THE UNIVERSITY OF MANITOBA

COMPARATIVE STUDIES OF THIOGLUCOSIDASE FROM *SINAPIS ALBA*, *CRAMBE ABYSSINICA* AND SEVERAL *BRASSICA* SPECIES.

by
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A THESIS

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ABSTRACT

Thioglucosidases from Sinapis alba, Crambe abyssinica, and several Brassica species were partially purified using three different preparative methods, namely ethanol, acetone, and ammonium sulfate fractionation from aqueous extracts of the seed. Substantial differences were found in the activity of the various preparations from different species. However, the method of purification had only minimal effects on the activity of the enzyme.

Heat stability tests indicated S. alba and B. napus (var. - turret) thioglucosidases were approximately equally resistant to thermal denaturation, both showing complete inactivation on heating for three minutes at 69°.

Activity in citrate-phosphate buffer was found to be similar for S. alba and turret rape thioglucosidase, with maximum activity observed at pH 5.5. On addition of ascorbic acid to turret rape thioglucosidase, greater activity was observed in phosphate buffer than in citrate-phosphate buffer. For these conditions, maximum activity was found to occur at pH 6.3 with a second peak at pH 7.1. Both enzymes were denatured on ten minutes exposure to acid levels below pH 2.15. Between pH 2.15 and 2.50, turret rape thioglucosidase was more resistant to acid denaturation than S. alba thioglucosidase.

Ascorbic acid was confirmed as an activator for S. alba thioglucosidase, producing an approximately threefold increase in activity. For similar enzyme preparations from Brassica species, a six-to-ten-fold increase in activity was observed in the presence of ascorbic acid.

Polyacrylamide gel disc electrophoresis was used to separate isoenzymes from seed extracts, and from the various enzyme

preparations. Each species exhibited a characteristic band pattern made up of three to six isoenzyme bands. Slight variations were noticed on comparing the isoenzymes from the seed extracts and the enzyme preparations.

Bands with comparable R_m values for S. alba and turret rape were developed in the presence and the absence of ascorbic acid. The rate of development of the band with R_m 0.25 in S. alba was unaffected by the presence of ascorbic acid. However, for the rape preparation, the rate of development of the band with R_m 0.25 was approximately doubled in the presence of ascorbic acid.

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INTRODUCTION

Quantities of Cruciferae oilseeds grown throughout the world have increased substantially over the past ten to fifteen years. This has come about mainly due to the increased interest in rapeseed, which has become the world's fifth largest oilseed crop (24). Initial interest in this seed was due to the value of the oil. However, of growing concern is the proper use of the high protein meal fraction. Extensive utilization of this potentially valuable food or feed ingredient has been hampered by the presence of potentially toxic substances in the seed. Extensive use of seed meals from Crambe abyssinica and several mustard species has been limited for the same reason.

The toxin precursors have been identified as thioglucosides, and the enzyme, thioglucoside glucohydrolase (EC 3.2.3.1) or alternatively thioglucosidase, or myrosinase, has been found to be responsible for the conversion of thioglucosides into toxic substances. These toxins include isothiocyanates (volatile mustard oils), L-5-vinyl-2-thiooxazolidone (goitrin) and several nitriles.

Conventional processing procedures have utilized heat to inactivate the enzyme, or steam stripping to volatilize the toxins. Although both methods produce meal of greater value, several disadvantages remain. As well as degrading some of the protein, heat treatment does not remove the thioglucosides. Subsequent reintroduction of thioglucosidase from other feeds, such as mustard or rape screenings, will cause formation of toxins. It has also been established that the microflora in the intestines of certain animals will cause the formation of toxins from thioglucosides. Steam stripping is only partially successful as several of the toxins cannot be removed at temperatures below the destruction level for the protein.

Over the past few years, several research groups have proposed different methods for oilseed processing to overcome these difficulties. These newer methods include water (3,5,20,25), or acetone (34)extractions, which remove the thioglucosides; or ammoniation (13), soda ash treatment (16), or treatment with iron or copper ions (39), all of which catalytically decompose the thioglucosides. Disadvantages have been shown for most of these methods, not the least of which is the excessive production cost involved.

Considerable effort has been placed on investigating the chemistry of the various thioglucosides and their degradation pathways and products. Relatively little is known, however, about thioglucosidase. Research carried out on this enzyme to date has been confined to Sinapis alba, one variety of Brassica juncea and to one fungous species, Aspergillus sydowi. Due to the important role thioglucosidase plays in this system, it seems prudent to attempt to expand the information in this area if foods and feeds of high quality are to be produced at minimum costs.

With this in mind, the following objectives were set for this study:

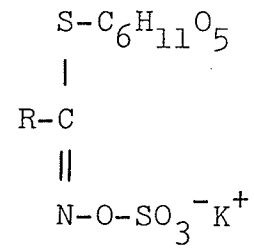
1. to extract active thioglucosidase from various Brassica species, as well as S. alba and C. abyssinica, and compare some of their kinetic properties.

2. to compare isoenzyme patterns for these species using polyacrylamide gel disc electrophoresis.

3. to relate any activity differences to differences in isoenzyme patterns.

LITERATURE REVIEW

Unlike other β - glucosidases, thioglucosidase shows a highly specific hydrolytic activity toward a thioglucosyl bond. This type of bond is found in nature in compounds referred to as thioglucosides (I).



(I)

More than fifty of these thioglucosides have been found to date occurring mainly in the plant family Cruciferae (35), but also in Tropacolaceae, Capparidaceae, Resedaceae, and a few individual plant species (11).

Natural thioglucosides contain a glucose moiety, a sulfate radical, and an R-group. The majority of the thioglucosides differ from one another due to variation of the R-group. These may take the form of aliphatic, aromatic, or hydroxy-substituted side chains.

The specific function of thioglucosides is not yet fully understood, but it is believed they play a part in the defense of the plant against microbial attack. This hypothesis is based on the toxic nature of the hydrolytic products. These include thiocyanates, isothiocyanates, oxazol compounds, and nitriles, as well as glucose and potassium sulfate.

The antinutritional properties associated with plant material containing mustard oils have been known for centuries, though the chemistry of the compounds, and the metabolic pathways involved have only recently been understood. Early workers

believed the isothiocyanates were the major cause of poor growth. However, in 1949, Astwood et al identified *l*-5-vinyl-2-thiooxazolidone (goitrin) as being the compound responsible (1). More recently, Van Etten et al have shown the toxicity of goitrin is relatively low compared to that of various nitriles, which may form under different reaction conditions (36).

In addition to being found in plants, thioglucosidases have been found in animals (12), in various strains of fungi (18, 19, 21), and in some bacteria (12). Interestingly enough, although Goodman et al reported finding thioglucosidase activity in mammalian tissues, no natural substrate could be found (12). These workers further reported that the animal thioglucosidases appeared to have similar activities to plant thioglucosidases.

The presence of thioglucosidase activity in bacteria found in the intestinal tract of animals has long been known. This was believed since feeds containing thioglucosides, but which have had all native enzymes destroyed, still cause characteristic depressed growth when ingested.

Considerable work has been carried out recently investigating fungal myrosinase (18, 19). Ohtsuru et al reported that thioglucosidase isolated from Aspergillus sydowi showed considerable similarity to plant myrosinase (18). This conclusion was based mainly on the fact that both enzymes are competitively inhibited by glucose, salicin, and *δ*-gluconolactone. In contrast to plant myrosinase, however, the fungal enzyme was not stimulated by L-ascorbate (18).

Although it is currently believed that thioglucosidase is a single enzyme and not two enzymes (35), much controversy and research have centered around this question. Early workers believed a thioglucosidase was responsible for the splitting of the thioglucosyl bond, and a sulfatase was responsible for the

removal of the sulfate fraction (6, 22). In 1956, Ettlinger and Lundeen proposed the currently accepted structure for thioglucosides (I), and postulated that the degradation of thioglucosides could be accomplished through the action of a thioglucosidase followed by a chemical rearrangement of the Lossen Type (6). Although this type of rearrangement was novel to enzymic reactions, it was well substantiated in chemical reactions (6). When Ettlinger and Lundeen reported the syntheses of thioglucosides in their laboratory the following year, their proposed structure and their degradation pathway became widely accepted (7).

The controversy resumed in 1960 when Gaines and Goering reported the separation and partial characterization of the two enzymes which were earlier believed present (9, 10). However, as these results could not be repeated by Calderon et al (4), or by Tsuruo et al (27), the two-enzyme system again lost support.

Much of the work carried out before 1956 in characterizing thioglucosidase has been discounted. Over the past fifteen years, some work has been carried out in characterizing the enzyme, but much more effort has been spent in investigating the nature and occurrence of the substrate and products.

One important discovery made in 1959 by Nagashima and Uchiyama was that L-ascorbic acid acted as a co-factor for thioglucosidase (17), while D-isoascorbic acid showed no effect. This was the first time that Vitamin C was shown to activate an enzymic reaction. Since extracts from various parts of a single plant, as well as enzymes from different sources, showed varying responses to this "coenzyme", Ettlinger et al concluded that two types of thioglucosidases were present (8). One of these was similar to the previously described fungal enzyme in that it showed no increased activity in the presence of L-ascorbic acid. The other, which was referred to as "an ascorbate-activated glucosinolase", did show increased activity. More than

three dozen enediols, or derivatives of ascorbic acid were also tested as cofactors by these workers. A few of these proved positive, but the most effective compound, 5,6-isopropylidene-L-ascorbate, was only one-half as effective as Vitamin C in increasing the rate of reaction. However, using the various rates of reaction as influenced by these cofactors, under variable conditions, it was established that the singly charged anion was responsible for the activation.

In 1961, Schwimmer reported a rapid spectrophotometric method for estimating thioglucosidase activity (23). At the same time he reported several of the kinetic properties of the enzyme, including the effect of enzyme and substrate concentration, the effect of pH, and the effect of ascorbic acid.

It was reported by Austin et al in 1968 that ferrous ion not only produced a more rapid reaction rate when added to the enzyme-substrate reaction mixture, but could in fact cause β-hydroxy isothiocyanate (epi-progoitrin) to be degraded in the absence of thioglucosidase (2). In addition, Austin et al showed that ferrous ion influenced the course of the reaction, causing epi-progoitrin to be converted preferentially to nitriles instead of goitrin (2). Van Etten et al had earlier shown this pathway to occur when freshly harvested seeds of Crambe, or several Brassica species were autolysed or when epi-progoitrin was degraded at an acid pH (33). These workers also showed temperature could affect the degradation pathway.

Tookey and Wolff have recently reported that the action of ferrous ion is quite specific, as several similar salts showed no effect on the rate or course of reaction. It was further shown that the influence of ferrous ion was not due to a direct reducing effect, since the oxidation-reduction

potentials of the metal ions tested spanned that of ferrous ion (29). These workers also noticed that the factor (or factors) which caused crambe thioglucosides to be degraded to cyano compounds at pH 4 - 8, was labile at extreme pH. This led to the conclusion that an enzyme(s) may be involved in the degradation pathway (26).

In attempts to duplicate the results of Gaines and Goering (9, 10), Tsuruo et al (27) could not find separate thioglucosidase and sulfatase activities in Sinapis alba, but did report finding two chromatographically distinguishable enzymes. In other characteristics, including their sulfatase activity, thioglucosidase activity, K_m and V_{max} values, and response to L-ascorbic acid, these chromatographically-separable fractions were found to be nearly identical (27). These results did not support Ettlinger et al (8) in that L-ascorbic acid acted as an activator for both fractions, and not as a coenzyme for one fraction as was suggested.

In attempts to further elucidate the nature of the enzyme-substrate-activator complex, Tsuruo and Hata investigated the effect of neutral salts on the reaction (29). Contrary to earlier work (17, 28), these tests showed that neutral salts could indeed inhibit the reaction in the presence of ascorbic acid, but not in its absence. They further proved that the effect of the salt was due to the anion, and not to the cation moiety. This difference in inhibition toward the activated and non-activated state proved the existence of separate activator and substrate sites on the enzyme.

In subsequent experiments, Tsuruo and Hata showed the inhibitory effect of several sugars and glucosides when present in excess quantities (30). Again differences were found depending on the presence or absence of ascorbic acid. These findings led to the conclusion that the substrate site on the enzyme was itself

comprised of an area which received the glycon, and an area which received the aglycon portion of the substrate. They also showed it was the aglycon area and not the glycon area which was affected by the ascorbic acid site.

These suggested enzyme sites were further substantiated by Tsuruo and Hata by showing the various effects of the previously mentioned activators and inhibitors on the degradation of p-nitrophenyl β-glucoside (p-NPG) (31). It had been previously reported by Reese et al (21) that this glucoside served as a substrate for myrosinase. The fact that the glycon portion of this substrate is the same as that for thioglucosides while the aglycon portion differed, allowed Tsuruo and Hata to prove their model.

Recently, polyacrylamide gel electrophoresis was employed for the separation of the isoenzymes of thioglucosidases (15). This was suggested as a method for investigating chemotaxonomic properties of plant species. Detection of the active enzyme bands in the gel was made by allowing a thioglucoside to be degraded in the presence of $BaCl_2$, thereby forming an insoluble $BaSO_4$ precipitate which can be photometrically detected. MacGibbon and Allison reported finding from one to four electrophoretically distinct bands from the seeds of various species (15). Although some of these bands appeared to have similar mobility in different species, each species had a characteristic band pattern. This method appeared to be more sensitive than the TEAE-cellulose column chromatography used by Tsuruo, by which only two separate fractions were found (27).

A similar study was carried out by Vaughan and Gordon using serological techniques to investigate the characteristics of thioglucosidase from various sources (37). This work indicated that the serological responses of thioglucosidase from Brassica juncea and from Sinapis alba were homologous, though the enzyme concentrations were considerably higher in Sinapis alba.

MATERIALS AND METHODS

I. Thioglucosidase Source

The enzyme was obtained from three genera of Cruciferae oilseeds, namely Sinapis alba (yellow mustard), Crambe abyssinica Hochst ex R.E. Fries (crambe), and seven species or varieties of Brassica. Of the Brassica species, four were mustards (B. nigra - var. black, B. juncea - var. brown, B. juncea - var. common oriental, and B. juncea - var. Lethbridge 22 A) and three were rape seeds (B. napus - var. turret, B. napus - var. bronowski, and B. campestris - var. echo.). The bronowski and echo samples had had their seed coats removed.

The crambe and all mustard samples were obtained from Northern Sales (1963) Ltd. of Winnipeg, Manitoba. The echo and bronowski rape were obtained from the Food Research Institute, Ottawa, Ontario, while the turret rape was supplied by the Department of Plant Science, University of Manitoba. All seeds came from 1970 plantings with the exception of the crambe which was from the 1969 crop grown in southern Manitoba. All samples were held at room temperature throughout the storage period.

II. Thioglucosidase Purification

A. Wrede's Method

The method proposed by Wrede in 1941 (38), and modified by workers at the Northern Regional Research Laboratories of the United States Department of Agriculture, Peoria, Illinois, (32), was carried out as follows.

Coarsely-ground seed from the various sources was defatted at 4 - 6° by extracting four to eight times with petroleum ether (b.p. 37.6 - 50.5°). After air-drying, 220 g. of this

meal were shaken periodically over two hours with 750 ml. of water at room temperature. After centrifugation, the supernatant was slowly decanted into 750 ml. of 90 percent v/v ethanol with constant stirring. After a second centrifugation, the supernatant was thoroughly stirred into 750 ml. of 70 percent v/v ethanol. After a third centrifugation, the precipitate was suspended in 250 ml. of water and left in a refrigerator overnight. The small amount of precipitate which remained undissolved was removed by centrifugation. The supernatant was frozen at -20° and then freeze-dried to yield a few grams of enzyme preparation. Activity was found to be moderately stable at -20° throughout the experimental period. Over a period of one year, a loss in activity of 20 percent was observed.

B. Schwimmer's Method

The procedure outlined by Schwimmer in 1961 was used as a second preparative technique. (23).

One hundred grams of coarsely-ground seed were extracted 10 times with 300 ml. portions of acetone at 4 - 6°. After air-drying, the defatted meal was shaken periodically over 30 minutes with 300 ml. of water. After centrifugation, the residue was shaken a second time with 300 ml. of water and then centrifuged. Fractionation was carried out on the combined supernatants by increasing the acetone level first to 30% and then to 70%. The 30 - 70% precipitate was taken up in a small portion of water and dialyzed for two days against distilled water. After centrifugation, the supernatant was frozen at -20° and freeze-dried, again yielding a few grams of enzyme preparation.

C. Salt Precipitation Method

Coarsely-ground seed was extracted 3 - 4 times with petroleum ether (b.p. 37.6 - 50.5°) over a period of about 2 hours. The solvent was decanted and the partially defatted meal allowed to air-dry overnight. After grinding to pass through a 25-mesh screen, the meal was water-extracted for 1 hour with shaking, and centrifuged. The supernatant was fractionated by precipitation with 60%, then by 80% saturation with ammonium sulfate. The 60 - 80% precipitated fraction was taken up in a small amount of water and dialyzed for 24 hours. After centrifugation, the supernatant was frozen at -20°, then freeze-dried to yield an enzyme preparation.

Protein was determined by the method of Lowry et al (14), with crystalline bovine serum albumin (British Drug Houses-fraction V) used as a standard.

III. Kinetic Studies

In order to quantitatively evaluate the effect of various conditions on enzyme activity, the spectrophotometric method described by Schwimmer (23) was adopted as the standard procedure for the determination of thioglucosidase activity. In this method, the thioglucoside, sinigrin, is enzymatically degraded under controlled conditions. The fall in absorbance at 227.5 nm (the wavelength of maximum light absorption for sinigrin) was monitored using a Pye-Unicam SP 800 recording spectrophotometer.

For this test, 5.0 mg. of enzyme preparation were dissolved in 46 ml. of water and 4 ml of 0.08 M citrate-phosphate buffer at pH 5.7. A second solution consisted of 7.5 mg. of sinigrin dissolved in 50 ml. of water. Both solutions were heated to 37° in a water bath. On stabilizing at this temperature, the two solutions were mixed thoroughly and 3 ml. rapidly transferred

into a 10 mm cuvette (also warmed to 37°, through the use of a thermostatically controlled cell holder.) The absorbance curve was recorded over a period of 10 - 20 minutes measured against a reference solution in which sinigrin was omitted. Enzyme activity is expressed in μM sinigrin degraded per minute per μM of enzyme preparation (assumed m.w. of 100,000).

