The Use of Non-Aqueous Chloroform/Methanol Extraction for the Delipidation of Brain with Minimal Loss of Enzyme Activities

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When freeze-dried brain was extracted at $-4-0^{\circ}\text{C}$ with dry chloroform/methanol (2:1, v/v), four of the five enzymes examined were recovered in the diethyl ether-washed residue without inactivation. By contrast, extraction with chloroform/methanol (2:1, v/v) in the presence of water destroyed activities of all the enzymes examined. The amounts of major lipids extracted were similar whether extraction was done in the absence or presence of water. The study was carried out with special interest in 2':3'-cyclic nucleotide 3'-phosphodiesterase (EC 3.1.4.37), which is firmly bound to the membrane structures of brain white matter.

In studies of membrane-bound enzymes, removal of lipid frequently affords an important clue. The most effective method for lipid extraction is that of Folch *et al.* (1951, 1957), using a mixture of chloroform and methanol, which is assumed to be a strong denaturant of proteins. If wet brain was extracted with chloroform/methanol (2:1, v/v), the activity of 2':3'-cyclic nucleotide 3'-phosphodiesterase (EC 3.1.4.37) was completely lost. However, it has been found that enzymes which are denatured by organic solvents added to the aqueous phase are frequently quite stable when exposed to dry organic solvents (Morton, 1955). In fact, if freeze-dried brain was extracted with chloroform/methanol (2:1, v/v) non-aqueously, 2':3'-cyclic nucleotide 3'-phosphodiesterase was recovered in the diethyl ether-washed residue without inactivation. Four of the five enzymes examined survived non-aqueous chloroform/methanol extraction and removal of major lipids seemed as complete as that achieved by the usual aqueous chloroform/methanol extraction.

Methods

Preparation of delipidated residues

Fresh bovine cerebral white matter (centrum semiovale) or grey matter (cortex) was homogenized at 0°C with 2 vol. of water in a Waring Blendor, freeze-dried completely and dried thoroughly at 4°C in a vacuum desiccator over silica gel. Chloroform, methanol and diethyl ether, all analytical reagents, were redistilled freshly before use.

Non-aqueous chloroform/methanol extraction. Freeze-dried white or grey matter (usually 0.6 or 0.3 g) was homogenized at the indicated temperature

with chloroform/methanol (2:1, v/v) in the proportion of 20 ml/0.3 g and left for a few minutes at that temperature. A Potter-Elvehjem-type homogenizer with a Teflon pestle was used for this and later homogenization; in large-scale preparations a Waring Blendor was more suitable. Care was taken to avoid any contamination by water. The residue was collected by centrifugation for 10 min at 15000g and -4°C in a polyethylene centrifuge tube. The supernatant fluid was decanted and used for lipid analysis. The residue obtained as the precipitate was immediately washed with diethyl ether at 0°C by homogenization as above and centrifugation (15000g for 5 min); the volume of diethyl ether used was about the same as that of the chloroform/methanol (2:1, v/v). The residue was then pressed between filter papers to remove diethyl ether as far as possible, suspended in water at 0°C by homogenization as above and finally freeze-dried. The enzyme activities in the freeze-dried residue were stable for at least 6 months when it was stored at -20°C in a sealed plastic bottle.

Aqueous chloroform/methanol extraction. Freeze-dried white or grey matter (0.3 g) was homogenized at the indicated temperature with 20 ml of chloroform/methanol (2:1, v/v) containing 5% (v/v) water. Alternatively, 0.3 g of freeze-dried white matter was homogenized with 2 ml of water at 0°C and then with 38 ml of chloroform/methanol (2:1, v/v) at the indicated temperature. The residue was collected by centrifugation at 15000g for 10 min at -4°C and the supernatant fluid was used for lipid analysis; if the residue did not precipitate, more chloroform/methanol (2:1, v/v) was added. The residue was washed with diethyl ether, suspended in water and

Fig. 1. Effects of non-aqueous ($\circ\Delta\nabla\Box\Diamond$) and aqueous ($\bullet\blacktriangle\blacksquare\blacklozenge$) chloroform/methanol extraction on enzyme activities in bovine cerebral white (a) and grey (b) matter

