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Chloral Hydrate: A Solvent for Biological Membranes

Abstract. *A buffer system containing chloral hydrate, taurine, and bromopyridinium lactate was used to dissolve several biological membranes and separate their protein components by polyacrylamide gel electrophoresis. This solvent system was capable of separating molecules of similar size on the basis of their charge and allows easy recovery of the proteins. Thus, aqueous chloral hydrate is an effective solvent for biological membranes.*

A major difficulty in studying biological membranes is the lack of solvent systems which permit complete disaggregation of membrane components and allow their recovery with biological activity. Present methods used to disaggregate the membranes involve sonication, detergents, and organic solvents (*1*), and they have not proved entirely satisfactory either because of incomplete dissolution or because when the components were totally dissolved it has been difficult to recover the dissolved components in a functional state. Buffer systems that contain sodium dodecyl sulfate (SDS) have been successfully used for completely dissolving membranes and subsequently separating the protein components by electrophoresis. However, in these systems complexing with SDS masks the net ionic charge of the native molecule, and separation on the basis of native ionic charge becomes difficult (*2*).

To study genetic variations in mammalian cell membranes we required a technique that can be used to dissolve membranes completely and also separate the components on the basis of their ionic charges because allelic variants of any kind would most often differ by amino acid substitutions and thus would not be separable on the basis of size. We here present a new method for the solubilization of membranes, which permits separation of the components on the basis of charge as well as size. In addition, in one complex system tested, biological activity of proteins was retained after the proteins were exposed to the solubilizing buffer.

Chloral hydrate (Mallinckrodt, U.S.P.) was recrystallized from chloroform, dried in a vacuum, and stored at −20°C. This step removes contaminating formic acid resulting from decomposition of chloral hydrate. Solubilizing buffer was prepared by dissolving 100 g of chloral hydrate in 33.3 ml of 0.6*M* taurine (2-aminoethanesulfonic acid), adding 0.58 ml of 3-bromopyridine (K & K Laboratories, Plainview, New York), and adjusting to *p*H 3.0 and a final volume of 100 ml by alternate addition of 85 percent lactic acid and water at room temperature. The membranes were dissolved in the solubilizing buffer as follows. A solution of 2-mercaptoethanol in water (40 percent by volume) was adjusted to *p*H 8.2 with 5*N* NaOH. Membranes were suspended at a concentration of 20 to 40 mg of protein per milliliter in dilute neutral buffer (or distilled water); seven parts of this suspension were mixed with one part of the mercaptoethanol solution (*p*H > 8), allowed to stand at room temperature for 5 to 10 minutes, and then dissolved in the solubilizing buffer to give a protein concentration of 1 mg/ml. The solid recrystallized chloral hydrate, 2.5 g per milliliter of the membrane-mercaptoethanol mixture, was added to give a final concentration of 1 g/ml. Neutral red (0.1 mg/ml) was added as a tracking dye, and 80 mg of dry Bio-Gel P6 (Bio-Rad, Richmond, California) was added per milliliter of solution to form a slurry.

Acrylamide gel buffer was prepared by the method described for the solubilizing buffer, except that the *p*H was adjusted to 2.8. Acrylamide (5 g) and 0.5 g of methylenebisacrylamide (Bio-Rad) were then dissolved in this buffer to a final volume of 100 ml. Gels were cast in nonsiliconized tubes (5 by 125 mm) with tight-fitting siliconized glass inserts to shape the gel (*3*). The tubes, sealed at the bottom with plastic holders (*3*), were flushed with nitrogen, the inserts were dropped to the bottom, and the tubes were again flushed with nitrogen and stoppered until ready for use.

