EXTRACTION, HYDROLYSIS, AND ELECTROPHORETIC ANALYSIS OF RIBONUCLEIC ACID FROM MICROSCOPIC TISSUE UNITS (MICROPHORESIS)

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ABSTRACT

A procedure for the purine-pyrimidine analysis of RNA in the 100- to 1000 $\mu\mu$g. range is presented. It includes hydrolysis and electrophoretic analysis of RNA, which is extracted from single isolated tissue units, like single cells. The quantitative determination of the separated compounds is carried out by a photographic-photometric procedure in ultraviolet light. The determined values show a coefficient of variation of about ± 7 per cent on test substance. Microelectrophoretic analyses of RNA from different sources have been performed and are compared to macrochemical analyses. The agreement is good in those cases in which it is possible to get any information at all through macrochemical analyses.

For nucleotide analysis of RNA in the 100 to 1000 $\mu\mu$g. scale, electrophoresis is carried out in a microscopic cellulose fibre and the separated compounds are determined by a photographic-photometric procedure in ultraviolet light (10, 7). For use on single tissue units it is necessary that the method is supplemented by procedures for extraction and hydrolysis of RNA in the micro-scale. Extraction has been described earlier (10, 9, 11) but the procedure for the hydrolysis of RNA in amounts down to the 100 to 1000 $\mu\mu$g. range has not yet been published.

Since it was first presented, microelectrophoresis or microphoresis, has been considerably modified and simplified. A complete description of the procedure, including extraction, hydrolysis, electrophoresis, and analysis of biological material will therefore be given.

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MATERIALS AND METHODS

Cells or other tissue parts are isolated as follows: 1 to 2 mm. thick pieces of the tissue to be used are fixed in Carnoy's solution (ethanol, chloroform, and concentrated acetic acid, 6:3:1) for 30 to 90 minutes at room temperature, either fresh or after freeze-drying. The fixed tissue is embedded in solid paraffin after passing through absolute ethanol and benzene (90 minutes in each). Twenty-five to 100 μ thick sections are cut and spread on 12 × 32 × 0.17 mm. coverglasses. For microdissection the sections are deparaffinized (chloroform-diethylether-70 per cent ethanol, 5 minutes in each) and hydrated with a 0.01 N acetic acid solution. The dissections are made under liquid paraffin (*paraffinum liquidum pro injectione*, Merck 7162) in the oil chamber of de Fonbrune (4). de Fonbrune's micromanipulator is used for the work. Using two needles it is possible to isolate and clean cells or other tissue parts. The isolated units

are placed on a second coverglass under liquid paraffin, where they can be kept until the analyses are performed. The question of RNA preservation in the tissue during these preparatory steps has been investigated quantitatively (6, 8). It has been found that there is no significant loss of RNA.

RNA is extracted as described earlier for quantitative RNA determinations (10, 9) using ribonuclease in a solution containing volatile electrolytes. The composition of this fluid is 0.4 mg. crystalline, protease-free ribonuclease (Worthington Corp., Freehold, New Jersey) per ml. of 0.2 N ammoniumbicarbonate solution adjusted to pH 7.6 with acetic acid. With a micropipette each tissue unit is extracted successively with three volumes (0.1 to 1 mμ l.) of the extraction fluid for a total time of 60 minutes at 20°C. Extracts are evaporated to dryness on a coverglass and those from the same unit are added to each other. After an extract has been delivered, the pipette is used for taking a new volume of enzyme solution to the same tissue component. In this way the pipette is washed, and the washings are added to earlier extracts. Coverglasses supporting dried extracts are put in stoppered test tubes and kept in this way for later use.

For hydrolysis, the extracted and digested dry RNA substance is dissolved in 4 N HCl under liquid paraffin in the oil chamber. The solution is hydrolyzed in a micropipette of about 10 μ diameter at the tip. To prevent evaporation of the hydrochloric acid from the pipette, columns of liquid paraffin and hydrochloric acid are arranged above and below the hydrolysate as shown in Fig. 1. The pipette is immersed into a bath of liquid paraffin in a cylindrical vial. The paraffin is saturated with 4 N HCl at 100°C. and is kept in boiling water. Hydrolysis is allowed to go on for 30 minutes, after which the hydrolysate is ready for microphoretic analysis.