The extracted residues were prepared as described in the Methods section. Extraction was done at the indicated temperature. Enzyme activities and protein content were calculated on a dry-weight basis. Relative enzyme activities are defined as enzyme activities of the residue divided by those of the original white or grey matter, and relative protein content defined as protein content of the residue divided by that of the original white or grey matter. Enzyme activities of the original white and grey matter were as follows: 2':3'-cyclic nucleotide 3'-phosphodiesterase, 6.20 (white) and 1.94 (grey) mmol/min per g; acid proteinase, 1.11 (white) and 5.56 (grey) μ mol/min per g; catechol *O*-methyltransferase, 7.90 (white) and 18.4 (grey) nmol/min per g; acetylcholinesterase, 1.82 (white) and 13.9 (grey) μ mol/min per g; monoamine oxidase, 0.486 (grey) μ mol/min per g. Protein content of the original white and grey matter was 0.27 and 0.49 g/g respectively. Each point represents the mean of duplicate determinations for a single extraction. $\circ, \bullet, \bigcirc, \blacklozenge$, 2':3'-Cyclic nucleotide 3'-phosphodiesterase or protein; $\Delta, \blacktriangle, \triangle, \blacklozenge$, acid proteinase; $\nabla, \blacktriangledown, \triangledown, \blacklozenge$, catechol *O*-methyltransferase; $\Box, \blacksquare, \boxtimes, \blacklozenge$, acetylcholinesterase; $\diamond, \blacklozenge, \blacklozenge, \blacklozenge$, monoamine oxidase; $\bigcirc, \blacktriangle, \triangledown$, non-aqueous chloroform/methanol extraction without washing with ether.

freeze-dried, as described for the non-aqueous chloroform/methanol extraction.

Analytical methods

Enzyme assays. Freeze-dried powders of the delipidated residues and of the original white or grey matter were homogenized at 0°C in a Potter-Elvehjem-type homogenizer with the following media: 0.2M-sodium acetate buffer, pH 3.8, for acid proteinase; water for catechol *O*-methyltransferase (EC 2.1.1.6) and monoamine oxidase (EC 1.4.3.4); 0.1M-sodium phosphate buffer, pH 8.0, for acetylcholinesterase (EC 3.1.1.7); and 20mM-imidazole/HCl buffer, pH 6.6 at 30°C, for 2':3'-cyclic nucleotide 3'-phosphodiesterase.

Acid proteinase was assayed by the method of Anson (1938) at pH 3.8 (Marks & Lajtha, 1963) and 37°C; the substrate solution, containing 2% (w/v) bovine haemoglobin in 0.06M-HCl, had been incubated for 1 h at 37°C and the pH adjusted to 3.8 with 2M-NaOH. Catechol *O*-methyltransferase was assayed by the method of McCaman (1965) as described by Jarrott (1974); *S*-adenosyl-L-[methyl-¹⁴C]meth-

ionine (sp. radioactivity 0.5mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) was used and the assay temperature was 37°C. Acetylcholinesterase was assayed by the method of Ellman *et al.* (1961), and monoamine oxidase by the method of Kraml (1965), except that HClO₄ was used in place of trichloroacetic acid.

2':3'-Cyclic nucleotide 3'-phosphodiesterase activity was determined by the following spectrophotometric method. Both sample and reference cells in a Hitachi 323 recording spectrophotometer contained a mixture (2ml) of Bromothymol Blue (0.12mM), hexadecyltrimethylammonium bromide (0.2%, w/v), imidazole/HCl buffer (10mM, pH 6.5 at 30°C), 2-mercaptoethanol (0.5mM) and potassium adenosine 2':3'-cyclic phosphate (7.5mM). The final pH of the mixture was 6.6 at 30°C. The mixture had been equilibrated at 30°C and was maintained at 30°C in the spectrophotometer by a thermostatically controlled cell-holder. Homogenates of the freeze-dried powders (6mg/ml) in 20mM-imidazole/HCl buffer, pH 6.6, were mixed with equal volumes of 0.4% (w/v) hexadecyltrimethylammonium bromide

