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DISC ELECTROPHORESIS OF ANALOGOUS ENZYMES IN *HORDEUM*

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Abstract—The genus *Hordeum* has been surveyed for electrophoretically distinct forms of esterase, peroxidase, catalase, glutamic acid (GDH) and malate dehydrogenase (MDH). "Critical" enzymes like GDH, MDH and catalase were the same in all species, but esterases and peroxidases, which have broad substrate specificities, exhibit both inter- and intra-specific polymorphism. No variant enzyme types were detected in a limited population of a cultivated "pure line" barley variety. The resemblances between zymograms of the species examined agree with the affinities based on cytogenetic analyses. The limited data obtained does indicate the potential usefulness of this information for taxonomic and evolutionary studies.

INTRODUCTION

ZONE electrophoresis, in spite of its limitations, provides a handy tool for the evaluation of gross structural similarities between the proteins from related taxa. Structural similarities between analogous (similar in function) proteins reflect genetic homologies. The importance of considering macromolecules for the evaluation of phylogenetic and evolutionary trends in living organisms have been stressed.[1] The usefulness of electrophoretic techniques in plant systematics has been described earlier.[2] Enzyme activity revealed as bands on the gels by specific enzymological methods, at least, implies functional similarity and analogy of the proteins being compared.[3] Other advantages of considering enzyme proteins have been discussed,[2,3] e.g. in the study of species relationships. Recently, electrophoresis of analogous enzymes has been carried out in the Fabaceae,[4] among the members of Triticinae[5,6] and in *Brassica* species.[7] The technique has also been used in taxonomic studies in fungi.[8] This report deals with electrophoretic study of esterase, peroxidase, catalase, glutamic acid (GDH) and malate (MDH) dehydrogenase in genus *Hordeum*. Attention was given to: (1) enzyme polymorphism during ontogeny, and tissue specific isoenzymes; (2) inter-species variation; and (3) assessment of esterase polymorphism in cultivated varieties of barley from different geographic regions.

RESULTS

Representative zymograms for the enzymes investigated are shown in Fig. 1. Esterase variation within a "pure line" population of *H. vulgare* CV 292 was studied in over 200 individual coleoptiles, but no variant forms were observed.

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[2] D. BOULTER, D. A. THURMAN and E. DERBYSHIRE, *New Phytol.* **66**, 27 (1967).
[3] G. E. HART and C. R. BHATIA, *Can. J. Genet. Cytol.* **9**, 367 (1967).
[4] D. A. THURMAN, D. BOULTER, E. DERBYSHIRE and B. L. TURNER, *New Phytol.* **66**, 37 (1967).
[5] C. R. BHATIA, *Proceedings* III *International Wheat Genetics Symposium* (edited by K. W. FINLAY and K. W. SHEPHERD), p. 111, Australian Acad. Sci., Canberra (1968).
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[7] J. G. VAUGHN, *J. Exptl. Botany* **18**, 269 (1967).
[8] B. G. CLARE, N. T. FLENTJE and M. R. ATKINSON, *Australian J. Biol. Sci.* **21**, 275 (1968).

