Two aged samples of the combination tablets were assayed by the native fluorescence method, the colorimetric method, and the sulfuric acid fluorescence method. The data in Table VI show that the results by the native fluorescence method are much lower indicating that there may be a quenching effect operative. When the ethinyl estradiol was extracted from these sample mixtures and then assayed by the native fluorescence method, the results agreed with those obtained by the other two methods. This quenching effect was not noted with tablets containing only ethinyl estradiol.

SUMMARY

Application of the automatic analyzer to the analysis of ethinyl estradiol has been accomplished. The nonvarying volume, time, and temperature characteristics of this system have enabled the development of a repeatable assay procedure even for very low concentrations of EE. The procedure has been shown to be specific for intact EE in the presence of thermal and photochemical decomposition products. The procedure was also demonstrated to be specific for EE in combination with large amounts of the usual progestational steroid hormones and in the presence of large amounts of other similar estrogenic substances. A number of trade packages of antifertility products were successfully analyzed by the automated method. Twenty samples per hour can be handled easily as opposed to one to two samples per hour by conventional methods. This laboratory has found the automated method very convenient for determining unit-to-unit variation in formulations.

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New System of Disk Electrophoresis Using Large Acrylamide Gels

By ROBERT C. PETERSON

The disk electrophoretic method has been modified to use gels of 22-mm. diameter. A gel tube base was designed to produce a gel with a convex base, and a special buffer cell was provided. With the future goal of preparative electrophoresis, a quenched fluorescence technique was developed which locates the major protein zones without dyeing. A transverse destainer was designed for rapid removal of unbound dye from stained protein patterns.

THE THEORY, methods, and applications of gel electrophoresis have been well reviewed (1, 2). It has been our purpose to develop a simple, convenient method of columnar electrophoresis which could be adapted to preparative electrophoresis. Preliminary work has indicated that this approach is very promising.

The method of Ornstein (3) and Davis (4) has been modified to give gels 22 mm. in diameter by about 11.4 cm. in length.

METHODS AND MATERIALS

The pilot compound for this work was N.F. trypsin crystallized reference standard, lot 6040, 3226 N.F. units/mg. (5). The pH 5 buffer, pH 4.3 gel, and solutions for a gel concentration of 15% have been described.[1] Acrylamide, *N,N'*-methylenebisacrylamide, and β-alanine were of good purity.[2] *N,N,N',N'*-Tetramethylethylenediamine was practical grade, redistilled.[3] The ammonium persulfate and riboflavin were reagent grade.[3] The acetic acid was reagent grade.[4]

The gel tube was placed in the new base (see Fig. 1), and 40 ml. of lower gel ingredients[1] were introduced with the usual water layering. Teflon was selected for the base material in order to give less wear and a minimum of contamination. The 12.7-cm. long glass tube fits snugly within the O-ring and on the shelf in plug C.

When plug C is threaded into block B, the concavity in plug C forms the gel base for glass tube A. This rounded gel base (convex on the gel) is neces-

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[1] Canal Industrial Corp., Bethesda, Md.
[2] Eastman Organic Chemicals, white label.
[3] Matheson Coleman and Bell.
[4] Baker and Adamson.

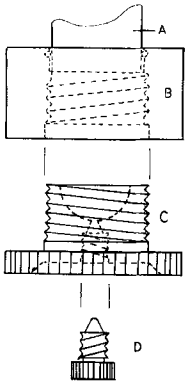

Fig. 1—Gel tube base. Key: A, glass gelation tube; B, Teflon block fitted with O-ring to hold glass tube and threaded to take plug C; C, Teflon plug milled to form base for gelation tube, threaded to fit block B and also to admit air plug D; D, plug to admit air.

sary. During polymerization, larger gels such as this tend to contract at the bottom and become concave. This cavity will collect gases during electrophoresis which stop the flow of current. A convex gel bottom as we have prepared will not hold these gases.

Plug C is made removable from block B in order to leave a minimum of surface holding the gel tube; this reduces the chances of detaching the gel from the tube when the tube is removed. Plug D is necessary to provide access of air to plug C before it is removed; otherwise, suction is created when plug C is removed which detaches the gel. This base has worked very satisfactorily.

The critical dimensions of this gel tube base assembly can be furnished by the author upon request.