The acrylamide was polymerized, with the use of a mixture of hydrogen peroxide and 1,1,3,3-tetramethyl-2-thiourea (Aldrich, Milwaukee, Wisconsin) (TMT) as catalyst. Because polymerization was very rapid under the conditions used, the gels were cast within 1 minute after the acrylamide solution was mixed with the catalysts. Oxygen was excluded from the system because it interferes with polymerization. For the preparation of five gels, 15 ml of the acrylamide solution in the gel buffer (*p*H 2.8) was gently degassed until bubbling began, and was then brought to atmospheric pressure with nitrogen. The process was repeated twice more, and then 6 ml was withdrawn into a 10-ml glass syringe that had been rinsed with the degassed acrylamide solution; 60 μl of 10 percent TMT in chloroform was added to the remaining 9 ml. A portion (6 ml) of the solution containing TMT was withdrawn into a second syringe which was rinsed as described above. Each solution was degassed by withdrawing the syringe plunger sharply while the syringe outlet was kept closed; the bubbles were then expelled. One-tenth milliliter of 6 percent hydrogen peroxide (freshly prepared) was added via a female-female Luer adapter to the first syringe; and the contents of the two syringes, now connected in the two syringes, were mixed by vigorous pumping back and forth. This mixture was quickly added to the gel tubes, which were then capped and kept at room temperature for 2½ hours to ensure complete polymerization of the gels. The inserts and any loose gel trapped by the inserts were then removed. At the time of electrophoresis, the gels were inverted and the sample was placed at the end originally con-

taining the insert. The other end of the gel column was secured against slippage by a cheesecloth square held by a silicon rubber band.

Electrophoresis was carried out with a conventional gel assembly. The electrode buffer consisted of 0.2*M* taurine and 0.06*M* 3-bromopyridine adjusted to *p*H 3.0 with lactic acid. The solubilized membrane–Bio-Gel P-6 slurry (20 to 200 μl) was then added to each tube.

To prevent the ends of the gels from swelling, the gels containing the chloral hydrate were isolated from the aqueous electrode buffer system by interposing a stable layer of solubilizing buffer containing chloral hydrate. The upper electrode chamber, which was made of Lexan polycarbonate (chloral hydrate dissolves Plexiglas, rubber, and many kinds of plastics), and the gel tubes were then connected by silicone rubber grommets (short segments of Silastic medical grade tubing; Dow Corning, Midland, Michigan). The samples in the gel tubes were overlayered with a slurry of Bio-Gel P-6 (8 g/100 ml) in a mixture of nine parts of solubilizing buffer and one part of electrode buffer. The same slurry was added to the upper tray to a height of 2 to 3 cm; the tray was then filled with electrode buffer, which was layered over the slurry. The lower ends of the gel were also separated from the electrode buffer by the same slurry used in the upper tray.

Electrophoresis was carried out at 0.5 ma per gel (110-mm gels) for 12 to 18 hours, depending on the sample, with a constant current power supply. During this period the power supply voltage increased from 400 to 500 volts to about 1000 volts. Gels were fixed and stained for 20 minutes at 70°C in 0.2 percent Coomassie blue in a mixture of ethanol, water, and acetic acid (9 : 9 : 2), and then destained for two 20-minute periods in the same solvent. Further destaining was done with

Table 1. Reconstitution of ribosomes after incubation of protein components in chloral hydrate. The effect of chloral hydrate on the biological activity of ribosomal proteins was studied by the method described (*8*) for the dissociation and reassociation of *E. coli* ribosomes into functional units. Individually purified 30*S* ribosomal proteins were used to prepare a total protein reconstitution mixture (TP 30) in buffer V (*8*). The TP 30 was diluted tenfold with solubilizing buffer and incubated for 24 hours either at room temperature or at 0°C. As a control a sample of ribosomal proteins was simultaneously incubated at room temperature in buffer V (room temperature control); the remainder was again frozen (TP 30 control). At the end of the incubation period all samples, except the TP 30 control, were dialyzed at 4°C first against buffer V (three changes, 2 hours each), next against buffer III [6*M* urea, 10 m*M* phosphoric acid (*p*H adjusted to 8.0 with methylamine), 3 m*M* 2-mercaptoethanol (½ hour)] and finally against buffer V (2 hours). All samples, including the TP 30 control, were then reconstituted with 16*S* RNA and assayed for activity in polyuridine-directed synthesis of polyphenylalanine (*8*) (reconstituted 30*S* ribosomes have a higher activity in this assay than standard nonreconstituted 30*S* ribosomes). Results are expressed as counts per minute of [^{3}H]phenylalanine incorporated into material precipitable by trichloroacetic acid per absorbancy unit of RNA at 260 nm. Standard 30*S* ribosomes were assayed at the same time. The percentage of activity was calculated after subtraction of background contributed by 50*S* ribosomes from the value obtained with each assay.