Fig. 2. Amounts of lipids extracted with non-aqueous (○△□) and aqueous (●▲■) chloroform/methanol from bovine cerebral white (a) and grey (b) matter

Extraction was done at the indicated temperature. Each point represents the mean of duplicate determinations for a single extraction. ○, ●, Total phospholipid; △, ▲, cholesterol; □, ■, total galactolipid.

at about 25°C and used within 1 h as enzyme solutions. The reaction was started by the addition of an enzyme solution (5–80 μ l) to the mixture in the sample cell and the A_{420} was automatically recorded with appropriate scale expansion. An A_{420} change of 0.100 corresponded to the hydrolysis of 1.13 μ mol of the substrate. This method has been developed from the previous potentiometric method (Kurihara & Takahashi, 1973) and is based on the use of an acid–base indicator and a buffer having identical pK_a values (Darrow & Colowick, 1962).

Total protein. Total protein was determined by the method of Lowry *et al.* (1951), after the samples were solubilized in 4% (w/v) sodium dodecyl sulphate. Bovine albumin (fraction V; Sigma Chemical Co., St. Louis, MO, U.S.A.) was used as a standard.

Lipids. Non-aqueous chloroform/methanol ex-

tracts were shaken with 0.25 vol. of 0.1 M-NaCl and aq.-chloroform/methanol extracts with 0.2 vol. of 0.125 M-NaCl. The upper phase was removed and the surface of the lower phase was washed three times with a small volume of chloroform/methanol/0.05 M-NaCl (3:48:47, by vol.). The lower phase was taken to dryness on a rotary evaporator. The solid was dissolved in chloroform/methanol (2:1, v/v) and again taken to dryness. This process was repeated four times. The solid was finally dissolved in chloroform/methanol (2:1, v/v), filtered and adjusted to an appropriate volume with chloroform/methanol (2:1, v/v) for analysis. The lipids were analysed for total lipid phosphorus (Bartlett, 1959), cholesterol (Zak, 1957) and total lipid galactose (Svennerholm, 1956). Phosphorus was determined after ashing with 60% (v/v) HClO_4 . The values for phosphorus and galactose were multiplied by 25 and 4.7

Fig. 3. Effect of the proportion of chloroform/methanol to freeze-dried white matter in non-aqueous chloroform/methanol extraction

The results were obtained by extraction at 0°C. Each point represents the mean of duplicate determinations for a single extraction. (a) Curve A, relative 2':3'-cyclic nucleotide 3'-phosphodiesterase activity; curve B, relative protein content; for the definitions see the legend to Fig. 1. (b) $\circ$, Total phospholipid; Δ , cholesterol; $\square$, total galactolipid.

respectively to obtain the weights of total phospholipid and total galactolipid.

Results

Fig. 1 shows the effects of non-aqueous and aqueous chloroform/methanol extraction on enzyme activities in bovine brain. When freeze-dried brain was extracted with chloroform/methanol (2:1, v/v) non-aqueously at $-4-0^{\circ}\text{C}$, 2':3'-cyclic nucleotide 3'-phosphodiesterase, acid proteinase, catechol *O*-methyltransferase and acetylcholinesterase were recovered in the ether-washed residue without inactivation. Most of the monoamine oxidase activity was, however, lost (Fig. 1*b*). Activities of the former four enzymes expressed on a dry-weight basis increased 3.0–3.4- and 1.3–1.4-fold for the white and grey matter respectively, and protein content increased 2.7- and 1.4-fold respectively. These increases corresponded roughly to the removal of lipids. For the white matter, the increases in enzyme activities were greater than the increase in protein content. Presumably this may be ascribed at least partly to the removal of proteolipid protein from the white matter.

In contrast with non-aqueous chloroform/methanol extraction, when freeze-dried brain was rehydrated before extraction or extracted with chloroform/methanol (2:1, v/v) containing 5% (v/v) water, activities of all the enzymes examined were destroyed (Fig. 1). Even in non-aqueous chloroform/methanol extraction, if washing with diethyl ether was omitted, enzyme activities in the residue were markedly decreased (Fig. 1*a*). This is obviously due to inactivation; enzymes must have been inactivated by the action of the chloroform/methanol remaining in the residue when the residue was suspended in water. Chloroform/methanol mixture is thus shown to cause severe denaturation of enzymes in the presence of water. The possibility that aq.-chloroform/methanol extraction had removed enzymes from the residue seems to be excluded.

