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DISC ELECTROPHORESIS OF CITRUS FRUIT PROTEINS

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Abstract—A new preparative procedure was used in conjunction with disc electrophoresis to study proteins in vesicles, segment membranes, albedo, and flavedo of the Washington Navel orange, and vesicles and peels of lemon and grapefruit. A cationic gel system and an anionic gel system were applied to each tissue, and produced characteristic and reproducible patterns for each source. Diagrams and densitometric tracings of the gels are presented.

INTRODUCTION

THE nitrogenous components of citrus fruits have been studied rather intensively, and the data have been summarized in several reviews.[1-3] In general, however, studies of proteins of different varieties of citrus have been hampered by those difficulties encountered with most fruits: low protein levels, low pH, and the presence of deleterious substances. Most investigations have not been concerned with isolation of proteins in their native form, but rather with the presumably denatured material present in expressed juices or extracted from macerated tissues. Thus, the data provide information regarding nitrogen distribution and amino acid compositions, but reveal little regarding the nature of the proteins in the intact fruit.

Recently the author reported a promising technique for the extraction of protein from fruit tissues.[4,5] This procedure, coupled with disc electrophoresis has produced strong, reproducible protein patterns from each citrus variety and tissue studied, and should provide a basis for further qualitative and quantitative investigations. The following report presents results of a preliminary study of different tissues of three varieties of citrus.

RESULTS AND DISCUSSION

Yields of solids, and Kjeldahl nitrogen contents, of powders and extracts (0·1 M phosphate buffer, pH 7·5, containing 25% sucrose and 50 μM EDTA) are presented in Table 1. Nitrogen content of the vesicle powders ranged from 3·2 to 3·7 per cent. If these values represent purely protein nitrogen, the usual 6·25 conversion factor indicates these powders contain over 20 per cent protein. The phosphate buffer medium extracted from 55 to 72 per cent of this nitrogen under the conditions employed. Peel preparations contained much less nitrogen, but 65 per cent or more was extracted. In general, 0·01–0·05 ml vesicle extract, or 0·05–0·30 ml peel extract, provided optimum loads for disc electrophoresis. From the foregoing values, this would represent approximately 100–500 μg protein, a range which approximates

[1] J. F. KEFFORD, *Adv. Food Res.* **9**, 285 (1959).
[2] L. B. ROCKLAND in *The Orange* (Edited by W. B. SINCLAIR), p. 230. University of California, Berkeley, Calif. (1961).
[3] R. L. CLEMENTS and H. V. LELAND, *J. Food Sci.* **27**, 20 (1962).
[4] R. L. CLEMENTS, *Anal. Biochem.* In press.
[5] R. L. CLEMENTS, *Am. Soc. Hort. Sci.*, Annual Meeting, Urbana, Ill. (1965).

that found useful for serum proteins. This is a rough indication that the nitrogen extracted was essentially proteinaceous. The effects of pH, ionic strength, and various additives have as yet to be determined, and these factors may be expected to have both qualitative and quantitative effects on the extraction.

TABLE 1. LOW-TEMPERATURE ACETONE POWDERS OF CITRUS FRUIT TISSUES YIELDS AND NITROGEN CONTENTS

Fruit	Tissue	Solids as % of fresh wt.	N in solids (%)	N in ext.* (mg/ml)	N extd. from solids (%)	N in solids as % of fresh wt
Navel orange	Vesicles	2·8	3·2	1·5	69	0·09
	Seg. membrane	-	1·7	-		
	Albedo	18·2	0·9	0·7	100	0·16
	Flavedo	17·0	1·7	1·1	65	0·29
Grapefruit	Vesicles	2·0	3·2	1·6	72	0·06
	Peel	16·2	1·0	0·5	70	0·16
Lemon	Vesicles	0·9	3·7	1·4	55	0·03
	Peel	12·3	1·4	0·7	78	0·17

* 0·1 M phosphate butter (pH 7·5) containing 25% sucrose and 50 μM EDTA.

