BBA 75113

STUDIES ON A MATERIAL WHICH INDUCES ELECTRICAL EXCITABILITY IN BIMOLECULAR LIPID MEMBRANES

I. PRODUCTION, ISOLATION, GROSS IDENTIFICATION AND ASSAY

LEO D. KUSHNIR

Department of Basic Research, Eastern Pennsylvania Psychiatric Institute, Philadelphia, Pa. (U.S.A.)

(Received October 6th, 1967)

SUMMARY

This report constitutes the first part of an investigation into the nature of the material which induces electrical excitability in experimental bimolecular lipid membranes. The excitability-inducing material (EIM) is released upon growth of *Aerobacter cloacae* ATCC 961 in a defined medium containing only low molecular weight substances. It has been isolated by adsorption on Kieselgel and subsequent elution with 1 % ammonia solution.

By TEAE-cellulose column chromatography of EIM solution, protein and RNA moieties, the only detectable components of EIM, have been separated. The separation of moieties results in loss of activity. Partial reactivation can be obtained by mixing 1 % solutions of the protein and RNA. This reactivation does not occur when 0.1 % solutions (or lower) are mixed. Both moieties obtained by TEAE-cellulose column chromatography show gross homogeneity by electrophoretic and sedimentation studies. The basic properties of the protein moiety, not evident with total EIM, become apparent after its separation from RNA. The chemical, chromatographic and electrophoretic studies reported in this paper raise the possibility that a ribonucleoprotein complex constitutes the functional entity of the material.

The paper also presents standardization of the EIM activity assay.

INTRODUCTION

Four decades ago, GORTER AND GRENDEL[1], drawing upon lipid extraction and monolayer studies, and later DAVSON AND DANIELLI[2], from studies of cellular permeability, proposed the bimolecular and pauci-molecular models of the living cell membrane. Over the following years, this model of a living-cell membrane not only withstood repeated scrutiny in the light of new techniques and observations but received additional support from a large number of studies[3,4]. However, pending experimental verification that such a structure was capable of independent physical existence and possessed properties ascribed to plasma membranes, it remained largely a theoretical concept.

Abbreviation: EIM, excitability-inducing material.

Recently, experimental evidence of physical stability and other properties of such bilayer structure was provided when it was constructed *in vitro* in 0.1 M saline solution from brain phospholipids dissolved in chloroform–methanol solvent[5]. Furthermore, an unknown substance, named "excitability-inducing material" (EIM), released by some strains of *Aerobacter cloacae* during growth in egg-white medium was found to adsorb to this bilayer, lower its resistance, and regularly induce an ionic switching or gating effect (a non-linear resistance change) under electrical stimulation[5]. This phenomenon is similar to those found in living cells[6,7] and tissues[8]. More recently complete action potential phenomena were developed with the EIM-modified lipid bilayers[9]. These observations extend the similarity of the EIM-modified films to the living-cell membranes and place the identity of EIM in the forefront of interest as a biochemical entity, possibly responsible for ionic permeability changes found in the living cells, and as a basic molecular component of action potentials.

Production and isolation of EIM solution from a defined medium with partial characterization of the material is described.

MATERIALS AND METHODS

All chemicals were chemically pure and were used without further purification. Kieselgel D-5 from Camag, Switzerland, was washed several times with distilled water (brought to pH 4.0 with HCl) before use. Protease (subtilopeptidase A) bacterial type VIII, crystallized and lyophilized; ribonuclease A from bovine pancreas 5 × crystallized, type I-A, protease-free; phosphodiesterase from *Crotalus adamanteus* venom, type II and yeast RNA, type XI, purified per CRESTFIELD *et al.*[10], were all obtained from Sigma Chemical Co., St. Louis, Mo. *A. cloacae* ATCC 961 was purchased from American Type Culture Collection, Rockville, Md. Thioglycollate medium was obtained from Difco Labs. and Cellogel, gelatinized cellulose acetate strips for electrophoresis were obtained from Colab Labs. Inc., Chicago Heights, Ill. Diaplex Ultrafil Membranes, types UM-1 and UM-2, were purchased from Amicon Corp., Cambridge, Mass. TEAE-cellulose (Serva, TEAE-cellulose ion exchanger) was purchased from Gallard-Schlesinger Chem. Mgr. Corp., Carle Place, New York.

A. cloacae ATCC 961 inoculum: The thioglycollate medium made up according to label instructions was inoculated with *A. cloacae* ATCC 961 and incubated overnight at 37°. The resulting growth, maintained at 5°, was used to inoculate "synthetic" media. Each batch of inoculum was used for about 2 weeks.

"Active" egg white: Fresh egg white brought to pH 7.0 with 0.1 M HCl was inoculated with *A. cloacae* ATCC 961 inoculum and incubated for about 20 h at 37°. The resulting growth, active at 1/50–1/500 dilution, is referred to as "active" egg white.

"Synthetic" medium: Contained in g/l distilled water: glucose, 5.0; citric acid, 3.0; $CaCl_2$, 1.0; NH_4NO_3, 3.5; lysine·HCl, 0.2; $MgCl_2$, 0.02; $Fe(NO_3)_3$, 0.002; and 20 ml of 0.1 M K_2HPO_4. The final solution was adjusted to pH 6.9–7.0 with 5 M NaOH.

Assay of EIM activity

The lowering of resistance and gating reaction obtained during subsequent electrical stimulation of EIM-modified bilayer is designated as EIM activity.

The activity of EIM solutions is expressed in terms of dilutions, such as 1/10, 1/100 *etc.* 1 unit of activity designates 1 ml of non-dilutable EIM solution, 1 drop of which added to approx. 3 ml of the environmental solution of the bilayer produces the phenomena within 5 min. Accordingly, the final concentration of EIM in the environmental solution of the bilayer which still produces the phenomena, will be approximately two orders of magnitude lower than in the tested EIM solutions.