The standard procedure for the liberation of purines and pyrimidine nucleotides from RNA is to use 1 N HCl at 100°C. for 1 hour (13). Under such conditions accurate analyses can be performed, provided a correction is made for the formation of pyrimidine nucleosides from the corresponding nucleotides. During microphoresis the nucleosides migrate together with their nucleotides. As they have the same light absorption characteristics, the extent to which nucleosides are formed during the hydrolysis is of no importance. It was found that it was unsuitable to use 1 N HCl for microphoresis, because the extracted RNA is dissolved only slowly in acid of this strength. Therefore an investigation was made to see if it is possible to use 4 N HCl, which dissolves RNA extracts instantaneously. Chromatographic RNA analyses in the macroscale were made of hydrolysates of yeast RNA using 1 N HCl at 100°C. during 60 minutes and 4 N HCl at the same temperature for 30 minutes. As is apparent from Table I both methods give the same results, and consequently 4 N HCl can be used for quantitative work.

The electrophoretic separation and the determination of the RNA constituents (purines and pyrimidine nucleotides after hot acid hydrolysis) is performed as follows. RNA hydrolysates containing 300 to 400 μμg. of RNA are placed on a fibre of regenerated cellulose of 20 to 25 μ diameter, using a micropipette. The fibre is the single filament of rayon-silk, produced according to the cuprammonium method (*40 denien Cupresa HW Naturglanz ungedreht im Strang*, Bayerwerk, Dormagen, Germany).

The silk is treated with alkali to make it jelly-like and is impregnated with buffer before use. The alkali treatment is carried out in the following way. About 20 pieces of silk thread 2 cm. long, are immersed for 2 minutes in distilled water, 2 minutes in 1.5 N NaOH, 5 minutes in 2.25 N NaOH at 10°C., and are finally washed in three changes of distilled water. They are then placed in a vial containing a special buffer solution.

The buffer is prepared as follows: 10 ml. 8 N H_2SO_4, 18 ml. distilled water, and 33.3 gm. of glycerol, sp.gr. 1.26 are mixed with 72 gm. of D-glucose

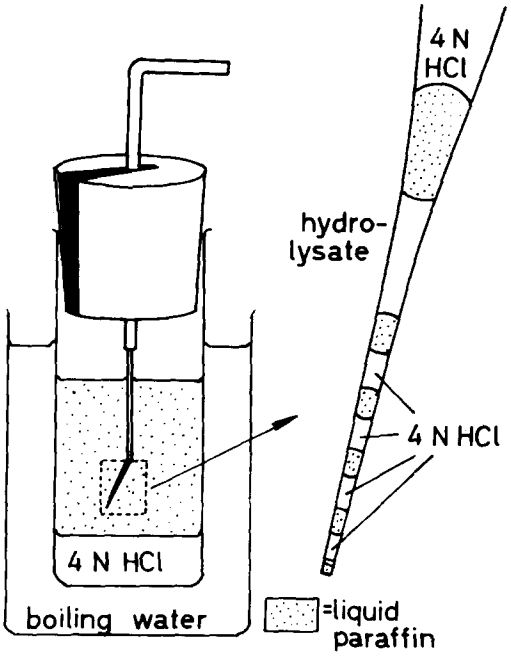

FIGURE 1

Illustration of the procedure for quantitative hydrolysis of RNA in amounts down to 100 to 1000 μμg.

TABLE I

Chromatographic Purine-Pyrimidine Analyses (Macroscale) of Yeast RNA Hydrolyzed in Acids of Different Strength

Molar proportions in per cent of the sum ± S.E.M.

