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Isoenzymes and polyploidy

I. Qualitative and quantitative isoenzyme studies in the Triticinae

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SUMMARY

Isoenzymes of alcohol (ADH), malate (MDH), glutamate (GDH) and isocitrate (IDH) dehydrogenases, and a fast migrating esterase (EST-1) were separated by disk electrophoresis from dry seed extracts of diploid, tetraploid, hexaploid and octaploid species or amphiploids belonging to the subtribe Triticinae. Only ADH and EST-1 isoenzymes showed inter-species variation; the other dehydrogenases, which show stringent substrate specificities ('critical' enzymes), revealed the same pattern in all diploid and polyploid species. The qualitative zymogram studies showed that (1) the number of variant enzyme bands increased with the level of ploidy, (2) the amphiploid isoenzyme pattern was additive of the parental species, (3) enhancement in the number of bands was due to the presence of not only parental bands, but also hybrid bands formed by association between heteromonomers. Quantitative data were obtained by densitometry of the enzyme bands as well as spectrophotometric measurements of enzyme activity in crude extracts. Increase in the level of enzyme activity was observed with ploidy level. In spite of the evidence that all duplicate/triplicate genes are expressed, increased enzyme activity observed in the polyploid species was not proportional to the level of ploidy or expected gene dosage. On the basis of ADH and EST-1 zymograms obtained in 2× and 4× wheat, probable zymograms for these enzymes in the B-genome donor to 4× wheat were extrapolated. Neither *Ae. speltoides* nor *Ae. bicornis* showed the extrapolated ADH pattern. Amphiploids involving *Ae. speltoides* and *Triticum monococcum* or *T. aegilopoides* fully reproduced the EST-1 zymogram of 4× wheat, but not the ADH. *Ae. bicornis* × *T. aegilopoides* amphiploid showed an ADH zymogram similar to that of 4× wheat, but the EST-1 bands were different.

1. INTRODUCTION

Comparative studies of proteins or analogous (similar in function) enzymes provide a rapid method of ascertaining genetic homologies, and thus inferring phylogenetic relationships among related taxa. In the past few years this criterion has been used by several workers to elucidate relationships and detect inter-specific hybrids in natural populations of plants and animals. In plants, these methods have recently been used to investigate phylogenetic relationships between

species of *Gossypium* (Cherry, Katterman & Endrizzi, 1970), *Nicotiana* (Sheen, 1970; Smith *et al.* 1970) and *Hordeum* (Mitra, Jagannath & Bhatia, 1970). References to previous publications can be found in these articles.

Protein and isoenzyme studies seem to be potentially useful in the identification of the probable genome donors in amphiploid species. This will only be possible if the protein and isoenzyme spectra of the amphiploids are entirely or nearly additive of the two parental species. Hall (1959) and Hall & Johnson (1963) did, in fact, observe additivity of the proteins in wheat × rye and *Stipa* × *Oryzopsis* amphiploids respectively. Barber, Driscoll & Vickery (1968) obtained similar results in wheat × rye amphiploids for an esterase. Johnson (1968) was able to pinpoint the A'A' genome donor in *Triticum zhukovskyi*, on the basis of electrophoretic data of seed proteins. Sing & Brewer (1969), using electrophoresis of seed and leaf enzymes, found an additive relationship for only one out of eight enzymes they investigated in a polyploid series of wheat species. Consequently, they were sceptical of identifying the diploid isoenzyme contributions in naturally occurring amphiploids.

An increase in the number of esterase-1 (EST-1) isoenzyme bands in 4 × wheat, in comparison to 2 × wheat and other diploid species of *Aegilops* and *Secale*, has been reported (Bhatia, 1968). Between 4 × and 6 × wheat, no further enhancement in the multiplicity of EST-1 bands was observed, as the 4 × wheat bands completely overlap those of *Aegilops squarrosa*, the widely accepted D-genome donor of 6 × wheat. Sing & Brewer (1969) did not observe any increase in the number of isoenzyme bands at higher levels of polyploidy.

In the present investigation, species belonging to the subtribe *Triticinae* at 2 ×, 4 × and 6 × ploidy levels and synthetic amphiploids at 4 × and 8 × levels were examined, using disk electrophoresis. Two classes of enzymes – class (A), with broad substrate specificities like esterases (E.C. 3.1) and alcohol dehydrogenase (E.C. 1.1.1.1), and class (B), with highly stringent substrate specificities like malate (E.C. 1.1.1.38), isocitrate (1.1.1.42) and glutamate (1.4.1.2) dehydrogenases – were considered. The specific object of this study was to ascertain the following:

(1) Increase or decrease in the number of isoenzyme bands and total enzyme activity in relation to polyploidy.