The number of activity units contained in EIM solution was obtained by multiplying the volume of EIM solution by its highest level of dilution. The activity units expressed per μg of total organic material, protein, or RNA contents of EIM solution will designate specific activities of the respective materials. The samples of pH lower than 6 (supernatants from Kieselgel adsorption and fractions eluted with 0.1 M acetic acid and subsequent H_2O, washes of the TEAE-cellulose) were brought to pH 8–9 with 1 % ammonia before assay.

EIM activity was measured by the method developed by MUELLER *et al.*[11]. A 5 % solution of total bovine white matter lipid[12] in chloroform–methanol (3:2, v/v), with 400 mg/ml α-tocopherol and 30 mg/ml cholesterol added as stabilizers, was used to form a lipid membrane between two compartments containing 5 mM histidine–0.1 M NaCl (pH 6.8) at 32–35°. After formation of the black film with a resistance greater than 10^8 $\Omega \cdot cm^2$, the temperature was raised to 38–40°. Then 1 drop of the sample to be tested was added to the solution on one side of the membrane and stirred with a magnetic stirrer. This side of the membrane is designated as the "inside". The effect of the added sample on the resistance of the film was measured every minute. Depending on the activity of the sample, the time for resistance to reach about 25 % of the original value took anywhere from a few seconds up to 10 min; samples requiring longer times were considered to be negative. At this point, voltages of increasing strength were applied across the bilayer until a gating reaction resulted. Generally, the gating reaction had a lower threshold when the inside electrode was negative. Therefore, to avoid inadvertently generating the gating effect, it was found best to

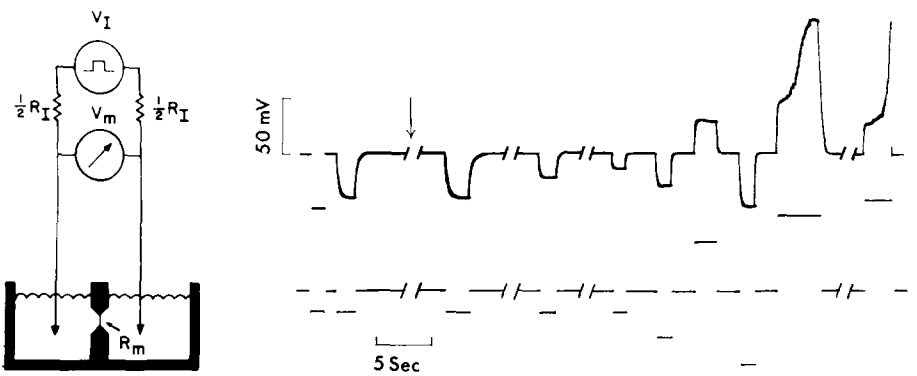

Fig. 1. Testing of EIM activity. The essential stimulating and recording circuit elements are shown in the insert. V_I stimulating voltage; R_I, series input resistor; V_m, recorded membrane potential, R_m, membrane resistance. The upper trace shows recordings of the membrane potentials in response to rectangular stimulating voltages (shown in lower trace) applied through R_I. After addition of EIM (arrow) test pulses were applied at intervals of 1 min. The membrane potentials which are proportional to the resistances show a progressive decrease and at 3 min reach 25 % of the original value. At this point applied potentials of opposite sign are increased until the threshold potential and the resulting gating reaction were obtained.

follow the steadily decreasing resistance by testing with the inside electrode positive and to reverse the polarity of stimulation to obtain the gating reaction, as shown in Fig. 1.

In the course of the purification studies of "active" egg white, it became evident that in the testing of EIM activity, sensitivity of lipid, *i.e.*, the receptiveness of the black film toward penetration by the active sample varied by as much as two orders of magnitude between various batches of lipid mixtures prepared in an identical manner. Moreover, lipid sensitivity was found to change with time. These observations made the standardization and periodic checks of lipid sensitivity imperative as a routine calibration procedure.

The sensitivity of the lipid mixture was determined at the beginning of each test series and also was spot-checked during the series by use of standard "active" egg white EIM preparation. Although the activity of "active" egg white in solution decreases with time, it was found that the activity of "active" egg white twice precipitated with ammonium sulfate (50 % satn.). dissolved in distilled water and stored at 5°, remained essentially constant up to 7 months or more. This solution, diluted to its limiting activity (about 0.001 % protein), was used as a standard. Only lipid mixtures sensitive to this standard were used in testing.

The method is sufficiently sensitive to detect a 25–50 % change in activity of samples which were diluted to the level of limiting activity. Because of occasional false negatives, rechecks should be performed routinely.

Production and isolation of activity

The activity in "synthetic" medium is produced by inoculation with *A. cloacae* inoculum (1–5 drops/l media) and incubation overnight (15–20 h) at 37°. This incubation yields media active up to a 1/50 dilution. The small amounts of active material released by bacteria into the medium necessitated a pilot-scale preparation. In routine preparations, 30 l of active media (six 5-l batches in erlenmeyer flasks) were grown and adsorbed by suspending the media in 2.5-l portions on washed Kieselgel (150 g, dry). The active medium–Kieselgel suspension was brought to pH 4.0–4.5 with 2 M HCl and equilibrated for 10–60 min with stirring at 5° and then centrifuged at approx. 850 × *g* for 5 min. A small portion of the resulting supernatant was checked for activity after additional centrifugation at 34000 × *g* and adjusting the pH to 9 with a 1% ammonia solution.