	Adenine	Guanine	Cytosine	Uracil
1 N HCl, 100°C., 60 min.	27.8 ± 0.1	26.9 ± 0.2	20.8 ± 0.2	24.6 ± 0.3
4 N HCl, 100°C., 30 min.	27.7 ± 0.3	26.7 ± 0.1	21.2 ± 0.1	24.4 ± 0.1

(analar, British Drug House). The mixture, kept in a flask, is placed in a water bath at 100°C. for 50 minutes under stirring till the sugar dissolves. When ready, it is cooled down as rapidly as possible. It is stored at −20°C. and can be used for several months.

Because of its function the sulphuric acid solution is called a buffer, although this is an unconventional designation. A low pH is necessary to keep guanine soluble during the separations. The dissolved glycerol and glucose serve to give the buffer a high viscosity during electrophoresis in order to reduce the diffusion of the migrating compounds. This makes possible sharp separations also of narrow bands. The increased resistance to electrophoresis can easily be compensated for by an increased voltage because electrical heating is of no importance during separations in the present small scale.

The buffer with silk threads is stirred gently for a few minutes and left at room temperature for a couple of hours after which the silk is ready for use, or can be stored in a refrigerator for later use. It is not advisable to use preparations older than 3 days, since the fibres show a tendency to become thinner with time. A single fibre is drawn out of the silk and freed from excess moisture by letting it adhere repeatedly to a clean glass surface. It is then stretched on a quartz slide, 24 × 30 × 0.5 mm. Buffer bridges are drawn from the ends of the fibre towards one of the long sides of the slide, where they end a few millimeters from the corners at the edge of the slide. A paste obtained by mixing buffer with powdered silica gel is used as buffer bridge material. The quartz slide is placed in an oil chamber free from liquid paraffin, the fibre facing downwards.

Hydrolysates are applied with a micropipette under continuous evaporation. To prevent local spreading in the fibre of the RNA constituents, a small electrical heater of 30 w., mounted on the microscope stand, is used to dry the fibre. 5 to 10 hydrolysates, containing 200 to 500 μμg. RNA components, are usually placed after each other on the same fibre. With two or more parallel fibres a large number of analyses can be run simultaneously. After the application of the extracts the quartz slide is taken to a constant humidity chamber (see Appendix) with the buffer bridges in contact with brass nails, which serve as electrodes. Forty-two per cent relative humidity is used and the fibre is left for 5 minutes for equilibration. After this time the fibre is covered with liquid paraffin and 2000 to 4000 v./cm. are applied for a time, which in minutes is about 2500 a/v, where a is the fibre length in mm. and v the applied voltage. At 4000 v and 10 to 15 mm. fibre length, 6 to 9 minutes are necessary. The current is of the order 2 to 3 μa. and is measured with a Pye galvanometer, 7904/S. The viscosity in the buffer during the separations lies at 5000 to 8000 c.p. After the electrophoretic runs, the fibre is photographed in monochromatic ultraviolet light of 257 mμ wavelength in the Köhler ultraviolet microscope with a cadmium spark as a light source. Finally the plates are investigated by photometry by recording the optical density in a track along the axis of the fibre (Fig. 2). The curves are integrated, which simply means that the areas under the curve peaks are determined by planimetry. A Moll recording photometer (Kipp and Zonen, Delft, Holland) was mostly used, although it is not ideal for the purpose. Towards the end of the work the automatic recording microdensitometer of Joyce, Loebl and Co., Newcastle upon Tyne, England, as described by Walker (15) became available, an instrument which was found to be well suited for the present work. For calculation of the purine and pyrimidine proportions, the following specific optical density constants × 10^{-3} at 257 mμ were used, taken from the curves of Beaven *et al.* (1): adenine 11.2, guanine 9.6, cytidylic acid 5.15, and uridylic acid 9.5. The values for the purines were taken from the curves recorded by Beaven *et al.* in 6 N HCl.