IV. Polyacrylamide Gel Electrophoresis

The procedure outlined by MacGibbon and Allison (15) was generally followed although several modifications were introduced.

A. Sample Preparation

Forty mg. of coarsely-ground seed were gently stirred for 2 minutes in a Sorvall Omni Mixer, with 2 ml. of water, containing 0.05 ml. of mercaptoethanol and 0.1 ml. of 0.089M tris-glycine buffer, pH 8.9. After filtering through glass wool, 0.8 g. of sucrose was added to the solution. Aliquots of 0.025, 0.05, and 0.1 ml. were applied to the upper gel using lambda pipettes.

B. Electrophoretic Separation

Separation of isoenzyme bands was carried out using disc electrophoresis apparatus obtained from Savant Instruments, Inc., New York. The procedure outlined in the Polyanalyst Instruction Manual (Buchler Instruments Inc., Fort Lee, New Jersey) was generally followed. Tests were carried out using a running pH of 9.3 and a gel concentration of 7.5%. The amounts of the ammonium persulfate in both gels, and the riboflavin in the upper gel, were double the amounts recommended in the Buchler manual.

Electrophoresis was carried out over a period of 90 minutes, maintaining the current as close to 4 mA per tube as possible. This was carried out by starting the run at 100V, increasing

to 150V and then to 200V as the resistance in the gels increased. Both the upper and lower buffers were cooled to 4^{o} before the start of the run, and the lower chamber of the apparatus was immersed in a circulating ice bath during the run. Bromophenol blue was added to the upper buffer to indicate the electrophoretic front.

C. Detection of Isoenzyme Bands

The gels were flooded in an aqueous solution of sinigrin, 2 mg/ml., barium chloride, 6 mg./ml., and ascorbic acid 0.003 M. Sufficient acetic acid was added to attain a final concentration of 1.0 N. The bands were allowed to develop overnight after which a densitometric tracing was made using a Photovolt Densicord Model #542 equipped with a Photovolt #5087 disc gel carriage. The response was set at L with a motor speed of 1/2" per minute, and a chart speed of 2" per minute.

RESULTS

I. Enzyme Preparation:

Three different enzyme partial purification procedures were attempted in order to obtain active preparations from the various seeds investigated. It was also thought that a comparison of the enzymes obtained through different methods of purification might have indicated varietal differences.

Table I summarizes some of the characteristics of the various preparations. These results show striking differences among species with regard to either the extractability of thioglucosidase, or to the activity of the extracted enzyme.

No attempt was made to quantitatively evaluate the enzyme activity after each step in purification, but a positive or negative response could be obtained through the use of a "taste test". This test was carried out by mixing the enzyme preparation with water, then adding mustard powder which had previously been heat-treated to inactivate the enzyme. After standing at room temperature for 15 - 20 minutes, the characteristic heat of the mustard isothiocyanates could be detected by the tongue if the preparation contained active enzyme.

Through the use of this test, it was found that activity appeared to be decreased, but not lost, in Brassica species and crambe, during the oil extraction step. The subsequent water extraction step resulted in very low or no activity in the supernatant. The protein precipitation steps were carried out, however, in the event that activity could be detected. The precipitation step also allowed quantitative testing, using the spectrophotometric procedure of Schwimmer (23), without interference from other u.v. - absorbing seed constituents.

Table I Summary of Enzyme Purification

Material	Method of Purification	Yield (g.freeze-dried material/100g seed)	Protein (%)	Activity (μM sinigrin degraded /min/μM protein)
S. alba (yellow)	Wrede	.83	35	94.0
B. juncea (oriental)	"	.55	58	n.a.*
B. juncea (brown)	"	.75	63	n.a.
B. napus (turret)	"	.20	42	6.3
B. napus (bronowski)	"	.68	66	2.6
B. campestris (echo)	"	.25	62	n.a.
C. abyssinica	"	1.14	47	n.a.
S. alba (yellow)	Schwimmer	1.22	88	65.2
B. napus (turret)	"	2.05	84	3.0
C. abyssinica	"	3.80	93	n.a.
S. alba	Salt Ppt'n	.064	62	71.0
B. napus (turret)	" "	.075	83	6.2
B. campestris (echo)	" "	.035	50	4.5

* enzyme activity could not be detected under standard test conditions.

Addition of ascorbic acid had very little or no effect on the samples reported as showing no activity.

The degree of purity and the activity of the yellow mustard were comparable to that reported by Schwimmer (23).

II. Standard Test

In order to check the validity of the test used to determine enzyme activity, both the substrate and the product, allyl isothiocyanate, were scanned through the u.v. range. From Figure I, it is seen that at equal molar concentrations, the absorbance at 227.5 nm due to allyl isothiocyanate is 8.2% that of sinigrin. This is slightly higher than the value of 7.2% reported by Schwimmer (23), but still sufficiently low to allow the rate of fall in absorbance at 227.5 nm to indicate the rate of reaction.

Figure II illustrates a typical reaction curve. It can be seen that the absorbance is linear with respect to time, until almost one half of the sinigrin has been degraded. Although the recording trace commences approximately 15 - 20 seconds after the start of the reaction, extrapolation of the linear portion of the curve to zero time produces the expected absorbance level for 7.5 mg. of sinigrin. It is further seen from Figure II that the curve levels off at an absorbance equal to that expected for the amount of allyl isothiocyanate that would be produced.

To test for the possibility of product inhibition, 1 mM of allyl isothiocyanate was added to the reaction mixture ten minutes before the addition of the substrate. No difference in enzyme activity could be detected compared with the reaction rate in the absence of allyl isothiocyanate.

FIGURE I SUBSTRATE AND PRODUCT ABSORBANCE CURVES THROUGH ULTRAVIOLET RANGE.

Curve A - 0.1 mM Sinigrin
Curve B - 1 mM Allyl isothiocyanate

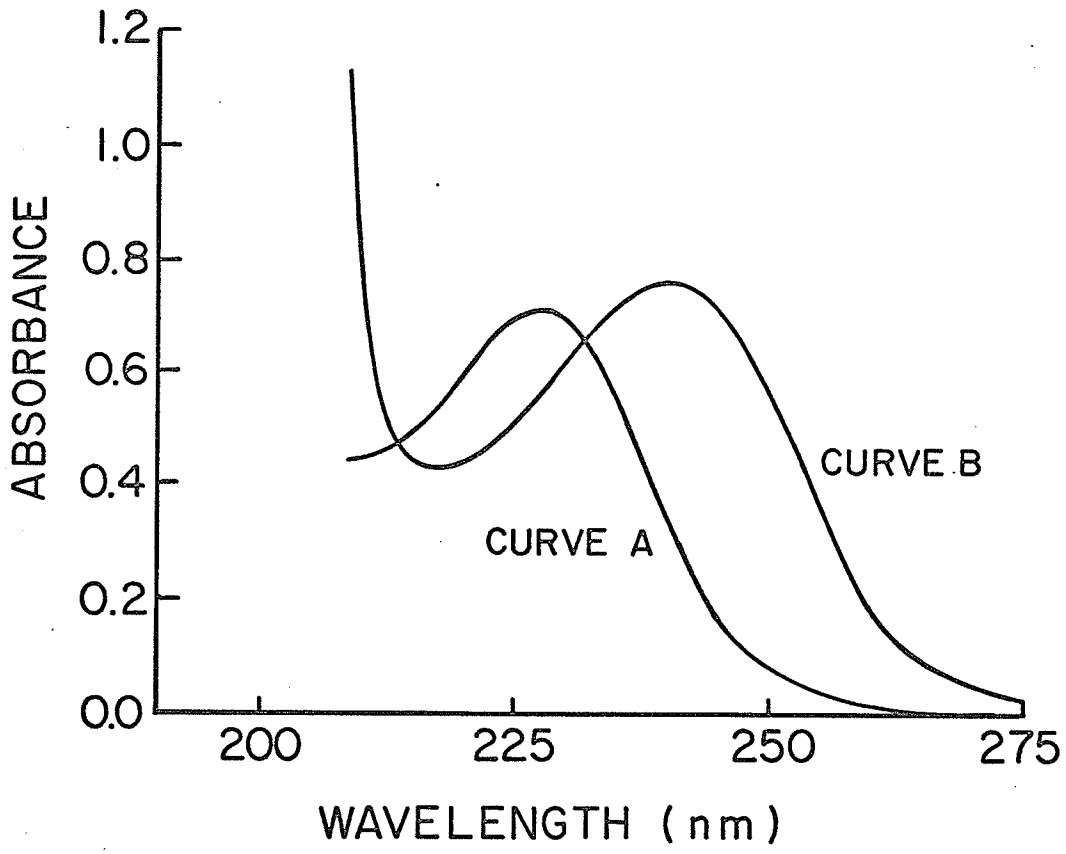
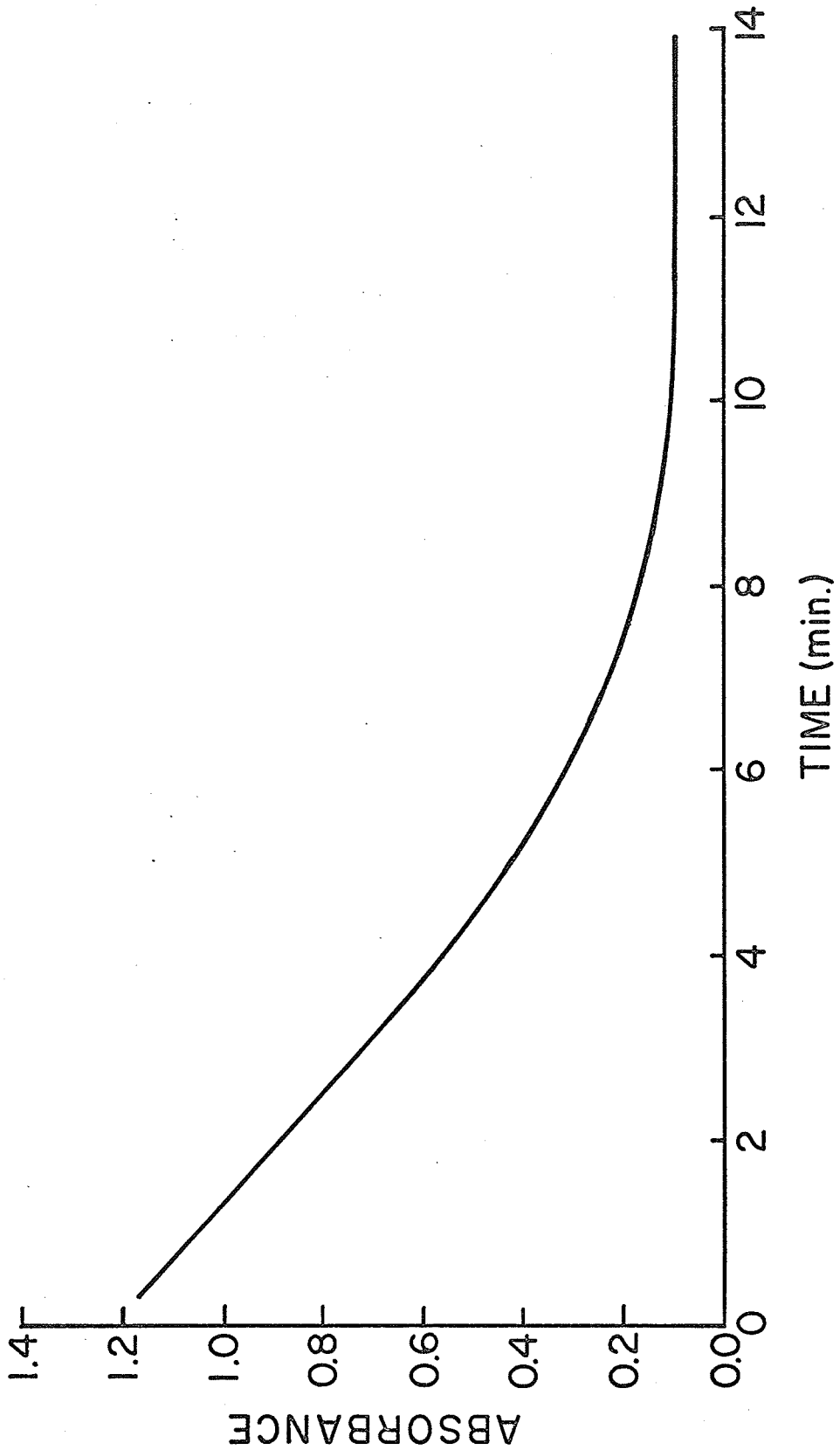

1.2
1.0
0.8
0.6
0.4
0.2
0.0
ABSORBANCE
CURVE B
CURVE A
200
225
250
275
WAVELENGTH (nm)

FIGURE II DISAPPEARANCE OF SINIGRIN

Monitored at 227.5 nm. with a chart speed of 1.4 cm per minute.


ABSORBANCE
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
14
TIME (min.)

The effect of enzyme concentration on the reaction rate is shown in Figure III. The linear relationship is similar to that reported previously (23).

III. Effects of Ascorbic Acid

In order to determine the effect of ascorbic acid on the rate of reaction, tests were run with different levels of acid added to the reaction mixture. The results of these tests are shown in Figure IV.

Preliminary results indicated that the rate of reaction was unaffected by pre-incubation of ascorbic acid with either substrate or enzyme.

It was also found that the activating effect of ascorbic acid was different for the various enzyme preparations. Table II shows there is a significantly greater activating effect by ascorbic acid on the rape preparations, than on the yellow mustard preparations. The difference in activation for different methods of preparation appears to be insignificant.

IV. Effects of pH

The reaction rates at various pH values for both mustard and rape enzyme preparations were determined using three different buffers. The influence of ascorbic acid on enzyme activity at various pH values was also studied. In each case, the pH of the reaction mixture was recorded approximately five minutes after the commencement of the reaction, and this value was taken as the final pH. Standard test procedures were followed except for the addition of ascorbic acid and the different buffers used.

FIGURE III EFFECT OF ENZYME CONCENTRATION ON THE RATE OF REACTION.

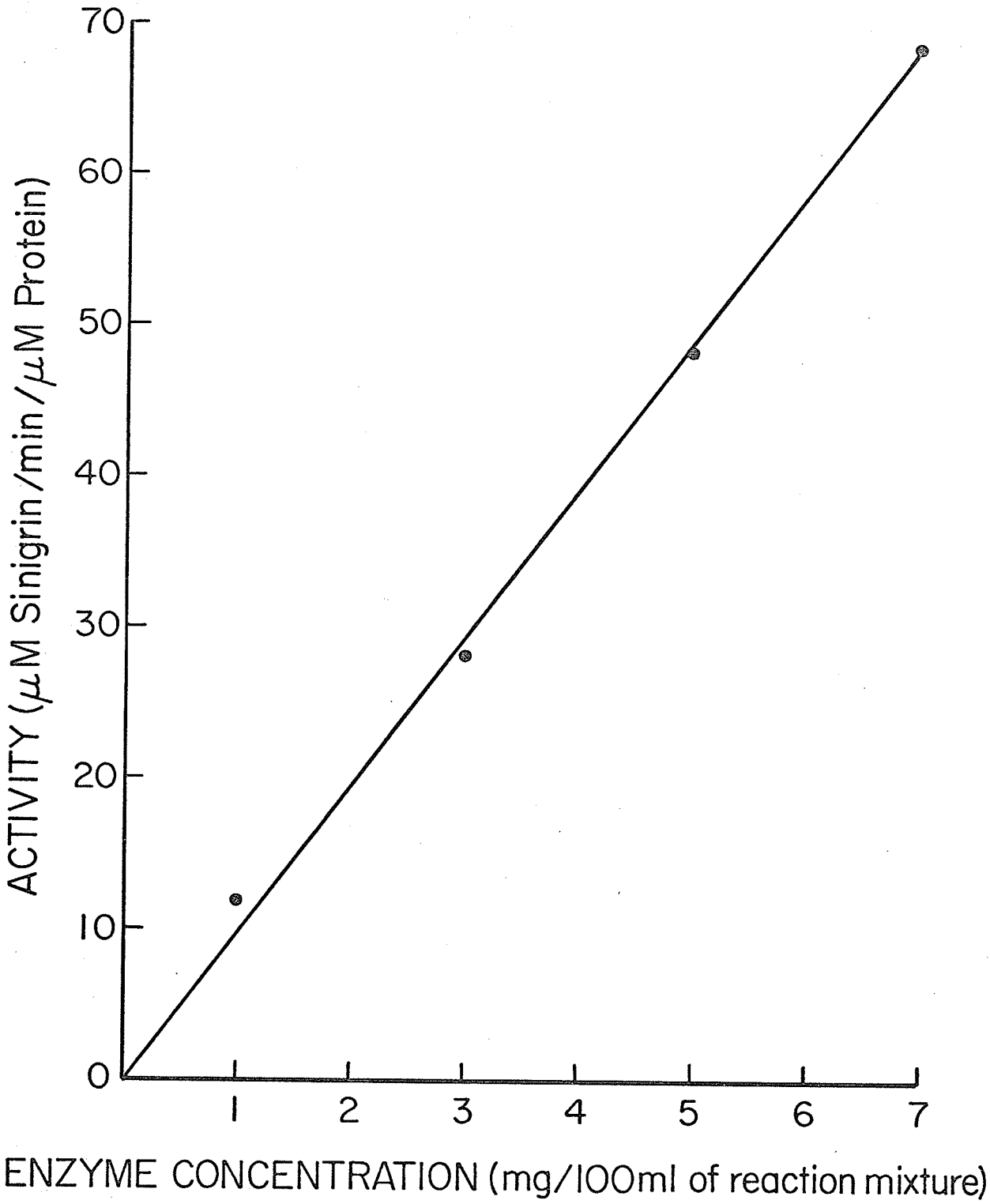

70
60
50
40
30
20
10
0
ACTIVITY (μM Sinigrin/min/μM Protein)
1
2
3
4
5
6
7
ENZYME CONCENTRATION (mg/100ml of reaction mixture)

FIGURE IV THE EFFECT OF ASCORBIC ACID ON THE RATE OF REACTION.