Anionic System

A diagrammatic presentation of the patterns of gels from the anionic system[6] adjusted to a common frontal distance is shown in Fig. 1. Since R_f values varied with frontal migra-

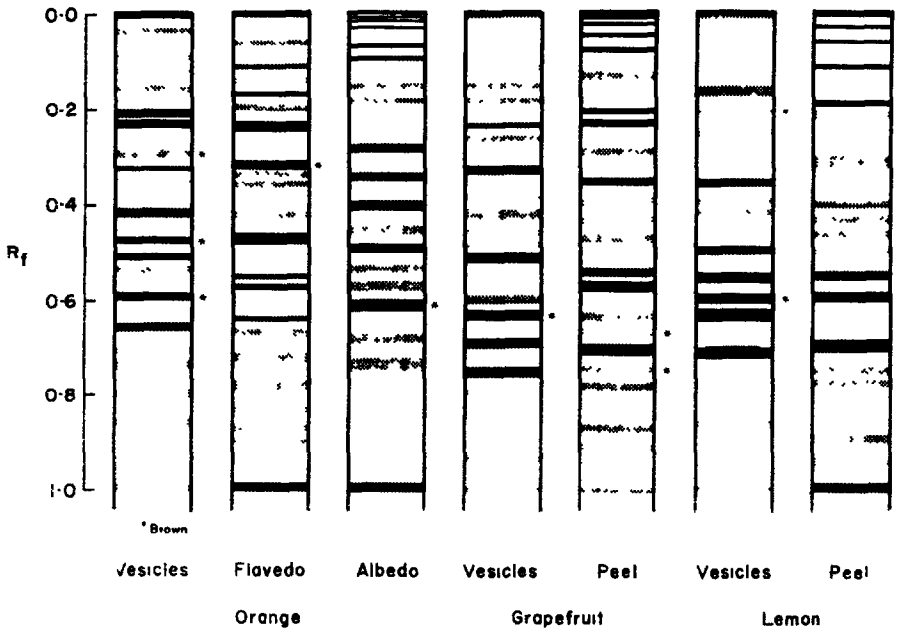

FIG. 1. DIAGRAMS OF DISC-ELECTROPHORETIC PATTERNS OF CITRUS PROTEINS, ANIONIC SYSTEM, ADJUSTED TO A COMMON FRONTAL DISTANCE.

B. J. DAVIS, *Ann. N.Y. Acad. Sci.* **121**, 404 (1965).

tion, the diagrams were prepared from gels in which the salt fronts had migrated equal distances (± 2 mm). Each diagram was prepared from a single specimen, but the specimens were selected as typical representatives of many gels obtained from each sample. Many bands which can be distinguished by visual inspection may not appear in photographs of the gels, the diagrams provide a means for illustrating these components. Figure 2 presents densitometric scans of typical gels.

In view of the limited sampling, the patterns are tentative. However, repeated analyses of each sample resulted in gel patterns which appeared to be characteristic with respect to both

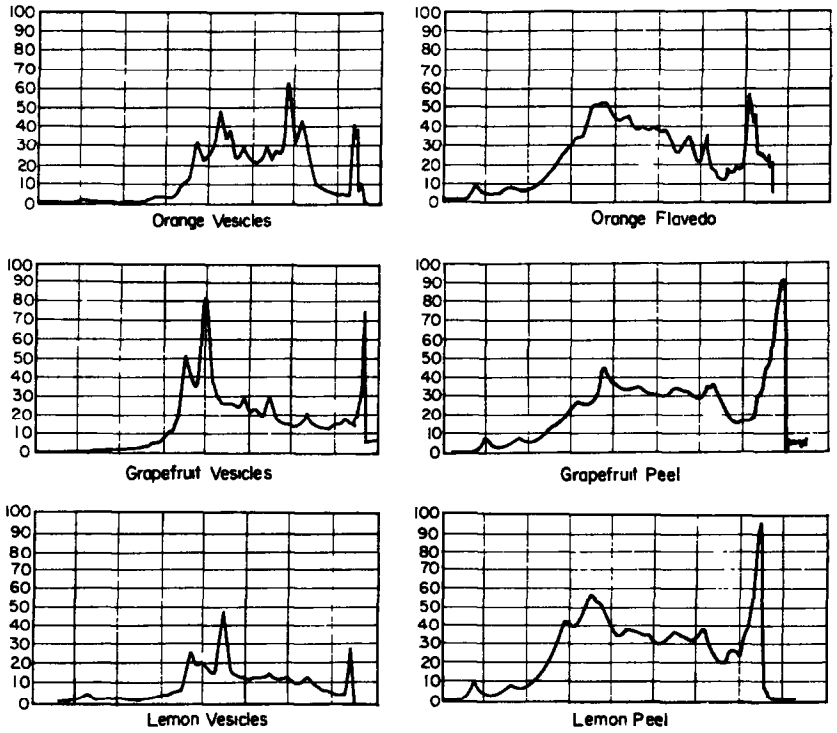

FIG. 2. DENSITOMETRIC TRACINGS OF DISC-ELECTROPHORETIC PATTERNS OF CITRUS PROTEINS: ANIONIC SYSTEM.