The polymerization of these larger gels produces considerable heat; this heat can form cavities in the gel from dissolved gases which separate the gel from the tube. However, if polymerization is carried out in a refrigerator (about 6°), it is successful very nearly 100% of the time. Gelation is accomplished in about 1 hr.; this can be detected by the disappearance of heat from the gel tube.

For use with trypsin, the gels were pre-run overnight without spacer gel to remove possible interfering contaminants. Fresh buffer was used for the actual run. It was found empirically that this treatment improved resolution and definition. The electrophoresis assembly is illustrated in Fig. 2. The vertical glass tube buffer compartment is 40 mm. in diameter (o.d.) by 153 mm. The lower rectangular buffer compartment is made from 1/4-in. Lucite and measures 152 mm. long, 114 mm.

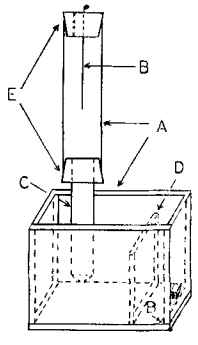

Fig. 2—Electrophoresis assembly. Key: A, buffer compartments; B, platinum electrodes; C, glass gel tube; D, baffle plate; E, rubber stoppers.

wide, and 108 mm. deep. The baffle in this compartment is just covered when the volume is 1100 ml. This baffle aids in keeping electrophoresis gases away from the gel.

Before the actual run, 8 ml. of spacer gel solution was added in the usual manner[1] above the small pore gel and photopolymerized for about 20 min. Trypsin, 5 mg., was added in 0.5 ml. of pH 5 buffer[1] containing 30% sucrose w/v.

Movement of trypsin components is downward when the upper electrode is made the anode. The d.c. power source was variable voltage regulated.[5] The fractionation was carried out for 9 hr. at 200 v. and about 24 ma. Both the pre-run and fractionation were done in the refrigerator (about 6°). Removal of the gel was accomplished by rimming with a 22-gauge hypodermic needle through which distilled water was continually forced from a syringe. Immediately upon removal, the gel was placed on a fluorescent plate (see Fig. 3) under a low wavelength (253.7 mμ) mercury lamp.[6] The gel must be lying over the slot with its long axis horizontal to the slot. Zones of highly concentrated protein appear as medium to dark blue zones where the fluorescence of the green fluorophor[7] is quenched. Figure 4 shows a sketch of the quenched pattern above the stained pattern of the same gel. The dye solution is 1% naphthol blue black[8] w/v in methanol–acetic

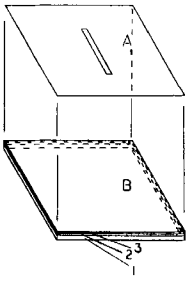

Fig. 3—Fluorescent plate. Key: A, black paper mask with slot (4 1/4 in. × 3/16 in.); B, glass plate with indicated layers (1, 6-in. square glass; 2, sprayed coating of fluorophor; 3, dipped coating of paraffin).

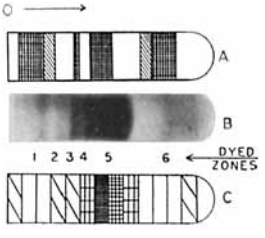

Fig. 4—Key: A, pattern from quenched fluorescence; B, same gel as A after staining protein zones; C, pattern of proteolytic activity of similar, unstained gel (see text).

acid–water (5:1:5 by vol.). The gel is kept in the dye solution for 48 hr. before destaining. The authors have found that an aqueous acetic acid solution of the dye is insufficient in its complexing of the protein to prevent zone removal during destaining. Also in this figure is shown a sketch of the pattern of proteolysis of gelatin[9] as given by a gel column run in identical fashion. The gel was not stained, and transverse sections about 0.25 in.

[5] Heath, model PS-3, Benton Harbor, Mich.
[6] Chromato-Vue Cabinet, model C-3, Ultra-Violet Products, San Gabriel, Calif.
[7] Luminescent Chemical No. 161 (p-1), lot CEX-3B; this was kindly donated by Sylvania Electric Products, Inc.
[8] C. I. No. 20470, Allied Chemical Corp., New York, N. Y.
[9] Peter Cooper Corp., Oak Creek, Wis.