(2) Expression of parental genes and identification of the contribution made by each of the parental species in amphiploids as revealed by zymograms.

(3) Extrapolation of the electrophoretic characteristics of the B-genome donor to 4 × and 6 × wheat.

2. MATERIALS AND METHODS

(i) *Species and amphiploids examined*

Species and amphiploides along with the number of accessions examined in the present investigation are given in Table 1. All plant materials were grown in pots, and seeds were harvested at maturity.

Table 1. *Species and amphiploids examined*

Species	Genome	No. of accessions examined
$2n = 2\times = 14$		
Triticum monococcum L.	AA	2
T. boeoticum Boiss. (*T. aegilopoides* (Link) Ball).	AA	5
Aegilops speltoides Tausch	SS(= BB)	1
Ae. bicornis (Forsk.) Jaub. et Spach	S^bS^b	4
Ae. squarrosa L.	DD	2
Secale cereale L.	RR	1
$2n = 4\times = 28$		
Triticum turgidum (L.) Thell. conv. *durum*	AABB	4
Aegilops bicornis × *T. aegilopoides** (A1)	AAS^bS^b	1 (TA 250)*
Ae. speltoides × *T. monococcum** (A2)	AASS	1 (TA 396)*
Ae. speltoides × *T. aegilopoides** (A3)	AASS	1 (TA 398)*
$2n = 6\times = 42$		
Triticum aestivum ssp *vulgare* (Vill., Host) MacKey	AABBDD	5
$2n = 8\times = 56$		
T. aestivosecale MacKey (*T. aestivum* × *Secale cereale*)	AABBDDRR	1

* Seeds under these numbers and names were received from Dr R. Riley, Cambridge, these names have been retained in the text.

(ii) *Preparation of seed extracts*

Dry seeds were homogenized with 0·1 M Tris-HCl buffer, pH 8·3, containing 0·1 % 2-mercaptoethanol, at 0–4 °C. In all extractions the ratio of seed weight to buffer volume was 1 g to 10 ml. The slurry was centrifuged in the cold at 12000 *g* for 15 min, and 200 μl of the supernatant was used for each electrophoretic analysis. Since Tris-HCl extracts of the dry seeds did not give satisfactory resolution of the fast-migrating esterase bands, seeds were homogenized with 0·01 M sodium pyrophosphate buffer, pH 9·3.

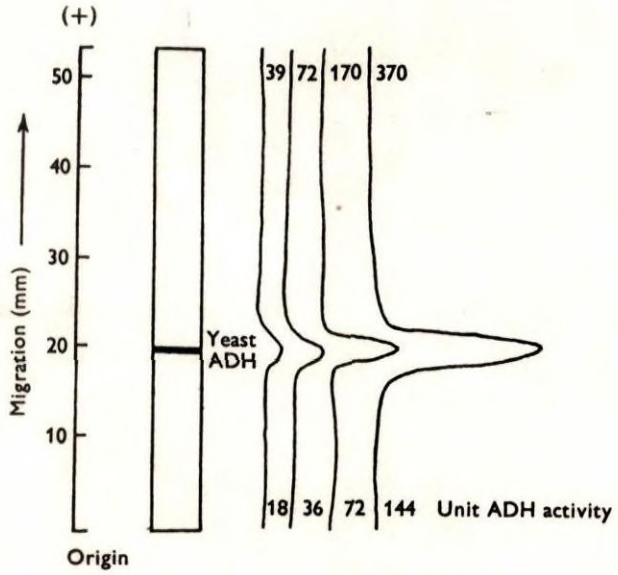
(iii) *Electrophoresis and location of enzyme bands*

Electrophoretic procedures and location of the enzyme bands on the gels, except for ADH and IDH, have been described previously (Mitra *et al.* 1970). ADH and IDH (NADP-dependent) bands were located following the procedures given by Scandalios (1967) and Henderson (1965), respectively. Electrophoresis was continued until the front formed by the tracking dye bromophenol blue had migrated 5 cm from the origin of the small-pore gel. Since the intensity of bands obtained depends upon several factors, namely the time of incubation and the concentration of each reactant (co-enzyme, electron acceptor, dye, substrate, etc.), great care was taken to control each of the above factors in order to obtain comparable data.

