THE GENETIC CONTROL AND BIOCHEMICAL MODIFICATION OF CATECHOL OXIDASE IN MAIZE

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ABSTRACT

Three isozyme variants of catechol oxidase have been shown to be determined by alleles of a gene, *Cx*, which has been located on chromosome 10 less than 0.1 recombination units from the endosperm marker du_1.——The extractable form of the enzyme is modified by an endogeneous "modifier" which appears to function as an enzyme substrate. Enzyme and modifier are functionally isolated in intact cells. Modified enzyme has altered kinetics, does not migrate in electrophoresis and most probably results from a "tanning" of the enzyme by reaction products. The content of modifier varies in different lines and is genetically determined by gene(s) independent of *Cx*. Treatment with maleic hydrazide causes a ten-fold reduction in the modifier content of seedlings, allowing the enzyme to be extracted in an unmodified form which will migrate in electrophoresis.——This system of enzyme and modifier fits the requirements of hypersensitive disease resistance in plants and may provide a test system to investigate the biochemical basis of disease resistance.

DIPHENOL oxidases have been detected in many eukaryotes and have been the subject of numerous reviews (e.g. MASON 1955, 1957, 1965; BONNER 1957; KERTESZ and ZITO 1962; MALMSTRÖM and RYDÉN 1968). Probable structural genes for the enzyme have been described in several cases (e.g. LEWIS and LEWIS 1963; HOROWITZ *et al.* 1961). In plants diphenol oxidases have been implicated in terminal oxidation, biosynthesis and most frequently in tissue responses to pathological and mechanical damage (MASON 1955; BONNER 1957).

In this paper we have termed the enzyme "catechol oxidase" rather than the recommended trivial name, o-diphenol oxidase for the enzyme o-diphenol-oxygen oxidoreductase (E.C. 1.10.3.1), because preliminary observations (unpublished) indicate that the enzyme shows considerable substrate specificity and is only one of several maize enzymes possessing o-diphenol oxidase activity.

The initial and unpublished observations which stimulated this present study were made by DR. TORU ENDO in our laboratory during the course of a study comparing the effect of γ-rays and maleic hydrazide treatments on enzyme systems in maize seedlings. ENDO recognized three classes of inbred maize lines with respect to their catechol oxidase (CX) isozyme content. Class I inbreds showed a

cathodally-migrating CX isozyme in starch gel zymograms. Class II inbreds did not show CX isozyme activity unless the seedlings had been pretreated with maleic hydrazide (MH). In this class MH caused the appearance of a CX isozyme with the same electrophoretic mobility as the isozyme observed in Class I. The inbreds of Class III were considered to be null variants, since no CX isozyme activity was observed even after MH treatment.

Superficially then, the three Classes were analogous to the now classical "constitutive", "inducible" and "null" classes of the lactose operon system (BECKWITH and ZIPSER 1970). This analogy proved to be fortituous, since we show in this paper that the mechanism of the apparent induction of CX by MH treatment does not involve a new production of enzyme, but rather is due to an effect of MH on decreasing the content of an endogenous modifier of CX which when present (in untreated seedlings) alters the CX into a form which does not migrate in electrophoresis.

MATERIALS AND METHODS

Maize seeds were soaked in water for 12–24 hours, washed thoroughly, and allowed to germinate for 4–5 days at 30° in the dark. MH treatment was achieved by soaking seeds in 0.01M MH instead of water.

Standard horizontal starch gel electrophoretic techniques (SMITHIES 1955) were used with gels containing 11% (w/v) hydrolyzed starch (Connaught) in 0.03M sodium borate (pH 8.3) with 0.3M sodium borate (pH. 8.3) for the electrode buffer. Samples adsorbed on filter paper pieces were placed in vertical slits in the gels. After electrophoresis (2–3 hours at 200 volts) the sliced gels were immersed overnight in 0.01M catechol in 0.02M sodium acetate buffer at pH 4.2. The location of the CX isozyme activity is revealed by the appearance of a band of dark brown catechol melanin presumably formed as a result of secondary non-enzymatic reactions of the o-benzoquinone product of catechol oxidation.

Electrophoresis on cellulose acetate strips was carried out on strips that had been presoaked (2 hours) in 0.03M sodium borate (pH 8.3). Samples were applied by capillary streaking across the center of the strips, which were then placed in an electric field for 2 hours at room temperature with a voltage drop of about 10 volts/cm. The strips were stained for CX activity by covering them for several hours with filter paper saturated with a solution of 0.01M catechol in 0.02M sodium acetate (pH 4.2).

Individual seedlings were scored by squashing the juice from epicotyl sections (about 1 cm in length above and including the coleoptilar node) directly onto filter paper pieces. Acetone powders from epicotyls of 4–5-day-old seedlings were prepared by first grinding the tissue in liquid nitrogen and then in cold (—20°) acetone. After filtering, the powder was washed with cold ether, air dried at 5° and finally stored at —20° until use. Enzyme extracts were prepared by extracting the acetone powder with cold 0.2M sodium phosphate buffer pH 6.2, (.1gm/ml) for 15 minutes with constant stirring. Cell debris was removed by centrifugation (30,000 g for 15 minutes) and the supernatant was then passed through a molecular exclusion column (P4 BioGel) with 0.01M sodium phosphate buffer. The CX in these solutions remained stable for at least a week at 5°, as judged by the presence of an enzyme band in starch gel zymograms. Protein content was ascertained according to the method of LOWRY *et al.* (1951).

CX enzyme activity was measured with an oxygen electrode (Y.S.I. Biological Oxygen Monitor). The reaction mixture contained 0.01M sodium phosphate buffer pH 6.2, 0.001M catechol and 0.1 ml of the enzyme extract in a final volume of 4.0 ml. The oxygen level measured after bubbling air through the reaction mixture for 30 seconds prior to enzyme addition was taken as 100% saturation. Controls run in the absence of catechol or enzyme showed negligible oxygen consumption.