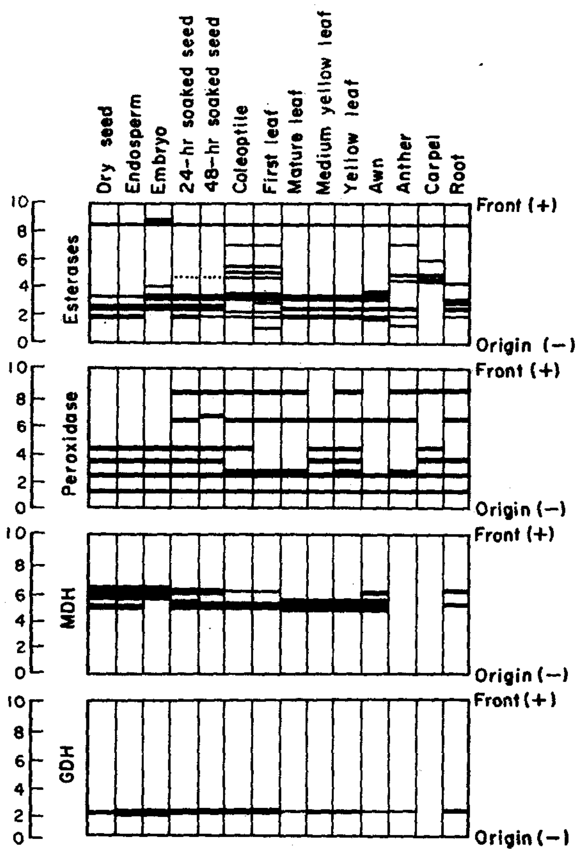

FIG. 1. DIAGRAMMATIC REPRESENTATION OF GDH, MDH, PEROXIDASE AND ESTERASE ZYMOGRAMS OBSERVED IN DRY SEEDS, CHANGES ACCOMPANYING GERMINATION, AND IN DIFFERENT PLANT ORGANS OF BARLEY VARIETY CV 292.

R_P values are indicated at the side. The solid black areas represent intense, broad bands; black lines indicate sharp narrow bands and dotted lines show faint bands. Drawings are based on mean values obtained from at least five different gels.

Ontogenic changes and tissue specificity. Enzyme bands observed in dry seeds, changes accompanying seed germination and in different organs of the barley plant are shown in Fig. 1.

Esterases. A fast migrating esterase band (Est-1) with an R_P of 8·5 was present in all the tissues examined. This band was very intense in embryonic tissue. Three slow migrating bands which appeared prominently in dry seed extracts were also present in most of the other tissues. With the soaking of seeds, and onset of germination, a new band of R_P 4·8 appeared; this showed a higher activity in coleoptiles and first leaf. In addition, three new bands with R_P 5·1, 5·5 and 7·0 appeared in coleoptile and first leaf. These bands were absent from extracts of mature leaves. In general, root, carpel, anther and embryo extracts showed esterase zymograms which were very different from those of other tissues.

Peroxidase. Two slow migrating peroxidase bands with R_P 2·5 and 3·6 were always present. Two additional bands appeared after 24 hr soaking of seeds. These bands were also observed in other tissues. Yellowing of leaves was accompanied by the appearance of two bands with R_p 4·7 and 5·6. These bands were also present in dry and soaked seed extracts but were absent from first and mature leaves.

GDH. A single GDH band with R_p 2·0 was observed in all the tissues except in carpel extracts; its activity varied in different tissues.

MDH. Two MDH bands with R_p 6·0 and 7·0 were observed, the first band being rather broad in dry seed extracts. With the onset of germination, the activity of this band decreased

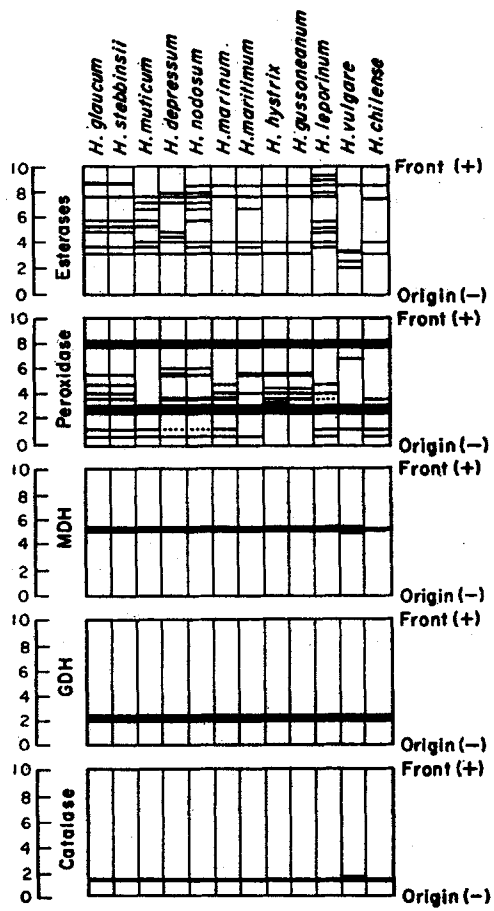

FIG. 2. DIAGRAMMATIC REPRESENTATION OF CATALASE, GDH, MDH, PEROXIDASE AND ESTERASE ZYMOGRAMS FROM LEAF EXTRACTS OF DIFFERENT *Hordeum* SPECIES.