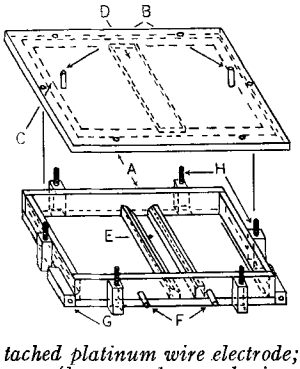

Fig. 5—Transverse destainer. Key: A, *Lucite cover and electrolyte compartment;* B, *gas vents and electrolyte inlets;* C, *rubber gasket (1/8 in. × 5/16 in.);* D, *Lucite strip (1/8 in. × 1 in. × 4-9/16 in.);* E, *gel compartment (see text);* F, *drains for removing electrolyte;* G, *banana plug socket with attached platinum wire electrode;* H, *brass bolts to hold cover (brass washers and wing nuts are not shown).*

thick were incubated on the plate of gelatin at room temperature for 25 hr. The gelatin, approximately 15% w/v, had been dissolved with heat in pH 7.6, 0.067 *M* phosphate buffer and hardened in a refrigerator before use. Correlation of most zones is seen with the dyed pattern and the quenched fluorescence and proteolytic pattern. These will be discussed later.

When using the transverse destainer, it was noticed that after most of the blue dye was removed some red dye remained. Multiplicity of the dye was suspected. Paper chromatography did indeed show 2 distinct zones, blue and red. Further investigation by cellulose acetate[10] electrophoresis showed 4 zones. The buffer was formic acid (90%)–pyridine–water (15:2.5:982.5 by vol.), pH 2.65. The main blue pigment stayed at the origin; at its tip was a small spot of brown pigment. Both of these pigments were preceded by 2 red pigments. Obviously this dye consists of at least 4 pigments. It appears that, in particular, those investigators who are quantitating proteins with this dye for clinical study should consider the possible variability in dye absorption by various proteins and the variability in wavelength of absorption among these pigments.

A transverse destainer has been designed and used in these laboratories for nearly 2 years. It is shown in Fig. 5. Other destainers using the same principle have been made (6–10), but to the author's knowledge these have been for 5–6-mm. gels or slabs. Although not shown in the figure, the 2 strips on either side of the gel compartment are each drilled through with about 100 holes, $^1/_{32}$ in. in diameter. The holes are staggered to give uniform current transfer. Destaining of the gel can be accomplished within 4 to 8 hr., or if more convenient, overnight at 50 to 100 v. (50 to 100 ma.). Four per cent acetic acid was the electrolyte.

RESULTS AND DISCUSSION

This system gives 6 to 7 zones in the dyed pattern of trypsin N.F. The electrophoretic pattern can be duplicated. This resolution can be compared with 2 zones by paper electrophoresis (11) and 4 zones by cellulose acetate electrophoresis (12). The variation in number of zones occurs with the light, lead zones in the region of zone 6. They vary from 1 to 2. These, to the best of the author's knowledge, have not been noted before. No attempt was made to identify these proteins, but from electrophoresis on cellulose acetate, followed by reaction with specific substrate (12), one would predict that the dark and largest zone would contain the tryptic activity.

Acrylamide gel electrophoresis of this same trypsin in the commonly used 5-mm. gels and with the same electrolyte system has shown only 4 or 5 zones. It seems that the larger gel gives much greater resolution partly due, no doubt, to the larger amount of material (5–15 mg.) which can be run and which results in being able to see what are probably mcg. quantities in a separated zone. It is felt that this approach is ideal for preparative electrophoresis. Since, in most cases, the desired compound already will be 80–90% pure, the major zone can be located by quenched fluorescence and sectioned out, and the purified compound can be removed by various means from the gel for further purification, if needed. Also, it is felt that this method will give better separation of zones both mechanically and electrically than the familiar continuous electrophoresis methods.

From Fig. 4, good correlation is seen for the major zone in quenching of fluorescence, staining, and proteolytic activity. A small amount of proteolytic activity is found in other areas of the gel. This indicates the probable presence of other proteolytic enzymes, such as chymotrypsin. The considerable quenching of fluorescence which is seen in the region of zone 6 routinely is found in the electrophoresis of trypsin. Its cause is not understood.

The broad, quenched area in the region of dyed zones 1 and 2 is considered to be the effect of scattered light from these zones.