Depending upon the activity of the incubated media, 15–300 l were required to saturate 150 g of Kieselgel, as determined by the absence of activity in the supernatants. All of the supernatants were discarded, and the saturated Kieselgel was suspended in 1–2 l of distilled water and stored in the cold (5°) or processed further. Following storage, the supernatant was decanted off or the suspension was centrifuged (850 × *g*), and the Kieselgel was washed 2 times more with 300 ml each of distilled water; during both washes the suspension was brought to pH 4.0 with 2 M HCl. The washed, saturated adsorbant was then extracted with 600 ml of 1 % ammonia solution by stirring for 2 h in the cold (5°). It was then centrifuged at approx. 850 × *g* for 10 min. The cloudy extracts showed activity at dilutions up to 1/2000. The second and third extracts of the Kieselgel obtained in a similar way showed little activity (about 1/10th and 1/100th of the first, respectively) and thus may be disregarded in routine preparations. The 1 % ammonia extracts dialyzed with stirring (cellulose dialyzer

tubing, flat width 1 inch, average pore size 4.8 mμ) against three changes of distilled water (1:30, by vol.) for 20 h at 5°. The dialyzed extracts were finally centrifuged at 100000 × *g* for 15 min, yielding water-clear solutions. These solutions are the starting materials for all studies and hereafter are referred to as EIM solutions. Dialysis and centrifugation may result in some loss of activity.

Electrophoresis

Solutions of lyophilized EIM and individual moieties were examined by electrophoresis on cellulose acetate strips in Tris–EDTA–boric acid (40.0, 2.0, and 7.0 mmoles/l, respectively) buffer, which was adjusted to pH 4.0, 6.0, 7.0, 8.9, and 9.5 with HCl or NaOH. Electrophoretic runs[13] were conducted on Colab apparatus according to recommended procedure (Colab brochure D 25-28, D 29). Strips were stained with Procion Brilliant Blue (0.5 g in 98 ml methanol and 2 ml conc. HCl) and destained with methanol. The conditions for electrophoresis were: 10–50 min, 3 mA, 60–340 V. A 1 % solution of yeast RNA type XI was run in parallel with each sample.

Enzymatic studies

Enzymes were added to EIM solutions to a concentration of 0.03 mg/ml, the solutions were brought to pH 7.0 and incubated at 37°. At intervals, aliquots were removed directly from the incubation flasks and tested for activity.

Chemical analysis

Total solids were obtained by the evaporation of EIM solutions in Pt crucibles to constant weight at 105°. These solids yielded ash when heated to dull-red. Nitrogen, phosphorus, and ribose were determined by micro-Kjeldahl[14], total phosphate[15], and orcinol[16] methods, respectively. The RNA content of the EIM solution was estimated on the basis of 9 % and 15 % P and N, respectively, and protein content was estimated on the basis of 17 % N. Protein-N was obtained from total N (Kjeldahl) *minus* RNA-N (the latter calculated from P content).

The analytical data were obtained in duplicate and repeated with extracts from different batches of saturated Kieselgel produced several weeks apart. Although the quantitative data vary, the qualitative trend was consistent with all batches.

Absorbances were determined on a Beckman DU spectrophotometer with distilled water as the reference.

TEAE-cellulose chromatography

TEAE-cellulose with rated capacity of 0.49 mequiv/g was freed of fines by repeated suspension in distilled water and decantation. The washed cellulose, suspended in distilled water and adjusted to pH 9–10 with 0.2 M Na_2CO_3, was packed into a column 2 cm × 18 cm (about 20 g dry TEAE-cellulose) by gravity flow. The packed column was vacuum-compressed and finally washed with distilled water. The elution of the adsorbed material, washed with distilled water (50–100 ml) was carried out with 0.1 M acetic acid, followed by 100 ml distilled water and distilled water brought to pH 8.5 with 0.02 M Na_2CO_3, until the effluent showed pH 5.5–6.0. Then the elution was continued with 1.0 M LiCl brought to pH 8.5 with 0.1 M NaOH.

RESULTS AND CONCLUSIONS

The flow diagram of the isolation procedure, together with the adsorbances of the resulting solutions at 235 mμ, 260 mμ, and 280 mμ, is presented in Table I.

All starting media are the same. The incubated, active media differ somewhat in the absorbances (A) and may differ greatly in activity. The activity levels of incubated media are not related to their absorbances nor to the amount of bacterial growth, as estimated by turbidity, and they may vary from an occasional negative to active at 1/50 dilution. Bacterial variations are suspected to be the cause of these differences. Although as many as 300 l of active media may be required to saturate 150 g of Kieselgel, in Table I, saturation of Kieselgel with 15 l of "very" active media is presented for convenience.

Each 5 l of active medium (A, B, C) yields two 2.5-l supernatants (A_1 and A_2 *etc.*) which show relatively small losses of absorbance, especially after adsorption of the first portion (medium A). In the case of medium B, the higher absorbances

TABLE I

ABSORBANCES AND ACTIVITIES OF MEDIA AND SUPERNATANTS AND EXTRACTS RESULTING FROM THEIR ADSORPTION ON 150 g KIESELGEL

Absorbances of all samples were determined *vs.* distilled water after centrifugation at 34000 × *g* in case of media, supernatants and water washes and after 20 h dialysis *vs.* 3 changes of distilled water (1:30, by vol.) and centrifugation at 100000 × *g* in case of 1% ammonia extracts. Precipitate is the sediment obtained from the dialyzed extracts at 100000 × *g*. Limiting activities of active media were assayed after dilution with distilled water. Supernatants and water washes were assayed for activity after centrifugation at 34000 × *g* and adjustment to pH 9.0 with 1% ammonia. Extracts were assayed after dialysis, centrifugation at 100000 × *g* and dilution with distilled water, d.c., dialyzed and centrifuged.