Details are as for FIG. 1. Note that some species names are regarded as synonyms (see Table 2).

and it was not observed in mature and yellowing leaves, which showed only one MDH band. MDH bands were not detected in anther and carpel extracts. The R_p 5·0 band was absent from embryos.

Hordeum *species.* Zymograms for nine species of *Hordeum* are shown in Fig. 2 and densitometer tracings for esterases in Fig. 3. Peroxidase and esterase zymograms showed variation between species, while catalase, MDH and GDH did not vary.

Esterases. H. glaucum and *H. stebbinsii* showed identical zymograms. *H. marinum, H. hystrix, H. gussoneanum* and *H. chilense* had identical zymograms which were different from those of *H. glaucum* and *H. stebbinsii*. The fast migrating Est-1, corresponding to that of *H. vulgare* CV 292, was present in all the species except *H. muticum* and *H. depressum*. Three fast migrating bands were observed in *H. leporinum*.

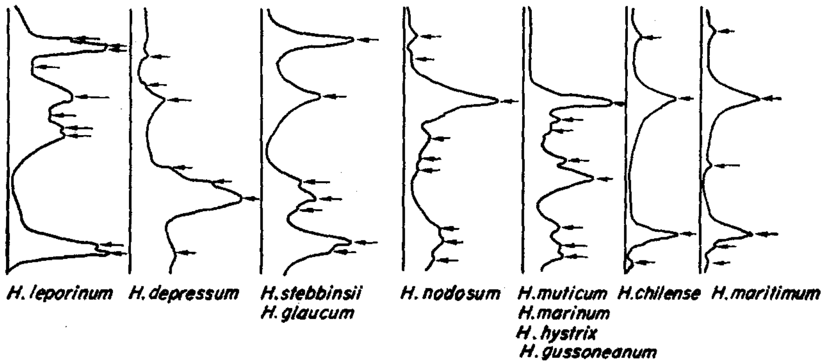

FIG. 3. DENSITOMETER TRACINGS OF ACRYLAMIDE GELS FOLLOWING ELECTROPHORETIC SEPARATION OF ESTERASES FROM LEAF EXTRACTS OF DIFFERENT *Hordeum* SPECIES.
Origin is at the bottom, migration was towards the positive electrode at the top.

Peroxidase. *H. glaucum* and *H. stebbinsii* showed identical zymograms, *H. depressum*, *H. nodosum* and *H. hystrix*, *H. gussoneanum* also had close similarities in peroxidase patterns. Two major bands with R_P 2·5 and 7·5 were common to all species; variation was observed in other minor bands.

Variation in Hordeum vulgare *for esterases*. Nine different esterase zymograms were observed (Fig. 4). Most of the variation was confined to slow migrating esterases. Types of esterase pattern for each variety is given in Table 1. In one line (EB 1265), the Est-1 band was absent. Auto-tetraploid stocks examined showed zymograms qualitatively identical to those of their parental diploids. However, quantitatively tetraploids showed higher enzyme activity.

DISCUSSION

Polymorphism in a "pure line". In the limited esterase data on seed and coleoptile of a morphologically homozygous *Hordeum vulgare* variety CV 292, variant types were not detected. Similar results were obtained with inbred populations of tetraploid and hexaploid wheat in our laboratory. Williamson *et al.*[9] have, however, reported considerable electrophoretic variation in esterases of three commercial "pure line" varieties of oats.

Ontogenic changes and tissue specificity. The results show that the enzyme patterns are tissue specific and change during differentiation and development. Studies with germinating seeds clearly show appearance of new enzyme bands in coleoptile, leaf and root, and concurrent disappearance of other bands. Similar changes in the zymograms in germinating barley seeds were reported.[10] The presence or absence of a band and its intensity on the gel is a reflection of the quantity of the active enzyme present in the extract sampled. Shannon,[11] and Efron and Schwartz[12] have considered possible regulatory control for tissue specific patterns. The latter have shown inactivation of alcohol dehydrogenase by a two factor system in maize.

