1956).

4. E. P. Kennedy, L. F. Borkenhagen and S. W. Smith, *J. biol. Chem.* **234**, 1998 (1959).
5. F. G. Wilgram, C. F. Holoway and E. P. Kennedy, *J. biol. Chem.* **235**, 37 (1960).
6. J. Y. Kiyasu and E. P. Kennedy, *J. biol. Chem.* **235**, 2590 (1960).
7. D. E. Vance and P. C. Choy, *Trends Biochem. Sci.* **4**, 145 (1979).
8. J. Pecker, B. Askar, A. Leclech and M. Robin, *L'Ouest Medical* **33**, 1075 (1980).
9. F. Cohadon, M. Rigoulet, B. Guerin and M. Vanderdriessche, *Nouv. Presse* **8**, 1589 (1979).
10. F. Boismare, M. Le Poncin, J. Lefrancois and J. C. Lecodier, *Pharmacol.* **17**, 15 (1978).
11. M. Fioravanti, A. E. Buckley, A. Agnoli, G. Nappi, A. Arrigo and F. Gerstenbrand, in *Cerebral Pathology in Old Age* (Eds. A. Cecchini, G. Nappi, A. Arrigo), p. 343. EmiRas, Pavia (1983).
12. T. Matsuoka, M. Kawanaka and K. Nagai, *Endocrinol. Japan.* **25**, 55 (1978).
13. P. Saba, G. Forli, F. Salvadorini, F. Galeone and G. Tunisi, *J. Neurosurg. Sci.* **21**, 239 (1977).
14. P. Saba, F. Salvadorini, F. Galeone, G. Tognetti and G. C. Saba, *Clinica Terapeutica* **74**, 425 (1975).
15. K. Shimamoto, T. Hirano and Y. Aramaki, *J. Takeda Res. Lab.* **34**, 440 (1975).
16. M. Alberghina, M. Viola, I. Serra, A. Mistretta and A. M. Giuffrida, *J. Neurosci. Res.* **6**, 421 (1981).
17. M. Rigoulet, B. Guerin, F. Cohadon and M. Vandendreissche, *J. Neurochem.* **32**, 535 (1979).
18. G. Benzi, E. Arrigoni, O. Pastoris, R. F. Villa, M. Dossena, A. Agnoli and A. M. Giuffrida, *J. Cereb. Blood Flow Metabol.* **2**, 229 (1982).
19. M. Martinet, P. Fonlupt and H. Pacheco, *Experientia* **34**, 1197 (1978).
20. M. Martinet, P. Fonlupt and H. Pacheco, *Biochem. Pharmac.* **30**, 539 (1981).
21. I. Serra, M. Alberghina, M. Viola and A. M. Giuffrida, *Neurochem. Res.* **6**, 595 (1981).
22. I. Serra, M. Alberghina, M. Viola, A. Mistretta and A. M. Giuffrida, *Neurochem. Res.* **6**, 607 (1981).
23. L. A. Horrocks, R. V. Dorman, Z. Dabrowiecki, G. Goracci and G. Porcellati, *Prog. Lipid Res.* **20**, 531 (1981).
24. G. Trovarelli, G. E. De Medio, R. V. Dorman, G. L. Piccinin, L. A. Horrocks and G. Porcellati, *Neurochem. Res.* **6**, 821 (1981).
25. V. Zappia, G. Romeo, G. Cocchiaria and M. De Rosa, *12th World Congress of Neurology*, Kyoto, Japan, abs. 1200 (1981).
26. H. G. Khorana, *J. am. chem. Soc.* **76**, 3617 (1954).
27. G. Romeo, G. Cocchiaria, M. De Rosa, F. Giordano and V. Zappia, *Boll. Soc. It. Biol. Sper.* **62**, 1175 (1981).
28. L. L. Miller, C. G. Blay, M. L. Watson and W. F. Bale, *J. exp. Med.* **94**, 431 (1951).
29. V. Zappia, F. Della Ragione, G. Cacciapuoti, M. Porcelli, P. Galletti and M. Cartenì-Farina, in *Biochemistry of S-Adenosylmethionine and Related Compounds* (Eds. E. Usdin, R. T. Bochardt and C. R. Creveling), p. 701. Macmillan, New York (1982).
30. M. Cartenì-Farina, F. Della Ragione, G. Cacciapuoti, M. Porcelli and V. Zappia, *Biochim. biophys. Acta* **727**, 221 (1983).
31. R. Sundler, G. Arvidson and B. Akesson, *Biochim. biophys. Acta* **280**, 559 (1972).
32. D. E. Vance, E. M. Trip and H. B. Paddon, *J. biol. Chem.* **255**, 1064 (1980).
33. J. D. Finkelstein, in *Transmethylation* (Eds. E. Usdin, R. T. Borchardt and C. R. Creveling), p. 49. Elsevier/North Holland, Amsterdam (1979).
34. H. B. Waynforth, *Experimental and Surgical Technique in Rats*. Academic Press, London (1980).