Sample	*Volume (ml)*	*Absorbance*			*Activity (dilutions)*
		235 mμ	*260 mμ*	*280 mμ*	
Media before growth		2.6	0.074	0.156	None
Active medium A	5000	2.95	0.43	0.42	1/25
Supernatant A_1	2500	2.88	0.28	0.22	None
Supernatant A_2	2500	>3.00	0.22	0.30	None
Active medium B	5000	2.90	0.20	0.25	1/25
Supernatant B_1	2500	2.95	0.31	0.33	None
Supernatant B_2	2500	2.80	0.29	0.31	None
Active medium C	5000	>3.00	0.28	0.33	1/25
Supernatant C_1	2500	>3.00	0.22	0.28	None
Supernatant C_2	2500	>3.00	0.27	0.32	1/1
H_2O wash 1	1000	2.70	0.30	0.20	None
H_2O wash 2	300	0.90	0.11	0.09	1/1
H_2O wash 3	300	0.38	0.12	0.11	None
1% ammonia extract 1, d.c. (EIM solution 1)	600	1.26	1.92	0.99	1/500
1% ammonia extract 2, d.c. (EIM solution 2)	600	0.29	0.45	0.22	1/100
Precipitate (in 1% suspensions)					1/1–1/5 (Several consecutive extracts with 1% ammonia)

of supernatants B_1 and B_2 resulting from adsorption of active medium B, can be explained by the displacement of non-active material adsorbed on fresh Kieselgel by EIM. The three water washes desorb additional, mostly non-active material. The first and second ammonia extracts are usually cloudy and cannot be clarified at 34000 $\times$ *g*. The absorbance values of these extracts presented in Table I pertain to solutions extensively dialyzed and centrifuged (d.c.) at 100000 $\times$ *g* for 15 min (EIM solutions). Precipitate is the distilled water washed, freeze dried sediment obtained from these dialyzed extracts at 100000 $\times$ *g*. It is only slightly soluble, but several consecutive extractions with 1 % ammonia yield active solutions. Although in routine preparations the second extracts were seldom used, the results of the second extraction are presented here to point out some correlations between activities and materials in two consecutive extractions.

The activity of incubated media deteriorates within 1 or 2 days on standing at room temperature or in the cold (5°). In contrast, activity adsorbed on Kieselgel was found to be essentially intact after 2 months (or more) or storage in the cold. The Kieselgel adsorption not only affords 25–200 fold increase of activity by a comparatively fast and simple procedure, but it also permits prolonged storage necessary for accumulation of active EIM.

Analytical data of EIM solutions 1 and 2 and the precipitate (Table I) are presented in Table II. The estimated protein and RNA moieties account for gravimetrically obtained total organic solids in both solutions and the precipitate. Similar correspondence was obtained with all EIM solutions spot-checked during several months of study.

Fairly pure nucleic acid as a constituent of EIM solutions is suggested by the absorbance ratios: $A_{280\,m\mu}/A_{260\,m\mu}$ and $A_{260\,m\mu}$/mole P per l. However, the molar N/P ratios indicate the presence of some other N-containing material. Hydrolysates of the lyophilized EIM solutions chromatographed on thin layer and paper (to be presented later) show the presence of large amounts of both amino acids and purine and pyrimidine bases. The R_F value of the pentose present in the hydrolysates corresponds to that of ribose[17]. No deoxyribose (diphenylamine test) was detected in the EIM solutions.

The EIM solutions and the precipitate which sedimented from them at 100000 $\times$ *g* show a strong chemical similarity, as evidenced by the N/P ratios.

The absorbance ratios and the ratios of chemical data of EIM solutions 1 and 2 (Table I) presented in Table IIa are shown to be related to the activities. These ratios suggest that either protein or RNA or both of these two components may constitute the EIM. The specific activities 1.52, 2.57 and 5.64 and 1.18, 1.60 and 4.86 (Table II) with respect to "complex" (total organic material; protein and RNA), protein and RNA in EIM solutions 1 and 2 show that 1 unit of activity, on the average, may be represented by either 0.75 μg of "complex", 0.50 μg of protein or 0.19 μg of RNA.

In spite of the relatively high degree of concentration on Kieselgel, the EIM solutions (first extracts) contain, on the average, 0.1–0.3 mg of solids per ml. Because most of the available techniques (electrophoresis, *etc.*) cannot be applied to samples of such low concentrations, attempts were made to increase their solid content.

The solid material obtained by lyophilization of the EIM solutions was only partly soluble (approx. 50 %) and only about 1 % of the original activity could be recovered. Partial lyophilization, with and without fillers such as glucose, glycerine,

TABLE II

CHEMICAL ANALYSES OF EIM SOLUTIONS 1 AND 2 AND THE PRECIPITATE PRESENTED IN TABLE I

RNA and protein values were estimated on the basis of 9% P and 15% N in RNA and 17% N in protein. N-protein was obtained from N-Kjeldahl *minus* N-RNA; the latter was calculated from P content. Precipitate was analyzed after several washes with distilled water and lyophilization.

Sample	*N*	*P*	*Ribose*	*RNA*	*Protein*	*Total*		*Molar ratios*		$A_{280\,m\mu}/A_{260\,m\mu}$	$A_{260\,m\mu}$/ mole P per l
						Solids	*Ash*	*N/P*	*P/ribose*		
	($\mu g/ml$)	($\mu g/ml$)	($\mu g/ml$)	($\mu g/ml$)	($\mu g/ml$)	($\mu g/ml$)	($\mu g/ml$)				
EIM solution 1	46.4	7.98	19.8	88.67	194.71	328.0	49.0	12.7	2.0	0.51	7384
EIM solution 2	13.7	1.85	4.2	20.56	62.47	85.0	4.0	16.3	2.0	0.49	7537
Precipitate*	84.06	12.52	20.0	139.11	371.71	1000.0	535.0	15.0	3.1	—	
Specific activities of materials in											
EIM solution 1				5.64	2.57	1.52					
EIM solution 2				4.86	1.60	1.18					