Fig. 2 shows the amounts of major lipids extracted with non-aqueous and aqueous chloroform/methanol from bovine brain. The amounts of total phospholipid, cholesterol and total galactolipid were similar with both extraction procedures. Interpretation of the data should take into account that the aqueous extraction usually used more solvent (including chloroform/methanol added to precipitate the residue) than the non-aqueous extraction. The amounts of the extracted lipids were not affected by varying the temperature in the range between -4° and 20°C .

Fig. 3 shows the effect of the proportion of chloroform/methanol to freeze-dried white matter in the non-aqueous extraction. The amounts of major lipids

extracted were near, but not at, the maximum at 20 ml/0.3 g; 2':3'-cyclic nucleotide 3'-phosphodiesterase activity and protein content clearly increased further when the proportion was increased to 40 ml/0.3 g.

Discussion

The results indicate that chloroform/methanol mixture acts as a strong denaturant only in the presence of water. Non-aqueous chloroform/methanol extraction can be used for the delipidation of brain with minimal loss of enzyme activities. However, after the non-aqueous extraction the remaining chloroform/methanol must be removed completely from the residue, e.g. by washing with diethyl ether as described here, otherwise enzymes will be inactivated when the residue subsequently comes in contact with aqueous media.

Radin (1969) suggested that the extraction of highly polar lipids would be incomplete with chloroform/methanol used in the absence of water. However, so far as total phospholipid, cholesterol and total galactolipid are concerned, the extraction with non-aqueous chloroform/methanol seems as complete as that achieved by the extraction with chloroform/methanol in the presence of 5% (v/v) water. At least, no great difference was found in the amounts of these lipids extracted.

2':3'-Cyclic nucleotide 3'-phosphodiesterase is firmly bound to the membrane structures of brain white matter (Drummond *et al.*, 1962; Kurihara & Tsukada, 1967), and has so far resisted purification, though some progress has been made (Guha & Moore, 1975). Neither phospholipase C (EC 3.1.4.3) treatment (T. Kurihara, unpublished work) nor non-aqueous chloroform/methanol extraction affected 2':3'-cyclic nucleotide 3'-phosphodiesterase activity, suggesting that the enzyme requires no lipid for its activity. The enzyme activity of the residue is over three times that of the original white matter on a dry-weight basis, and the fact that the residue is free of lipid will facilitate greatly the solubilization and purification of the enzyme from the residue. Similar applications of non-aqueous chloroform/methanol extraction may be possible for membrane-bound enzymes that do not require lipid for their activities. It is uncertain, however, whether non-aqueous chloroform/methanol extraction would be of any use for lipid-requiring enzymes. According to Noguchi & Freed (1971), the activity of ($\text{Na}^+ + \text{K}^+$)-stimulated adenosine triphosphatase (EC 3.6.1.3) that has been lost by extraction with chloroform/methanol (1:1, v/v) at -75°C can be restored by recombining the residue and the filtrate in chloroform/methanol (1:1, v/v) at -75°C . These authors used a powder of wet brain prepared under liquid N_2 , and it is not clear how brain-derived water

contributed to their system. In a dry state, ($\text{Na}^+ + \text{K}^+$)-stimulated adenosine triphosphatase would be more stable to chloroform/methanol and similar reconstitution might be possible at higher temperature.

It is well known that prior chloroform/methanol treatment of central nervous tissue prevents the breakdown of encephalitogenic basic protein during later acid extraction (Kies, 1965). This effect has been explained by inactivation of acid proteinase present in central nervous tissue, and the observation of Carnegie *et al.* (1967) that acid proteinase activity was lowered after non-aqueous chloroform/methanol extraction has been regarded as evidence for the explanation. In our hands, however, acid proteinase survived the non-aqueous extraction, although it was inactivated completely or almost completely by the aqueous extraction. In the experiments of Carnegie *et al.* (1967), acid proteinase activity may have been lowered by the action of the remaining chloroform/methanol when the residue was homogenized with 0.02M-HCl.

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