(iv) *Quantitative estimation of enzyme activity*

Each gel was scanned for quantitative estimation of enzyme activity in a Chromoscan mark II (Joyce Loebl) recording-integrating densitometer, using visible reflectance. All densitometer recordings were made using × 3 gear ratio, i.e.

the recordings obtained were three times the original length of the gel. Under our conditions, amphiploids A 1, A 2 and A 3 produce viable but not very well filled kernels. Therefore, the integration values obtained for these amphiploids may not be truly representative. Variation ranging from 1 to 3 % in the integration values of the bands was found for the same lot of seeds, under standard conditions of extraction, electrophoresis, incubation and densitometry.



Text-fig. 1. Densitometer recordings of yeast ADH zymogram showing relationship between enzyme activity and integration values. Integration values are given at the top.

Purified yeast ADH (Schwarz, sp. act. 360 u./mg protein) was used to demonstrate that the variation in the integration values is proportional to the enzyme concentration. Different concentrations of yeast ADH were subjected to electrophoresis, like any other sample, and ADH bands were located. Densitometer tracings of these gels, along with the integration values, are shown in Text-fig. 1. A linear relationship between the enzyme concentration and integration values is apparent.

(v) *Estimation of total enzyme activity*

The dehydrogenase activity was measured in the seed extracts of 2×, 4×, 6× wheat species and 8× *Triticale*, prepared as described in (ii). A larger seed sample used for extraction minimizes seed-to-seed variation and provides an average estimate of enzyme activity. Four replicate extractions were made. ADH activity was estimated by the method of Efron & Schwartz (1968). MDH and GDH activity were measured by the change in O.D. (340 mμ) from 0–15 min, at 5 min intervals, at room temperature (24 ± 2 °C). One unit of enzyme activity represented a change

of 0·001 O.D./min. The composition of the reaction mixture for the assay was as follows:

MDH: 0·25 ml 1 M Tris-HCl, pH 10·5; 0·25 ml 0·88 M L-malic acid neutralized with NaOH; 0·075 ml 0·01 M NAD; 1·825 ml distilled water; and 0·1 ml extract.

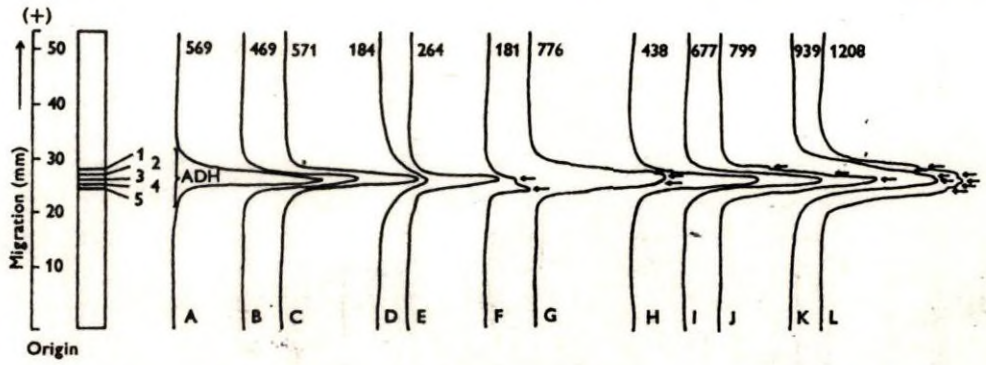
GDH: 1·25 ml 0·2 M phosphate buffer, pH 8; 0·075 ml 0·01 M NAD; 0·25 M Na-glutamate; 0·825 ml distilled water; and 0·1 ml. extract.

The enzyme activity is expressed as activity/mg buffer-soluble protein and activity/100 mg tissue. The total as well as buffer-soluble protein content varies in different species and in strains of the same species. The enzyme activity/100 mg tissue was therefore used to show the increase in catalytic activity in the same amount of tissue, irrespective of the variation in the amount of buffer soluble proteins.