* All values in μg/mg

TABLE IIa

RATIOS OF ANALYTICAL DATA OF EIM SOLUTION 1 : EIM SOLUTION 2 PRESENTED IN TABLES I AND II

	N	*P*	*Ribose*	*RNA*	*Protein*	*Organic solids*	*Absorbance*			*Activity*
							235 mμ	*260 mμ*	*280 mμ*	
EIM solution 1 / EIM solution 2	3.4	4.3	4.7	4.3	3.1	3.4	4.3	4.3	4.5	approx. 5.0

or NaCl, as well as vacuum evaporation to about 1/4th of the original volume at 30–40°, resulted in some precipitation and appreciable loss of activity. Concentration of the EIM solutions by ultrafiltration[18] produces similar results. The precipitates formed by attempts to concentrate EIM solutions can be brought into solution with 10 % ammonia, but the activity is then largely destroyed. It appears that EIM has limited solubility and also loses activity upon concentrating above a certain level. In view of these findings, except for electrophoretic and sedimentation analyses, further studies were conducted on EIM solutions as obtained from Kieselgel (approx. 0.01 % total solids).

Solubility

The loss of activity and precipitation of the material upon a relatively small increase in concentration of 0.01 % EIM solutions was discussed above. Approx. 0.6 % solutions of lyophilized EIM were used in electrophoretic and sedimentation studies without regard to activities. However, because only part of the lyophilized material was soluble, it was necessary to determine the identities of both soluble and insoluble parts.

TABLE III

CHEMICAL ANALYSIS OF LYOPHILIZED EIM AND ITS SOLUBLE AND INSOLUBLE PARTS AS OBTAINED DURING SOLUBILIZATION

1 % suspension of lyophilized EIM in distilled water (brought to pH 8.5 with 0.2 M Na_2CO_3) was stirred for 1 h then neutralized to pH 7.0 with 0.1 M HCl and centrifuged at 100000 × *g* for 15 min.

Sample	*N* (*μg/mg*)	*P* (*μg/mg*)	*Ribose* (*μg/mg*)	*Molar ratios*		*% Dissolved*		
				N/P	*P/ribose*	*N*	*P*	*Ribose*
Lyophilized EIM	101.93	13.08	22.58	17.3	2.8			
Soluble part	64.60	7.99	16.30	17.7	2.4	63.4	61.1	72.2
Insoluble part	38.30	5.07*	6.28*	17.1	4.0			

* Values obtained by difference (lyophilized EIM *minus* soluble part).

Chemically, the soluble part of lyophilized EIM appears to be identical to the total lyophilized EIM and to its insoluble part, as is evidenced by N/P ratios (Table III). Although the P/ribose ratios differ somewhat and may indicate RNA's of different Pu/Py in both parts, they are at present disregarded, because of unknown effects of lyophilization *etc*, on the orcinol test. The P/ribose ratio of lyophilized precipitate (Table II) is also high. It appears that lyophilization of EIM affects the ribose test.

The N, P, and ribose of lyophilized EIM and its dissolved part and the N value of the insoluble part of EIM were obtained by direct analysis, whereas P and ribose of the insoluble part of EIM were obtained by difference. The agreement of the sum of N values of soluble and insoluble parts with total EIM renders satisfactory the calculation of P and ribose by difference.

The effect of enzymatic digestions on the activity of EIM solutions

Protease digestion of EIM solutions resulted in the complete destruction of activity within 2 h. On the other hand, nucleases (ribonuclease and phosphodiesterase

II) showed essentially no effect on activity of EIM, even after prolonged incubation (20 h). However, in no case did the losses of RNA estimated by absorbances and determined analytically after extensive dialysis of EIM solutions incubated without or with nucleases, differ significantly. In both cases only about 10 % of RNA become dialyzable (about 94 % of protein become dialyzable after digestion with protease). The immunity of EIM-RNA to digestion with nucleases may indicate a complex in which RNA is protected by protein. It is conceivable that the protein moiety of the complex forms a protective coating on the RNA, rendering it inaccessible to digestion by nucleases. A protective effect of proteins on RNA and DNA was reported in several cases of intact viruses[19].

EIM chromatography on TEAE-cellulose

The separation of protein and RNA, the only analytically detectable components of EIM solution, by TEAE-cellulose column chromatography results in complete loss of activity. None of the activity was recovered despite the fact that all of the material was eluted as indicated by the absorbances of eluted regions.

In Fig. 2 is shown the elution profile of 25 ml of EIM solution from TEAE column. The absorbances of the eluted fractions recorded at two wavelengths (260 and 280 mμ) show that the two eluted regions are very different in their optical properties. The fractions eluted with acetic acid show essentially identical readings at both wavelengths, whereas the optical properties of the fractions eluted with 1.0 M LiCl are those of a nucleic acid solution.