variety and morphology. Each tissue produced approximately twenty bands, including from one to five intense bands. The patterns can best be compared by inspecting the figures, and description is largely superfluous. However, some interesting points of comparison should be mentioned. Most of the bands appeared in a uniform shade of blue-black, while others appeared as a bright blue; other bands were conspicuous for a characteristic brown tone. The latter have been indicated in the diagrams.

Vesicles of the orange produced a pattern containing several intense bands interspersed with secondary bands. The flavedo, however, presented a continuous spectrum of bands of rather uniform intensity, with three appearing somewhat more prominently. The albedo profile was of similar (but weaker) quality, with a single strong band near R_f 0·5 appearing

to correspond to one of the more intense bands in the vesicle patterns. The membrane powder produced a pattern essentially the same as that of the albedo, and is not shown. Resolution was excellent in gels from each of the four tissues, but close inspection was required to distinguish individual bands in the rather homogeneous patterns from the peel and membrane tissues.

The grapefruit vesicles produced a profile similar to that from the orange vesicles. However, a strong brown band near R_f 0·5 in the orange was weak or lacking in the grapefruit pattern. The lemon vesicles produced a very strong blue band near R_f 0·65, followed closely by a brown band near R_f 0·60 and another blue band near R_f 0·55. A somewhat weaker band near R_f 0·70 was usually conspicuous because of a tendency to diffuse. In earlier investigations,[4] two of these bands occasionally appeared as two sets of doublets, but this did not occur in the present study.

The grapefruit and lemon peels represent albedo combined with flavedo, and produced many relatively sharp bands. The two profiles were strikingly similar, with the most obvious differences appearing in the upper part of the gels, and near R_f 0·65. Although separation of albedo from flavedo may be quite arbitrary, the differences exhibited by these two fractions from the orange indicate protein studies of citrus peel will be more significant if this preliminary separation is performed.

Under standardized conditions, a particular fruit powder yields a characteristic and reproducible pattern. However, experiments indicate the R_f value of a protein is subject to variation and is apparently influenced by non-protein matter in the sample. Thus, the same protein may appear in two different samples, but may exhibit different R_f values. This has been substantiated by preliminary experiments in which extracts from two different sources (e.g. albedo and flavedo) were combined, and coincidence or non-coincidence of bands was determined. In comparing profiles, these variations must be considered. In general, it appears that the patterns have more bands in common than the diagrams indicate. Preliminary fractionations, and the use of mixed samples, specific stains, and other techniques, should permit more valid comparisons.

Cationic System

Application of the cationic system[7,8] provided an interesting contrast (Figs. 3 and 4). Since the pore size in this system was presumably identical to that of the anionic system, the differences in patterns must be primarily due to the effects of pH on mobilities. The patterns illustrated were obtained by using the cationic "upper gel" (pH 5·8) rather than phosphate buffer (pH 7·5) as an extraction medium. However, the phosphate medium produced essentially the same results, indicating differences in pH of the extraction medium were not responsible for the variation in patterns. In each specimen, a very heavy band appeared at the salt front, and it was assumed that this band represented a mixture of unresolved proteins. However, a number of slower bands appeared, and provided distinctive patterns for each source. Because of the simplicity of these profiles, the differences are immediately evident.

Interesting points of comparison include a strong band near R_f 0·95 which appeared in orange vesicles, but which appeared only faintly in grapefruit vesicles and was lacking in gels from the other sources. Gels from lemon vesicles were characterized by a prominent band near R_f 0·83 (but which did not correspond to a band near R_f 0·85 present in gels from

[7] R. A. REISFELD, U. J. LEWIS and D. E. WILLIAMS, *Nature* **195**, 281 (1962)
[8] Y. NAGAI, J. CROSS and K. A. PIEZ, *Ann. N.Y. Acad. Sci.* **121**, 494 (1965)