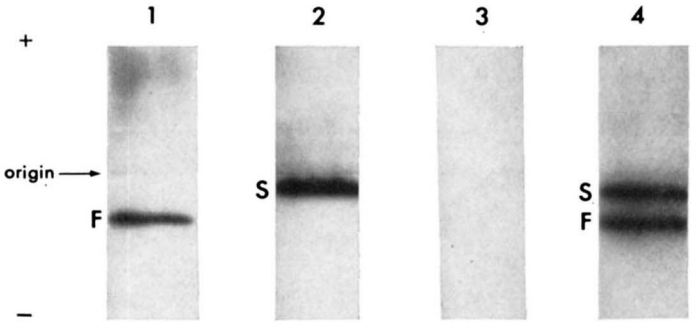

FIGURE 1.—Electrophoretic variants of catechol oxidase in MH-treated seedlings. (1) Fast isozyme (P22); (2) Slow isozyme (P21); (3) Null (P4); (4) Fast and Slow isozymes in a Fast/Slow hybrid. (P22/P21).

Ascending chromatography was carried out with Whatman No. 1 paper and two solvent systems: A, t-butanol : acetic acid : water (3:1:1) and B, t-butanol : water (3:2).

RESULTS

Inheritance of electrophoretic variants: MH-treated seedlings from 40 inbred lines were screened for CX isozyme content. Of these 28/40 carried the same isozyme that has previously been observed by ENDO, 10/40 were nulls while the remaining two possessed an isozyme variant with a slow migration rate. Each inbred line bred true. F_1 hybrids between lines carrying the Fast isozyme (F) and lines carrying the Slow variant (S) showed both the F and S isozyme bands in zymograms prepared from MH-treated seedlings (Figure 1). Hybrids between a Null (N) and either S or F lines showed only the S or F isozyme band, respectively. The backcross and F_2 progeny from some of these hybrids were assayed for CX isozyme content and the classes of progeny observed were in agreement in number and frequency with those expected on the hypothesis that the S, F

TABLE 1

Inheritance of catechol oxidase isozyme variants

F_1	Tester	Number of progeny F	FS	S	N	Total	χ^2	P
F/S	× N	65	—	51	—	116	1.68	0.1–0.2
S/N	× N	—	—	58	56	114	0.02	0.8–0.9
F/N	× S	—	60	72	—	132	0.54	0.3–0.5
F/S	× F/S	19	52	25	—	96	1.41	0.7–0.8

The F_1 hybrids were between inbred lines that were true breeding for one of the catechol oxidase isozyme variants.

F = Fast isozyme (Line P13); S = Slow isozyme (P21), N = Null (P4).

χ^2 was calculated on the hypothesis that F, S, and N are inherited as alleles of a single gene, *Cx*.

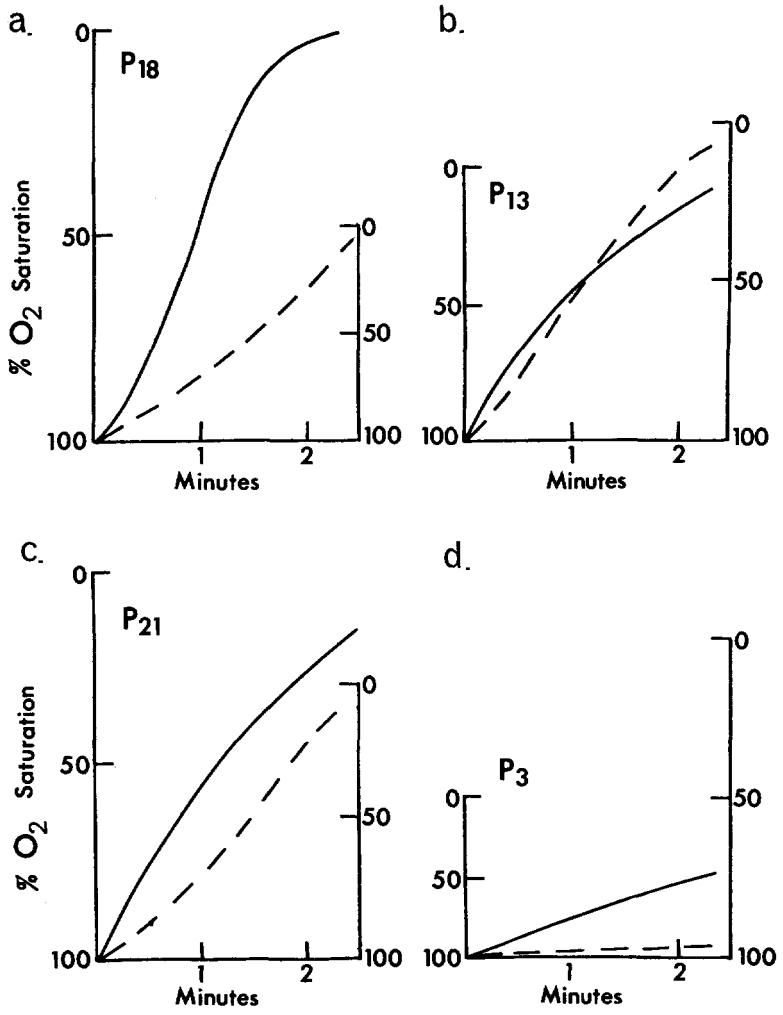

FIGURE 2.—Rate of O_2 consumption in extracts from untreated and MH treated seedlings. Untreated: ———— MH-treated: — — — — — —
The height of the percent O_2 saturation axis is adjusted according to the reciprocal of the protein content of the extract. The left axis on each graph is for extracts from control seedlings, and the right axis is for extracts from MH-pretreated seedlings. (a) Class I: P18 (Cx^F); (b) Class II: P13 (Cx^F); (c) Class II: P21 (Cx^S); (d) Class III: P3 (Cx^N).

and N variants are inherited as alleles of a single gene which is designated as *Cx* (Table 1). On this basis the three alleles are:

Cx^S—the allele which determines the Slow isozyme variant

Cx^F—the allele which determines the Fast isozyme variant

Cx^N—a Null allele which produces no recognizable gene product.