Sample	[^{3}H]Phenylalanine incorporated (count/min)	Activity (%)
Standard 30*S* ribosomes	24,467 24,412	88
TP 30 control	27,085 28,310	100
TP 30 incubated in solubilizing buffer at 0°C	25,587 26,007	93
TP 30 incubated in buffer V at ~25°C	15,953 16,135	56
TP 30 incubated in solubilizing buffer at ~25°C	10,308 11,099	35
50*S* control	1,594 1,231	

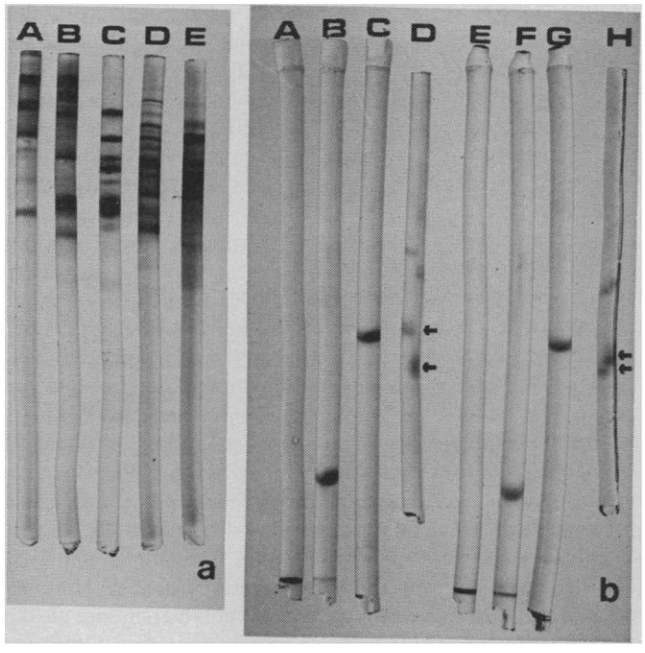

Fig. 1. Electrophoretic patterns in polyacrylamide gels. (a) Electrophoresis of membrane components in chloral hydrate gels. Mouse thymocyte plasma membranes were prepared by a modification of the method of Wallach and Kamat (*11*). Human erythrocyte stromata (*12*), spinach chloroplasts (*13*), beef heart mitochondria (*14*), and *Azotobacter vinlandii* phosphorylating particles (*15*) were prepared as described. The samples and the chloral hydrate gels were prepared as described in the text. Electrophoresis was carried out for 18 hours. Samples contained the specified amount of protein: (A) 20 μg of human erythrocyte stromata, (B) 50 μg of mouse thymocyte plasma membrane, (C) 200 μg of whole spinach chloroplasts, (D) 50 μg of beef heart mitochondria, and (E) 100 μg of *Azotobacter vinlandii* phosphorylating particles. (b) Separation on the basis of charge in chloral hydrate gels. Myeloma proteins MOPC 321 and MOPC 63, and the derivatives 5-carboxamidomethyl MOPC 321 and aminoethyl MOPC 321, were prepared as described (*5*). Electrophoresis in SDS gels (5 by 110 mm) was performed by the method of Lämlli (*16*). Chloral hydrate gel electrophoresis was carried out as described in the text. The split gel technique (*17*) was used for all gels: a glass partition (cut from No. 1 cover slips) was placed at the top of the gel, dividing it into two parts, and a sample was placed on each side. In all cases 3 μg of protein was placed on each side of the gels. For SDS gels, the ratio of acrylamide to methylenebisacrylamide was 75 : 1. Gels A, B, C, E, F, and G are SDS gels; gels D and H are chloral hydrate gels. (A) SDS, 7.5 percent acrylamide; left, carboxamidomethyl MOPC 321; right, aminoethyl MOPC 321; (B) samples as in gel A, 10 percent acrylamide; (C) samples as in A, 12 percent acrylamide; (D) chloral hydrate gel; left, carboxamidomethyl MOPC 321; right, aminoethyl MOPC 321; (E) SDS, 7.5 percent acrylamide; left, MOPC 321; right MOPC 63; (F) samples as in gel E, 10 percent acrylamide; (G) samples as in gel E, 12 percent acrylamide; and (H) chloral hydrate gel; left, MOPC 321; right, MOPC 63. Arrows indicate separated protein bands in gels D and H.

several portions of 10 percent acetic acid, first at 70°C and then at room temperature (*4*).