S. alba thioglucosidase, which was partially purified by Schwimmer's method, was used.

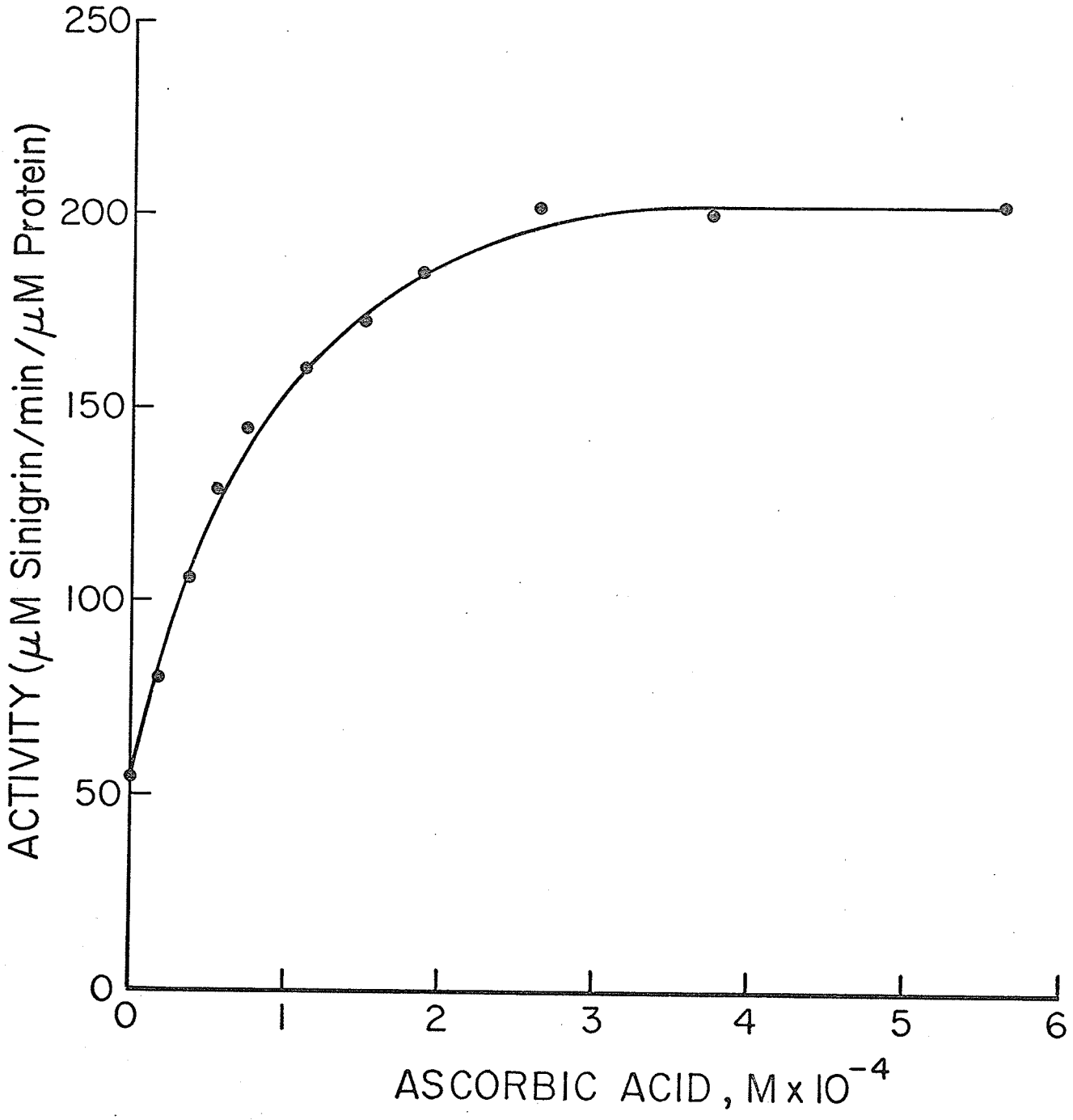

250
200
150
100
50
0
ACTIVITY (μM Sinigrin/min/μM Protein)
0
1
2
3
4
5
6
ASCORBIC ACID, M x 10⁻⁴

Table II Effect of Ascorbic Acid on Various Enzyme Preparations

Material	Method of Purification	Activity - (Specific activity Units) A (control)	B (with 3.75×10^{-4} M ascorbic acid)	Ratio B/A
S. alba (yellow)	Wrede	94.0	243	2.6
B. napus (turret)	"	6.3	52.4	8.3
B. napus (bronowski)	"	2.6	25.4	9.8
S. alba (yellow)	Schwimmer	65.2	200	3.1
B. napus (turret)	"	3.0	18.1	6.0
B. napus (turret)	Salt Ppt'n	6.2	48.0	7.7
B. campestris (echo)	" "	4.5	47.6	10.6

Figure V shows the effects of the three buffers on mustard thioglucosidase. No sharp increase in activity was recorded at pH 6.96 as reported by Schwimmer (23). It was noted that the activity in phosphate buffer increased slightly as the pH was raised from 5.6 to 7.5, while over the same pH range, the activity in citrate-phosphate buffer decreased.

Of further interest is the considerable difference in the rate at which activity increased in citrate-phosphate buffer as compared to the hydrochloric acid - sodium acetate buffer, within the pH range of 3.3 to 5.3. A slight plateau is noted in both buffers at about pH 3.5. It is further noted that activity is lost completely at pH 3.1 in citrate-phosphate buffer, while at this pH in hydrochloric acid - sodium acetate buffer, nearly one-half maximum reaction velocity is still evident.

Of the three buffers employed, it is shown that the maximum reaction rate for S. alba is attained in citrate-phosphate buffer at pH 5.5.

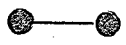
Figure VI represents the effect of citrate-phosphate buffer on thioglucosidase from yellow mustard, using two different methods of enzyme purification. Although the two curves exhibit similar ranges for maximum activity, the ratio of activities through pH 3.5 - 5.0 shows some difference from those through pH 6.0 - 7.5.

The effect of pH on rapeseed thioglucosidase is shown in Figure VII. The activity with phosphate buffer in the presence of ascorbic acid is the mean of three experiments. Several interesting findings are shown by this curve. Contrary to previous observations for mustard, activity within a pH range of 6.0 to 7.2 was shown to be greater in phosphate buffer than in citrate-phosphate buffer. It was also demonstrated that a

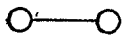
FIGURE V

THE EFFECT OF pH ON S. alba THIOGLUCOSIDASE ACTIVITY.

The enzyme was partially purified by Schwimmer's method.



0.05 M citric acid - 0.1 M Na_2HPO_4 buffer



0.02 N sodium acetate - HCl buffer

0.1 M sodium phosphate buffer

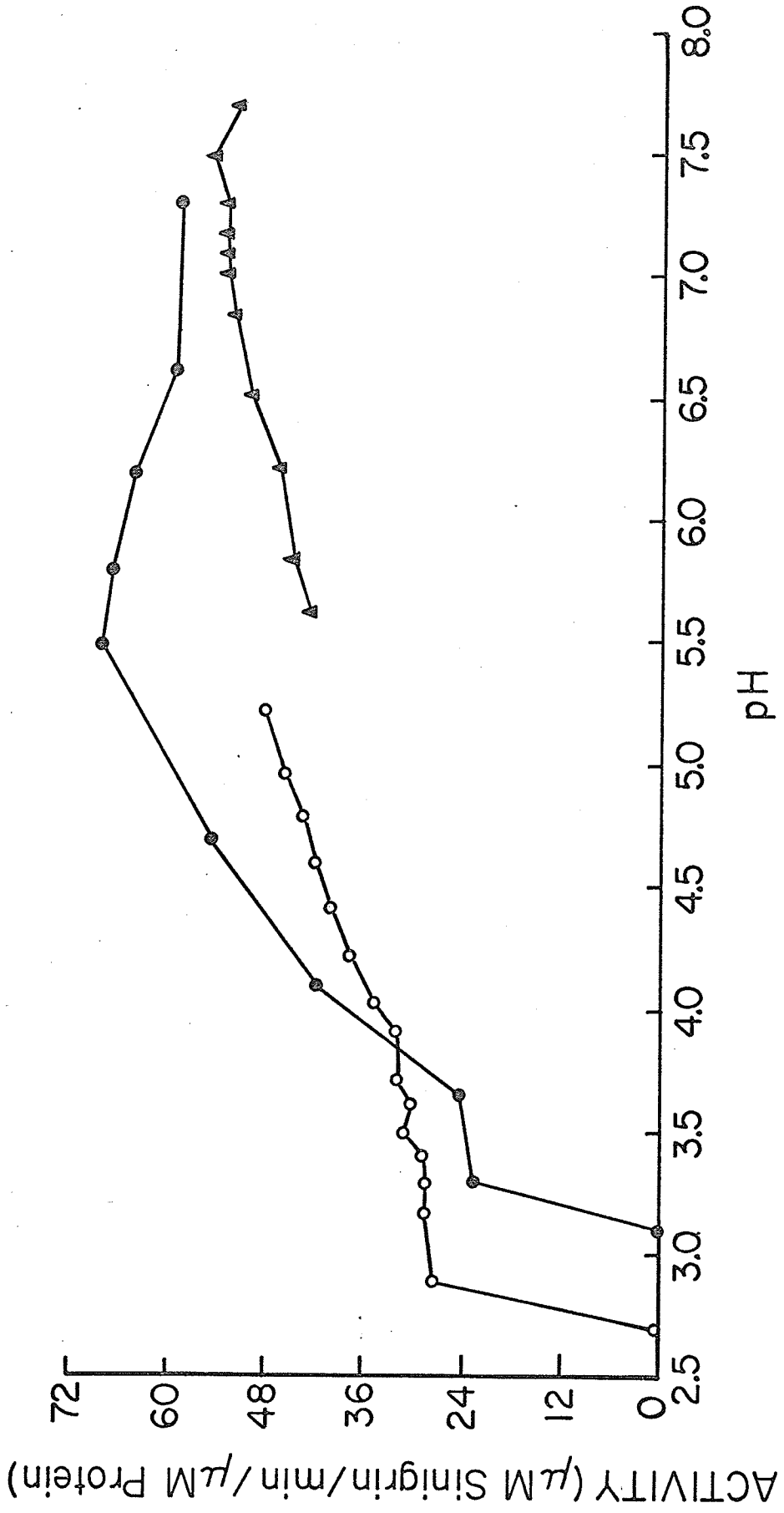

ACTIVITY (μM Sinigrin/min/μM Protein)
72
60
48
36
24
12
0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
pH

FIGURE VI

THE EFFECT OF pH ON THIOGLUCOSIDASE ACTIVITY FOR DIFFERENT METHODS OF ENZYME PREPARATION.

0.05 citric acid - 0.1 M Na_2HPO_4 buffer was used.

●——● S. alba - Schwimmer's Method

○——○ S. alba - Wrede's Method

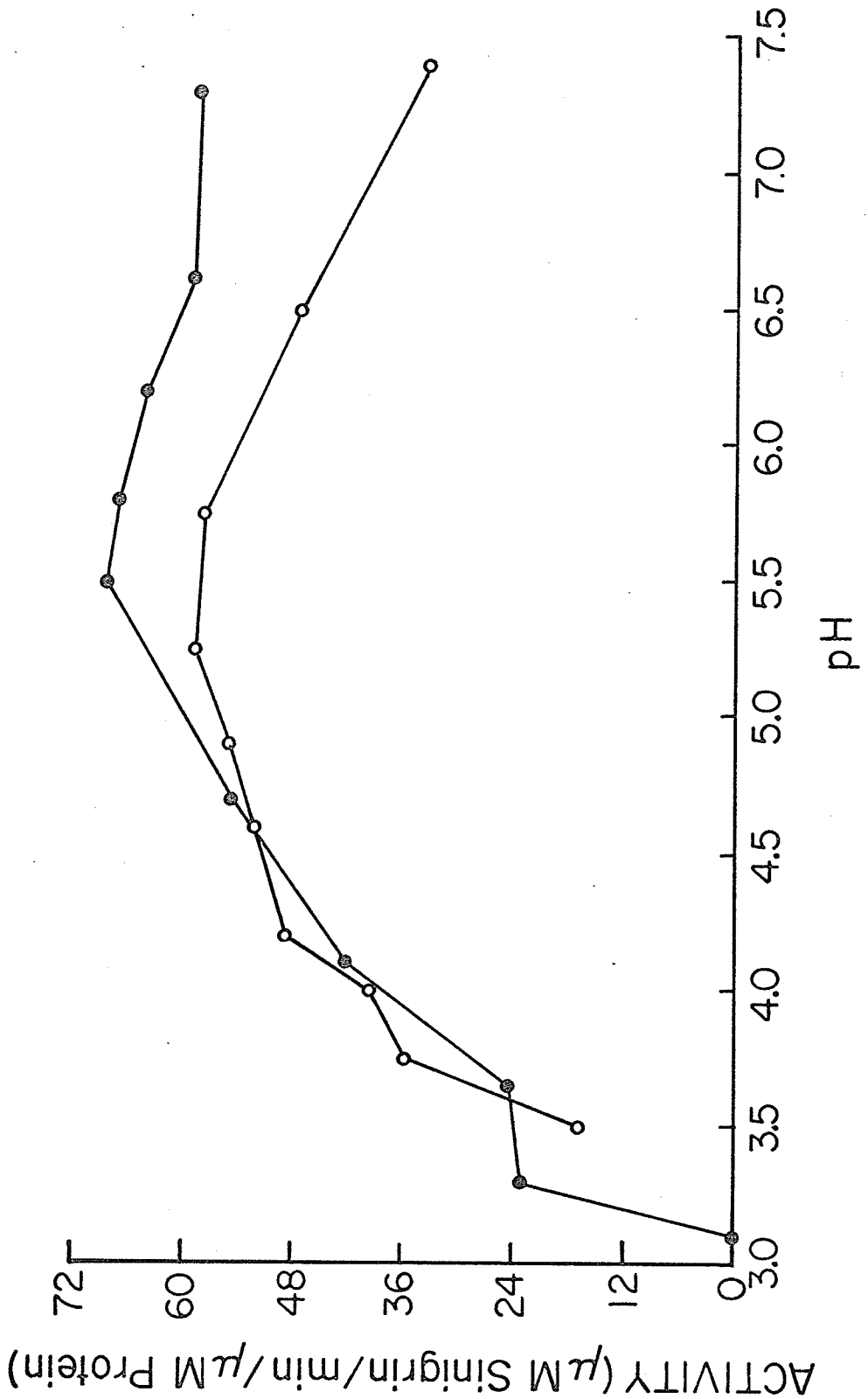

ACTIVITY (μM Sinigrin/min/μM Protein)
72
60
48
36
24
12
0
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
pH

FIGURE VII

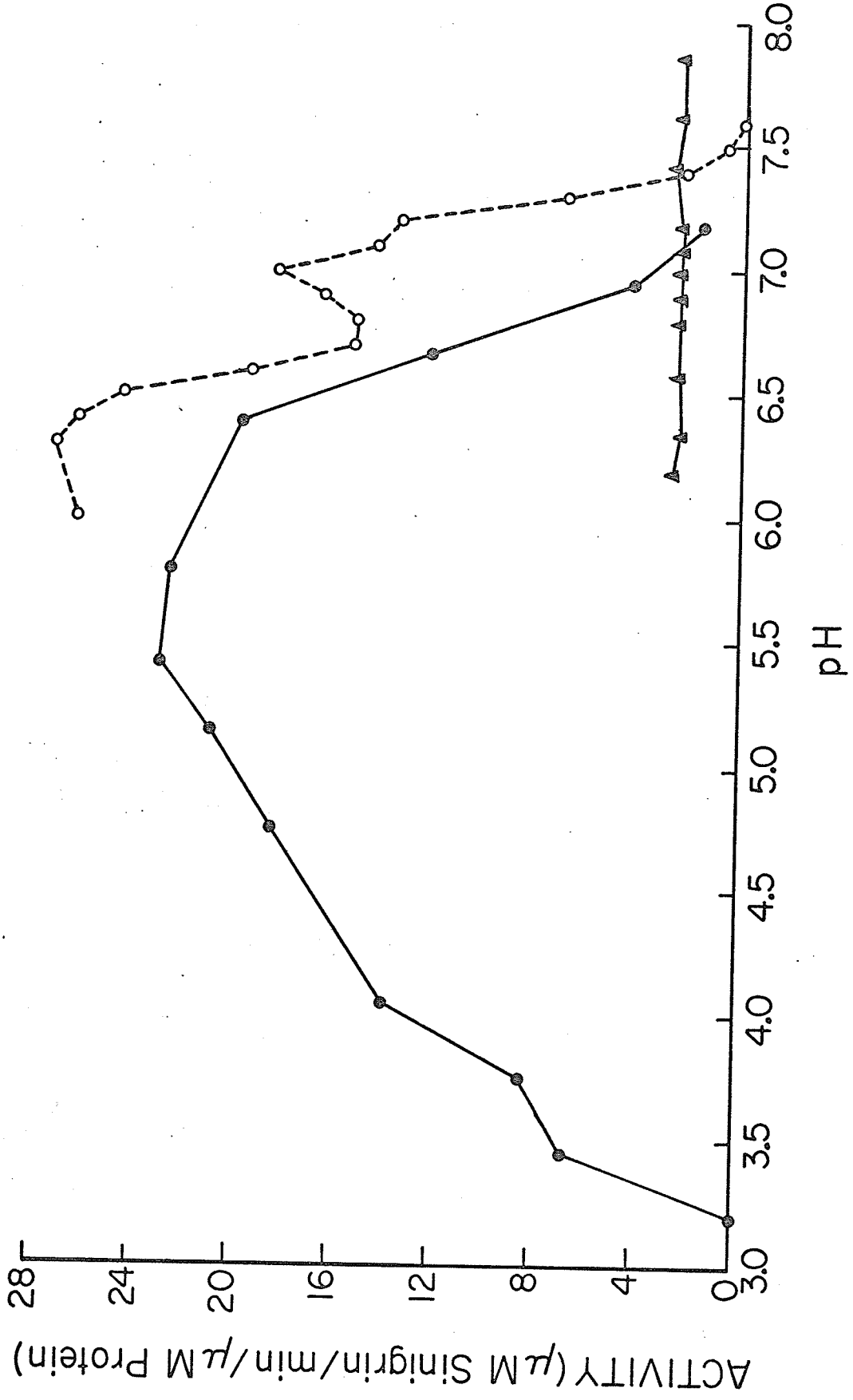
THE EFFECT OF pH ON B. napus (var. - turret) THIOGLUCOSIDASE ACTIVITY.