Mapping of the catechol oxidase gene Cx: The linkage group to which *Cx* belongs was determined by the waxy translocation method (ANDERSON 1938) utilizing a series of stocks each homozygous for a reciprocal translocation between

chromosome 9 and a particular chromosome under test. The translocation point was marked by the waxy mutant (*wx*) on chromosome 9. All the translocation stocks used were homozygous for the Cx^F allele. Close linkage was observed between *wx* and *Cx* with the T9–10b translocation, but not with either T1–9c to T6–9b (Table 2) indicating that *Cx* was probably located on chromosome 10. This conclusion was further strengthened by linkage studies with the gene for dull endosperm (du_1) which is carried in the proximal region of the long arm of chromosome 10 at position 33. Plants from seed homozygous for du_1 and Cx^F were crossed by Du_1, Cx^S pollen. Backcross progeny from this F_1 were scored for both markers and only two recombinations between du_1 and *Cx* were observed in a total of 2115 individuals tested (Table 3). Thus *Cx* and du_1 are closely linked showing less than 0.1% recombination.

Genetics of Class Type: Fifteen inbred lines were selected as being representative of the three classes described in the introduction (Table 4). These inbreds

TABLE 2

Location of Cx *on chromosome 10*

Translocation	Tx-9, *wx*, Cx^F/*Wx*, Cx^S × *wx wx*, Cx^N Cx^N				Total	χ^2	P
	Wx, *Cx*	*Wx*, *Cx*	*wx*, *Cx*	*wx*, *Cx*			
T1–9c	35	34	27	36	132	1.56	0.5–0.7
T6–9b	40	51	31	37	149	5.3	0.1–0.2
T9–10b	4	154	147	6	311	9.81	<0.01

χ^2 was calculated on the hypothesis that the genes *Wx* and *Cx* are unlinked. The *Wx* Cx^S genes in the translocation heterozygote came from Line P21 and the *wx wx* Cx^N Cx^N backcross parent was P4.

TABLE 3

Linkage between Cx *and* Du_1

	$\frac{du_1\ Cx^F}{Du_1\ Cx^S}$	×	$\frac{du_1\ Cx^F}{du_1\ Cx^F}$		
	$Du_1\ du_1$		$du_1\ du_1$		
Experiment 1	984		1027		2011
Experiment 2	778		743		1521
Total	1762		1770		3532

	$Du_1\ du_1$		$du_1\ du_1$		
	$Cx^F\ Cx^S$	$Cx^F\ Cx^F$	$Cx^F\ Cx^S$	$Cx^F\ Cx^F$	
Experiment 1	699	1	1	472	1173
Experiment 2	566	—	—	376	942
Total	1265	1	1	848	2115*

The Du_1 Cx^S genes in the heterozygote came from line P21.

* Not included in this total were 19 dull and 14 nondull seedlings which failed to show isozyme activity on the zymograms, presumably due to technical error.

TABLE 4

Frequency of CX *isozymes bands in different inbred lines with and without* MH *treatment*

	Line	Isozyme variant	No. of individuals scored for CX isozymes Untreated Absent	Untreated Present	MH-treated Absent	MH-treated Present
Class I	P18	Fast	8	100	—	70
	P19	Fast	11	25	—	36
	P26	Fast	8	22	—	30
Class II	P13	Fast	35	1	—	36
	P16	Fast	12	—	—	12
	P21	Slow	30	—	2	27
	P22	Fast	30	—	—	29
	P23	Fast	34	2	1	35
	P25	Slow	17	1	—	18
	P28	Fast	30	—	—	38
Class III	P3	Null	36	—	36	—
	P4	Null	36	—	36	—
	P9	Null	36	—	36	—
	P10	Null	36	—	36	—
	P15	Null	36	—	36	—

were maintained for a further five to six generations during which time no change in Class Type was observed. This suggests that Class Type is under genetic control.

The expression of Class Type in F_1 was observed from the incidence and intensity of CX isozymes in starch gel zymograms. This was found to be extremely variable and seemingly dependent on which particular inbred from each class was used and the direction of the cross. However the following observation was made. Whenever CX isozyme activity was observed in hybrids which had not been MH treated, such as between a Class I inbred carrying Cx^F and a Class II carrying Cx^S, both the F and S isozyme bands were present and in equal intensity. Thus Classes I and II are not determined by the *Cx* allele since, if this had been the case, these hybrids would have shown only the F isozyme band and the S band would have appeared only after MH treatment.

Attempts to follow the segregation of Class Type in genetic crosses, particularly of those hybrids involving Class I and II, were unsuccessful. Zymograms showed every grade of intensity, from complete absence to the presence of very strong bands of activity. Quantitation by using densitometer measurements of isozyme intensities failed to reveal any distinct classes of progeny.

From the genetic data it can be concluded that the difference between Class I and Class II is an inherited trait probably controlled by more than one gene inherited independently of the gene *Cx* specifying catechol oxidase isozymes. Class III, on the other hand, appears to be determined by the presence of the Cx^N allele.