[9] J. A. WILLIAMSON, R. A. KLEESE and J. R. SNYDER, *Nature* **220**, 1134 (1968).
[10] M. D. UPADHYA and J. YEE, *Phytochem.* **7**, 937 (1968).
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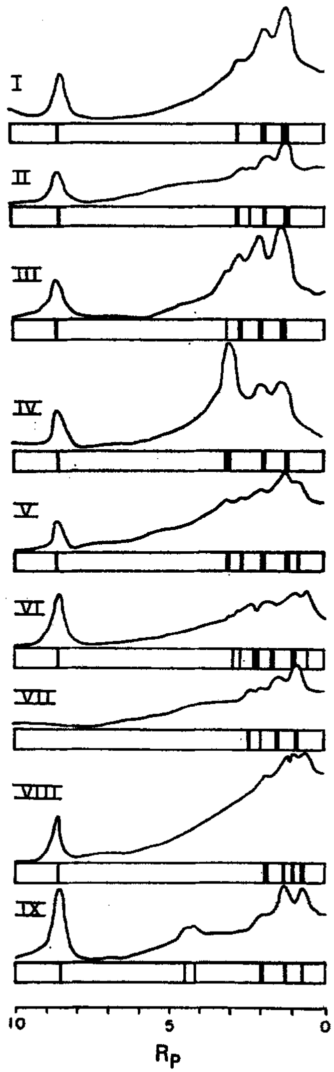

FIG. 4. DENSITOMETER TRACINGS ALONG WITH DIAGRAMMATIC REPRESENTATION OF DIFFERENT ESTERASE TYPES OBSERVED IN DRY SEED EXTRACTS OF DIFFERENT CULTIVATED VARIETIES OF BARLEY.

Origin is at the right hand, migration was towards the positive electrode on the left. Other details as in FIG. 1.

Inter-species variation. The zymograms shown in Fig. 2 for the 12 stocks of *Hordeum* species (Table 2) reveal that MDH, GDH and catalase do not show any inter-species differences; variation between species was, however, observed for esterases and peroxidases. Of the five enzymes investigated, GDH and MDH are highly substrate specific. GDH deaminates L-glutamic acid oxidatively to α-ketoglutaric acid and thus provides the key link between nitrogen metabolism and the tricarboxylic acid (TCA) cycle. Similarly, MDH is one of the vital enzymes in the TCA cycle. Catalase decomposes H_2O_2 and it plays an important part in plant metabolism by keeping redox balance. On the other hand, esterases and peroxidases have broad substrate specificities and their precise role in plant metabolism is unknown.

TABLE 1. BARLEY VARIETIES INVESTIGATED FOR ESTERASE POLYMORPHISM

Origin	EB No.	Grain	Esterase type	Origin	EB No.	Grain	Esterase type
Ethiopia	532	H	I	Afganistan	18	H	III
	639	H	II		20	H	II
	849	H	V		24	HL	I
	1254	HL	I		47	H	I
	1261	HL	II		52	H	II
China	16	H	III	Egypt	234	H	I
	61	HL	II		235	H	I
	119	H	II		299	H	V
	114	H	I		520	H	V
	662	H	II		521	H	I
Hindukush	852	HL	VIII	Manchuria	760	H	VI
	860	H	I		880	H	II
	1173	H	II		992	H	I
	1229	H	V		1381	H	II
	1265	HL	VII		1436	HL	II
Nepal	696	HL	I	Turkey	178	H	II
	787	H	I		189	H	II
	1069	H	I		195	H	II
	1077	H	I		300	H	IV
	1104	H	I		304	H	II
India	N.P. 13			Argentina	Malteria Heda	H	IX
	Diploid	H	I				
	Tetraploid	H	I		MC 20	H	IX
	Himalaya	HL	I		E-6	H	IX
	CV 292	HL	I				

H = Hulled. HL = Hull less.

TABLE 2. *Hordeum* SPECIES INVESTIGATED

Section/species	PI No.	Chromosome No. n =
I *Stenostachys* Nevski		
H. depressum Rydb.	247051	14
H. glaucum Steud.*	220521	7
H. stebbinsii Covas*	204847	7
H. muticum Presl.	269211	7
H. nodosum L.	247841	14
II *Hordeastrum* Doll.		
H. hystrix Roth.*	185155	14
H. gussoneanum Parl.*	203462	14
H. leporinum Link	168258	14
H. marinum Huds.*	200341	7
H. maritimum With.*	247056	7
IV *Cerealia* Ands.		
H. vulgare L. emend. Lam, CV 292		7
Not assigned to the four sections:		
H. chilense Brongn.	255751	7

* According to Nilan,[13] the following pairs of species names are synonymous: *glaucum* and *stebbensii: marinum* and *maritimum:* and *hystrix* and *gussoneanum*. The latter pair are more correctly referred to as *H. geniculatum*. The original names are retained here, because some of the "species" pairs (see text) show differences in enzyme patterns.