3. RESULTS

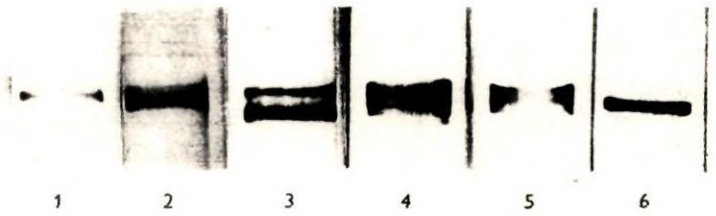
(i) *Qualitative variation*

ADH. All diploid species (Text-fig. 2; Plate 1, fig. 1), with the exception of rye, showed one band (ADH-3). Rye had one additional band (ADH-5). Three closely spaced bands (*ADH-1, 2* and *3*) were observed in 4× and 6× wheat. Amphiploids



Text-fig. 2. Densitometer recordings along with integration values of ADH zymograms. A = *T. monococcum* (2×), B = *T. boeoticum* (2×), C = *Ae. speltoides* (2×), D = *Ae. squarrosa* (2×), E = *Ae. bicornis* (2×), F = *Secale cereale* (2×), G = *T. turgidum* (4×), H = *Ae. speltoides* × *T. monococcum* (4×, A2), I = *Ae. speltoides* × *T. aegilopoides* (4×, A3), J = *Ae. bicornis* × *T. aegilopoides* (4×, A1), K = *T. aestivum* (6×) and L = *T. aestivosecale* (8×). Integration values are given at the top against each tracing. The diagrammatic representation on the left shows the location of respective ADH bands on the gel.

A 2 and A 3 had only the ADH-3 band, the same as in their parental species. Three bands, ADH-1, 2 and 3, similar to those of 4× and 6× wheat, were observed in A 1. In this amphiploid the ADH-3 band was intense, while others were relatively faint *Triticale* revealed five bands (ADH-1 to 5), of which ADH-4 had no homologue in wheat or rye. This band, presumably a hybrid band, was also observed following freezing and thawing of a mixture of wheat and rye crude extracts.

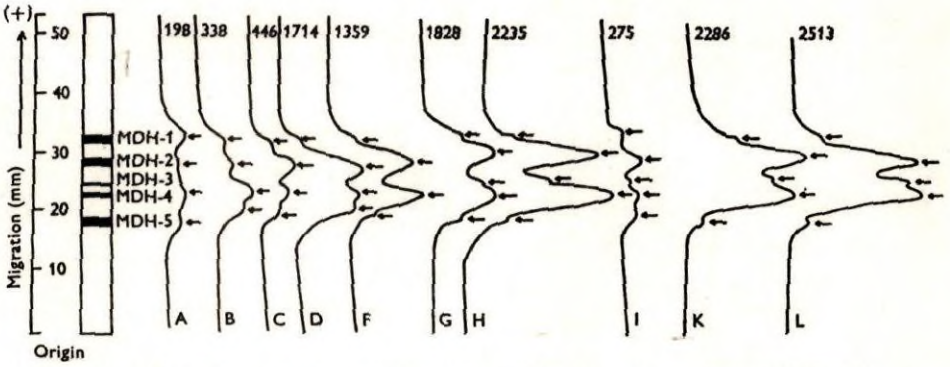


(a)

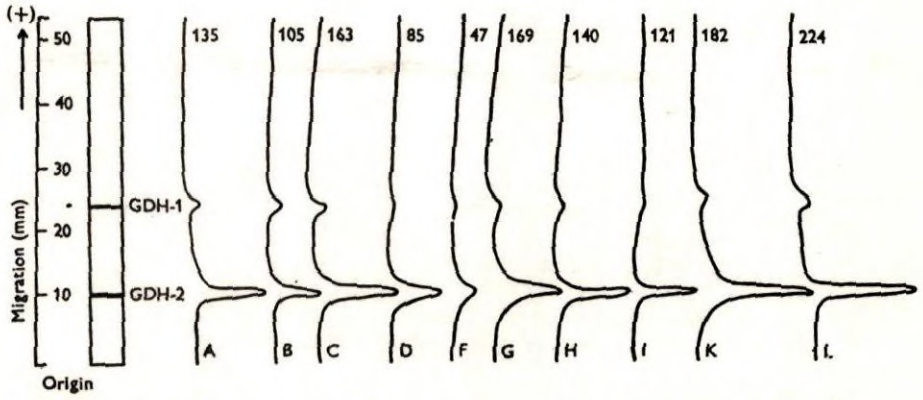
Fig. 1. ADH zymograms. (1) *Ae. bicornis*. (2) *Ae. bicornis* × *T. aegilopoides* (A1), (3) *Secale cereale*, (4) *T. aestivosecale*. (5) *T. turgidum* and (6) *T. monococcum*. Origin is at the bottom and the migration was towards the + ve electrode at the top.