Activity of CX in different inbred lines: Measurement of the CX activity in enzyme extracts from seedlings was undertaken to determine whether the MH-

mediated appearance of an isozyme band does represent a net increase in the amount of o-diphenol oxidase activity. The measurement of total o-diphenol oxidase activity will reflect not only that activity attributable to the CX isozyme but also to any other enzymes having some ability to oxidize catechol. Evidence for the existence of such enzymes is occasionally seen in the anodal region of starch gel zymograms; however they are of low activity, variable incidence and occur independently of Class Type or CX allele. Extracts from control and MH-treated seedlings of representative lines from each Class were measured: Class I—P_{18}, P_{26}; Class II—P_{13}, P_{21}, P_{22} and P_{28}; Class III—P_3, P_4 and P_{10}. All measurements were duplicated and repeated at least twice with different extracts, and the results from each line were fully consistent, allowing us to make a number of general conclusions.

There is no indication that MH-treated Class II seedlings (those showing a distinct CX isozyme band) have higher enzyme activity than control seedlings (no CX band), (Figure 2b.c.).

Notably the kinetics of oxygen consumption varied according to whether or not the extract showed a migrating band of CX activity in electrophoresis. Extracts from control Class II seedlings (no migrating isozyme) consumed oxygen with a constantly decreasing velocity. However those extracts which show the migrating CX isozyme, and this includes both control and MH extracts of Class I as well as MH extracts from Class II lines, consumed oxygen with sigmoidal kinetics.

The Class III lines showed reduced levels of activity compared to Class I or Class II lines (Figure 2d). It is conceivable that the Cx^N allele carried by these lines does not produce any active enzyme and the low activity is attributable to other enzymes which possess some catechol oxidase activity.

Electrophoresis on cellulose acetate strips: Ignoring, for the moment, the difference in kinetics of O_2 consumption between control and MH extracts in Class II, the above measurements indicate that the induced appearance of a CX isozyme band by MH treatment is not associated with any real overall increase in CX activity. Electrophoresis on cellulose acetate strips was performed to test the possibility that the CX of the Class II control extracts was in a non-migrating form. If this were the case, a band would not be observed in starch gel zymograms, for the enzyme would remain in the sample filter paper.

As in starch gels, extracts from MH pretreated Class II seedlings had the cathodally migrating CX band (Figure 3). Extracts from control Class II seedlings showed a band of CX activity which did not migrate but remained at the origin (Figure 3). Class III extracts on cellulose acetate still do not show any CX activity, further supporting the hypothesis that the Cx^N allele does not produce any active product.

The effect of MH on the migration and activity of CX: Treatment of seeds with MH causes the CX in five-day seedlings to be in a form which migrates in electrophoresis, but does not cause any significant increase in enzyme activity. This treatment is only effective when applied four to five days before assaying for CX. MH itself had no effect on the migration of CX in extracts, nor did extracting

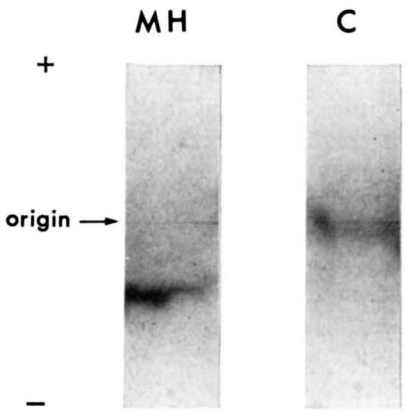

FIGURE 3.—Electrophoresis on cellulose acetate strips. MH: Extracts from maleic hydrazide treated seedlings (P22). C: Control (untreated).

control seedlings in buffer containing 0.01M MH produce any effect. Similarly the presence of 0.01M MH does not alter either the rate or kinetics of O_2 consumption of a given extract.

The effect of catechol on the electrophoretic migration of CX: Melanin formation during the course of o-diphenol exidase activity can lead to the complexing of protein and phenols ('tanning') (BENDALL and GREGORY 1963). For this reason we investigated the effect of the *in vivo* substrate catechol on the electrophoretic migration and kinetics of oxygen consumption of CX.

Extracts from lines P_{13} or P_{22} containing the Fast migrating form of CX were incubated with an equal volume of 0.01M catechol in 0.01M sodium phosphate buffer pH 6.2 prior to electrophoresis. Within twenty seconds' incubation time the CX isozyme was absent from the zymogram. If, however, the incubation mixture in addition contained 0.005M NADH a new, more slowly migrating band (G_1) was generated in samples subjected to electrophoresis after ten seconds of incubation (Figure 4a). (NADH was added as a reducing agent in order to decrease the effective concentration of o-benzoquinone product without altering the substrate concentration.) This G_1 band has a faster electrophoretic mobility than the Slow CX isozyme variant. With increasing time of incubation the intensity of this G_1 band increased at the expense of the F band until after several minutes the F band was no longer observed. With longer incubation times the G_1 band itself decreased while an anodal smear of CX activity became evident. After about ten minutes' incubation no CX activity was seen on starch gel zymograms (Figure 4a), although enzyme activity in the incubation mixture continued, as judged by increasing production of melanin.

