Phytochemistry, 1976, Vol. 15, pp. 1589–1594. Pergamon Press. Printed in England.

ENZYME SYNTHESIS BY CONSERVED MESSENGERS IN GERMINATING WHEAT EMBRYOS

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(*Revised received* 21 *May* 1976)

Key Word Index—*Triticum aestivum*; Gramineae; wheat embryos; conserved message; translational control of *o*-diphenolase; peroxidase; RNase.

Abstract—The stimulation of *o*-diphenolase (× 13), peroxidase (× 6) and ribonuclease (× 25) activities was demonstrated in excised wheat embryos cultured for 48 hr. Cycloheximide (5 μg/ml) completely blocked leucine-[^{3}H] incorporation into protein fractions and simultaneously inhibited the activity of the three enzymes. Increasing concentrations of cycloheximide (2 and 4 μg/ml) caused a progressive inhibition of *o*-diphenolase activity of fast migrating multiple forms together with a proportional loss of leucine-[^{3}H] incorporation in the region of these activity bands. This indicated the involvement of fresh protein synthesis for enhanced *o*-diphenolase activity. However, fresh transcription did not seem obligatory for enzyme stimulation. Actinomycin D failed to inhibit the activities of *o*-diphenolase, peroxidase and RNase, although it caused a strong inhibition (70%) of uracil-[^{14}C] incorporation into the RNA fraction without affecting leucine-[^{3}H] incorporation into proteins. This suggested a translational control of enzyme stimulation which is supported by conserved messengers present in the ungerminated wheat embryos. Another inhibitor of RNA synthesis (6-methylpurine) strongly inhibited (78%) uracil-[^{14}C] incorporation into RNA fraction, but unlike actinomycin D, it caused a severe inhibition (75%) of *o*-diphenolase activity and also retarded (76%) leucine-[^{3}H] incorporation into protein fractions. Cordycepin caused a mild inhibition (25%) of *o*-diphenolase activity together with a simultaneous inhibition of adenine-[^{14}C] incorporation into RNA (78%) and that of leucine-[^{3}H] incorporation (30% inhibition) into protein fraction. The inhibition of *o*-diphenolase activity by 6-methylpurine and cordycepin is probably due to a direct block of translation of conserved message in wheat embryos.

INTRODUCTION

In a previous communication [1], we reported enhanced *o*-diphenolase activity in embryos excised from germinating wheat grains which was inhibited by cycloheximide, but not by Actinomycin D (Act D). Other workers have shown that Act D is ineffective in blocking the activities of protease and isocitratase in cotton cotyledons [2,3], β-1,3-glucanase in *Phaseolus* [4], peroxidase in sweet potato [5,6] and wheat seedlings [7] and phenylalanine ammonia lyase in *Citrus* fruit [8]. However, caution must be exercised in interpreting negative responses of Act D, since this may be due to lack of its penetration in some plant tissues. The presence of stable *m*RNA capable of supporting general protein synthesis has been reported in embryos of wheat [9–12], rice [13] and cotton [2,14,15] and seeds of *Pisum* [16] and soybean [17]. However, information is lacking on the nature of specific enzyme proteins whose translation is supported by stable messengers in wheat embryos. In this communication, evidence is presented for the occurrence of conserved messengers of *o*-diphenolase, peroxidase and RNase in ungerminated wheat embryos which are capable of supporting enzyme protein synthesis under conditions of strong inhibition of RNA synthesis.

RESULTS AND DISCUSSION

Effect of inhibitors of RNA and protein synthesis on embryo germination

Cycloheximide (5 μg/ml) proved to be a strong inhibitor of embryo germination, while inhibitors of RNA synthesis had a variable effect on germination. Actinomycin D (100 μg/ml) failed to interfere with the normal germination, although it did partially retard (20%) the growth of embryos in terms of fr wt. On the other hand, 6-MeP (2×10^{-4}M) strongly retarded the germination and maintained the embryos in a dormant phase. Cordycepin (2×10^{-4}M) caused nearly 80% inhibition of embryo germination, while the remaining 20% showed the emergence of a small root protuberance. Higher concentrations of cordycepin (3×10^{-4}M and above) brought about a complete inhibition of embryo germination.