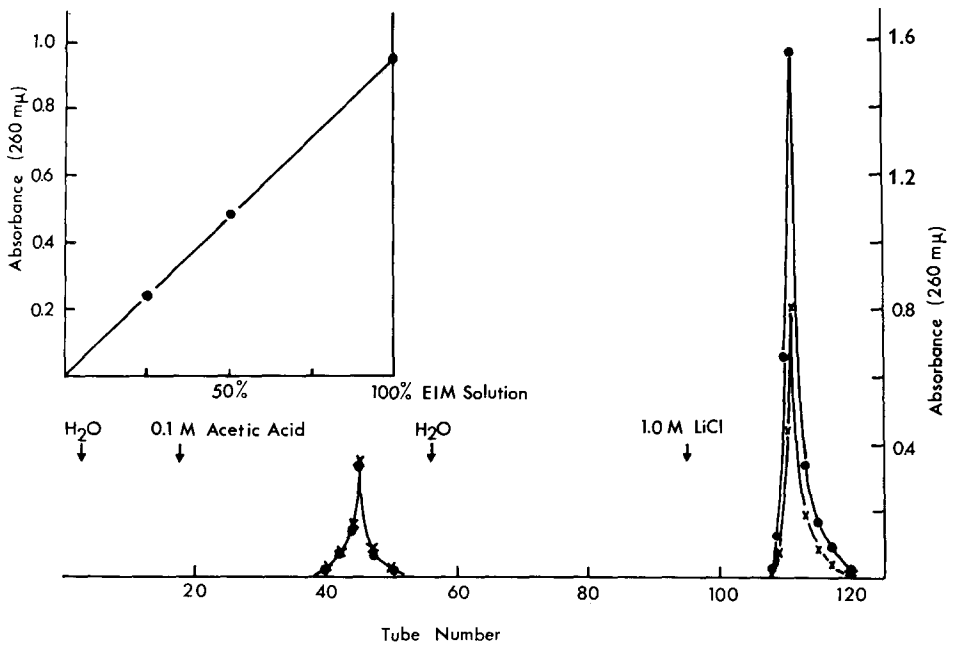

Fig. 2. Elution pattern of the EIM solution from TEAE-cellulose column. The adsorption and elution procedure as described in the text. ●—●, absorbance at 260 mμ; ×—×, absorbance at 280 mμ. Insert shows proportionality of absorbance at 260 mμ to concentrations of EIM solutions diluted with distilled water.

TABLE IV

"ANALYTICAL" CHROMATOGRAPHY OF EIM SOLUTION ON TEAE-CELLULOSE COLUMN (FIG. 1)

25 ml of EIM solution were introduced on the TEAE-cellulose column (2 cm × 18 cm). The absorbances of EIM solution and its 1/2 and 1/4 dilutions with distilled water at 260 mμ as a function of concentration are shown in the insert in Fig. 2. The EIM content of applied EIM solution and its recovery from the column was calculated by multiplying the volume of respective solutions by their absorbance (A) at 260 mμ.

Sample	*Volume (ml)*	*Tube No.*	*Absorbance*		*Recovery* $ml \cdot A_{260\,m\mu}$
			260 mμ	*280 mμ*	
EIM solution diluted 1:4			0.241	0.149	
EIM solution diluted 1:2			0.485	0.290	
EIM solution	25		0.95	0.555	23.75
0.1 M acetic acid region	38	42–49	0.099	0.091	3.76
H_2O wash	390	50–108	—	—	
1.0 M LiCl region	45	109–117	0.45	0.22	20.25
Σregions					24.01

Separately and when combined, none of the fractions nor the protein and RNA regions was active. The chromatography resulted in approx. 1/2 and 1/3 dilution of individual components and when combined of the total material, respectively. However, because the starting EIM solution was active at 1/200 dilution, the dilutions during chromatography are insignificant and cannot be the cause of lost activity. Complete elution of the material is indicated by the absorbances of eluted regions at 260 mμ as is shown in Table IV and by the insert in Fig. 2 (which shows linear correspondence of absorbance *vs.* dilution of EIM solution). Larger volumes (> 50 ml) of EIM solution introduced on the column cause aggregation of the material and consequently incomplete elution of moieties, ("preparative" chromatography).

The optical characterization of the EIM solution, RNA, and protein moieties is presented in Fig. 3. The comparison of EIM and RNA spectrograms shows that absorbance of EIM solution is largely governed by its RNA component. Protein moiety at the concentration present has a small contribution. Furthermore, the protein does not exhibit the $A_{280\,m\mu}/A_{260\,m\mu}$ ratio of 1.75 characteristic of most proteins, indicating a low content of tyrosine and tryptophan residues.

Electrophoretic and sedimentation studies

The electrophoretic patterns of lyophilized EIM, and RNA and protein moieties obtained by TEAE-cellulose chromatography of EIM, are presented in Fig. 4. All three samples show two homogeneous spots tentatively identified as nucleoprotein and RNA in case of EIM and EIM-RNA and as nucleoprotein and protein in case of EIM-protein. The essentially immobile nucleoprotein spot, very intense in the EIM sample, is only indicated in RNA and protein samples, similarly as in yeast RNA. Still, in view of a relatively high degree of purity of the separated moieties indicated by chemical analysis (less than 1 % contamination of moieties by each other), the apparently high content of nucleoprotein in both moieties (in the order of 10 %, as estimated by comparison with yeast RNA) may be fortuitous and due to strong non-stoichiometric complexing or to different staining capacities of the individual moieties and the complex.

The RNA moiety shows a pattern similar in spot mobilities and intensities to yeast RNA and in spot mobilities to EIM (the EIM shows reversed intensities of spots). On the other hand, the protein moiety shows a high color intensity spot with cationic properties, not evident with EIM, and a low intensity spot corresponding

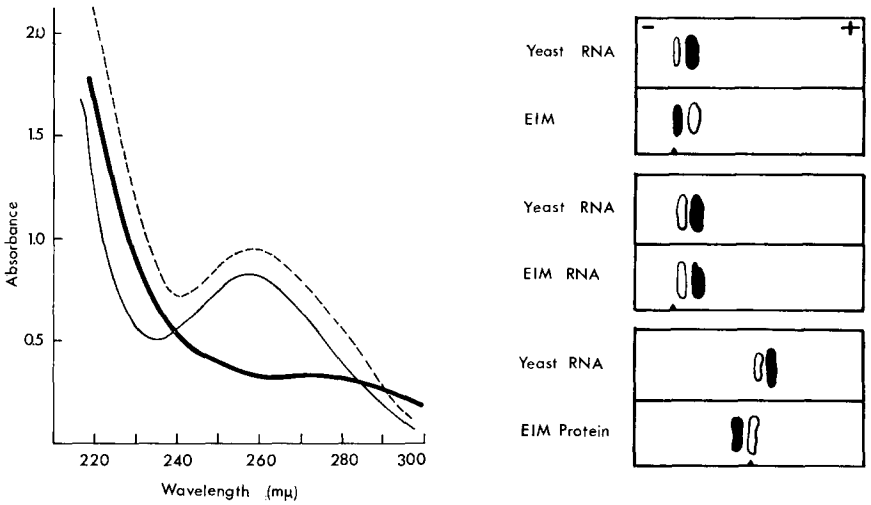

Fig. 3. Absorption spectra of EIM solution(- - - -), and its components, RNA (———) and protein (▬▬) obtained by TEAE-cellulose chromatography of EIM solution. Contents of the scanned solutions estimated from N, P and ribose values are in μg/ml: EIM solution: 250 protein, 56 RNA; RNA solution: 58 RNA, no protein; protein solution: 230 protein, 1.4 RNA.