At the same time that samples were removed from the incubation mixture for electrophoresis, a second sample was mixed with an equal volume of 0.01M sodium metabisulfite ($Na_2S_2O_5$). This strong reducing agent inhibits CX activity and also reduces the o-benzoquinone products back to catechol. Within about five minutes these samples were subjected to electrophoresis, and the resulting zymo-

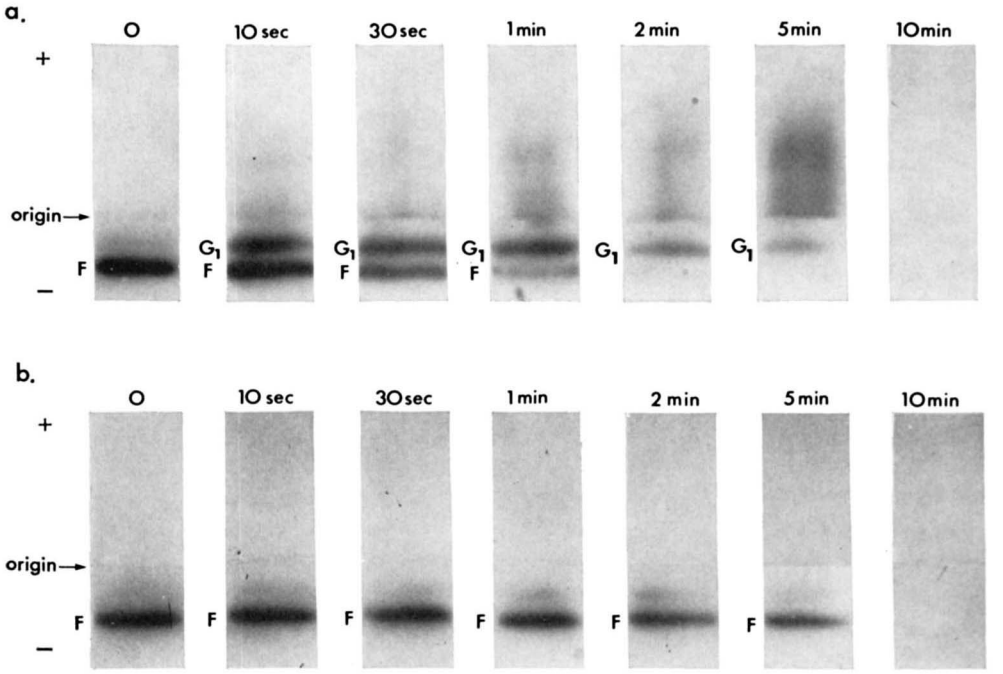

FIGURE 4.—(a) Starch gel zymograms showing the effect of catechol on the migration of Catechol Oxidase. Extracts containing the F isozyme mixed with an equal volume of 0.01M catechol and 0.005M NADH in 0.01M $NaPO_4$ buffer pH 6.2 were incubated for various times before electrophoresis. (b) Zymograms showing the effect of $Na_2S_2O_5$ on the G_1 band. At the same time samples were placed in gels in 4a, samples were also mixed with an equal volume of 0.01M $Na_2S_2O_5$ allowed to stand for 5 min, and then subjected to electrophoresis.

grams indicated that this treatment had largely reconverted the G_1 band back to the original F band (Figure 4b).

Thus during the course of reaction with catechol, CX was converted to a form which no longer migrated in electrophoresis. During this conversion definite intermediates appear, the first of which, G_1, can be reconverted to the parental form by $Na_2S_2O_5$.

The effect of catechol on the kinetics of O_2 consumption: Enzyme extracts from control and MH-pretreated seedlings of any Class II line (P_{13}, P_{22} or P_{21}) were incubated with an equal volume of 0.01M catechol in 0.01M sodium phosphate buffer pH 6.2. At various times aliquots were assayed for their CX activity (Figure 5).

MH extracts, which initially consume oxygen with sigmoidal kinetics, showed a marked stimulation in their rate of O_2 consumption after preincubation in catechol for 30 seconds (Figure 5a). Moreover, the kinetics of O_2 consumption were altered to a form similar to those of control extracts, i.e. a constantly decreasing velocity (Figure 5b). With increasing time of preincubation in catechol both control and MH extracts showed decreasing rates of O_2 consumption. This presumably reflects inactivation of CX by its reaction product(s), a phenomenon

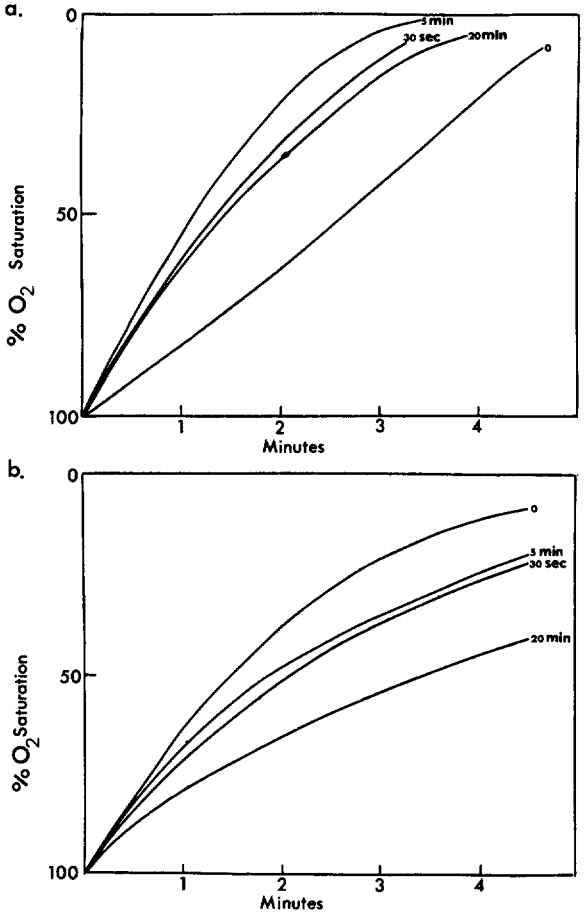

FIGURE 5.—The effects of incubation in catechol on the *Cx* activity in extracts from Class II seedlings (P22). (a) MH-pretreated seedlings; (b) Untreated seedlings. The incubation mixture contained equal volumes of the extract and 0.01M catechol in 0.01M $NaPO_4$ buffer pH 6.2. At different times 0.2 ml samples were assayed for *Cx* activity. The data presented are from extracts of P22 epicotyls but the same general results were obtained using lines P13 or P21 (Cx^S). The presence of 0.005M MH in the incubation had no effect on the ability of catechol to alter the enzyme kinetics.

which has frequently been observed with o-diphenol oxidases (SUSSMAN 1961). However the general shape of the O_2 consumption curve remained the same—a curve with a constantly decreasing velocity.