The enzyme was prepared by Schwimmer's method.

●——● 0.05 M citric acid - 0.1 M Na_2HPO_4 buffer with 3.75×10^{-4} M ascorbic acid.

O---O 0.1 M sodium phosphate buffer with 3.75×10^{-4} M ascorbic acid

▲——▲ 0.1 M sodium phosphate buffer- No ascorbic acid.


ACTIVITY (μM Sinigrin/min/μM Protein)
28
24
20
16
12
8
4
0
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
pH

second pH optimum occurred at 7.1 when phosphate buffer was used. The rapid decrease in activity from pH 7.1 - 7.5 was also not observed for the mustard preparation.

The curve obtained on testing the same rape enzyme with citrate-phosphate buffer, again in the presence of ascorbic acid, shows only a single optimum activity at pH 5.5. The shape of this curve through the pH range of 3.1 - 6.4 is almost identical to that seen in Figure V for mustard thioglucosidase, but above 6.4, a rapid decrease in activity is seen for the rape, in contrast to the mustard enzyme.

In the absence of ascorbic acid, the activity with phosphate buffer, over a pH range of 6.2 - 7.9, is almost constant. This is similar to the curve for mustard under the same conditions, though at a much lower level of activity. There appears to be no correlation, however, between these values and the activity shown over this pH range when ascorbic acid is present. In fact, it would appear at pH values above 7.4 that ascorbic acid inhibits activity.

Figure VIII indicates that the effect of ascorbic acid on mustard thioglucosidase is similar to that observed for rape. A slight increase in activity is seen at about pH 7.1, although not as marked as the second peak for the rape thioglucosidase. Again there appears to be no direct correlation between activity without ascorbic acid and activity in the presence of ascorbic acid.

V. pH Stability

The values obtained from the pH activity tests with various buffers showed that activity was lost at approximately pH 3.0. To ascertain if this was due to irreversible denaturation,

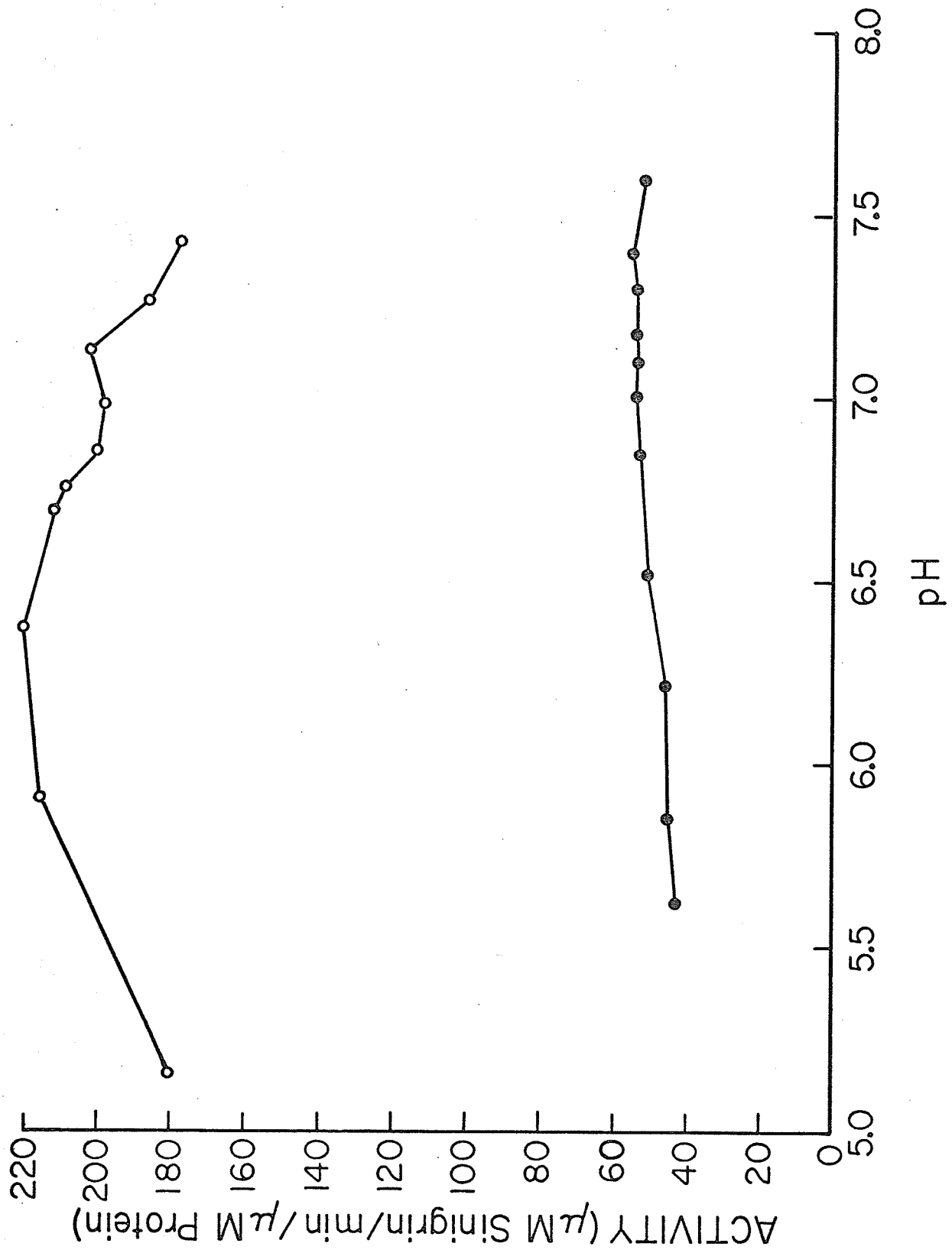
FIGURE VIII

THE EFFECT OF ASCORBIC ACID, AT VARIOUS pH LEVELS, ON *S. alba* THIOGLUCOSIDASE.

The enzyme was prepared by Schwimmer's method. 0.1 M sodium phosphate buffer was used.

●——● No ascorbic acid.

○——○ 3.75×10^{-4} M ascorbic acid


ACTIVITY (μM Sinigrin/min/μM Protein)
220
200
180
160
140
120
100
80
60
40
20
0
5.0
5.5
6.0
6.5
7.0
7.5
8.0
pH

samples of both rape and mustard enzyme preparations were subjected to varying levels of acidity, after which the pH was adjusted to 5.3 $\pm$ .1 and the residual activity determined.

For this test, 12.5 mg. of enzyme preparation were dissolved in 5 ml. of .5N hydrochloric acid - sodium acetate buffer within a pH range of 2.15 - 2.90. After standing for exactly 10 minutes at room temperature, the pH was adjusted to 5.3 by the addition of 5 ml. of 1N sodium acetate. To determine residual activity, 4 ml. of this solution replaced the citrate-phosphate buffer and the 5 mg. of enzyme in the standard activity test. Preliminary testing indicated there was no increase in inactivation for a 30 minute exposure as compared to a 10 minute exposure.

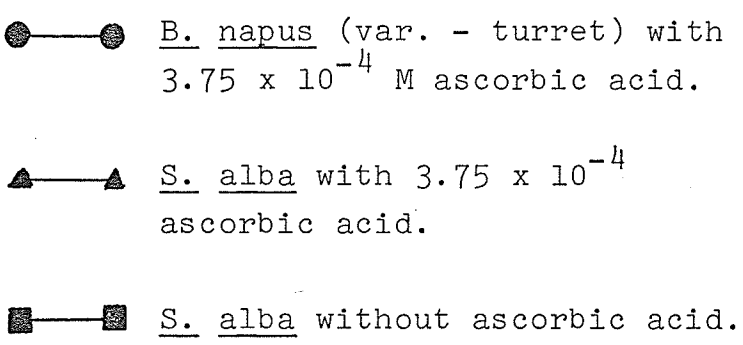
Another 4 ml. of the enzyme-buffer solution were used to test residual activity in the presence of 3.75×10^{-4} M ascorbic acid. This was required in the case of the rape preparation in order to obtain significant activity. The mustard preparation was run with ascorbic acid for comparison.

It is interesting to note in Figure IX that the rape preparation appears to be more resistant to acidic denaturation under the conditions of this test. For exposure to acid levels between pH 2.15 and 2.50, the rape preparation shows greater residual activity than the mustard preparation. The addition of ascorbic acid to the mustard thioglucosidase after exposure to low pH had little effect on the residual activity curve.

VI Effects of Temperature

Figure X shows the effect of temperature on the rate of reaction over a temperature range of 25 - 70°. Between 25° and 60° a positive correlation was observed between the temperature and

FIGURE IX ENZYME STABILITY ON EXPOSURE TO LOW pH FOR 10 MINUTES AT ROOM TEMPERATURE.



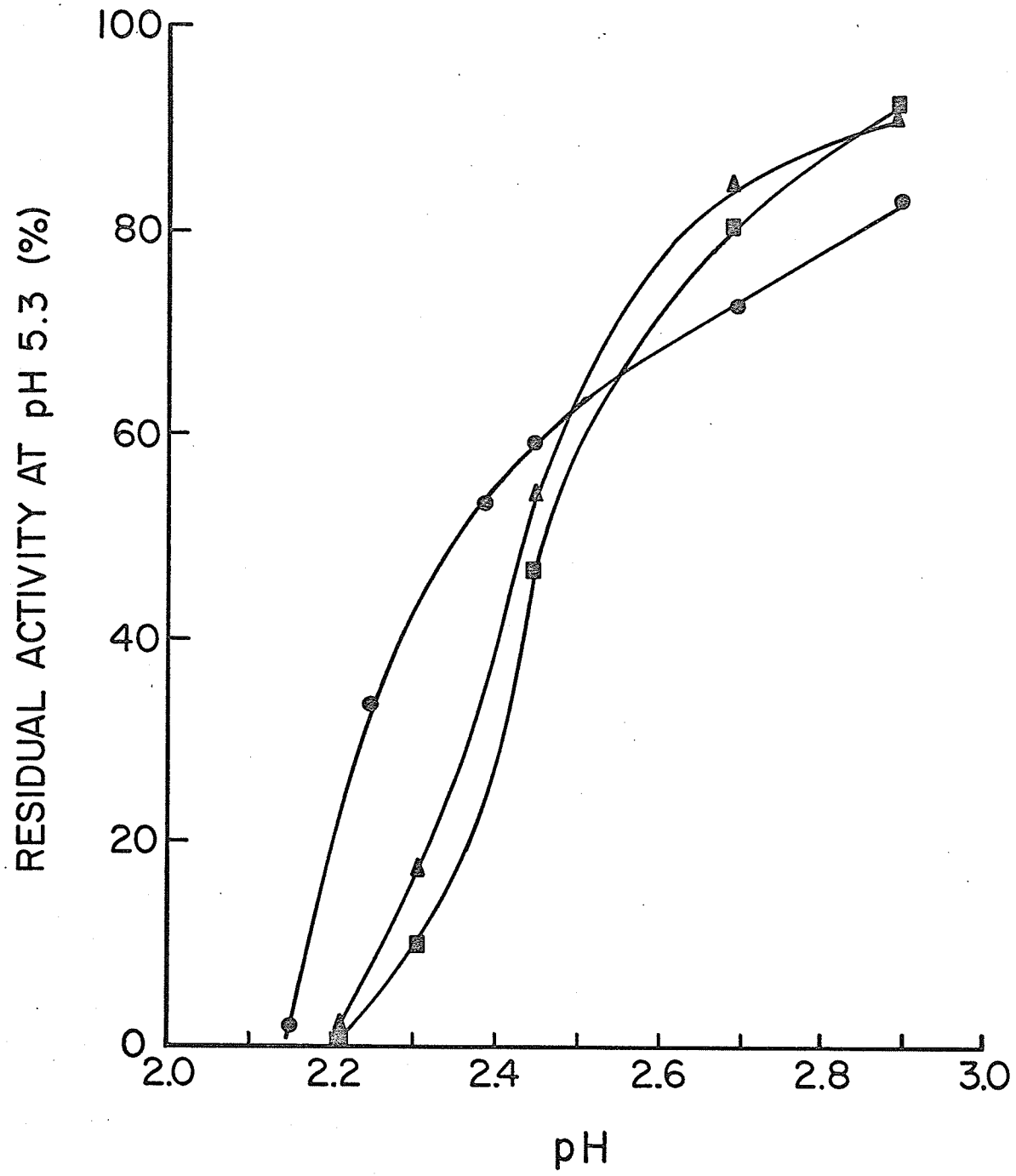
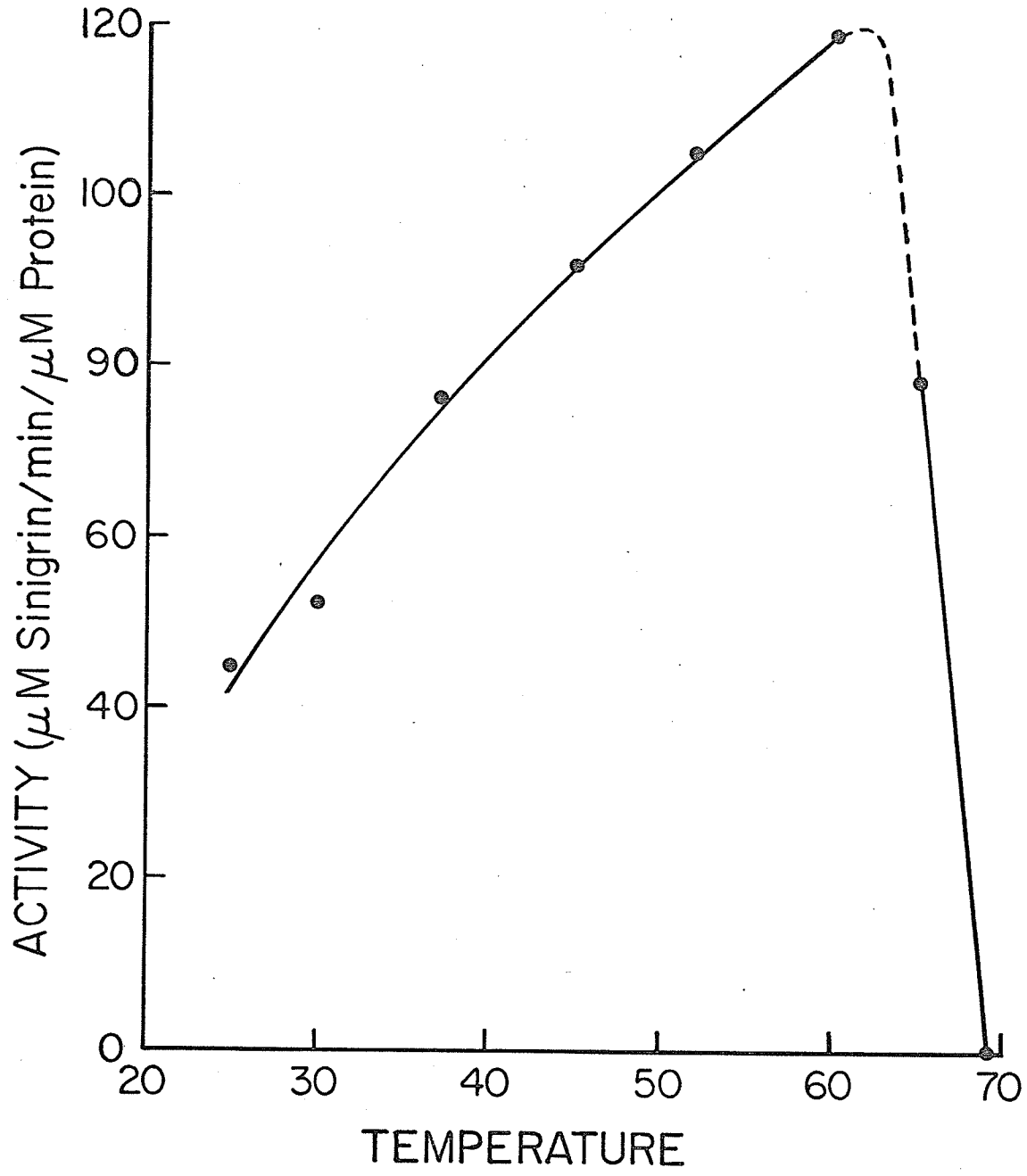

100
80
60
40
20
0
RESIDUAL ACTIVITY AT pH 5.3 (%)
2.0
2.2
2.4
2.6
2.8
3.0
pH

FIGURE X THE EFFECT OF TEMPERATURE ON ENZYME ACTIVITY.

Thioglucosidase from S. alba prepared by Wrede's method.


120
100
90
60
40
20
0
ACTIVITY (μM Sinigrin/min/μM Protein)
20
30
40
50
60
70
TEMPERATURE

the reaction rate, with activity approximately doubling for every 20° rise in temperature. Above 60° a sharp decrease in activity was observed. This decrease in reaction rate was presumably due to thermal denaturation. As the degree of denaturation is dependent on the time of exposure as well as the temperature, accurate reaction rates become increasingly difficult to record as the temperature was increased above 60°. At 69° no activity could be detected.

In attempts to ascertain if the denaturation was irreversible, both mustard and rape preparations were tested for heat stability. For this test, 2.5 ml. of enzyme solution were pipetted into a test tube held in a heated water bath at the temperature shown. After exactly three minutes, the tube was removed from the water bath and rapidly cooled to room temperature. The residual activities were determined at 37° and the result is shown in Figure XI.

These data indicate similar stabilities towards heat for the two preparations, although between 57° and 68°, the mustard enzyme is slightly more stable.

VII. Isoenzyme Patterns

A. Comparison of Seeds

Results from polyacrylamide gel electrophoresis of crude extracts from several species of Cruciferae oilseeds are shown in Figures XII and XIII. Densitometric tracings from the isoenzyme bands are shown in Figure XII. The R_m values for each species were reproducible to within $\pm$ 5%. The variance in the rate of migration of the solvent front for all trials was as much as 20%. For purposes of comparing relative mobilities, the isoenzyme bands, shown as line

FIGURE XI HEAT STABILITY DETERMINATION

Exposure of the enzyme for 3 min. to the temperature shown. Both enzymes prepared by Wrede's method.