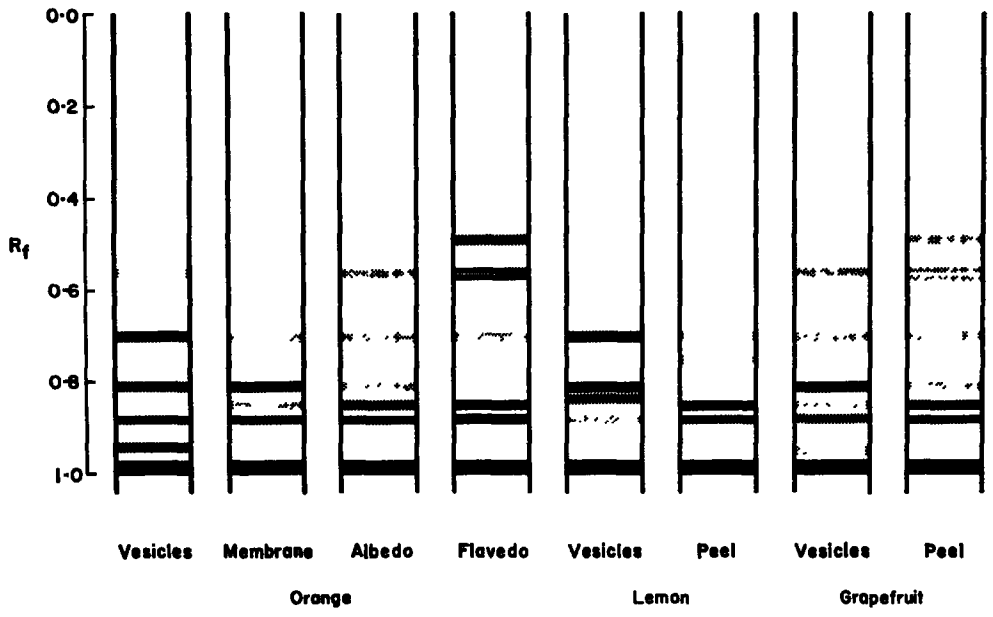

FIG. 3. DIAGRAMS OF DISC-ELECTROPHORETIC PATTERNS OF CITRUS PROTEINS: CATIONIC SYSTEM, ADJUSTED TO A COMMON FRONTAL DISTANCE.

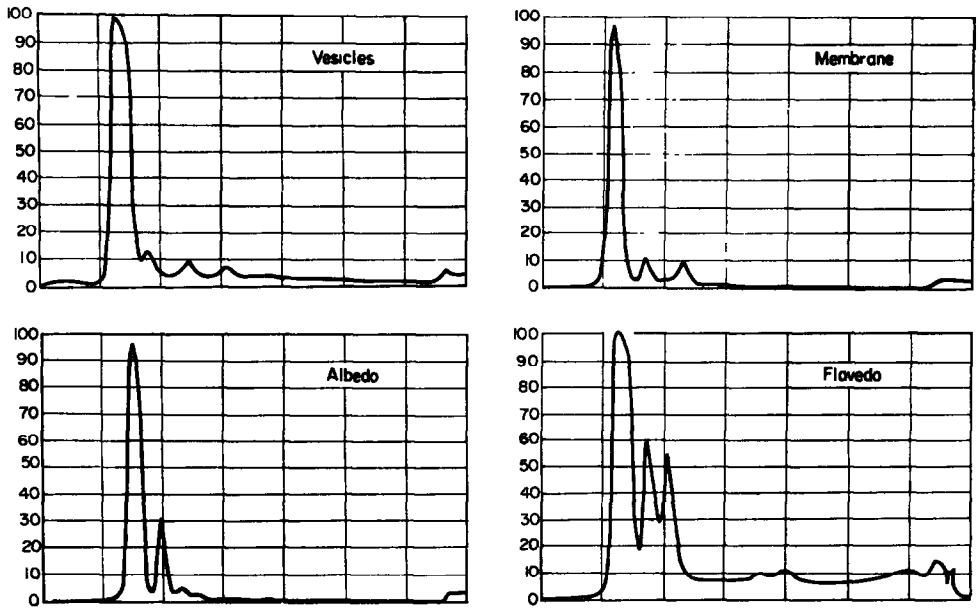

FIG. 4. DENSITOMETRIC TRACINGS OF DISC-ELECTROPHORETIC PATTERNS OF PROTEINS FROM VARIOUS TISSUES OF NAVEL ORANGE: CATIONIC SYSTEM.

most of the other sources). A characteristic of orange flavedo and peels from lemon and grapefruit was a pair of intense bands in the range R_f 0·85–0·90. Orange flavedo and grapefruit peel produced a weak band near R_f 0·50, and a sharply defined doublet near R_f 0·55. Negligible staining material remained at the origin in gels from this system.