RESULTS AND CONCLUSIONS

Comparison between Chromatographic and Microphoretic Analyses of Yeast RNA

The accuracy of the microphoretic method, including the hydrolytic procedure was first tested on a preparation of RNA of known composition. Yeast RNA (Bios Laboratory, Inc., New York)

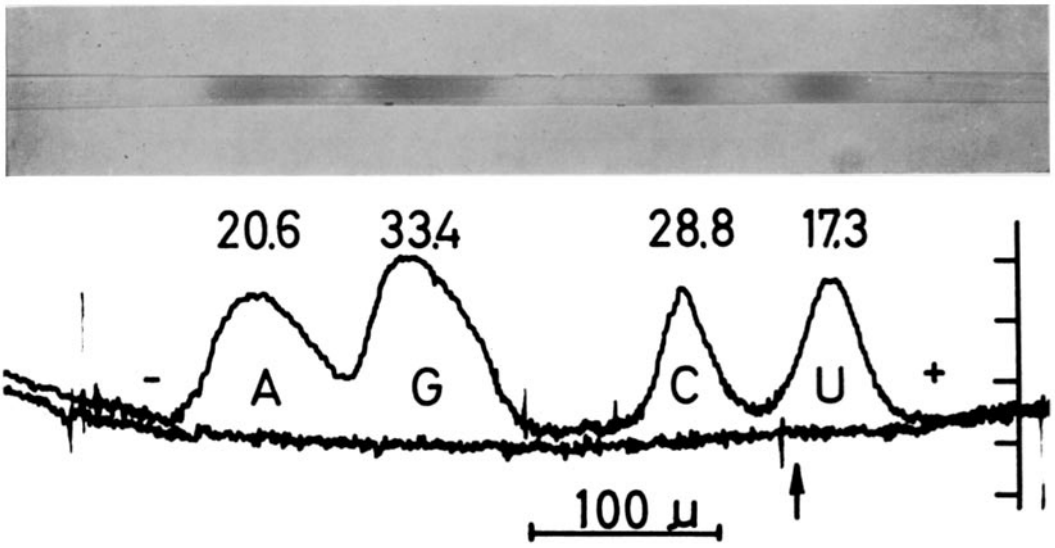

FIGURE 2

A microphoretic separation of a hydrolysate containing about 350 μμg. of RNA from isolated sensory ganglion cells. The values shown are the molar proportions in per cent of the sum. *A*, *G*, *C*, and *U* stand for adenine, guanine, cytidylic acid, and uridylic acid, respectively. The arrow shows the starting point. The distance between two scale divisions on the vertical scale shown to the right corresponds to 0.1 optical density units.

TABLE II

Purine-Pyrimidine Analysis of Yeast RNA by Means of Microphoresis after Hydrolysis in the Macro- or Microscale

200 to 400 μg. of hydrolyzed RNA has been used for the chromatographic macroscale analyses, 200 to 400 μμg. for each microphoretic analysis. Figures in per cent of the ± sum S.E.M. Molar proportions.

	Adenine	Guanine	Cytosine	Uracil	No. of analyses	No. of fibres
Composition as determined by chromatography	27.8 ± 0.1	26.9 ± 0.2	20.8 ± 0.2	24.6 ± 0.3	3	—
Microphoresis, macroscale hydrolysis	27.7 ± 0.5	27.6 ± 0.2	20.6 ± 0.5	24.1 ± 0.7	42	6
Microphoresis, microscale hydrolysis	27.9 ± 0.4	27.0. ±.0.4	21.4. ±.0.2	23.8 ± 0.3	39	6

was chosen for the purpose. Its nucleotide composition was first determined by chromatography according to Markham and Smith (13). Then, one series of microphoretic analyses was made on material hydrolyzed in the macroscale. In a second series the nucleic acid was digested in the macroscale by enzyme, after which it was hydrolyzed in the micropipette under standard conditions. In this way both the hydrolytic procedure and the electrophoresis, including the photographic-photometric determination was tested. As can be seen from Table II, the results of both series are consistent with the macrochemical results. The average value for the coefficient of variation in these microphoretical analyses is about ±7 per cent.