[13] R. A. NILAN, *The Cytology and Genetics of Barley* 1951–62, p. 278, Monographic Suppl. 3, Washington State University (1964).

Variation for a group of enzymes having broad substrate specificities and unknown physiological function were compared with another group of "critical" enzymes in two *Drosophila ananasse* populations[14] and greater variability was observed in the non-specific group. Though our sampling was not so extensive, our results in *Hordeum* agree with those obtained in *Drosophila*.

The variations in esterases and peroxidases in *Hordeum* provide data that can be used for comparing similarities or dissimilarities between species. *H. glaucum* and *H. stebbinsii* show exactly similar zymograms both for esterase and peroxidase. They are also reported to have similar karyotype[15] and as stated earlier[13] are regarded as synonyms. It is to be noted that their reported karyotype is very similar to that of *H. vulgare* but the zymogram patterns show considerable differences.

H. marinum, *H. maritimum*, *H. hystrix* and *H. gussoneanum* showed matching esterase zymograms, but for two minor additional bands in *H. maritimum*. Peroxidase zymograms of these species are also very similar; *H. hystrix* and *H. gussoneanum* differ by only one minor peroxidase band. Variation between *H. marinum* and *H. maritimum* was greater. Diploid *H. marinum*, *H. maritimum*, *H. hystrix* and *H. gussoneanum* are reported to possess a similar karotype.[16] However, the stocks of *H. hystrix* and *H. gussoneanum* investigated were tetraploid. Close similarities especially for esterases between these tetraploid and diploid species are of interest. Induced auto-tetraploid *H. vulgare* did not differ qualitatively from its diploid stock. A survey of the diploid and polyploid species of the *Triticinae* revealed that diploid and auto-tetraploid species had fewer esterase bands than the amphidiploid species.[5] There is evidence for the presence of parental forms, and additional hybrid esterases in allopolyploid wheats and wheat × rye amphidiploids.[5,6] In the *Hordeum* species examined *H. nodosum* and *H. leporinum*, the two tetraploids, do show more esterase bands than other species. However, other tetraploid species, especially *H. hystrix* and *H. gussoneanum* show only four esterase bands, the minimum observed in any diploid species. This suggests that, either *H. hystrix* and *H. gussoneanum* are auto-tetraploids or have arisen as a result of hybridization between closely related species having iso-allelic genes for esterases. The latter seems more probable as the cytological data indicate that tetraploid *H. gussoneanum* is not auto-tetraploid.[16]

Variation in Hordeum vulgare *for esterases.* As esterases are relatively stable and easy to locate on the gels, over 40 cultivated varieties of different geographical origin were examined for esterase variation. These varieties showed nine different zymogram types (Fig. 4), of which type I and II were most frequent (Table 1). No definite association between geographical origin and the esterase type could be established from the available data, but further sampling is in progress.

EXPERIMENTAL

Materials. Stocks of *Hordeum vulgare* were obtained from Dr. J. S. Bakshi of the Co-ordinated Barley Improvement Program, Indian Agricultural Research Institute, New Delhi. These, with their geographical origin, are listed in Table 1. Seeds of other *Hordeum* species were obtained from Dr. G. A. Weibe and Dr. D. A. Reid of the U.S. Department of Agriculture, Crops Research Division, Beltsville and are listed in Table 2 along with the PI numbers. The chromosome numbers given in Table 2 were obtained from root meristem preparations.