Effect of cycloheximide on enzyme activity

The present work has revealed that the stimulation of *o*-diphenolase, peroxidase and RNase activities in germinating excised wheat embryos is not dependent on fresh transcription, but does require protein synthesis. Embryos cultured for 48 hr showed stimulation of *o*-diphenolase (× 13), peroxidase (× 6) and RNase (× 25) activities. Cycloheximide (CHI, 5 μg/ml) caused a strong inhibition of leucine-[^{3}H] incorporation into protein fractions. This was associated with a significant inhibition of enzyme activities (Table 1), indicating the possible requirement of fresh protein synthesis for enzyme stimulation. Since CHI inhibited the formation of several multiple forms of *o*-diphenolase, an experiment was designed to confirm if the appearance of newly formed activity bands was dependent on fresh protein synthesis. The dialyzed *crude extracts* prepared from control and CHI-treated embryos (cultured in presence of leucine-[^{3}H])

Table 1. Effect of cycloheximide (CHI) on L-leucine-[^{3}H] incorporation and enzyme activity of *o*-diphenolase, peroxidase and RNase in wheat embryos

Additions	L-Leucine-[^{3}H] incorporation		*o*-Diphenolase activity		Peroxidase activity		RNase activity	
	cpm/mg protein	% inhibition	Enzyme units/mg protein	% inhibition	Enzyme units/mg protein	% inhibition	Enzyme units/mg protein	% inhibition
Control	33450	—	317	—	870	—	536	—
CHI, 5 μg/ml	4	99 9	51	84	220	75	230	57
CHI, 10 μg/ml	4	99 9	44	86	155	82	280	48

Excised wheat embryos were cultured in dark at 25° for 48 hr with and without the addition of CHI. Leucine-[^{3}H] (1 μCi/ml, sp act 7600 mCi/mmol) was present throughout the period of germination. The enzyme activities were assayed in *crude extracts*.

were fractionated on acrylamide gels and the region of two fast migrating activity bands was eluted with Pi buffer (*eluted fraction*). The *eluted fraction* was examined for *o*-diphenolase activity and radioactivity. Cycloheximide (2 and 4 μg/ml) caused nearly 40% and 100% inhibition of *o*-diphenolase activity with a proportional decrease in the radioactivity (57 and 94%) of leucine-[^{3}H] in the *eluted fraction* [18]. This suggested that protein synthesis was essential for the formation of new multiple forms of *o*-diphenolase in germinating embryos.

Effect of inhibitors of RNA synthesis on enzyme activity, RNA and protein synthesis

In order to determine the role of transcription in the stimulation of *o*-diphenolase, peroxidase and RNase enzymes, the effect of inhibitors of RNA synthesis (Act D, 6-methylpurine, cordycepin) on enzyme activities was studied.

Excised embryos cultured on a medium containing Act D (100 μg/ml) failed to arrest the stimulation of *o*-diphenolase, peroxidase and RNase activities in 48 hr old seedlings. Also, embryos germinated for a longer duration (96 hr) revealed no appreciable inhibition of enzyme activities (Table 2). The lack of inhibitory response by Act D was not due to its ineffectiveness in the embryonic tissue. The drug proved to be a potent inhibitor of RNA synthesis* as evident from uracil-[^{14}C] incorporation studies (Tables 3 and 5). The inhibitor caused no reduction in the free pool of uracil-[^{14}C] at different stages of embryo germination. Also, the ratio of uracil-[^{14}C] incorporation into the RNA fraction/free pool of uracil-[^{14}C] in Act D-treated embryos was lower than the controls (Table 3). Obviously Act D caused a true inhibition of RNA synthesis which could not be ascribed to decreased uptake of uracil-[^{14}C] in excised embryos. Acrylamide gel electrophoresis of RNA, isolated from Act D-treated embryos revealed significant inhibition of uridine-[^{3}H] labelled polydisperse RNA fraction (unpublished results). The drug, however, failed to inhibit leucine-[^{3}H] incorporation into protein fractions (see Table 5). Thus the experiments with Act D indicated that fresh transcription was not mandatory for the stimulation of *o*-diphenolase, peroxidase and RNase activities and the enzyme production was possibly supported by long lived messengers present in the ungerminated wheat embryos. Similar results have been reported in cotton cotyledons [14,19] and sea urchin eggs [20]. Fertilization of the sea urchin egg cell brings about the activation of protein synthesis [21]. Actinomycin D failed to inhibit the activation of protein synthesis, although it retarded *m*RNA formation [20]. In germinating cotton cotyledons, the *de novo* synthesis of protease and isocitratase was unaffected by Act D and supported by preexisting *m*RNA [2].