●——● S. alba

○---○ B. napus (Var. - bronowski)

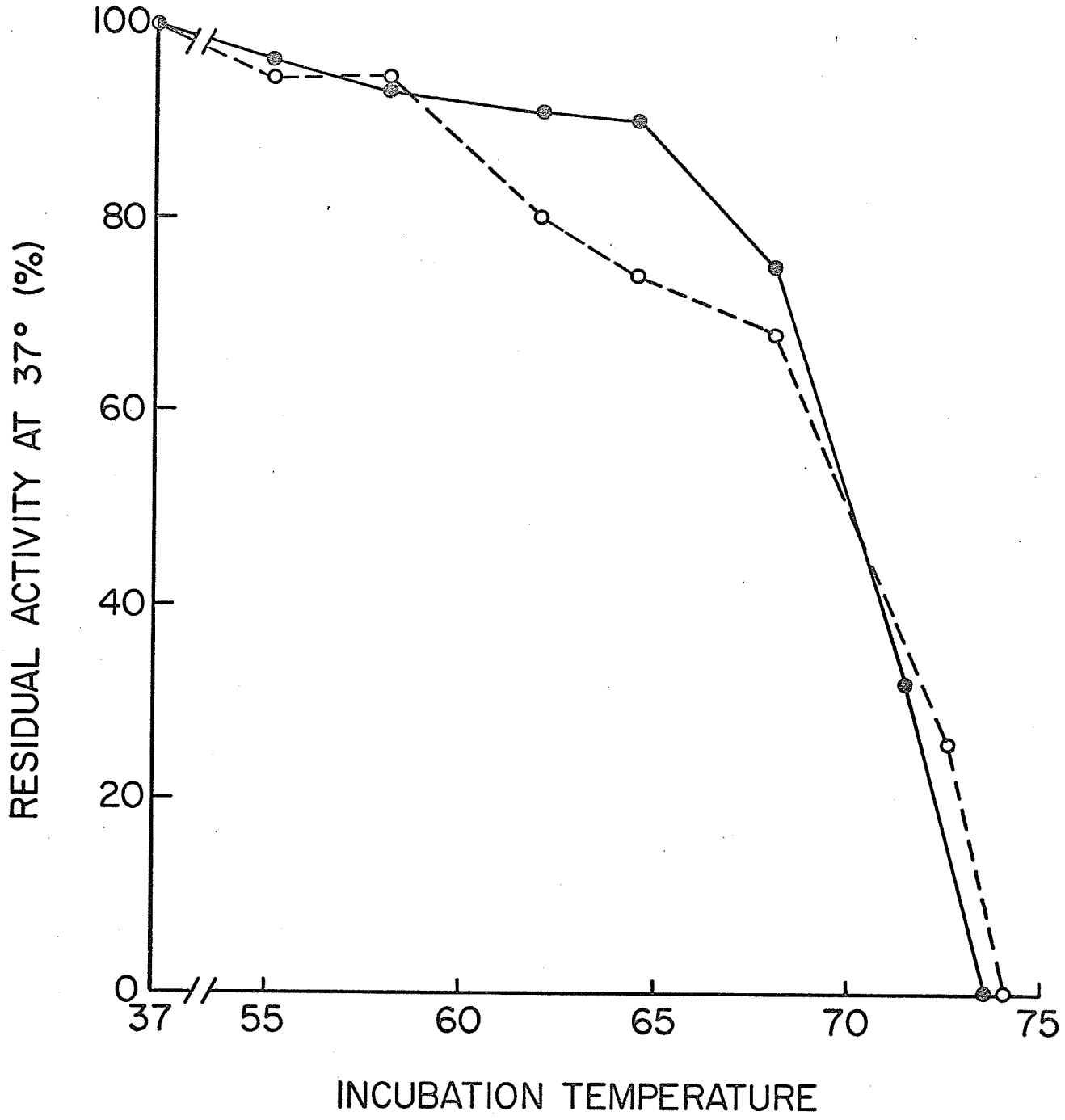

100
80
60
40
20
0
RESIDUAL ACTIVITY AT 37° (%)
37
55
60
65
70
75
INCUBATION TEMPERATURE

FIGURE XII DENSITOMETRIC TRACINGS OF ISOENZYMES FROM SEED EXTRACTS.

Disc electrophoresis was performed on 7.5% polyacrylamide gel at pH 9.3

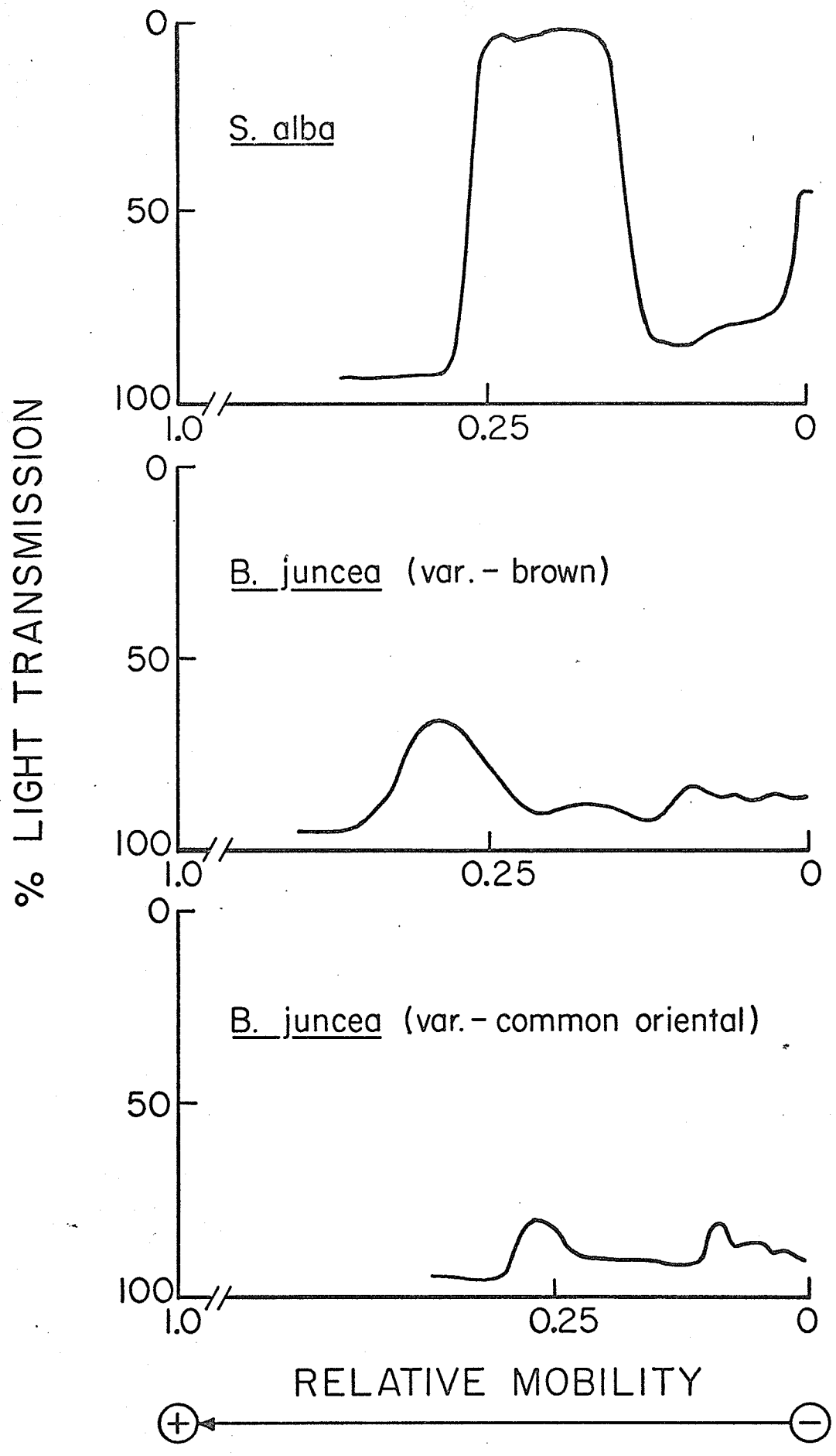

% LIGHT TRANSMISSION
0
50
100
S. alba
1.0
0.25
0
B. juncea (var. - brown)
B. juncea (var. - common oriental)
RELATIVE MOBILITY
+
−

FIGURE XII Continued

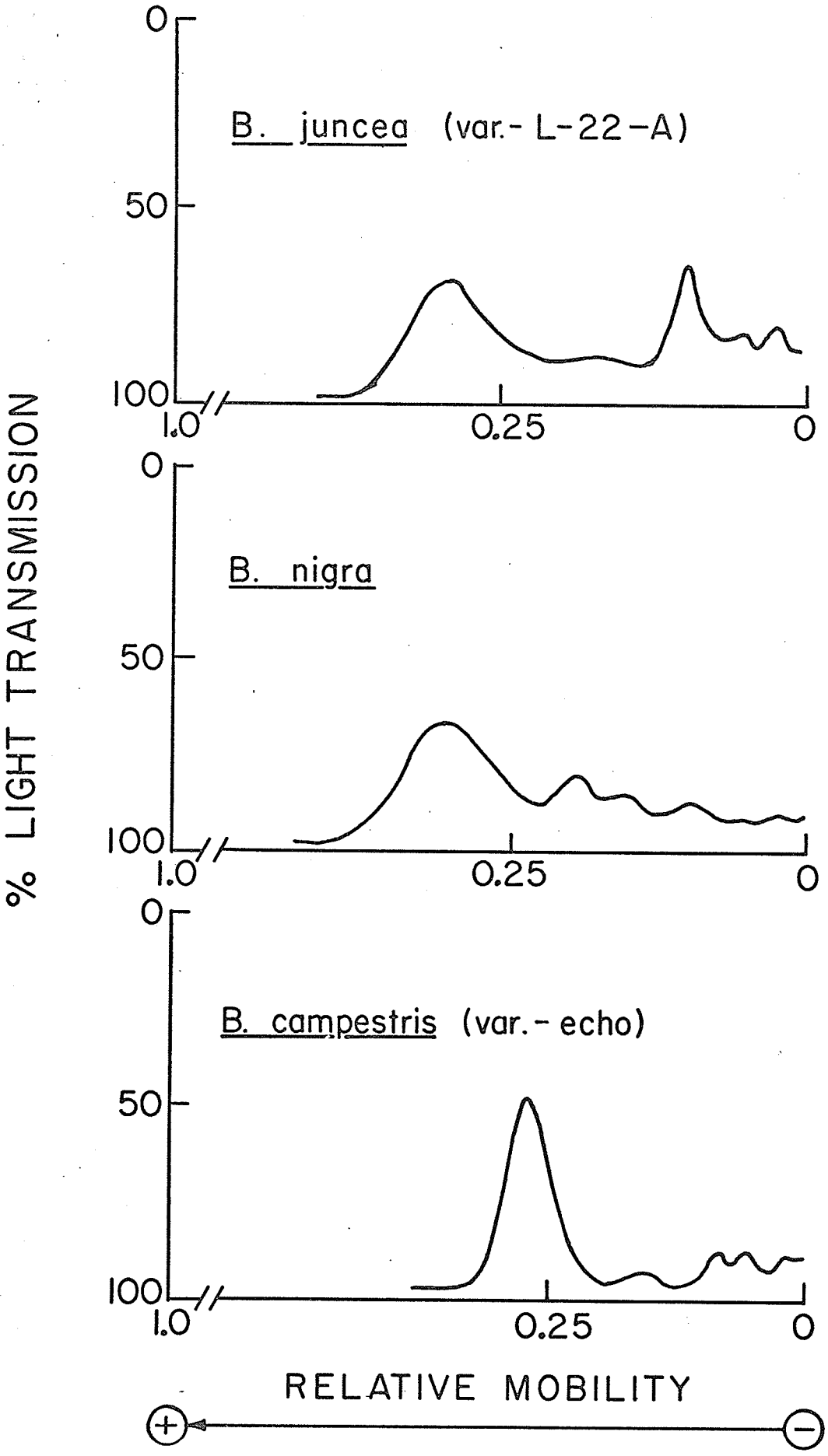

% LIGHT TRANSMISSION
0
50
100
B. juncea (var.- L-22-A)
1.0
0.25
0
B. nigra
B. campestris (var.- echo)
RELATIVE MOBILITY
+
−

FIGURE XII Continued

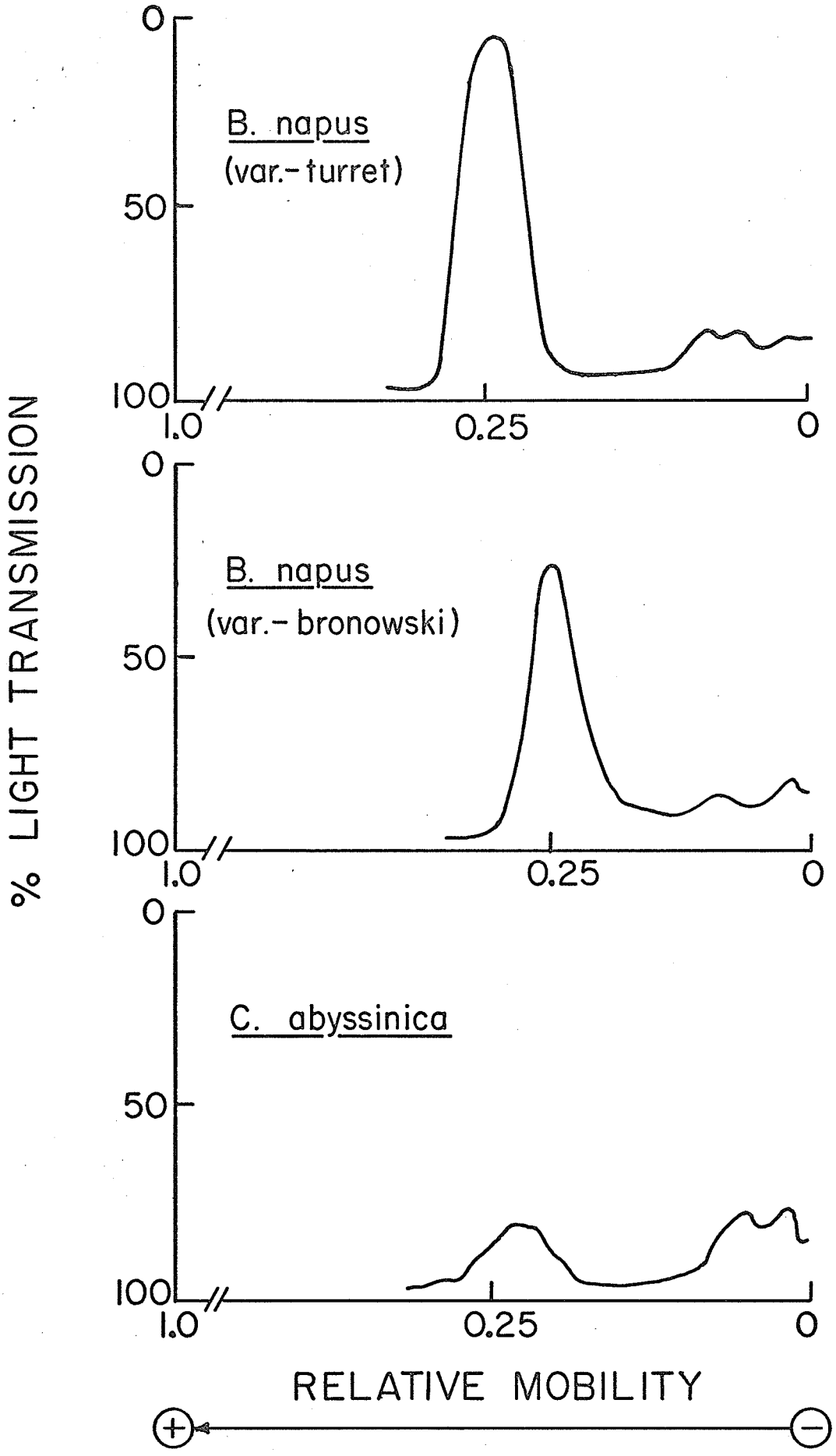

% LIGHT TRANSMISSION
0
50
100
B. napus
(var.- turret)
1.0
0.25
0
B. napus
(var.- bronowski)
C. abyssinica
RELATIVE MOBILITY
+
−

FIGURE XIII LINE DRAWINGS OF ISOENZYMES FROM SEED EXTRACTS.

Band width based on peak height as shown in Figure XII.

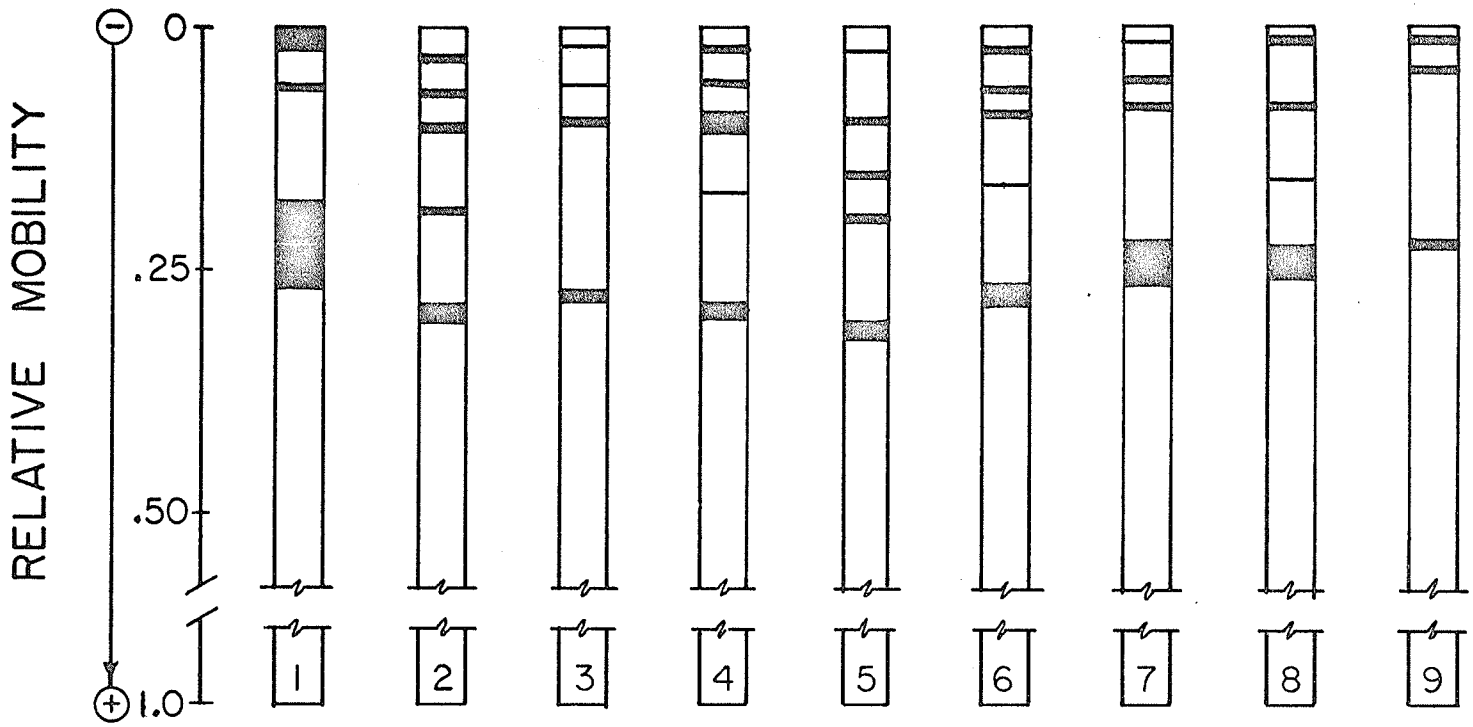


Fig. XIII Isoenzyme bands from nine varieties of Cruciferae oilseeds.