Any possibility of the zones, other than the main one, being artifacts seems very unlikely. The pattern can be duplicated. In addition, the proteolytic pattern indicates the difference in activities shown by the various zones. Possibly more conclusive is an experiment in which the major zone (zone 5) was cut out using the fluorescent plate (a butter and cheese cutter is excellent) and placed on top of a new gel column. Conditions were the same except that the run time was slightly longer. The stained pattern had 2 zones, the major one and a very light following zone which probably was a portion of zone 4 that was cut out with it. The gel section which had been placed on top also was dyed and destained. It showed no protein zones.

It has not been a purpose here to quantitate by densitometry or other means the stained protein zones of these larger gels. The aim is to use large gels for preparative electrophoresis of practical amounts of various compounds of high purity, and the work noted in this paper is considered to be a step in this direction. With the fluorescent plate method of locating the major zone, the greatest hurdle has been eliminated. It should be mentioned that gels 2 in. in diameter have been used with good results.

[10] Oxoid. Colab Laboratories, Inc., Chicago Heights, Ill.

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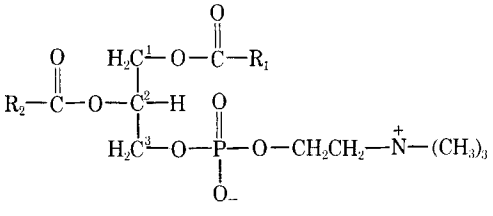
Notes

Solubilization and Rate of Dissolution of Drugs in the Presence of Physiologic Concentrations of Lysolecithin

By THEODORE R. BATES*, SONG-LING LIN, and MILO GIBALDI†

The micellar solubilizing properties of physiologic concentrations of lysolecithin for hexestrol, dienestrol, and griseofulvin are demonstrated. The extent of solubilization was found to decrease in the following order: hexestrol > dienestrol ≫ griseofulvin. Dissolution rate studies showed that lysolecithin significantly enhances the rate of solution of hexestrol, dienestrol, and griseofulvin. A role for this physiologic surfactant in the absorption process of hydrophobic drug molecules is explored.

HUMAN BILE is chiefly composed of cholesterol, calcium, bile salts, and phospholipids. Lecithin is the major phospholipid component of bile. It belongs to the class of compounds referred to as phosphatidylcholines (I).



R_1, R_2 = alkyl chains of fatty acids

I

The enzyme, phospholipase A, is capable of removing only one of the fatty acids from the lecithin molecule to form either 1- or 2-acyl lysolecithins. Phospholipase A is found in abundance in snake venom (*Crotalus adamanteus*) and also occurs in humans and animals. Its presence has been established in the mucous membrane of the small intestine (1), in duodenal fluid (2, 3), in the pancreas (4–6), and in blood serum (7). Commercially available lysolecithin is usually prepared by the enzymatic action of snake venom on purified egg lecithin. This material has palmitic or stearic acid in the 1-position of the glycerol moiety, since snake venom phospholipase A specifically catalyzes the hydrolysis of the fatty acid linkage at the 2-position (8–11).

Aqueous solutions of lysolecithin exhibit a more or less abrupt change in their physical properties over a narrow concentration range suggesting the formation of aggregates or micelles. The marked surface activity of this compound as well as other physical properties of its solutions have been reported by a number of investigators (9, 11–18). Surface tension *versus* concentration curves indicate that the critical micelle concentration (CMC) for this compound is in the concentration range of 1–2 $\times 10^{-3}$% (11, 14).

One of the most interesting properties of surface-active agents is their ability to bring into aqueous solution, at concentrations above the CMC, otherwise water-insoluble compounds. This phenomenon is known as micellar solubilization (19). There have been only a limited number of reports demonstrating the solubilizing ability of lysolecithin. Robinson and Saunders (20) found that aqueous solutions of lysolecithin possess a marked solubilizing power for cholesterol, triolein, and monostearin.

Lysolecithin appears to occur in small concentrations in human bile (21) and in high concentrations in the contents of the duodenal region of the small intestinal lumen (2). On examination of human small intestinal contents, following a test meal free of phospholipids, Borgström found that most of the phospholipids contained therein were in the form of monopalmitoyl-lysolecithin. This material was identical to that prepared by the action of snake venom on purified egg lecithin. The presence of phos-

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* Present address: Pharmaceutics Department, School of Pharmacy, University of Connecticut, Storrs, CT 06268