Methods. Hull-less barley variety CV 292 was used for the developmental studies. Seeds sterilized with 0·1% $HgCl_2$ for 2–3 min were washed with distilled water and then germinated in sterilized petri dishes or

[14] H. Gillespie and K. I. Kojima, *Proc. Natl. Acad. Sci. U.S.* **61**, 582 (1968).
[15] T. Rajhathy and J. W. Morrison, *Can. J. Genet. Cytol.* **4**, 240 (1962).
[16] J. W. Morrison, *Can. J. Botany* **37**, 527 (1959).

vermiculite at 25° ± 2, under continuous illumination. Embryos, roots, coleoptiles and the first leaves were excised from the germinating seeds at different stages of growth. Mature, green, and yellowing leaves, anthers, carpels and awns were obtained from plants grown in pots.

1 g of tissue was homogenised with 5 vol. of 0·01 M sodium pyrophosphate buffer (pH 9·3) containing 0·7% 2-mercaptoethanol, at 0–4°, this temp being maintained during the subsequent procedures. Since chlorophyll pigments interfered with the electrophoretic separations, the slurry was treated with cold acetone at −15° and the acetone was removed by filtration. The dry powder obtained was extracted with pyrophosphate buffer for 15 min, extracts were centrifuged at 10,000*g* for 15 min and the supernatant was dialyzed against 0·01 M phosphate buffer pH 7·8 containing 0·2% 2-mercaptoethanol, for 24 hr. Protein concentration in the dialysed extracts, determined by the Biuret method, ranged between 0·3–0·6 mg/ml for seed extracts and 0·09–0·15 mg/ml for leaf and root extracts respectively. Anthers plucked before anthesis and carpels before pollination (0·1 g) were extracted using the same tissue to buffer ratio. Single coleoptiles from 4–5-day-old germinating seeds were homogenised in 0·1 ml pyrophosphate buffer for investigating variation within "pure line" population. These extracts were used directly for electrophoresis.

Samples of 0·1 to 0·2 were applied to polyacrylamide gels. Disc electrophoresis using standard 7% acrylamide gels was performed in an apparatus similar to that described by Davis.[17] After electrophoresis, enzyme bands were located by incubating the gels in appropriate mixtures given below. A control gel for each enzyme was incubated in the mixture without the substrate. For the dehydrogenases, two control gels were incubated, one in the reaction mixture omitting NAD and the other one without the substrate. In such control gels, no bands were observed.

Esterase. Gels were incubated in 50 ml phosphate buffer pH 5·9 containing 1 ml of 1% α-naphthyl acetate in 60% acetone and 25 mg Fast Blue RR, at room temp. for 10–30 min.

Peroxidase. Saturated solution of benzidine in 25% acetic acid was mixed with an equal amount of 1% H_2O_2 and gels were incubated at room temp. for 2 min. Since the intense blue colours fade very rapidly, the band positions were immediately recorded.

Dehydrogenases. Gels were incubated at 25°, in dark, for 3–24 hr with 2 ml of 0·5 M substrate, nicotinamide adenine dinucleotide (NAD) 5 mg, nitroblue tetrazolium 0·5 ml (2 mg/ml), phenazine methosulphate 0·5 ml (2 mg/ml) in 10 ml of appropriate buffer. The buffers were tris–HCl 0·05 M, pH 7·4 for MDH and 0·1 M phosphate buffer, pH 6·5, for GDH.

Catalase. Soluble starch (0·25%) was incorporated into the separating gel. After electrophoresis, gels were incubated for 1 min with 0·5% H_2O_2, washed with distilled water and dipped in 1% KI solution acidified with acetic acid. Region of catalase activity on the gel remained unstained.

Variation in esterases among cultivated varieties of *H. vulgare* was investigated using dry seed extracts. For inter-species comparisons, mature green leaves, picked just before flowering were used, except for two species, *H. muticum* and *H. chilense*. Although the leaf tissue is not an ideal material for comparing inter species homologies,[3] this was used because of the non-availability of seeds in sufficient quantities. At least two independent extractions were made for all the materials examined. For each group of enzymes, 2–4 replicate runs were made. Tissues showing variant patterns were rechecked. At least five separate gels have been used to calculate the relative migration (R_F) of each band with respect to the front formed by the tracking dye bromophenol blue. Homology of the bands in different stocks was established by loading the gels with equal amounts of the two samples to be compared. Densitometer tracings of the gels were obtained on a Joyce Loebl Chromoscan MK II using visible reflectance.

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[17] B. J. DAVIS, *Ann. N.Y. Acad. Sci.* 121, 404 (1964).