1. S.alba
2. B. juncea (Var.-brown)
3. B. juncea (Var.-common oriental)
4. B. juncea (var.-L-22-A)
5. B. nigra
6. B. campestris (var.-echo)
7. B. napus (var.-turret)
8. B. napus (Var.-bronowski)
9. C. abyssinica

drawings in Figure XIII, are presented with each species adjusted to a standard absolute mobility.

Differences in comparative peak heights for repetitive tests on the same seed were found to vary up to 30%. This was believed to be due both to the method of enzyme extraction from the seeds, and to the small quantities of extract applied to the gels. Thus, comparisons of peak areas between gel tracings in Figure XII should be made with some reservations. It is safe to say, however, that the amount of precipitate observed for S. alba far exceeds that shown for the other species.

No attempt was made to determine if the differences in mobility of the various bands among species were statistically significant. It was observed, however, that the band patterns for S. alba, B. campestris and B. napus (var. - turret), closely resembled those reported by MacGibbon and Allison (15) for these three species.

To determine the effect of mercaptoethanol, samples of crambe were extracted without the addition of mercaptoethanol to the extracting solution. On comparing these results with those seen in Figure XIII for crambe, it was observed that the bands at R_m 0.015 and 0.045 remained, while the band at R_m 0.23 disappeared.

B. Comparison of Enzyme Preparations

Several of the enzyme preparations were separated into isoenzyme bands using polyacrylamide gel electrophoresis. All conditions were similar to those for the whole seed studies, except for a proportional reduction in sample size based on the activity of the preparation. Using this procedure, it was possible to locate active isoenzymes in the crambe and B. juncea preparations even though their

activity could not be detected by the standard method for estimation of thioglucosidase activity. Figures XIV and XV show the densitometric tracings and the comparative bands, respectively, for these tests. Results from the whole seed experiments have been included in Figure XV for comparison.

If significant alterations of the enzyme had been brought about by the various purification steps, it was believed this would have become evident in the isoenzyme patterns. Although some changes are observed in the band patterns for each preparation, these are, in most instances, considered insignificant.

It is interesting to note that two distinct bands appear in the R_m 0.2 - 0.3 range for the enzyme preparations from turret rape and from crambe, where only one band was observed in each case for the whole seed. Further studies are necessary to determine whether these bands arise from the dissociation of high molecular weight aggregates, or from some other type of alteration.

Two faint bands exhibiting R_m values of 0.35 and 0.41 were observed in the crambe preparation. Bands in this relative mobility range were not observed for any of the other preparations. Further work is necessary to characterize these isoenzyme bands.

As it had been noted that the bands of slower mobility from yellow mustard were lost using Wrede's method of purification, samples were taken from a subsequent preparation at various stages throughout the purification procedure and their isoenzyme patterns determined. The activity of these bands was shown to be lost during the second ethanolic precipitation step. Similar tests carried out on bronowski rape at the

FIGURE XIV

DENSITOMETRIC TRACINGS OF ISOENZYMES FROM PARTIALLY PURIFIED ENZYME PREPARATIONS.

Disc electrophoresis was performed on 7.5% polyacrylamide gel at pH 9.3.

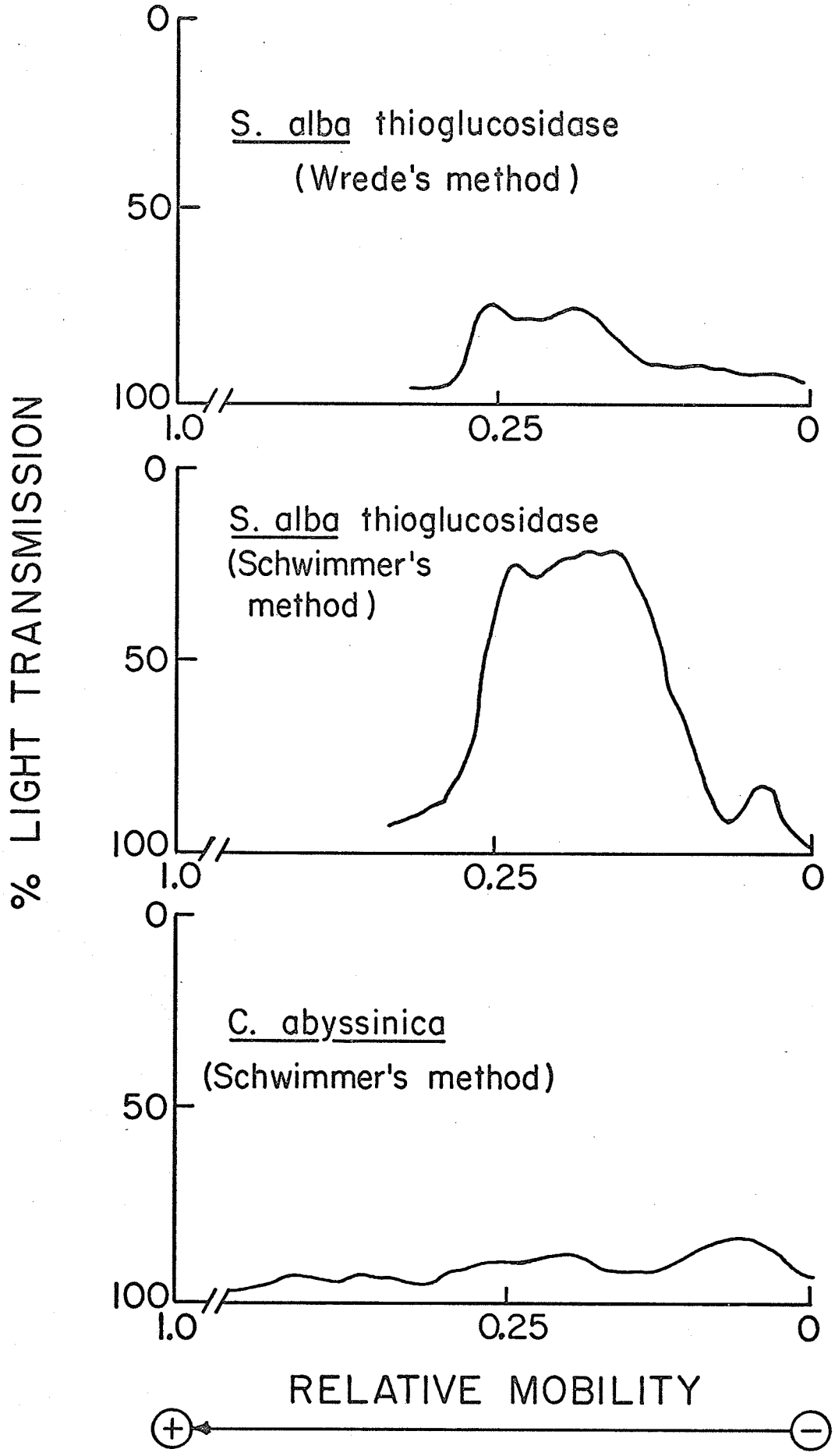

% LIGHT TRANSMISSION
0
50
100
S. alba thioglucosidase
(Wrede's method)
1.0
0.25
0
S. alba thioglucosidase
(Schwimmer's
method)
C. abyssinica
(Schwimmer's method)
RELATIVE MOBILITY
+
−

FIGURE XIV Continued

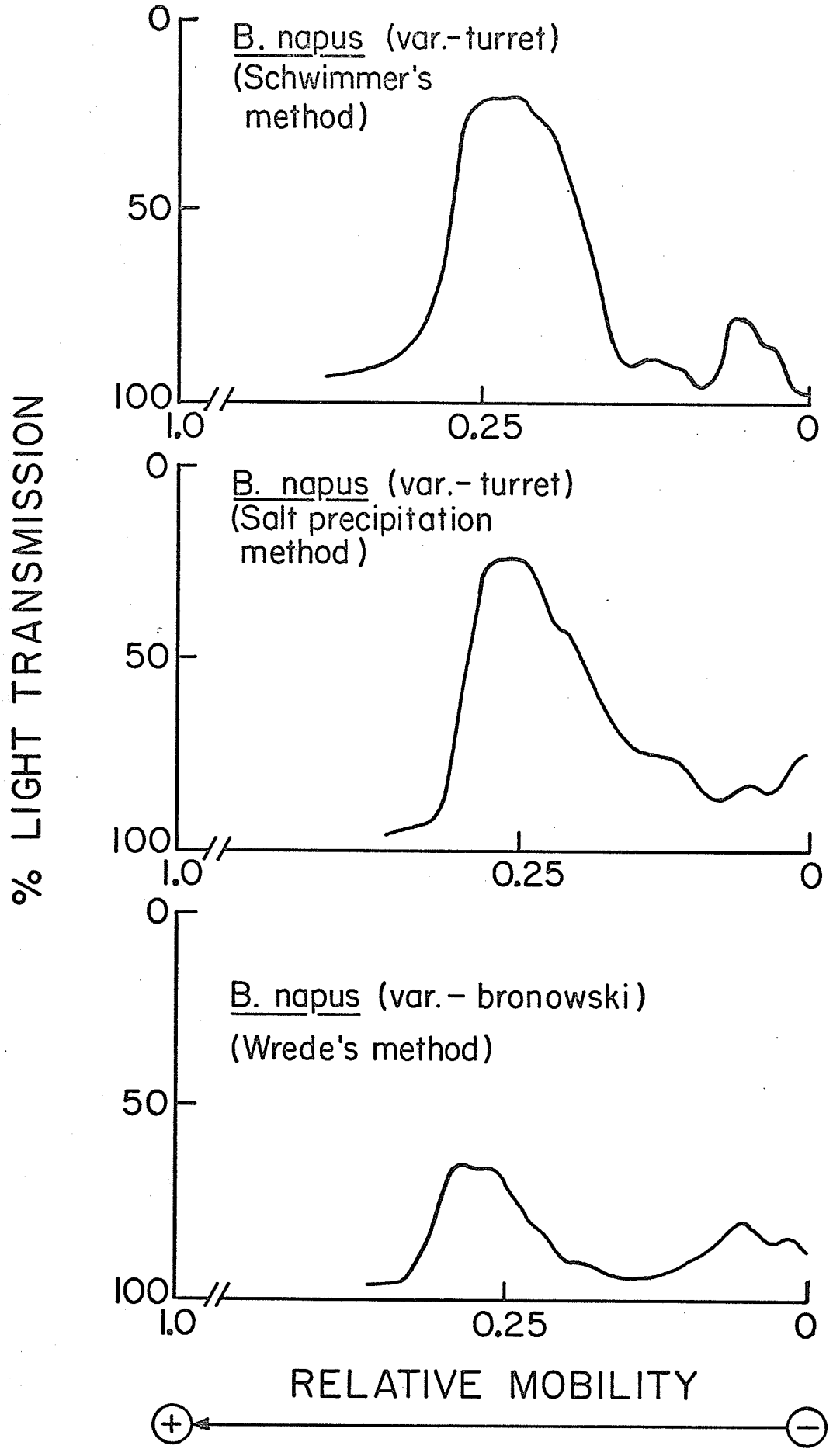

B. napus (var.-turret)
(Schwimmer's method)
B. napus (var.- turret)
(Salt precipitation method)
B. napus (var.- bronowski)
(Wrede's method)
0
50
100
1.0
0.25
0
% LIGHT TRANSMISSION
RELATIVE MOBILITY
+
−

FIGURE XIV Continued

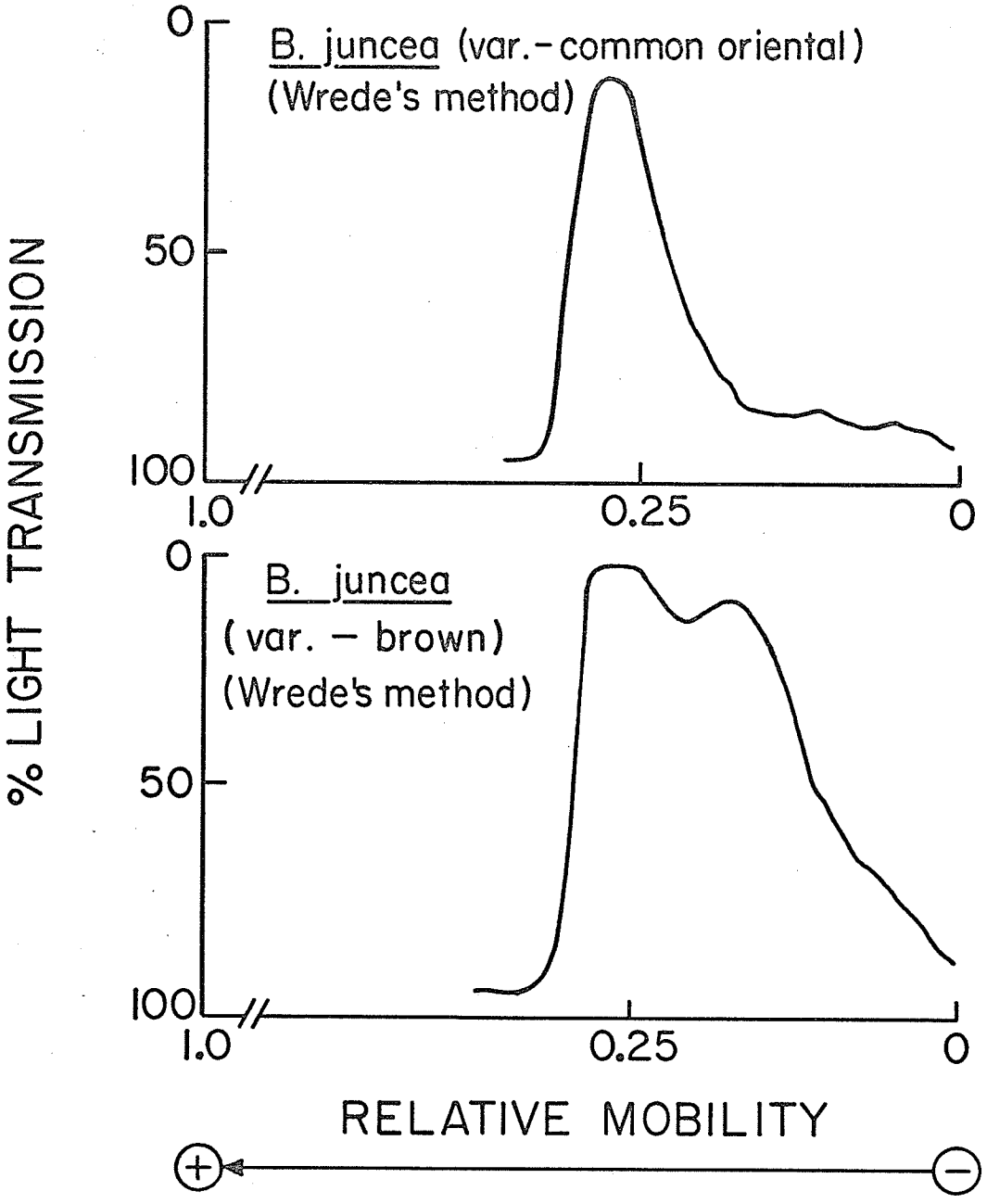
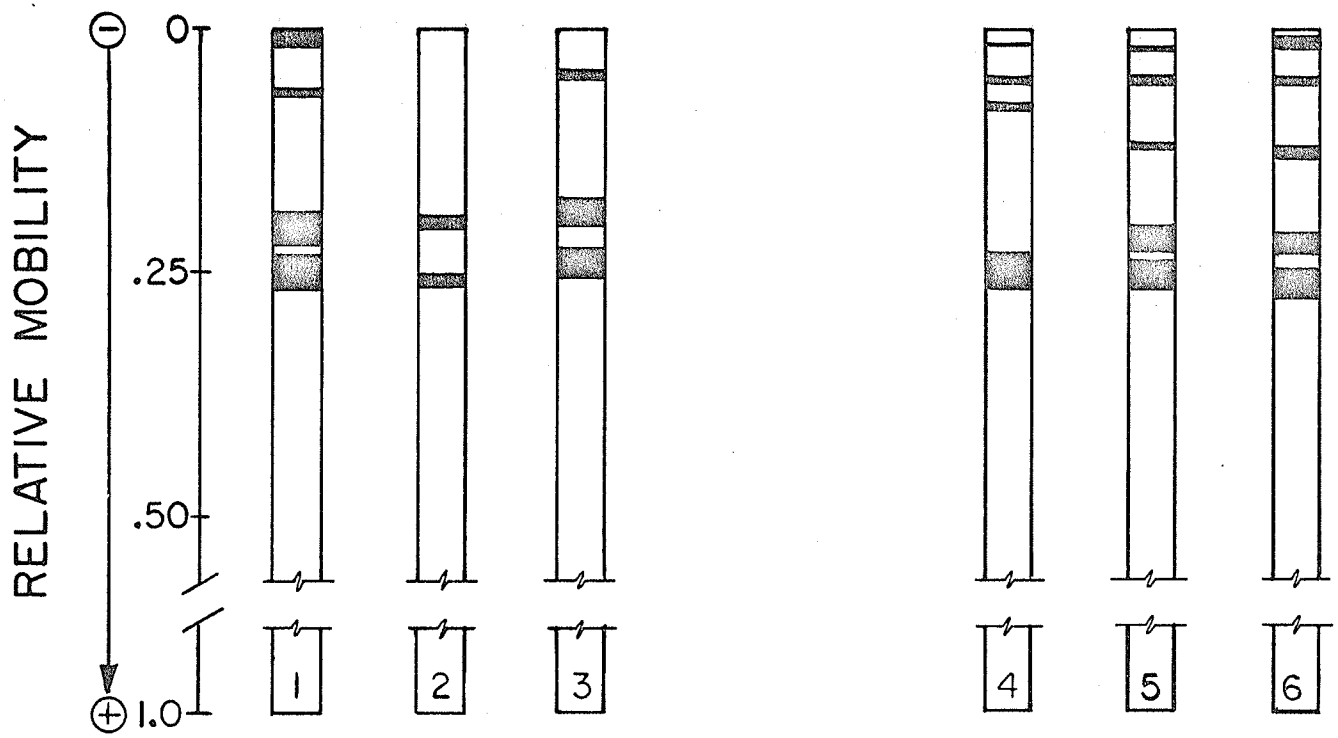