(b)

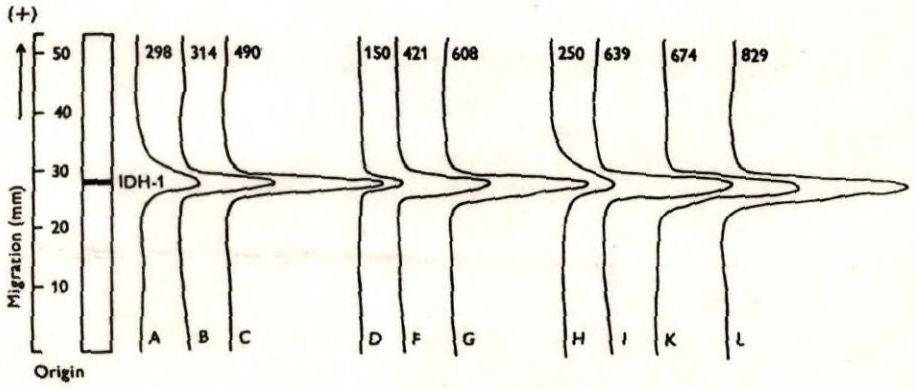
Fig. 2. Esterase-1 zymograms. (1) *T. turgidum* (4 ×), (2) *Ae. speltoides* × *T. monococcum* (4 ×, A2), (3) *T. monococcum* (2 ×), (4) *Ae. speltoides* (2 ×), (5) *T. aegilopoides* (2 ×). (6) *Ae. bicornis* × *T. aegilopoides* (4 ×, A1). and (7) *Ae. bicornis* (2 ×). Other details as for Fig. 1.



Text-fig. 3. Densitometer recordings along with integration values of MDH zymograms. Details as for Fig. 2.



Text-fig. 4. Densitometer recordings along with integration values of GDH zymograms. Details as for Fig. 2.

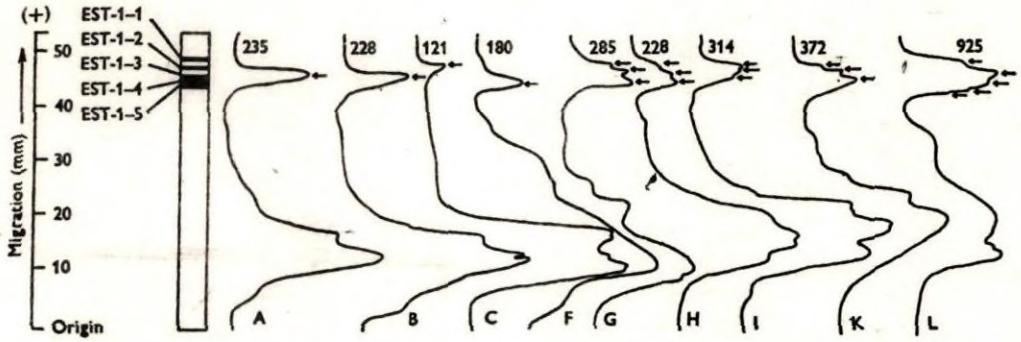


Text-fig. 5. Densitometer recordings along with integration values of IDH zymograms. Details as for Fig. 2.

MDH. All five diploid species exhibited four MDH bands (MDH-1, 2, 4 and 5) without any variation (Text-fig. 3). One additional band (MDH-3) was observed in 4× and 6× wheat, *Triticale* and the three amphiploids. This band was noted only when the enzyme concentration was high, and could also be obtained on electrophoresing a larger volume of extracts from diploid species.

GDH. Two bands, GDH-1 and 2 (Text-fig. 4) was observed in all diploid and polyploid species examined.

IDH. One IDH band (Text-fig. 5) was observed in all the stocks investigated.



Text-fig. 6. Densitometer recordings along with integration values of esterase zymograms. Details as for Fig. 2. The location of EST-1 bands is shown only on the left.