The presence of 0.005M MH in the incubation mixture has no effect on the ability of catechol to cause these changes.

The amount of oxygen consumed during the oxidation of catechol is generally more than the theoretical one atom (FORSYTH and QUESNEL 1957), the additional oxygen being consumed by a number of secondary non-enzymatic reactions which involve the o-benzoquinone product and lead to the formation of a 'catechol melanin' (MASON and WRIGHT 1949). Thus the kinetics of CX activity

are difficult to interpret. At least two factors may be considered as contributing to the kinetics with a constantly decreasing velocity. Reaction or product inactivation, presumably resulting from the reactivity of the four and five positions of the o-benzoquinone with amino and sulphydryl groups on the protein (MASON 1955; WOOD and INGRAHAM 1965), will become progressively greater during the course of the reaction. Another factor precluding linear kinetics is the low affinity of phenol oxidase for oxygen (BENDALL and GREGORY 1963), for as oxygen is consumed it increasingly limits the rate of the reaction.

The sigmoidal kinetics of the migrating form of CX and the marked stimulation of the rate by prior incubation in catechol could be indications of enzyme activation. However a more likely possibility can be considered. Since the secondary non-enzymatic reactions of the quinone product also consume oxygen it could be argued that initially the possibility for these secondary reactions is low, but that during the course of the reaction the number of potential oxygen-consuming sites increases. These sites of secondary reactions are involved in melanin formation, and the net result would be a CX-melanin complex (i.e. 'tanning') (BENDALL and GREGORY 1963).

Both the increased rate of O_2 consumption and loss of electrophoretic migration of CX during the course of reaction with catechol might be expected as the enzyme is progressively 'tanned' by the quinone reaction products. Although the data presented here are not considered sufficient for this conclusion they appear to be consistent with this interpretation.

Evidence for an in vivo *modifier:* By treatment with its substrate, catechol, the CX in extracts from MH-pretreated seedlings was changed from a form which had sigmoidal kinetics and migrated in electrophoresis, to a form which did not migrate and consumed oxygen with a constantly decreasing velocity. Thus *in vitro*, catechol can cause the sorts of modification in CX that distinguish the CX from control and MH-pretreated seedlings. This suggested the occurrence of an *in vivo* 'modifier' which, like catechol, can change the form of CX. Evidence for a modifier is provided in the following experiments.

I. Epicotyls from untreated Class II seedlings (P_{22}, Cx^F) do not normally show the F isozyme band, while the S isozyme is present in MH-pretreated Class II seedlings of line P_{21} which carries the Cx^S allele. Mixtures of these epicotyls were ground together at ice bath temperature and the resulting slurry was assayed for CX isozymes. As the weight ratio of MH-treated (Cx^S)/untreated (Cx^F) epicotyls increased, the F isozyme band started to appear in the zymograms (Figure 6). The same result was obtained if, instead of MH-pretreated epicotyls, the untreated P_{22} seedlings were ground with increasing concentration of an S enzyme extract from which all low molecular weight species had been removed (P4-BioGel).

These experiments show that untreated Class II seedlings do contain an electrophoretically mobile form of CX, but normally it is not seen in the starch gel. They suggest the presence of a modifier which is present in limited concentration in untreated Class II seedlings. On cell disruption the modifier interacts with the CX altering it to a form which no longer migrates. If additional electrophor-

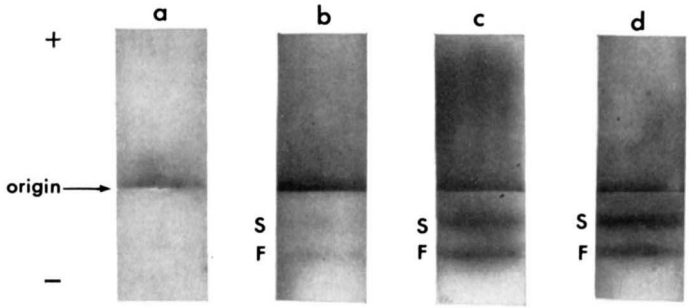

FIGURE 6.—The appearance of the F band in zymograms from untreated Class II (Cx^F, line P22) epicotyls extracted in the presence of increasing weights of MH-treated Class II epicotyls (Cx^S, line P21). Ratio untreated/MH-treated epicotyls—(a) 1:1; (b) 1:1; (c) 1:2; (d) 1:4. The reciprocal experiment using untreated P21 (Cx^S) epicotyls with MH-treated P22 (Cx^F) epicotyls produced the same result except that here, increasing amounts of MH-treated F epicotyls caused the appearance of the S band. Line P22 could also be replaced with lines P13 or P28.

etically-distinguishable CX is added, the limited amount of modifier is no longer sufficient to alter all of the CX protein available and the isozyme present in the untreated seedlings is now observed in starch gels.

II. Epicotyls from untreated Class II seedlings (P_{22} Cx^F) were ground in phosphate buffer containing 0.01M $Na_2S_2O_5$ ($S_2O_5^{2-}$ is a strong inhibitor of CX and will rapidly reduce any quinone products). The resulting zymograms contained the F isozyme band (Figure 7). This is further evidence that in the intact cell, CX occurs as the migrating form of the enzyme. The modification that occurs on cell disruption is inhibited by the presence of $S_2O_2^{2-}$. Thus the action of the modifier is analogous to the effect of the *in vitro* substrate, catechol, leading one to the conclusion that the modifier is an *in vivo* substrate of CX which is functionally isolated from the enzyme until after cell disruption. Class III seedlings again behave in a manner consistent with their carrying a null allele for CX. No isozyme activity is revealed by their extraction in $S_2O_5^{2-}$ buffer.