Analyses of Isolated Cells

Although the sensitivity of the method is high enough for determinations on single large nerve cell bodies, like motor anterior horn cells and

TABLE III

Purine-Pyrimidine Composition of RNA from Various Sources

Molar proportions in per cent of the sum.

Source	Adenine	Guanine	Cytosine	Uracil	Method	Reference
Liver, rabbit	19.3	32.6	28.2	19.9	*	(2)
Liver, rabbit	22.1	30.9	29.6	17.4	m	—
Pancreas, calf	14.1	48.7	23.4	13.4	‡	(14)
Pancreas, exocrine, calf	19.3	38.5	27.6	14.7	m	—
Pancreas, exocrine, rabbit	21.8	39.4	25.0	13.9	m	—
Pancreas, endocrine, rabbit	24.4	32.1	24.7	18.8	m	—
Brain, cat	21.6	31.8	26.0	20.6	§	(5)
Brain, rabbit	22.5	33.8	25.9	17.7	*	‖
Motor anterior horn cells, rat	20.9	36.2	24.9	18.1	m	‖
Deiters' nerve cell bodies, rat	20.5	33.7	27.4	18.4	m	‖
Sensory nerve cell bodies (ganglion Gasseri), rat	18.7	34.8	27.8	18.7	m	‖
Neurosecretory cell bodies, hypothalamus, rat	19.1	35.3	27.1	18.6	m	‖

* Davidson and Smellie (3).

‡ Volkin and Carter (14).

§ Magasanik *et al.* (12).

‖ Unpublished results by Edström, Eichner, Hartmann, Hydén, and Jarlstedt.

m, microphoresis.

sensory ganglion cells, several cells were always extracted and analyzed together to save time. This could be done since it was desirable to obtain values representative only for the different kinds of cells. Results, which have been obtained with the method, are collected in Table III, with some macrochemical analyses for reference. It can be seen that there is a fairly good agreement between the macro- and microchemical analyses. A further fact of interest is that, in the nervous system, RNA from different sources is very similar and agrees in composition with macrochemical preparations.

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APPENDIX

A CONSTANT HUMIDITY CHAMBER FOR MICROPHORESIS

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In microphoresis the concentration gradients of the migrating compounds are many thousand times steeper than in conventional zone electrophoresis. Consequently, the tendency to concentration equilibration through diffusion is increased to a corresponding extent if not met by special measures. To a moderate degree this tendency can be neutralized by using shorter times for the runs (increased voltage), but a radical effect is obtained only by performing the separations in media of high viscosities (5,000 to 10,000 c.p). An increased viscosity is obtained by the addition of glycerol and glucose to the fibre used for microphoresis. The viscosity value will depend on the relation between the amounts of these compounds, the electrolytes in the buffer, and water. The amount of water in relation to the other constituents is subject to variations during the preparatory steps. In these highly viscous solutions large variations in viscosity may result even from small variations in the water content. The reason for these variations is that the fibre during part of the procedure must be in direct contact with the surrounding atmosphere, with which the buffer in the fibre will rapidly equilibrate. This means that the relative humidity of the surrounding air will determine the mole fraction of water in the buffer and, consequently, its viscosity. During the applications of the hydrolysates the fibre is furthermore heated, which causes loss of water from the buffer and increased viscosity.

In order to obtain reproducible results it is essential to have a reproducible and constant water content and viscosity in the buffer during the electrophoretic migrations. Furthermore, perfect separations are obtained only within a relatively narrow viscosity range. To achieve this result one has to place the fibres used for microphoresis in a constant humidity chamber before the separations are performed. One technical difficulty is to introduce them without changing the relative humidity in the chamber perceptibly, another one to carry out the separations in the chamber.

In the following description of a chamber designed to meet the requirements outlined above, Fig. 1 should be used as a reference.