Esterases. An overall increase in the number and activity of esterase bands was observed with an increase in the level of ploidy. However, the slow-migrating esterase bands do not resolve well, due to overlapping mobilities, and very likely represent a family of enzymes showing esterase activity rather than true isoenzymes. Therefore, we have only considered the fast-migrating esterase (EST-1) in detail. All diploid species had a single EST-1 band (Text-fig. 6; Plate 1, fig. 2). In *Ae. speltoides* and *Ae. squarrosa* this band migrated farthest from the origin (EST-1-1), while in *T. monococcum*, *T. boeoticum* and *Ae. bicornis* (EST-1-3) and rye (EST-1-5) it migrated a shorter distance. Three closely spaced bands (EST-1-1, 2, 3) were observed in 4× and 6× wheat and in amphiploids A 2 and A 3. Amphiploid A 1 showed only the EST-1-3 bands, the same as in diploid wheat and *Ae. bicornis*. Five bands in this region (EST-1-1 to 5) were recorded in *Triticale*, of which EST-1-1 to 3 were homologous to 4× or 6× wheat bands, and EST-1-5 to that of rye. EST-1-4 was presumably a hybrid band.

(ii) *Quantitative variation*

The areas under the respective peaks in Text-fig. 2–6 and the integration values show that, for the enzymes considered, diploid species among themselves have considerable variation. A marked increase in the integration value for 4× and 6× wheat in comparison to 2× species was noted. Further increase in the integration values was observed for 8× *Triticale*. ADH, MDH and GDH activity in 2×, 4× and

6× wheat and 8× *Triticale*, as measured by the more precise spectrophotometric method, is given in Table 2. The increase in enzyme activity from 2× to 8× levels of ploidy is evident.

Table 2. *Dehydrogenase activity in* Triticum *species of different ploidy*

Species...	*T. monococcum*	*T. turgidum*	*T. aestivum*	*T. aestivosecale*
Ploidy...	2×	4×	6×	8×
Enzyme activity/mg protein				
ADH	3070 ± 91	4440 ± 26	4380 ± 18	4900 ± 73
MDH	246 ± 1·7	381 ± 7·0	384 ± 9·3	458 ± 2·4
GDH	257 ± 3·3	395 ± 9·0	366 ± 6·2	363 ± 7·3
Enzyme activity/100 mg tissue				
ADH	2070 ± 53	2126 ± 29	2456 ± 32	3529 ± 53
MDH	167 ± 3·7	183 ± 5·1	212 ± 4·6	330 ± 1·7
GDH	173 ± 4·6	186 ± 3·0	207 ± 4·7	262 ± 5·0

3. DISCUSSION

(i) *Species relationships in the* Triticinae

All diploid species are believed to have evolved from a common unknown progenitor. Hybridization between *T. monococcum* (AA) and a diploid species of *Aegilops*, possibly *Ae. speltoides* (SS) or a species closely related to it, followed by doubling of the chromosome number, gave rise to tetraploid species. Hybridization between a tetraploid form and *Ae. squarrosa* (DD) and subsequent amphidiploidization accounted for the origin of 6× wheat. Thus, the 21 pairs of chromosomes in the 6× wheat belong to three genomes, each contributed by a different but closely related species. The chromosomes of 6× wheat can also be placed in seven homoeologous groups. The genetic content of the homoeologous chromosomes is believed to be nearly identical, and they substantially compensate for each other in nulli-tetra combinations (Sears, 1968*a*). Consequently most if not all genetic loci are duplicated in 4× and triplicated in 6× wheat.

(ii) *Variant and non-variant enzymes*

Of the five enzymes studies, MDH, GDH and IDH did not show any isoenzyme variation among the species examined. In a study of two *Drosophila ananassae* populations, Gillespie & Kojima (1968) reported greater variability for non-specific enzymes in comparison to 'critical' enzymes, which have more specific substrate affinities. Our earlier results with *Hordeum* (Mitra *et al.* 1970) and now with the species belonging to the Triticinae suggest that isoenzyme variation for 'critical' enzymes may be rare in closely related species of plants. However, these enzymes do show variation in electrophoretic mobilities at the genus level. In contrast, enzymes with broad substrate specificities, like esterases, or an enzyme like ADH, which apparently is not essential for the normal development of the plant (Schwartz, 1969), may show greater inter-species variation.

(iii) *Inter- and intra-species variation*

In diploid species, both inter- and intra-species isoenzyme variation is greater than in polyploid species (Bhatia, Mitra & Jagannath, 1968). Different species of 4× and 6× wheat, now classified as sub-species of *T. turgidum* and *T. aestivum*, did not show any significant variation for esterases and ADH (Bhatia, 1968). Similar zymograms were also observed for MDH, GDH and IDH in these species. Hence, the *durum* and *vulgare* subspecies were chosen as representative of the *turgidum* and *aestivum* species in the present study.