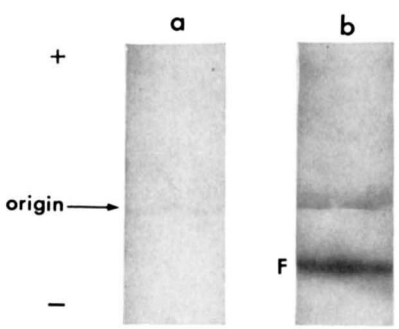

FIGURE 7.—The appearance of the *Cx* isozyme in extracts prepared by grinding Class II epicotyls in buffer containing $Na_2S_2O_5$. (a) Control; epicotyls extracted in phosphate buffer; (b) Epicotyls extracted in phosphate buffer containing 0.01M $Na_2S_2O_5$. Two zymograms prepared from line P13 epicotyls are shown but similar results were obtained with lines P22 and P28 as well as with P21 which carries the Cx^S allele.

III. The modifier was extracted by homogenizing epicotyls in a large excess of ethanol and evaporating the supernatant to a residue which was dissolved in 1 ml of water for every 4 gm of epicotyls. An aliquot of this aqueous solution was incubated with an equal volume of F isozyme extract for 2 minutes at room temperature. Absence of the F isozyme in starch gel zymograms indicated the presence of modifier in the seedling extract.

By making dilutions of modifier extracts which just caused the disappearance of the isozyme from the zymogram it was possible to estimate the relative amounts of modifier present in different inbred lines with and without MH treatment. To increase the sensitivity of this assay, enzyme extracts were diluted to the point where only a very light band of enzyme activity was seen on the zymogram, but this correspondingly produced a degree of uncertainty in determining when the isozyme was completely absent. Even so, duplicate assays gave consistent results and the results were repeatable on different extracts. The data in Table 5 were obtained from material prepared on the same day using the same F isozyme extract for each assay. All assays were run in duplicate and the results, although lacking precision, are clear-cut.

The Class I line P_{18} showed half the modifier activity of the Class II lines P_{22} and P_{28}. Thus the difference between Classes I and II seems due to the amount of modifier. The half level of modifier activity is insufficient to alter all the CX into the non-migrating form and in Class I lines, after cell disruption, some CX remains in the migrating form.

Epicotyls pretreated with MH contain approximately 1/10 the modifier activity of the controls (Table 5), indicating that the apparent induction of CX by MH treatment in Class II lines is due not to an effect of MH on the enzyme, but to an effect on the amount of modifier. Presumably a modifier content reduced tenfold is insufficient to alter all the enzyme and thus a migrating isozyme is seen in starch gel zymograms.

It should be noted that Class III or the Null Class was determined by the presence of the Cx^N allele and this was independent of the level of modifier activity. Thus P_4 showed low modifier activity (like a Class I) while P_3 contained high activity (Class II), yet both carried the Cx^N allele and thus could not show CX activity in zymograms.

TABLE 5

Relative amounts of modifier activity in epicotyls from control and MH-treated seeds

Class	Line	Modifier activity* Control (untreated)	MH-treated
I	P18	50	5
II	P22	100	3–4
	P28	100	10
III	P3	100	10
	P4	10	0(?)

* Arbitrary units with P22 = 100.

TABLE 6

R_f *of modifier activity in paper chromatography*

		Solvent A		Solvent B
		I	II	I
Class I	P18	.72–.79	.89	.80–.84
Class II	P13	.75–.79	.89–.93	.80–.85
	P21	.79	.90	.80–.84
	P22	.79	.89–.93	.80–.84
	P28	.75–.79	.89–.93	.84–.88
Class II MH treated	P22	.79	not detectable	.79–.87
Class III	P3	.72–.78	.93	.79–.83
	P4	not detectable	not detectable	not detectable

Two regions with modifier activity were observed with solvent A, only a single region with solvent B.

Preliminary characterization of the modifier: Ascending chromatograms of the various modifier extracts were run for 12–13 hours. The modifier activity was located in the chromatogram by cutting a ¾-inch-wide longitudinal strip extending from the origin to the solvent front. This strip was cut into ¼-inch horizontal sections and 0.01 ml of an F isozyme extract was pipetted onto each section. After a five-minute incubation at room temperature the chromatogram sections were placed in starch gels for electrophoresis. The absence of the F isozyme band in zymograms indicated the location of modifier activity in the chromatogram.

All lines tested had modifier activity at approximately the same R_f values (Table 6). In solvent system A two regions of modifier activity were observed: a major region with an R_f of .72 to .79 and a minor region with an R_f of .89 to .93. With solvent B only one region of activity, with an R_f .79 to .85, was observed.

Although the amount of modifier activity from MH-treated epicotyls of Class II (P_{22}) is only 1/10 that from control seedlings, the R_f of the active substance is apparently the same (Table 6). (The minor region found using solvent A was not detected, most probably because of its low concentration.) A partially purified extract of modifier was obtained by sequential chromatography insolvents A and B. The ultraviolet absorption spectrum of this fraction in ethanol contained maxima at about 270, 290 and 335 mu. Although this fraction is certainly not pure, the u.v. absorption spectrum is suggestive of a quercetin derivative (see HARBORNE 1967).

The R_f's of quercetin and rutin (quercetin-3-rutinoside) in solvent A were both different from that of modifier activity. However, when chromatograms containing either quercetin or rutin were assayed for modifier activity, it was found that quercetin did function as a modifier but rutin did not.