The chamber (*C* in Fig. 1) is double walled, has internal dimensions 100 × 100 × 100 mm., and is provided with a lid (*A* and *B*).[1] The outer bottom plate is thicker and larger than the other walls. The outer and inner wall plates are joined at the level of the rim of the interior wall by horizontal, 18 mm. broad plates. As is evident from *B*, the lid, which is screwed onto the chamber, is made to fit the upper profile of the connected chamber walls. The dotted part in *C* represents a rubber strip providing airtightness. The outlet tube of a separatory funnel has been fitted to pass through the lid as shown in *A* and *B*.

In the middle of two opposite vertical walls rectangular openings, 24 × 51 mm., have been made in all four wall plates for the passage of a guide (*C* and *D*). In the section, 30 mm. long, where the guide passes through the chamber walls, horizontal 27 × 30 mm. plates have been fastened between the outer and inner wall plates, fitting into the space between the free edges of the guide shanks, leaving free a rectangular 6 × 35 mm. canal in the guide. In this way the space between the outer and inner wall plates remains a closed compartment and there is also no direct communication between the exterior and the chamber when a slide, (35 × 6 × 310 mm.), has been inserted (*E*, *F*, and *G*). With its central point 90 mm. from one end of the slide, there is a rectangular, 3 mm. deep excavation or trough for housing the quartz glasses used for carrying the fibres. The glasses are supported by 1 mm. high, 3 mm. broad ridges along both short sides. Symmetrically and 20 mm. from each other, on one of the long sides, two cylindrical brass nails, serving as electrodes, have been fixed through the wall of the trough (*G*). Facing outwards they are provided with conical depressions. In the central part of the guide, at corresponding locations, two brass nails, mobile in canals, traverse one of its vertical shanks. These nails are pressed against the slide by means of elastic bronze plates and have conical terminations which fit into the depressions in the nails of the slide. This arrangement fixes the position of contact between the two nails of the slide and those of the guide but also allows free movement of the slide. The two bronze plates are fastened laterally to the guide with screws

[1] Unless otherwise stated 6 mm. plexiglas (lucite) has been used for construction of chamber etc.