Fig. 4. Electrophoresis of EIM and its components, RNA and protein, on cellulose acetate strips (3.5 cm × 12 cm) in Tris–EDTA–boric acid (40.0, 2.0 and 7.0 mmoles/l, respectively) buffer (pH 7.0). Yeast RNA type XI was run in parallel with each sample. Notch indicates the origin of the electrophoretic run.

to the nucleoprotein spot of EIM. It is evident that the cationic properties of the protein moiety of EIM are masked by RNA and become apparent only when most of the RNA is removed.

The electrophoretic analyses of lyophilized samples and yeast RNA type XI were run in pairs. The yeast RNA pattern identifies the intense, faster moving spot as RNA and the essentially immobile spot as protein or nucleoprotein; the nucleoprotein is more likely, because the proteins associated with nucleic acids are usually basic, and this characteristic is not shown here. Furthermore, the basic properties of EIM protein are shown with the separated moiety.

The presence of protein in yeast RNA type XI was shown by analysis. A molar ratio of N/P = 4.15 (P/ribose = 1.83) was obtained for this RNA in our laboratory, which, according to the estimates used (9 % and 15 % P and N, respectively, in RNA, 17 % N in protein) indicates approx. a 10 % protein content in this RNA preparation.

The electrophoretic analyses were conducted in buffers pH 4.0, 6.0, 7.0, 8.9 and 9.5. Two cellulose acetate strips, one with the respective sample and one with yeast RNA were analyzed together in each run. Two spots were obtained with the samples and yeast RNA at each pH. The best resolutions were obtained at pH 6.0 and 7.0.

The preliminary sedimentation velocity experiments were kindly performed by Dr. MARK S. LEWIS (National Institutes of Health, Bethesda, Md.) in a Spinco Model E analytical ultracentrifuge with schlieren optics at a maximum rotor speed 59780 at 20°. As shown in Fig. 5, the sedimentation of protein, RNA and EIM shows one component in each case. In view of the distinctly different sedimentation velocities of protein and RNA moieties, the gross homogeneity of EIM indicates complexing. The observed rates of sedimentation suggest relatively low molecular weight of EIM[9].

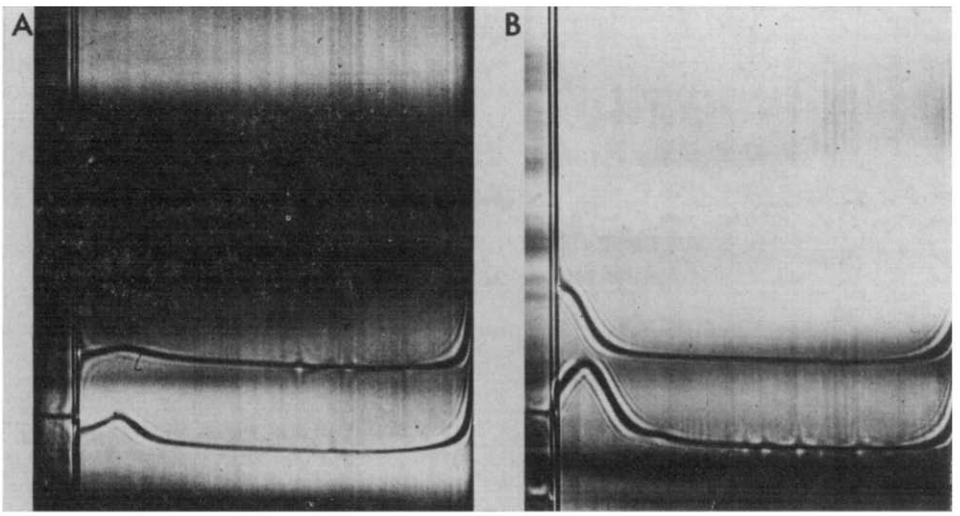

Fig. 5. Sedimentation pattern of EIM and its components, RNA and protein. A. Standard cell (lower pattern) EIM solution, and wedge cell (upper pattern) deproteinized EIM solution (dp-EIM) in 0.1 M NaCl. Photograph was taken 32 min after attaining 59780 rev./min at bar angle of 75°. dp-EIM solution was obtained by shaking equal volumes of EIM solution and chloroform–octanol (9:1, v/v). It contains approx. 1:1 weight ratio of protein to RNA. Discussion of its properties will be presented later. B. Wedge cell protein, and standard cell RNA in 0.1 M NaCl at 32 min, bar angle of 75°. Both moieties were obtained from EIM solution by TEAE-cellulose column chromatography.

Reactivation of separated moieties upon mixing

The capability of combined moieties to yield active material is now under investigation. Preliminary results obtained under randomly selected conditions of recombination are presented here.