In contrast to Act D, 6-methylpurine (6-MeP) significantly inhibited the enzyme activities of *o*-diphenolase, peroxidase and RNase. There was 83–84% inhibition of *o*-diphenolase and peroxidase activities, while RNase activity was curtailed to the extent of 56% (6-MeP, 5×10^{-4}M in Table 4). 6-Methylpurine (2×10^{-4}M) revealed an almost identical magnitude of inhibition of uracil-[^{14}C] incorporation (78%), leucine-[^{3}H] incorporation (76%)(Table 5) and *o*-diphenolase activity (75%) in germinating embryos. This would normally implicate the requirement of fresh transcription for enzyme stimulation. However, the above hypothesis seems untenable, as a similar extent of inhibition (73%) of uracil-[^{14}C] incorporation into RNA by Act D was not accompanied by the retardation of enzyme activities. Since 6-MeP caused a strong inhibition (76%) of leucine-[^{3}H] incorporation into the protein fraction, which was not the case with Act D (Table 5), it is likely that 6-MeP inhibited enzyme activity through its direct action on translation of conserved message.

* Marcus and Feeley [9] were unable to observe any inhibition of ^{32}P-incorporation by Act D in imbibing wheat embryos and this was ascribed to lack of its penetration, or ineffectiveness in plant systems. However, our studies clearly indicated that Act D was an effective inhibitor of RNA synthesis (in terms of uracil-[^{14}C] incorporation) in germinating wheat embryos.

Table 2. Effect of actinomycin D on the activities of *o*-diphenolase, peroxidase and RNase in excised wheat embryos

Additions	*o*-Diphenolase activity Enzyme units/mg protein		Peroxidase activity Enzyme units/mg protein		RNase activity Enzyme units/mg protein	
	48 hr	96 hr	48 hr	96 hr	48 hr	96 hr
Control	640	1020	1500	1850	400	580
Act D, 100 μg/ml	640	840	1420	1800	360	580

Excised wheat embryos were cultured in dark at 25° for 48 hr and 96 hr. Actinomycin D (Act D) was present throughout the period of germination. *Crude extracts* prepared from germinating embryos were used for the assay of enzyme activities.

Table 3. Effect of actinomycin D on uracil-[^{14}C] incorporation into RNA fraction and on the free pool of uracil-[^{14}C] in wheat embryos

Time of incubation (hr)	Uracil-[^{14}C] incorporation into RNA cpm/mg protein		Free pool of uracil-[^{14}C] cpm/mg protein		Ratio of uracil-[^{14}C] incorporation/ Free pool of uracil-[^{14}C]	
	Control	Act D	Control	Act D	Control	Act D
6	1460	842	5740	7160	0.25	0.12
12	4760	1040	4950	5910	0.94	0.17
24	4120	1620	2250	2740	1.83	0.58
48	4980	1750	1660	1770	3 0	0.99

Excised wheat embryos were cultured in dark at 25° for different intervals of time. Uracil-[^{14}C] (0.5 μCi/ml, sp act 49.3 mCi/mmol) was added to the control and Act D (100 μg/ml)-treated embryos for determining the free pool of uracil-[^{14}C] and the incorporation of labelled precursor into RNA fraction.

Administration of 6-MeP to pregerminated embryos (48 hr old) significantly curtailed the inhibitory effect of the drug both on *o*-diphenolase activity and leucine-[^{3}H] incorporation, while the severe inhibition of uracil-[^{14}C] incorporation remained unaltered (Table 6). Thus, the above data did not favour any correlation between enzyme stimulation and fresh transcription in germinating embryos. Instead, it appeared that some factor necessary for translation had already accumulated in pregerminated embryos which prevented the severe inhibitory action of 6-MeP on leucine-[^{3}H] incorporation and *o*-diphenolase activity.

Further support for the existence of stable message of *o*-diphenolase was sought by testing the effect of different concentrations of cordycepin (3′-deoxyadenosine) on excised wheat embryos. Low concentration of cordycepin (10^{-4}M) showed no inhibition of *o*-diphenolase activity, at 3×10^{-4}M there was 15% inhibition of enzyme activity*. Further increase in the concentration of cordycepin to 4×10^{-4}M and 5×10^{-4}M decreased the *o*-diphenolase activity by 20 and 25% respectively. As shown in Table 7, there was a steady decline in the incorporation of adenine-[^{14}C] into the RNA fraction with increasing levels of cordycepin. The inhibition of RNA synthesis in cordycepin-treated embryos was not due to the decrease in the free pool of adenine-[^{14}C]. Instead, a substantial increase in the free pool of labelled precursor was witnessed in embryos cultured in the presence of inhibitor (Table 7). Also, a decrease in the ratio of adenine-[^{14}C] incorporated into RNA upon free pool of adenine-[^{14}C] by cordycepin is a clear indication of true inhibition of RNA synthesis. Cordycepin at 4×10^{-4}M caused 78% inhibition of RNA synthesis (Table 7), while the *o*-diphenolase activity was affected only to an extent of 25%. Thus it became evident that under conditions of strong inhibition of RNA synthesis, there was no proportional decrease in *o*-diphenolase activity.