(iv) *Number of isoenzyme bands and ploidy level*

The variant enzymes, ADH and EST-1, showed an increase in the number of isoenzyme bands in polyploid species. Sing & Brewer (1969) failed to observe an enhancement in the mean number of isoenzyme bands in polyploid wheat species. On the contrary, they reported a decrease in the mean number of bands in 4× and 6× wheat. The presence of co-dominant, duplicate or triplicate genes in allopolyploid species may cause an increased number of isoenzyme bands, provided the gene products of the two parental species are electrophoretically distinct from each other. In addition to the parental bands, hybrid bands may also result from the association between heteromonomers.

That the above expectations are, in fact, realized is obvious from the results obtained with *Triticale* and the other amphiploids A 1, A 2 and A 3. In *Triticale* ADH and EST-1 showed the presence of parental bands and, in addition, new bands (ADH-4 and EST-1-4), presumably formed by the association of wheat and rye monomers. The dimeric nature of EST-1 and ADH is known (Barber *et al.* 1968; Hart, 1969). The ADH-4 band could also be obtained by freezing and thawing of a mixture of 6× wheat and rye extracts. Though EST-1 monomers do not form dimers *in vitro*, evidence for the formation of hybrid dimers between wheat and rye EST-1 monomers was presented by Barber *et al.* (1968).

In amphiploids A 2 and A 3, the parental species *T. monococcum* and *T. boeoticum* differ from *Ae. speltoides* with respect to EST-1 but not for ADH. In the amphiploids, it was possible to distinguish the EST-1 isoenzymes contributed by *T. monococcum* or *T. boeoticum* and *Ae. speltoides*. A new band (EST-1-2), intermediate in mobility between EST-1-1 of *Ae. speltoides* and EST-1-3 of *T. monococcum* was also observed in A 2 and A 3. The EST-1-2 band also occurs in 4× and 6× wheat.

Amphiploid A 1 showed three ADH bands similar to those of 4× and 6× wheat. The parental species of this amphiploid, *Ae. bicornis* and *T. aegilopoides*, each had a single (ADH-3) band. In this respect, the *Ae. bicornis* amphidiploid was very different from those involving *Ae. speltoides*. The three bands of 4× and 6× wheat have been attributed to the presence of duplicate genes in 4×, and triplicate genes in 6× wheat, located on chromosome 4 A, 4 B and 4 D (Hart, 1969, 1970). The presence of three ADH bands in A 1 would also require two genes, each producing a monomer of different electrophoretic mobility. The parental stocks of *Ae. bicornis*

and *T. aegilopoides* used in synthesizing this amphiploid showed functional dimers of the same mobility, like other accessions of these species. Thus, three bands in the amphiploid can only be explained if it is assumed that interaction between *Ae. bicornis* and *T. aegilopoides* chromosomes leads to the activation of an existing locus, possibly in *aegilopoides*. Such chromosomal interactions are known for other characters in wheat. These genes do not have any effect in diploid species, but when combined together in one system they become effective (Kihara, 1965).

(v) *Expression of parental genes in amphiploids*

In the amphiploids *Triticale*, A 1, A 2 and A 3 it was possible to observe the expression of parental genes where the two gene products were electrophoretically distinct. There is no reason why multiple genes producing electrophoretically identical proteins should not also function in a polyploid. This, however, is difficult to demonstrate. Since a quantitative measurement of the end-product (enzyme) may provide some clue, the activity of each band was estimated by densitometry and total activity by the spectrophotometric method. Variation in the intensity of isoenzyme bands on the gels reflects either changes in specific activity or the total amount of the material (Shaw, 1970). We fully realize the limitations of the enzyme activity data. In higher organisms control of enzyme activity is complex, and regulation can operate at several levels (Wyngaarden, 1970). Synthesis and breakdown of some enzyme molecules goes on simultaneously in actively metabolizing tissues. In dry seeds, however, all life processes are at a very low metabolic level and probably no new proteins are synthesised. *A priori*, there is no reason to expect that specific activity would change with the level of ploidy. Therefore, the increased enzyme activity in polyploids can be attributed to an increase in the number of active enzyme molecules. Whether this is due to a larger number of enzyme molecules initially synthesized or is the result of regulation remains to be established. Very little is known about gene regulation in polyploids or for that matter in any higher form. It has been pointed out that polyploids, besides having duplicated structural genes, also have an increased number of regulatory genes (Becak, 1969).