Individually, approx. 0.8–1 % solutions in 0.1 M NaCl of both lyophilized moieties with 20 mM NH_3 per l added, show no, or only trace, activities, even if added one after another (in both sequences). On the other hand, these solutions, mixed in a 3:1 protein to RNA ratio and stored for 0.5 h (or longer) at 5°, show a dramatic increase in activity, being active when diluted 10–50 fold. Although the specific activities of the mixtures are quite low as compared to EIM solutions (3–4 orders of magnitude lower), a definite reactivation takes place under these conditions.

The individual components do not show reactivation when diluted to 0.1 % mixed and stored at 5°. It appears that diluted moieties lose the ability to reactivate. A similar result was found by FRAENKEL-CONRAT AND WILLIAMS[20] in attempting to reconstitute tobacco mosaic virus.

It may be of interest to note that in their reconstitution, the specific activities of reconstituted viruses were 2–3 orders of magnitude lower than of the original virus.

DISCUSSION

The objective of this investigation was to isolate and to characterize chemically the material which is responsible for inducing excitability in lipid bilayers under electrical stimulation. By extensive elimination, a defined "synthetic" medium, adsorbent, and proper conditions were selected, permitting solutions of active material to be obtained which have approx. 10000 times higher specific activity (per total organic material) than the crude "active" egg white and 100 times higher than purified "active" egg white.

Two moieties, which constitute the total analytically detectable material of EIM solutions, were found: protein and RNA. At present, gross homogeneity of both, indicated by electrophoretic studies and further supported by sedimentation studies is tentatively assumed. The data presented in this study suggests that these two moieties of opposite charge constitute the functional entity; a ribonucleoprotein "complex". The crucial importance of protein moiety in the production of the phenomena was shown by proteolytic digestion studies. Still, its only involvement is questionable in view of loss of activity when it is separated from RNA by column chromatography. The separation of the protein and RNA moieties from EIM solutions under relatively mild conditions of TEAE-cellulose column chromatography results in complete loss of activity. 1 % solutions of separated moieties show very poor, or no activity; but, when combined and stored at 5° for 0.5 h or more, show partial reactivation. Barring a possibility of some inhibition effect being present with individual moieties, these results imply that both moieties are part of the functional material.

The stability of EIM solutions to digestion with nucleases (isolated EIM-RNA was found sensitive to nucleases; to be reported later) indicates protective action of the protein and, consequently, some organization of the "complex", possibly not unlike in some RNA viruses. Furthermore the apparent homogeneity of EIM solutions during sedimentation studies also indicates strong complexing of the two components.

Although the presented data suggest that both detected moieties are necessary for the activity of the EIM solutions it is not possible at present to make definite conclusions as to the function of either component in the process. Several other possibilities may be envisioned in addition to nucleoprotein being the functional entity such as one of the moieties facilitating the penetration of the other or preventing its aggregation and thus making the process feasible. Furthermore it may be that an undetected functional trace component adhered strongly to TEAE-cellulose and was mostly lost on the column during chromatography thus causing loss of activity. These problems are now being investigated.

The activity of EIM solutions shows a complex relationship with the temperature and time of heating (to be reported) in contrast to the reported heat stability of EIM[5].

ACKNOWLEDGEMENTS

Thanks are given to Drs. D. O. RUDIN and P. MUELLER for helpful discussions during the course of this work. I wish to thank Mrs. M. GALE for excellent technical assistance and Mrs. T. ARTYMYSHYN for testing of samples.

REFERENCES

1 E. GORTER AND F. GRENDEL, *J. Exptl. Med.*, 41 (1952) 439.
2 H. DAVSON AND J. F. DANIELLI, *Permeability of Natural Membranes*, Cambridge University Press, London, 2nd ed., 1952.
3 A. FISHMAN, *Symp. on The Plasma Membrane, New York, 1961*, American Heart Assoc. Inc., New York, 1962.
4 W. R. LOEWENSTEIN, *Ann. N.Y. Acad. Sci.*, 137 (1966) 403.
5 P. MUELLER, D. O. RUDIN, H. T. TIEN AND W. C. WESCOTT, *Nature*, 194 (1962) 979.
6 L. R. BLINKS, *J. Gen. Physiol.*, 19 (1936) 633.
7 P. MUELLER, *J. Gen. Physiol.*, 42 (1958) 137.
8 A. FINKELSTEIN, *Nature*, 190 (1961) 1119.
9 P. MUELLER AND D. O. RUDIN, *Nature*, 213 (1967) 603.
10 A. M. CRESTFIELD, K. C. SMITH, AND F. W. ALLEN *J. Biol. Chem.*, 216 (1955) 185.
11 P. MUELLER, D. O. RUDIN, H. T. TIEN AND W. C. WESCOTT, in J. F. DANIELLI, K. C. A. PANKHURST AND A. C. RIDDIFORD, *Recent Progress in Surface Science*, Vol. 1, Academic Press, New York, 1964, p. 379.
12 J. FOLCH AND M. LEES, *J. Biol. Chem.*, 191 (1951) 807.
13 J. R. SARGENT, *Methods in Zone Electrophoresis*, The British Drug Houses Ltd., Dorset, England, 1966, p. 43.
14 L. MILLER AND J. A. HOUGHTON, *J. Biol. Chem.*, 159 (1945) 373.
15 G. R. BARTLETT, *J. Biol. Chem.*, 234 (1959) 466.
16 A. H. BROWN, *Arch. Biochem.*, 11 (1946) 269.
17 E. STAHL AND U. KALTENBACH, *J. Chromatog.*, 5 (1961) 351.
18 W. F. BLATT, M. P. FEINBERG AND H. B. HOPFENBERG, *Science*, 150 (1965) 224.
19 S. GARD AND O. MAALØE, in F. M. BURNET AND W. M. STANLEY, *The Viruses*, Vol. 1, Academic Press, New York, 1959, p. 414.
20 H. FRAENKEL-CONRAT AND R. C. WILLIAMS, *Proc. Natl. Acad. Sci. U.S.*, 41 (1955) 690.