% LIGHT TRANSMISSION
0
50
100
B. juncea (var.-common oriental)
(Wrede's method)
1.0
0.25
0
0
50
100
B. juncea
(var. – brown)
(Wrede's method)
1.0
0.25
0
RELATIVE MOBILITY
+
–

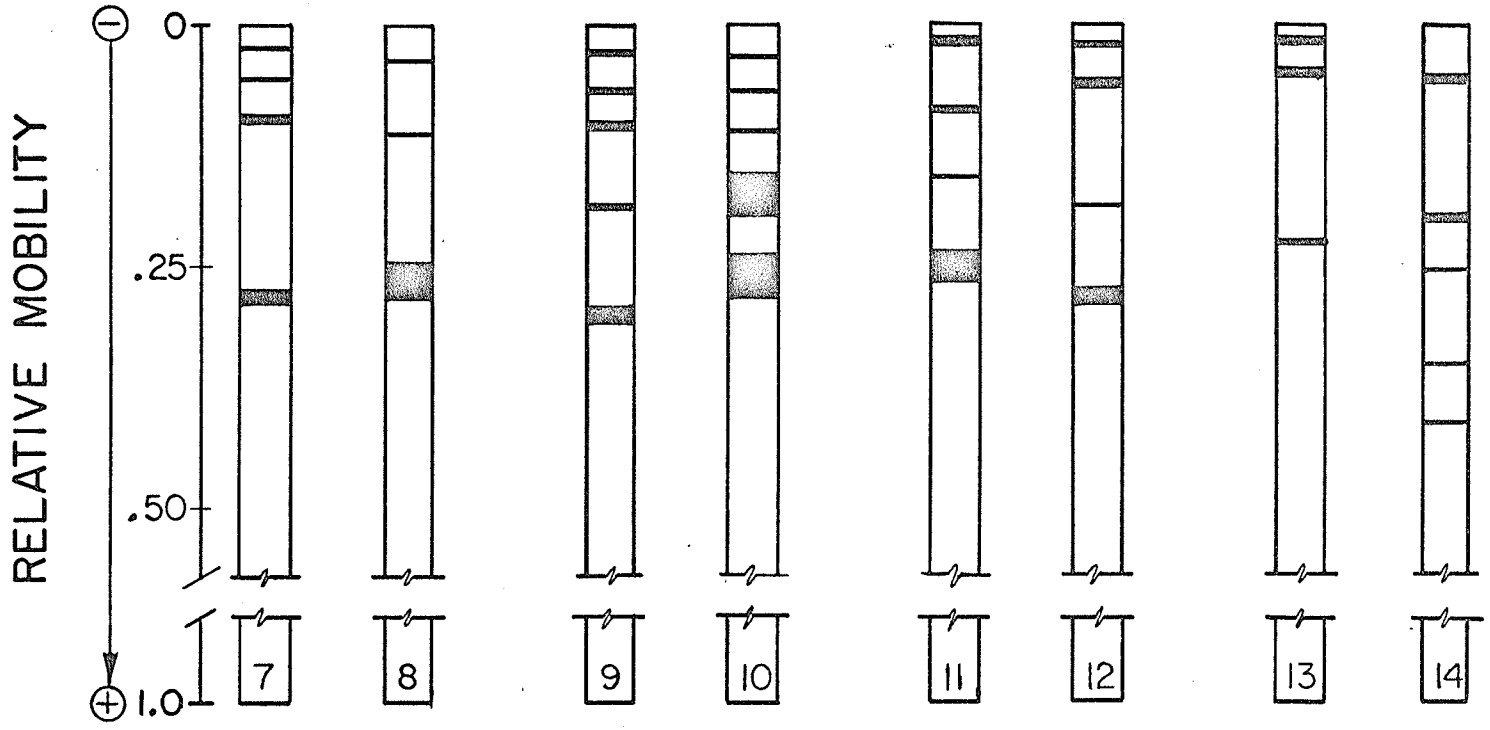
FIGURE XV LINE DRAWINGS OF ISOENZYMES FROM PARTIALLY PURIFIED ENZYME PREPARATIONS.

Band width based on peak height as shown in Figure XIV.



1. S. alba (whole seed)
2. S. alba thioglucosidase (Wrede's method)
3. S. alba thioglucosidase (Schwimmer's method)
4. B. napus (var.-turret, whole seed)
5. B. napus (var.-turret, Schwimmer's method)
6. B. napus (var.-turret, salt ppt'n method)

FIGURE XV Continued



7. B. juncea (var.- common oriental, whole seed)
8. B. juncea (var.- common oriental, Wrede's method)
9. B. juncea (var.-brown, whole seed)
10. B. juncea (var.-brown, Wrede's method)
11. B. napus (var.-bronowski, whole seed)
12. B. napus (var.-bronowski, Wrede's method)
13. C. abyssinica (whole seed)
14. C. abyssinica (Schwimmer's method)

same time, however, showed no loss of the slower moving bands throughout purification.

C. Effects of Ascorbic Acid on Specific Isoenzymes

Due to the variation in ascorbic acid activation in different species, experiments were conducted to determine whether ascorbic acid preferentially activated specific isoenzymes. As ascorbic acid was included in the standard developing solution, a control solution was prepared in which ascorbic acid was omitted. Gels, on which enzyme preparations from yellow mustard and turret rape had been electrophoretically separated, were developed in the two solutions. At various intervals, gels of each species were removed from the solutions and scanned on the Densicord. The tracings from these gels are shown in Figures XVI, XVII, XVIII and XIX.

Although no marked changes were observed among the band patterns, two slight effects of ascorbic acid can be shown. First it was observed that even after eight hours, none of the slower-moving bands in the turret rape preparations had appeared when ascorbic acid was absent (Figure XIX). These bands began to appear forty-five minutes after developing began, when ascorbic acid was present (Figure XVIII). This same observation might be made for the mustard preparation, but the observations are inconclusive at the enzyme concentration used.

A second change is illustrated in Figure XX. These figures were constructed by comparing the rate of development of the band with the most rapid mobility for the two preparations. This band exhibited an R_m value of .24 - .25 in both species. Comparisons were based on peak height, with the height after eight hours of development arbitrarily taken as 100 percent development. The ratio of the peak height after thirty minutes, forty-five minutes, seventy-five minutes, and two hours, compared with that after eight hours, was determined.

FIGURE XVI RATE OF ISOENZYME DEVELOPMENT IN *S. alba*, WITH ASCORBIC ACID.

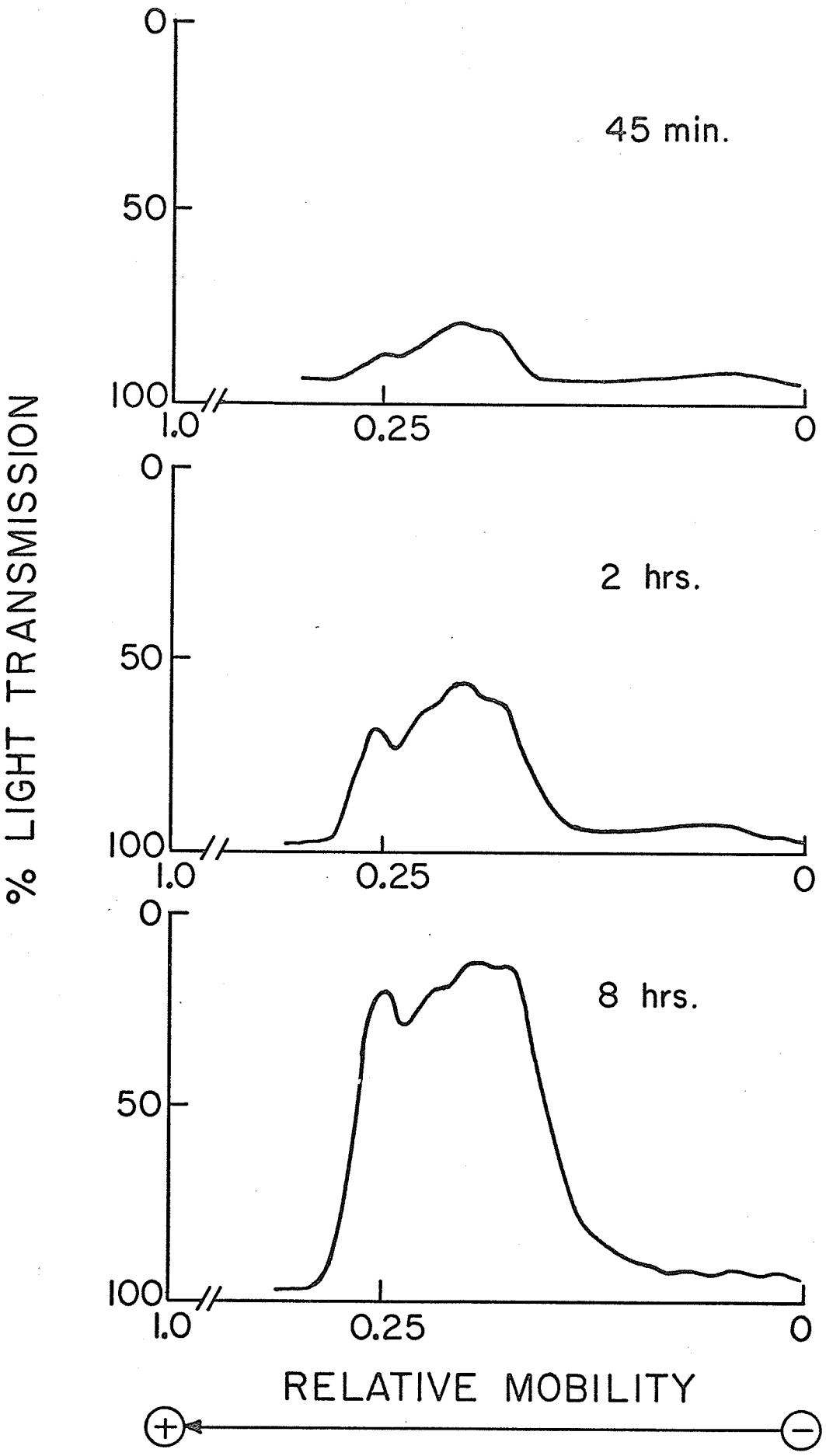

% LIGHT TRANSMISSION
0
50
100
45 min.
1.0
0.25
0
2 hrs.
8 hrs.
RELATIVE MOBILITY
+
−

FIGURE XVII RATE OF ISOENZYME DEVELOPMENT IN *S. alba*, WITHOUT ASCORBIC ACID.

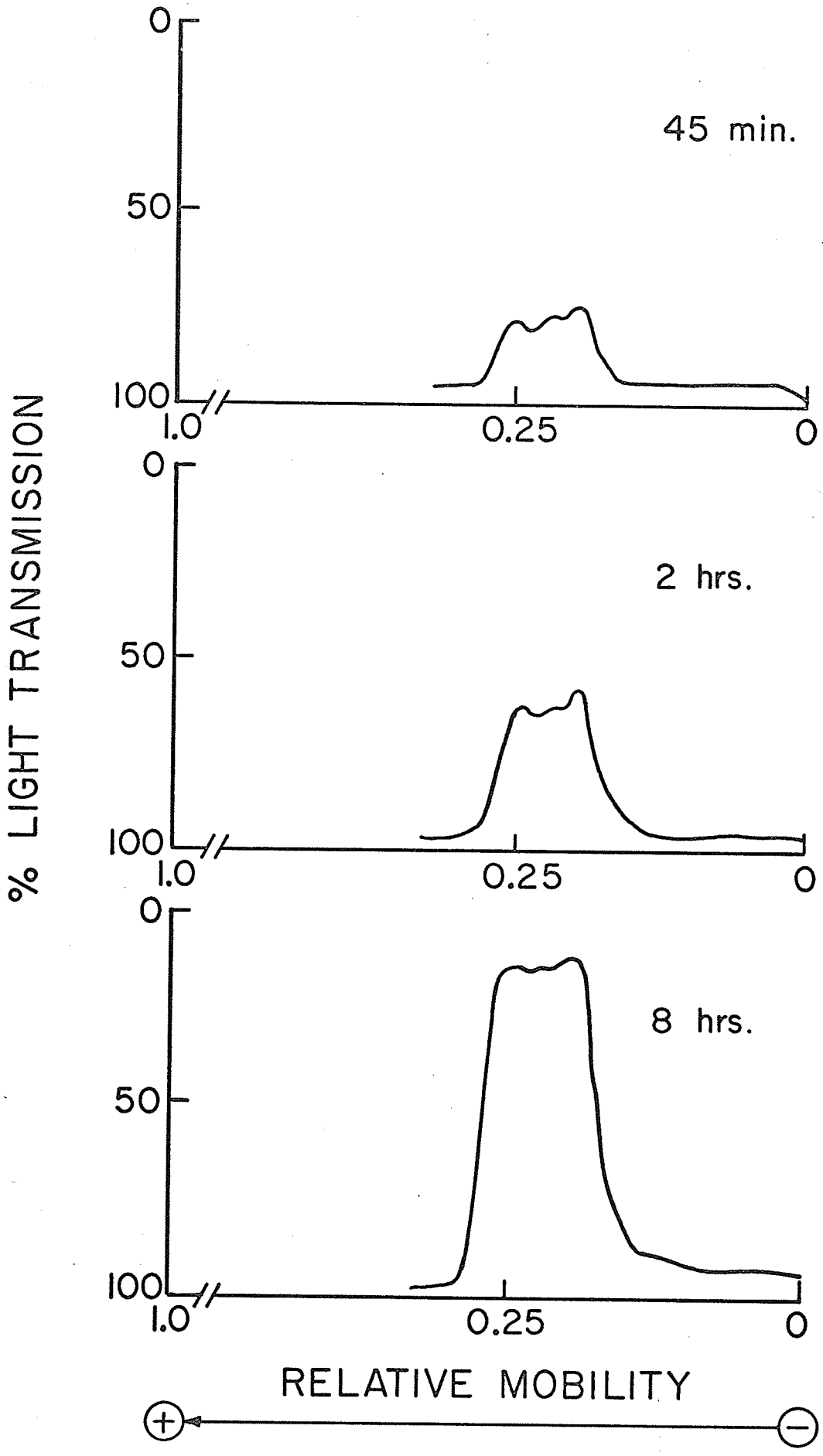

0
50
100
45 min.
1.0
0.25
0
2 hrs.
8 hrs.
% LIGHT TRANSMISSION
RELATIVE MOBILITY
+
−

FIGURE XVIII RATE OF ISOENZYME DEVELOPMENT IN *B. napus* (var. - turret), WITH ASCORBIC ACID.

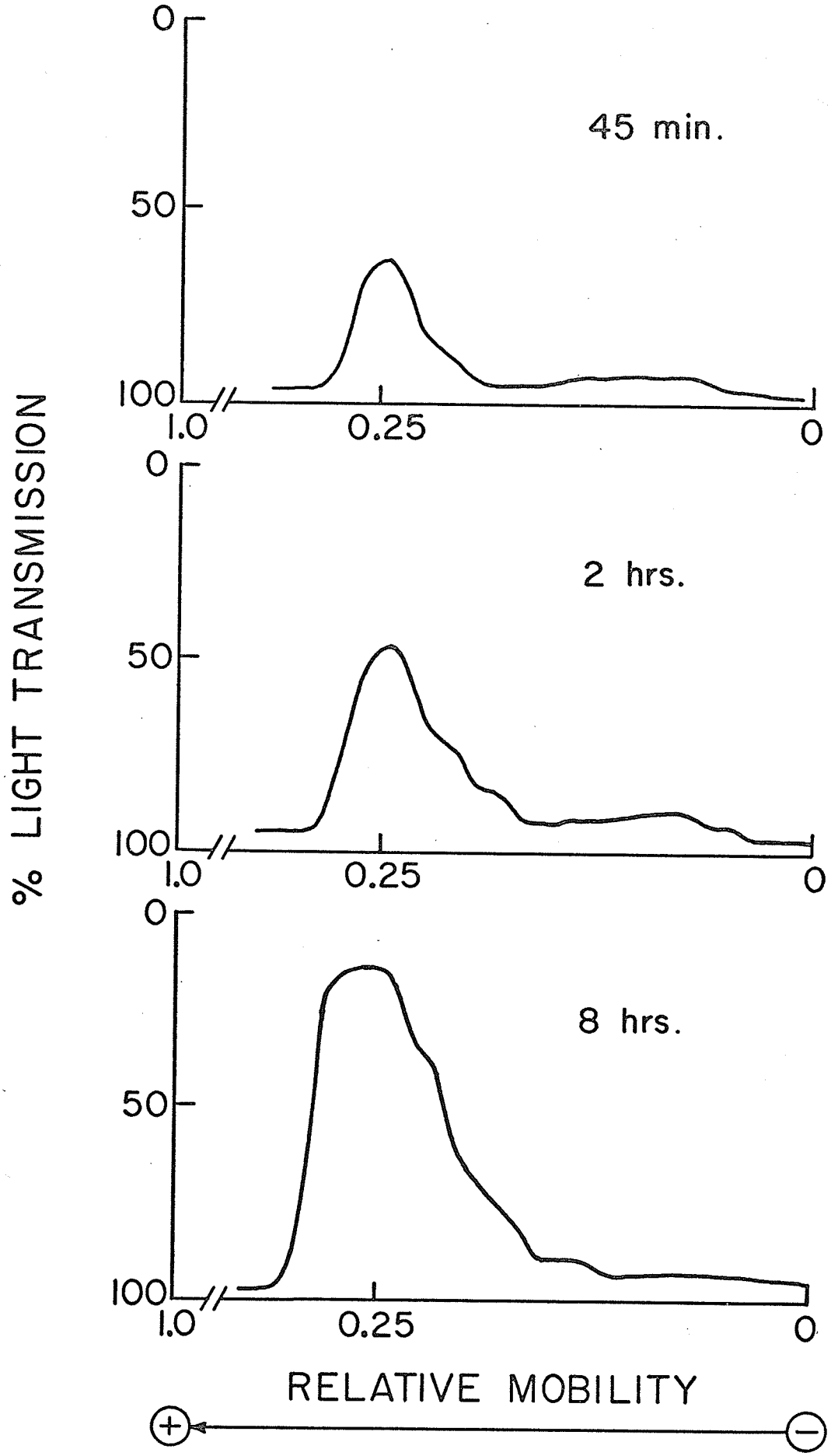

% LIGHT TRANSMISSION
0
50
100
45 min.
1.0
0.25
0
2 hrs.
8 hrs.
RELATIVE MOBILITY
+
−

FIGURE XIX RATE OF ISOENZYME DEVELOPMENT IN *B. napus* (var. - turret) WITHOUT ASCORBIC ACID.