The apparent difference in the response of Act D and cordycepin on *o*-diphenolase activity was resolved by comparing their effect on protein synthesis. As stated earlier, Act D (100 μg/ml) failed to inhibit leucine-[^{3}H] incorporation into protein fraction, whereas cordycepin (4×10^{-4}M) retarded protein synthesis to the extent of 30% (Table 7). The mild inhibition of *o*-diphenolase activity by cordycepin could be due to partial blocking of translation of conserved message. However, in cotton cotyledons, cordycepin completely blocked the stimulation of carboxypeptidase by preventing the polyadenylation of stored messenger of this enzyme [22,23]. In wheat embryos, the lack of strong inhibition of *o*-diphenolase activity by cordycepin suggests the nonrequirement of polyadenylation of conserved message for enzyme stimulation. Support for this postulation is derived from the report of Spiegel and Marcus [24] that the conserved *m*RNA of wheat embryos does not require polyadenylation prior to the formation of the polyribosomal complex. Since all species of *m*RNA which are active in protein synthesis are not polyadenylated both in animal [25,26] and plant cells [27], it appears that the stimulation of *o*-diphenolase in wheat embryos could be supported by a conserved message which is either polyadenylated during grain development, or perhaps does not require polyadenylation. Thus the slight inhibition

* In our previous communication [18], we reported lack of inhibition of *o*-diphenolase activity at 3×10^{-4}M concentration of cordycepin. Subsequent experiments have revealed a slight inhibition (15%) of enzyme activity at this concentration of the inhibitor.

Table 4. Effect of 6-methylpurine (6-MeP) on the activities of *o*-diphenolase, peroxidase and RNase in wheat embryos

Additions	*o*-Diphenolase activity		Peroxidase activity		RNase activity	
	Enzyme units/ mg protein	% Control	Enzyme units/ mg protein	% Control	Enzyme units/ mg protein	% Control
Control	530	100	1550	100	430	100
6-MeP:						
(a) 10^{-3}M	510	96	1500	97	480	111
(b) 10^{-4}M	320	60	900	58	176	41
(c) 5×10^{-4}M	86	16	240	17	190	44

Excised wheat embryos were cultured in the continuous presence of different concentrations of 6-MeP in dark at 25° for 48 hr. The enzyme activities were assayed in *crude extracts*.

Table 5. Effect of 6-methylpurine and actinomycin D on RNA and protein synthesis of wheat embryos

Additions	Uracil-[^{14}C] incorporation		Leucine-[^{3}H] incorporation	
	cpm/mg protein	% inhibition	cpm/mg protein	% inhibition
1. Control	6560	—	22500	—
6-MeP, 10^{-4}M	2600	61	8840	61
6-MeP, 2×10^{-4}M	1550	78	5420	76
2. Acetone-Control	5800	—	15800	—
Act D, 10^{-4}M	2290	61	18300	—
3. Acetone-Control	6360	—	15300	—
Act D, 2×10^{-4}M	1710	73	15200	0.5

Excised wheat embryos were cultured in presence of uracil-[^{14}C] (0.5 μCi/ml, sp act 49.3 mCi/mmol) or leucine-[^{3}H] (1 μCi/ml, sp act 7600 mCi/mmol) in dark at 25° for 48 hr. Actinomycin D (Act D) and 6-methylpurine (6-MeP) were present throughout the period of germination. Actinomycin D was dissolved in small aliquot (20 μl) of Me_2CO (79%) and the desired concentration was prepared by the addition of sterile H_2O. Appropriate acetone controls were maintained for each concentration of Act D.

of *o*-diphenolase by cordycepin in wheat embryos appears to be linked with the direct inhibition of translation of conserved message.