Typical separation patterns obtained with different biological membranes are shown in Fig. 1a. Very little, if any, stained material remains at the origin of these gels, an indication that the chloral hydrate system was disaggregating the biological membranes into their components to the extent that almost all the components entered the gel during electrophoresis. The buffer system allows separation of protein components into distinct bands. Banding patterns obtained with the same membrane preparations tested at different times were reproducible. The mobility of the lipid components has not been investigated.

The results shown in Fig. 1b demonstrate that in the chloral hydrate system separation can be obtained on the basis of charge as well as size. Light chains of mouse myeloma proteins of known sequences and having equal numbers of amino acid residues (*5*) were effectively separated on chloral hydrate gels, but not on SDS gels. Similarly, light chains from MOPC-321 proteins, either aminoethylated or carboxamidomethylated at five cysteine residues (*5*), were easily separated in the chloral hydrate system, whereas no detectable separation was achieved on SDS gels.

The possibility of modifications of proteins by chloral hydrate was investigated as follows. (i) A standard amino acid mixture was incubated in solubilizing buffer containing 1/100 volume of 2-mercaptoethanol, 1/200 volume of thiodiglycol, and 1/200 volume of dithiodiglycol for 18 hours at 25°C. No loss of any amino acid was observed on analysis in an amino acid analyzer (*6*). (ii) Ribonuclease A, chymotrypsinogen, and ovalbumin were incubated for 24 hours at 25°C in the solubilizing buffer plus 1/100 volume of 2-mercaptoethanol and then subjected to electrophoresis in urea–potassium acetate gels at *p*H 4.5 (*7*). No alteration in the electrophoretic pattern was observed as a result of incubation in chloral hydrate. (iii) Proteins extracted from the 30*S* ribosomal subunit of *Escherichia coli* (*8*) retained 93 percent of full biological activity after a 24-hour incubation period in solubilizing buffer at 0°C. Incubation in the same buffer at room temperature resulted in a loss of 65 percent of activity; however, incubation in a chloral hydrate–free buffer at room temperature also resulted in a marked loss of activity (Table 1).

Chloral hydrate is effective in disaggregating biological membranes at the high concentration used, but is not effective at lower concentrations tested. Whether lower concentrations may be adequate for dissolving selected proteins and protein aggregates is not known. The bromopyridinium lactate constitutes a double buffer system (*9*) that aids in achieving separation of the proteins into sharp bands. Several other buffer systems at the same *p*H (tris lactate, tris formate, tris phosphate, and bromopyridium formate) gave poorer resolution. The *p*H was chosen to be close to the *pK*'s of the two buffering counterions bromopyridinium ($pK_a = 2.8$) and lactate ($pK_a = 3.8$). Taurine, at the *p*H used, makes very little contribution to the conductivity; in some cases it appears to facilitate membrane dissolution. The *p*H difference between the acrylamide gel and sample provides some stacking effect. Thus up to 200 μl of sample can be used while retaining good resolution.

Many biochemical and biological studies depend on the availability of methods to analyze membrane components and isolate them in a biologically active state. The chloral hydrate system is a powerful solvent for biological membranes. Solubilization is sufficiently complete to allow all, or nearly all, protein components to enter a 5 percent polyacrylamide gel and separate into well-defined bands during electrophoresis. Membrane solubilization and electrophoretic separation on the basis of charge and size can be accomplished without prior removal of lipids and under mild conditions. Proteins can be recovered with biological activity in at least the one system tested (Table 1) after incubation with the chloral hydrate–containing buffer. Moreover, Houssainy, Zweidler, and Bloch (*10*), who introduced the use of chloral hydrate for isopycnic centrifugation of chromatin, reported no electrophoretically detectable changes in histones that had been exposed to chloral hydrate.

Buffer systems containing SDS, which are commonly used to solubilize biological membranes, allow separation of components essentially on the basis of size, while the chloral hydrate system allows charge-based separation. Whether the method described here will be useful in the development of two-dimensional gel electrophoresis for the complete analysis of membrane components remains to be seen. Our findings suggest that chloral hydrate is a powerful nonionic agent that should find wide application in many biochemical studies involving the disruption of tertiary structures of proteins and lipid-protein interactions.

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