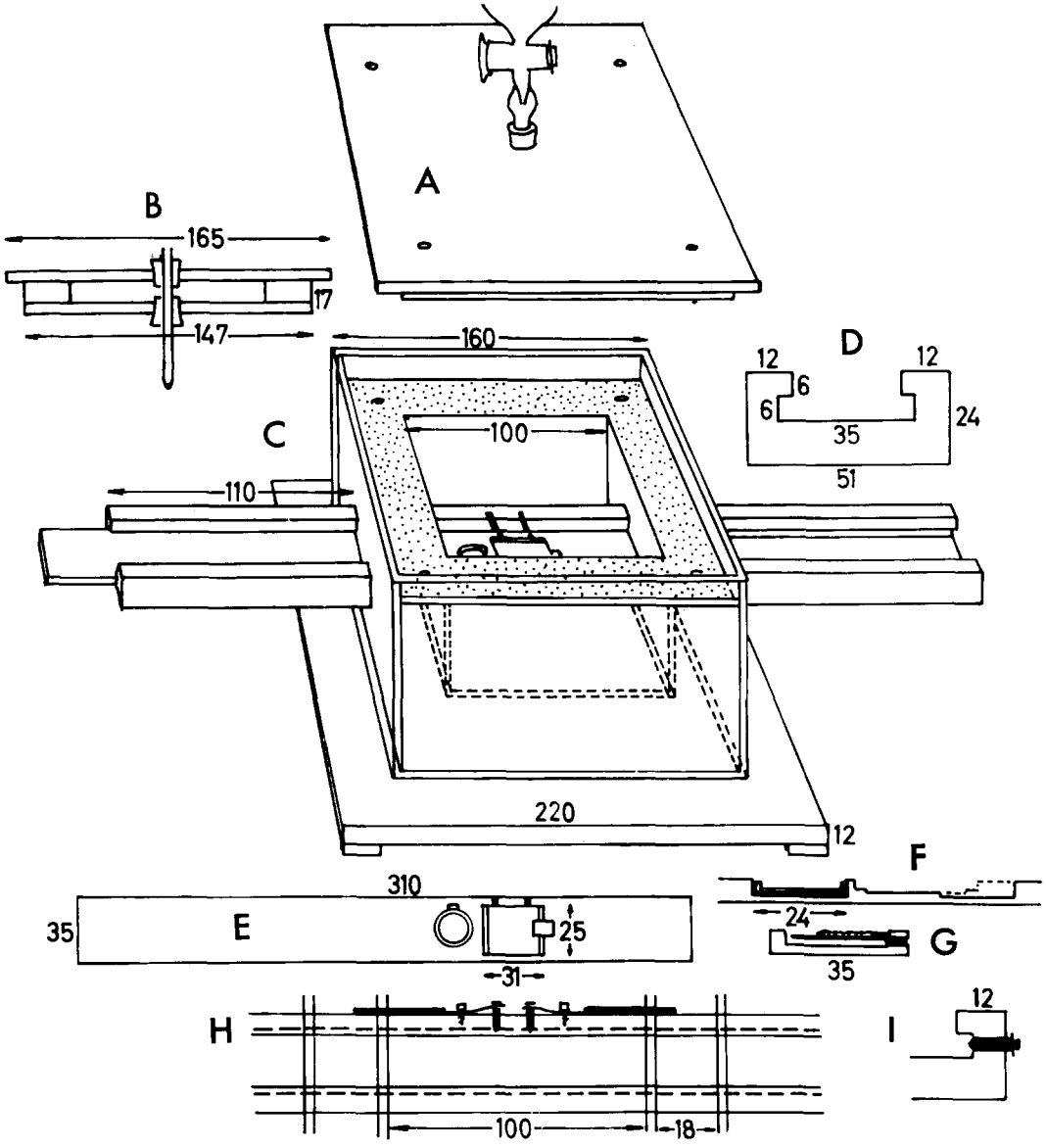

FIGURE 1

Constant humidity chamber for microphoresis: *A*, the lid in perspective; *B*, cross-section through the lid at the center; *C*, the chamber in perspective with guide and slide in position for microphoresis; *D*, cross-section of the guide; *E*, the slide with trough and cup for collecting liquid paraffin; *F*, longitudinal section through the slide along the axis. The profile of the trough at an out-of-axis section indicated by a broken line; *G*, cross-section of the slide through the trough and one of the brass nails. A quartz glass in position with a buffer bridge is also shown; *H*, view from above of the central part of the guide and the electrical connections; *I*, cross-section of the guide through one of the brass nails. *D*, *F*, *G*, and *I* have been drawn twice the size of *B*, *E*, and *H*. *A* and *C* are shown in approximately the same scale as the latter three.

as shown in *H* and are united to electrical cables, which pass into the closed compartment between the wall plates. From here they are taken down through the bottom plate and connected to plugs on the bottom plate lateral to the chamber.

In the bottom of the chamber there is a 1 cm. layer of saturated $Zn(NO_3)_2$ solution with salt in excess, giving a humidity of 42 per cent at 20°C. The separation funnel contains liquid paraffin, a few drops of which can be let down onto the quartz glass to cover the fibre before the separations are begun, and after it has achieved equilibrium (after 5 minutes).

A small cup can be placed in a round excavation in the slide close to the trough (*E* and *F*). It is used for collecting the first few drops of paraffin from the funnel, since these may have absorbed ultraviolet-absorbing material, if in contact with the atmosphere of the chamber for some time.

The chamber is kept in a temperature controlled room, the arrangement with double walls serves to eliminate small temperature fluctuations, which, if they are rapid enough, may cause changes in relative humidity.

The chamber permits the introduction of a quartz glass, carrying a fibre with buffer bridges, into an atmosphere of constant humidity without any risks of disturbing the equilibrium. With the aid of the pasty buffer bridges electrical connection between the ends of the fibre and the nails of the slide is obtained (*G*).