Though maximum activity for all the enzymes was observed in 8× *Tricitale*, the increase was not proportional to the level of polyploidy or gene dosage. The present data suggest that in polyploids individual genes are not expressed (or are not 'turned on') to the same level as in diploids. Further experimental data are of course needed to support this idea.

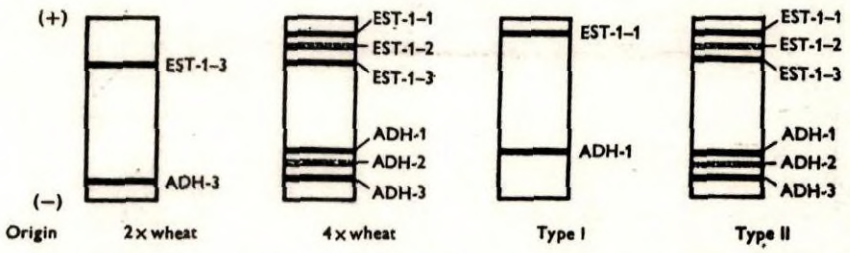
(vi) *Identification of parental contribution in amphiploids*

Zymograms of the amphiploids examined were a summation of the two parental species, a result similar to those reported by Johnson & Hall (1965) for seed proteins. Sing & Brewer (1969) were sceptical that parental contributions to polyploid species could be inferred from comparative isoenzyme studies. Our data indicate that isoenzymes can provide extremely useful information on genetic homologies in establishing putative genome donors of the contemporary polyploid

species, provided (1) intra-species isoenzyme polymorphism is limited both at diploid and polyploid level; (2) inter-species differences are greater than intra-species variation, and the zymogram patterns do not overlap. Members of the Triticinae apparently meet the above requirements, at least for the two enzymes ADH and EST-1. Isoenzymes provide information on gross homology or otherwise for a single or at the most for a few genetic loci. However, such studies with different enzymes can provide an estimate of the genetic loci altered in the process of speciation in closely related groups.

(vii) *Extrapolated zymogram of the B-genome donor*

On the basis of zymograms observed in 2× and 4× wheat, it is possible to extrapolate to ADH and EST-1 zymograms of the probable B-genome donor to 4× wheat. The two possible types are shown in Text-fig. 7. The following facts have been considered in making these extrapolations: (1) the functional ADH and EST-1



Text-fig. 7. Schematic representation of probable electrophoretic characteristics of B-genome donor to 4× wheat with respect to ADH and EST-1. Hybrid bands are shown hatched. For details refer to text.

molecules are dimers; (2) functional dimers are formed in amphiploids by association of hetero-monomers; (3) amphiploid zymograms are additive of the parental species. The available evidence in support has been discussed earlier in this paper. The type-II zymogram has not been found in any of the diploid species. Type-I is much closer to what has been observed at the diploid level.

Aegilops speltoides is considered to be the closest match for the B-genome donor on the basis of morphological characters (Sarkar & Stebbins, 1956) and karylogical evidence (Riley, Unrau & Chapman, 1958). Sears (1968*b*) has pointed out that the amphiploid between *Ae. bicornis* × *T. monococcum* is as similar to *T. dicoccum* as the amphiploid between *Ae. speltoides* × *T. monococcum*. Neither *Ae. speltoides* nor *Ae. bicornis* showed the extrapolated ADH pattern, though the former species had the required EST-1-1 band. The amphiploids involving *Ae. speltoides* (A 2, A 3) reproduced the EST-1 zymogram of 4× wheat, but not for ADH. The *Ae. bicornis* amphiploid (A 1) showed an ADH pattern similar to 4× wheat but its EST-1 pattern was different.

Extensive sampling of contemporary *Ae. speltoides* and *Ae. bicornis* or other members of the Sitopsis section may lead to the identification of a genotype showing both ADH and EST-1 bands similar to the extrapolated type-1. It has been

shown by Zohary & Feldman (1962) that, in addition to amphidiploidy, hybridization and exchange of genetic material between newly formed amphiploids have played an important role in the evolution of the polyploid species in the Triticinae. Such genetic exchange involving ADH or EST-1 loci between *Ae. speltoides* × *T. monococcum* and *Ae. bicornis* × *T. monococcum* or *T. boeoticum* amphiploids could also fully account for the observed zymograms of the 4× wheat.

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