General Conclusions

In general, previous studies of citrus proteins were performed on tissues which received rather drastic treatment prior to, or during, protein isolation. This has been particularly true of pulp tissues, in which the acidic contents of vacuoles have been released and mixed with cytoplasm, particulates, cell walls, and other protein-bearing components. The resulting solid and liquid fractions were often subjected to further extremes in pH, temperature, chemical environment, and other factors which might be expected to alter the native state of the proteins. The procedures utilized in the present study were designed to minimize these effects in so far as possible to provide a valid picture of protein composition of the intact fruit. It is significant that each source produced a characteristic, reproducible pattern. These results indicate that further studies of the citrus proteins will be complex, but that, under a particular set of conditions, a rather finite and unique array of components will be involved. The evidence suggests, however, that refinements in methods will reveal many additional components. This picture is in contrast to that presented by previous studies in which the protein of a tissue or extract was "isolated" and characterized as a single component.

It will undoubtedly be necessary to modify both the preparative procedure and the electrophoretic systems to adapt to the requirements of a particular study. Electrophoresis, in conjunction with gel filtration and ion-exchange methods, should be especially useful. Preliminary fractionation of fruit into various morphological and cytological units will aid materially in determining distribution of proteins within the fruit, and will simplify individual profiles. Characterization of profiles and individual proteins will permit application of the technique to many aspects of citrus biochemistry, including studies of enzymes, growth and maturation, postharvest behavior, pathology, and systematics.

EXPERIMENTAL PROCEDURE

The preparative and analytical procedures have been described in a previous paper.[4] For study of the citrus fruits, the procedures were modified or extended as described.

Preparation of Tissue

All fruit was purchased at local markets. The Washington Navel orange and lemon (variety unknown) were from California; the grapefruit (Marsh seedless) was Florida-grown.

Preparation of orange tissues was performed by first removing the flavedo from chilled fruit with a stainless-steel knife, followed by quick-freezing and homogenizing in acetone at −65 as previously described.[4] This slurry was allowed to stand several hours over dry ice, filtered and washed with acetone without elevation of temperature. The acetone was replaced with fresh acetone at −65 , and the suspension was stirred prior to elevation of temperature to −25 . This preliminary washing removed most of the oil and pigment before extraction of water and water-soluble components. The final precipitate was washed several times with acetone and acetone–ether (1:1) before drying. Albedo, segment membranes, and vesicles (without seeds) were processed separately, without the preliminary low-temperature washing. In each instance, the proportion of acetone to fresh tissue was approximately

3:1, but in some preparations (e.g. membrane) the sample was less than the usual 250 g. The procedure for preparation of lemon and grapefruit peels was identical to that used for orange flavedo. Vesicles were processed like those of the orange, but the segment membranes were discarded.

Disc Electrophoresis

Two different polyacrylamide systems were employed. The first method was essentially the anionic system of Davis,[6] as used in the initial studies. The second method was the cationic system developed by Reisfeld *et al.*,[7] as applied by Nogai *et al.*,[8] for collagen studies. Tube dimensions were either the standard or the elongated tubes used in the previous fruit study. Lower gels were 7·5% polyacrylamide, and maximum current was 3 mA per tube.

Fruit powders were extracted immediately after drying for application to the tubes. For the anionic system, the extraction medium was 0·1 M phosphate buffer, pH 7·5, containing 25% sucrose and 5×10^{-5} M EDTA. The powder (0·5 g) was stirred into the buffer (7·5 ml), and after 1–2 hr at 0°, the slurry was centrifuged at 20,000 *g* for 30 min. Supernatant (0·01–0·30 ml) was applied directly to the spacer gel without addition of large-pore gel, and voltage was applied immediately. For the cationic system, the same extraction and application procedures were used initially. However, sharper separations (but qualitatively identical) were obtained by substituting cationic upper-gel solution (pH 5·8) for the phosphate buffer. After centrifuging, the supernatant was applied to the tubes and photopolymerized prior to application of voltage.

Gels were stained with 1% Amido Schwartz in 7% acetic acid for 1 hr and excess stain was removed by electrophoresis from the anionic gels, and by washing from the cationic gels. For photography and densitometry, gels were placed in glass tubes. The densitometric tracings were made with a Photovolt Densicord Model 542, used without a filter, and at a response setting of "3". The slit width was 0·1 mm.

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