DISCUSSION

In this paper we have described an o-diphenol oxidase system in which a gene *Cx* determines the particular catechol oxidase isozyme present while another

genetic system controls the amount of a modifier of the enzyme. Both the modifier and the *in vitro* substrate catechol can alter CX from a form which has sigmoidal kinetics and migrates in electrophoresis to a non-migrating form which consumes oxygen with a constantly decreasing velocity. Sodium metabisulfite inhibits the effects of both catechol and the modifier. These observations suggest that the modifier is an endogenous substrate and that the modification is a result of CX activity which produces reactive quinones and subsequent 'tanning' of the enzyme. In addition the data indicate that the modifier is present in limited amount and is functionally isolated from CX in intact cells. (Grinding cells in the presence of additional electrophoretically recognizable enzyme or in the presence of $Na_2S_2O_5$ reveals the presence of endogenous unmodified CX isozyme). There is specificity between CX and the modifier since in chromatographic studies of phenolic extracts only one or two regions possess modifier activity.

The distinction between Classes I and II can be explained by their relative modifier contents. Class I contains only 50% of the modifier activity of Class II, this presumably being insufficient to modify all the CX in these plants and thereby allowing some CX to remain in the migrating form (i.e., 'constitutive'). Treatment with MH causes a tenfold reduction in modifier content in Class II seedlings and results in the apparent 'induction' of a CX isozyme. Class III is distinguished by the presence of the Cx^N allele which produces no recognizable CX isozyme.

On the role of CX in maize: Interaction of CX with the modifier would not only cause a 'tanning' of CX but also of other cellular proteins, depending on the availability of their amino or sulfhydryl groups. The end result would be a rapid death of the cell and this seems to be exactly what is required for hypersensitive resistance of plants to pathogens. Invasion of a host call by a pathogen might provide the necessary cellular disruption to bring CX and modifier out of isolation, initiating an overreaction by the plant which results in the death of the infected tissue and thus inactivation and isolation of the pathogen.

Other than a degree of specificity between CX and modifier we have no information on the significance of the modification of CX to the role of the enzyme in maize. Phenol oxidase activity has frequently been implicated in the mechanism of hypersensitive response (see MÜLLER 1959; WOOD 1967; STAHMANN 1967) and further study may also support this in maize. It might be expected that lines lacking CX (i.e., those carrying the Cx^N allele or Class III) may show reduced resistance and, similarly, there may be a correlation between resistance and modifier content.

This hypothesis would predict that, since MH treatment reduces the level of modifier activity, it would also cause a decrease in disease resistance. In fact this has been observed; MH-treated tomato plants were more severely stunted by Fusarium (WAGGONER and DIMOND 1952); MH-treated wheat seedlings are consistently more susceptible to stem rust than are untreated plants (BROMFIELD and PEET 1954; SAMBORSKI and PERSON 1960).

As a result of the substantial studies by FLOR the genetics of rust resistance in flax is well understood (see FLOR 1959). SAXENA and HOOKER (1968) have described an analogous genetic system in corn. The biochemical basis for hyper-

sensitive resistance to rust is still largely unknown, but if the CX-modifier system described in this paper can be related to disease resistance it may provide a system for investigating a number of possible models. Any model must explain, among other things, the gene-for-gene relationship that exists between host genes for resistance (*R*) and pathogen genes for avirulence (*Av*), the fact that these relationships can be altered by single mutational events, and the evidence that argues for virulence genes in the rust being loss functions. (Virulence is generally recessive to avirulence [FLOR 1959] and multivirulent rust strains compete poorly with strains having a narrow host range [WATSON 1958]).

The functional isolation required between CX and modifier in the intact cell could be achieved not only by restricted cellular location but also by chemical isolation, as in a phenolic glycoside. Thus potato phenol oxidase can utilize quercetin as a substrate but rutin (quercetin-3-rutinoside) shows very low substrate activity (PATIL and ZUCKER 1965). This is in agreement with our preliminary observation for CX in which quercetin functioned as a modifier while rutin did not. Invasion of a cell by a pathogen could lead to the hydrolysis of the glycosidic linkage releasing the phenolic substrate for CX and initiating the hypersensitive reaction.

A number of possibilities can be considered for the hydrolysis of the glycoside, the simplest of which is hydrolysis by a glycosidase produced by the fungal pathogen. MOLOT (1969) has suggested that the β-glucosidase produced by *Fusarium sp.* is responsible for the release of phenolic resistance factors from glucosides in corn. In fact, the idea of phenolic glycosides existing in plants as latent antimicrobic substances has been considered for some time (PRIDHAM 1960). From this line of argument, one can develop a model for rust resistance in which the rust gene Av_1 is producing a glycosidase which has the specificity to hydrolyze a particular phenolic glycoside whose biosynthesis is determined by information carried by the host gene R_1. The model would account for the gene-gene relationship between host and pathogen and could be easily expanded to include additional alleles and loci. Selection would be against Av_1 in the rust unless it performed a vital function. Genes coding for glycosidases, pectinases, cellulases, etc. are required for successful growth of the rust in the host tissue.

Another possibility might be the induction of host glycosidase activity as a response to cell invasion. In pear leaves, arbutin is hydrolyzed to glucose and hydroquinone by a β-glucosidase. Variation in antibiotic activity of pear leaves against *Erwinia amylovora* was primarily related to β-glucosidase activity rather than arbutin concentration (HILDEBRAND and SCHROTH 1964). A model for rust resistance based on this argument would require that the product of the Av_1 gene in some manner induced a glycosidase activity coded for by the R_1 gene of the host cell.

A number of other models could be imagined for hypersensitive resistance to rusts based on the hydrolysis of a phenolic glycoside, thereby producing a substrate for CX and subsequent cell death. The general principles put forward in these models are not particularly new, but if the CX-modifier system that has been described in this paper is involved with hypersensitive resistance in maize

it seems very likely that it may provide a sensitive technique for critically testing these sorts of models.

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