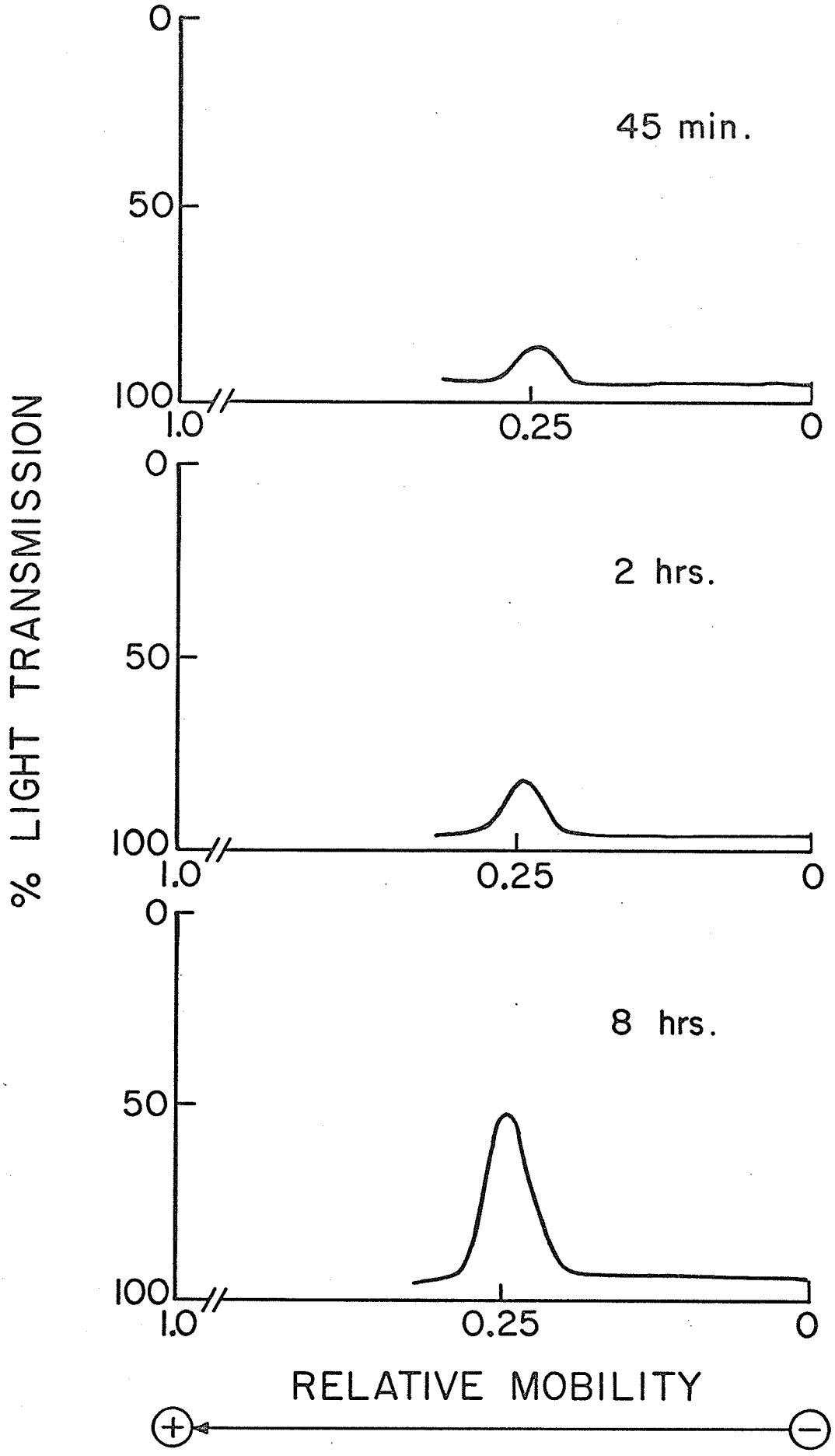
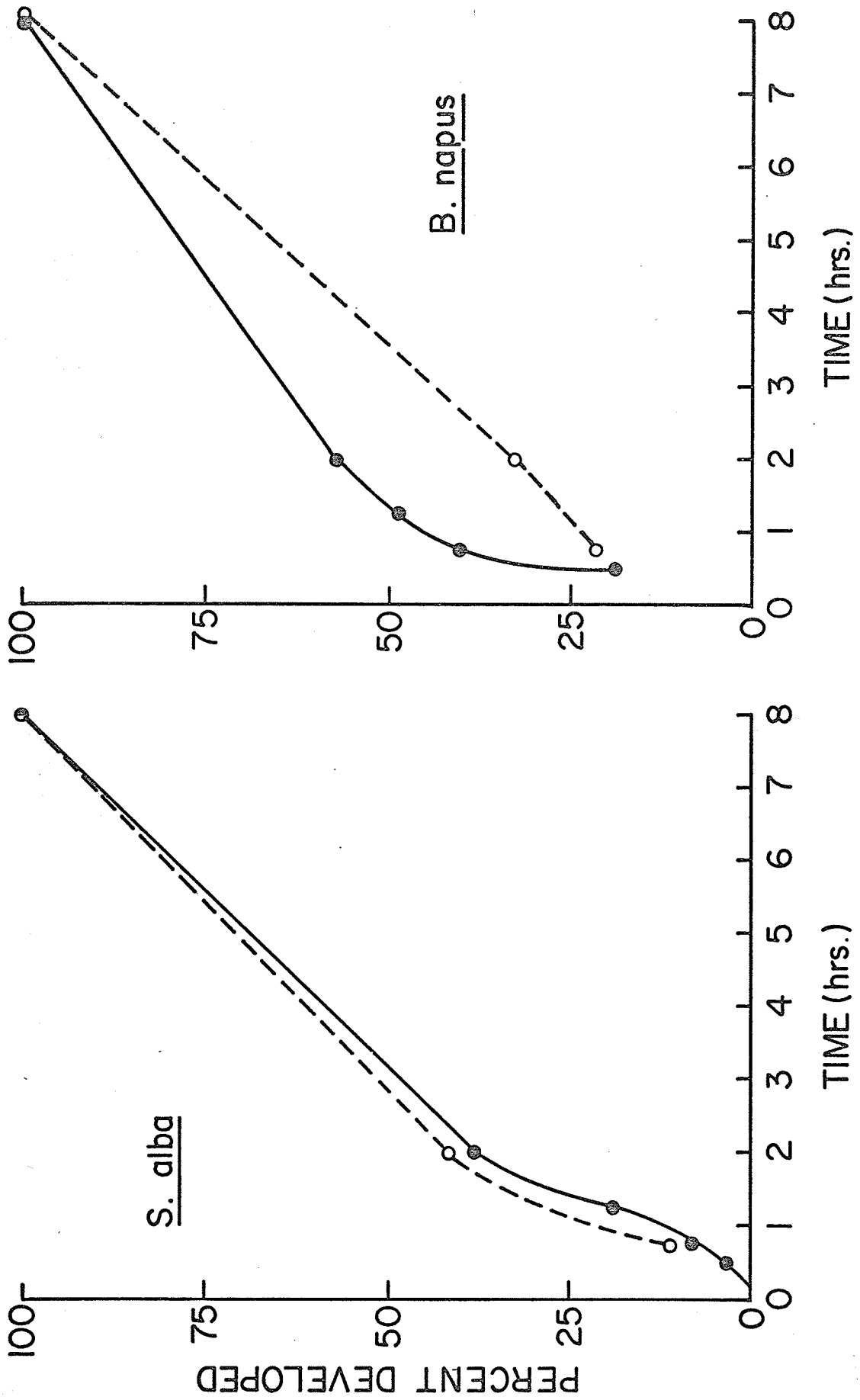

% LIGHT TRANSMISSION
0
50
100
45 min.
1.0
0.25
0
2 hrs.
8 hrs.
RELATIVE MOBILITY
+
−

FIGURE XX

COMPARATIVE RATE OF DEVELOPMENT OF THE ISOENZYME AT R_m 0.25 SEEN IN FIGURES XVI, XVII, XVIII AND XIX.

●——● with ascorbic acid

O---O without ascorbic acid


B. napus
S. alba
PERCENT DEVELOPED
100
75
50
25
0
0 1 2 3 4 5 6 7 8
TIME (hrs.)

This ratio was calculated separately for each species when the bands were developed in the presence and absence of ascorbic acid.

It was shown that ascorbic acid has no effect on the rate of development of this band for yellow mustard over the reaction period. For turret rape, however, this band develops much more rapidly in the presence of ascorbic acid.

On comparing absolute peak heights (Figures XVIII and XIX), the activating effect of ascorbic acid on this isoenzyme band in turret rape is further substantiated. After each comparable time period, development in the presence of ascorbic acid is approximately double that in the absence of ascorbic acid. For the mustard sample this band develops at the same rate or even slightly faster in the absence of ascorbic acid.

DISCUSSION

Several differences have been shown to occur among the thioglucosidases from various species of Cruciferae oilseeds. These include differences in enzyme stability under various preparative methods, in kinetic properties, and in electrophoretic characteristics.

There are at least four possible ways of accounting for the low activity in Brassica and crambe enzyme preparations. Firstly, it is possible that the amount of enzyme in these species is very low. Secondly, the thioglucosidase in these seeds may be relatively insoluble in water. Thirdly, the preparative conditions may have caused denaturation of these enzymes, and finally, it is possible that these species may contain thioglucosidase inhibitors.

The fact that a greater amount of precipitate was observed for crude extracts from S. alba than any of the other species in the electrophoresis tests, indicates that yellow mustard could contain a greater proportion of thioglucosidase. The quantitative differences observed in the amount of precipitate cannot, however, explain the large differences in activity among the enzyme preparations. Taste tests on crushed seed from each species also indicate that the differences in activity are not as great as the activity tests indicated.

Tookey and Wolff reported that crambe thioglucosidase was found to be relatively insoluble in water or in 0.035 M 2-mercaptoethanol, suggesting only about 10% may be dissolved upon simple extraction (26). This may explain some of the activity differences, but even if total solubility is assumed for yellow mustard thioglucosidase, the differences in solubility alone cannot fully explain the activity differences in the respective enzyme preparations.

The fact that little or no activity was observed in the bronowski or echo preparations, purified from seed coat-free meal, suggests that inhibition is not due to substances found exclusively in this portion of the seed. As activity is observed at comparable levels for the three preparative procedures, it appears that the dialysis step in the salt precipitation method is not serving to remove any activators or inhibitors. Thus, if inhibitors are present in the Brassica or crambe species, they are either firmly bound to the enzyme, or are of high molecular weight.

As the activity for the three yellow mustard preparations was found to be similar, it appears that S. alba thioglucosidase is fairly stable to the various purification steps employed.

The time available precluded further investigation of alternative methods of enzyme preparation. Different preparative methods, and quantitative testing for enzyme activity at each stage during preparation would certainly have led to a greater understanding of the mode of enzyme inactivation in the species examined.

Although the activities of the turret rape preparations were low compared to those obtained from yellow mustard, comparisons of kinetic properties could be made. The response to varying pH, buffers, temperature, and ascorbic acid activation showed the two thioglucosidases were similar in many ways, but certainly not identical.

The effect of pH and specific buffers on enzyme activity appears to be more complex than previously reported. Although no sharp rise in activity was observed for yellow mustard at pH 6.96 as reported by Schwimmer (23), a second activity peak for turret rape was observed only when ascorbic acid was present, supporting the theory of Tsuruo and Hata that the binding of

ascorbic acid affects the sensitivity of the active site towards anions (29). A similar change in the active site of turret rape thioglucosidase due to the presence of ascorbic acid is probably the cause of inactivation at pH levels above 6.8 for citrate-phosphate buffer, and above pH 7.3 for phosphate buffer. A different configuration is evident for S. alba thioglucosidase as very little inactivation was observed at similar alkaline pH in the presence of ascorbic acid and phosphate buffer. The differences in pH and temperature stability, further suggest dissimilar molecular configuration between these two species.

The variability in response to ascorbic acid is the most striking evidence suggesting differences among species with regard to their enzymic action. This variability was found not only among species, but also among specific isoenzyme fractions within a species. It was shown for turret rape and yellow mustard that isoenzymes with comparable mobility did not respond in the same way to ascorbic acid.

The comparison of isoenzyme fractions from seed extracts prepared both with and without 2-mercaptoethanol indicates that disulfide bonds play a part in the quaternary structure of crambe thioglucosidase. However, since at least two isoenzyme bands were observed in the absence of 2-mercaptoethanol, binding of subunits through disulfide bridges does not appear to fully explain the exhibited isoenzyme patterns. The observance of activity in the absence of 2-mercaptoethanol suggests that the active site is not affected by subunit binding.

Although active enzyme preparations were not obtained from several of the species investigated, it is believed that the information gained from the polyacrylamide gel electrophoresis experiments and the investigations of the kinetic properties

of the turret rape enzyme, may aid further research in this area. The response of turret rape to various temperatures and pH levels may also help to optimize processing conditions for this seed.

Much additional research could have been carried out had time permitted. In addition to extended studies on thioglucosidase purification as previously mentioned, pH activity studies with different buffers, especially above pH 7.5, should be undertaken. The effects of δ-gluconolactone, ferrous ion, salicin, neutral salts, and several sugars, known to affect thioglucosidase activity in S. alba (2, 8, 18, 26, 29, 30), should be determined for other Cruciferae species. Tests should also be carried out on intact seeds in order to more closely simulate processing conditions.

Isoenzymes could be fractionated using proper gel concentrations and buffers which would allow greater separation. This would permit further investigation into the effect of ascorbic acid on different isoenzyme fractions. Polyacrylamide gel electrophoresis could also be used as a preparative method for isoenzymes for testing their kinetic properties. This would also be useful for preparing purified fractions for molecular weight determinations.

SUMMARY

1. Attempts were made to purify thioglucosidase from seven different species of Cruciferae oilseeds, using three different methods of purification.

2. Active preparations were obtained for all species as determined by polyacrylamide gel disc electrophoresis. Extreme differences were observed in activity among species, as measured by their rate of degradation of sinigrin.

3. Enzyme activity was not greatly affected by the method of purification.

4. Different buffers were shown to inhibit activity at variable rates between pH 2.6 and 7.5.

5. Optimum activity was obtained at pH 5.5 in citrate-phosphate buffer for S. alba thioglucosidase.

6. Activity for turret rape thioglucosidase was observed to be fairly constant within the pH range of 6.2 to 7.9 with phosphate buffer.

7. In phosphate buffer, and with ascorbic acid present, maximum thioglucosidase activity for both S. alba and turret rape was observed at pH 6.3, with a second activity peak observed at pH 7.1.

8. A second activity peak at pH 7.1 was not observed for turret rape in the presence of citrate-phosphate buffer and ascorbic acid.

9. Turret rape thioglucosidase was shown to be slightly more resistant to acid denaturation than S. alba thioglucosidase.

10. For S. alba, thioglucosidase activity is lost at pH 3.1 in citrate-phosphate buffer, while at this pH in hydrochloric acid - sodium acetate buffer, nearly one-half maximum reaction velocity is still evident.

11. Ascorbic acid is shown to act as an inhibitor for pH values above 7.4.

12. Both turret rape and S. alba thioglucosidases were shown to be totally inactive on exposure to temperatures above 74° for 3 minutes. For heat treatments between 57° and 68°, S. alba was slightly more resistant towards inactivation than was turret rape.

13. Activity of S. alba thioglucosidase was shown to approximately double for every 20° rise in temperature.

14. Nine different species of Cruciferae oilseeds were examined for thioglucosidase isoenzyme patterns using polyacrylamide gel disc electrophoresis.

15. Each species exhibited a characteristic band pattern made up of three to six isoenzyme bands.

16. Slight variations were noticed on comparing isoenzyme patterns from seed extracts and the enzyme preparations.

17. Little or no change was observed in isoenzyme patterns of enzymes prepared by three different preparative methods.

18. Mercaptoethanol was shown to affect the isoenzyme pattern of thioglucosidase.

19. Turret rape isoenzymes with low R_m values appeared to be active only in the presence of ascorbic acid.

20. The isoenzyme band at R_m .25 found in S. alba developed at the same rate both in the presence and absence of ascorbic acid, whereas the band at R_m .25 in turret rape developed much more rapidly in the presence of ascorbic acid.

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