Effect of inhibitiors of RNA and protein synthesis on o-diphenolase multiple forms

Crude extracts of embryos (0 hr germination) revealed four multiple forms of *o*-diphenolase on acrylamide gels. Following germination of excised embryos (48 hr), six additional multiple forms appeared on gels which were completely inhibited by cycloheximide (CHI, 5 μg/ml). Addition of Act D (100 μg/ml) failed to alter the pattern and intensity of *o*-diphenolase multiple forms. Cordycepin (4×10^{-4}M) slightly diminished the intensity of newly formed *o*-diphenolase multiple forms, whereas 6-MeP (2×10^{-4}M) completely blocked their appearance on acrylamide gels. Thus the inhibition of *o*-diphenolase activity by CHI, 6-MeP and cordycepin was also reflected in the altered pattern of its multiple forms.

Table 6. Effect of 6-methylpurine (6-MeP) on *o*-diphenolase activity, uracil-[^{14}C] and L-leucine-[^{3}H] incorporation in germinating wheat embryos

Additions	*o*-Diphenolase activity		Uracil-[^{14}C] incorporation		Leucine-[^{3}H] incorporation	
	Enzyme units/ mg protein	% inhibition	cpm/mg protein	% inhibition	cpm/mg protein	% inhibition
Control	950	—	1760	—	8630	—
6-MeP						
(a) 10^{-4}M	800	16	1300	27	5790	33
(b) 2×10^{-4}M	820	14	636	64	5570	36
(c) 5×10^{-4}M	730	23	339	81	5400	37

Excised wheat embryos were pregerminated in dark at 25° on control nutrient medium for 48 hr. Thereafter, the embryos were transferred to a medium containing different concentrations of 6-MeP and allowed to grow for another 24 hr. The embryos were simultaneously fed with uracil-[^{14}C] (0.5 μCi/ml, sp act 49.3 mCi/mmol) or L-leucine-[^{3}H] (1 μCi/ml, sp act 7600 mCi/mmol) for studying the effect of inhibitor on RNA and protein synthesis.

Table 7. Effect of cordycepin on the free pool of adenine-[^{14}C] and on the incorporation of adenine-[^{14}C] and L-leucine-[^{3}H] into RNA and protein fractions respectively in germinating wheat embryos

Additions	Free pool of adenine-[^{14}C]	Adenine-[^{14}C] incorporation into RNA		Ratio of adenine-[^{14}C] incorporated	L-Leucine-[^{3}H] incorporation	
	cpm/mg protein	cpm/mg protein	% inhibition	Free pool of adenine-[^{14}C]	cpm/mg protein	% inhibition
Control	22500	47330	—	2.10	20000	—
Cordycepin						
(a) 5×10^{-5}M	30500	32000	33	1.04	18360	8
(b) 10^{-4}M	30700	21400	55	0.69	19770	1
(c) 2×10^{-4}M	61800	16900	65	0.27	17390	13
(d) 4×10^{-4}M	80300	10400	78	0.13	14000	30

Excised wheat embryos were germinated in dark at 25° for 48 hr in continuous presence of cordycepin. Adenine-[^{14}C] (0.5 μCi/ml, sp act 12.4 mCi/mmol) and L-leucine-[^{3}H] (1.0 μCi/ml, sp act 7600 mCi/mmol) were added from the inception of embryo germination for studying the effect of cordycepin on RNA and protein synthesis.

EXPERIMENTAL

Culture of excised embryos. Wheat seeds (*Triticum aestivum*, var. Shera) were soaked in cold (4°) H_2O for 10 hr. Embryos were scooped manually, surface sterilized with 0.02% $HgCl_2$ soln for 10 min and rinsed thoroughly with chilled sterile H_2O. Embryos were cultured in the dark at 25° on aseptic Nitsch's basic liquid medium [28] supplemented with White's vitamin soln [29], 2% sucrose and chloramphenicol (50 μg/ml).

Extraction and assay of enzymes. Embryos (40) were homogenized with 8 ml of 50mM Pi buffer (pH 6.6). Homogenate was centrifuged at 30000 *g* for 10 min and the supernatant (*crude extract*) employed for the assay of *o*-diphenolase, peroxidase and RNase. A slightly modified procedure of ref [30] was followed for the assay of *o*-diphenolase activity, that of ref [31] for the assay of peroxidase activity and ref [32] for the assay of RNase activity.

Enzyme units. A unit of enzyme activity of *o*-diphenolase and peroxidase is defined as the amount of enzyme that brings about a change in *A* of 0.01/min at 430 nm. In case of RNase, one unit of enzyme activity is equivalent to that amount of acid soluble oligonucleotides which cause a change in *A* of 0.01/min at 260 nm.

Uracil-[^{14}C] and adenine-[^{14}C] incorporation into RNA fraction. The effect of Act D, 6-MeP and cordycepin was studied on RNA synthesis by feeding uracil-[2-^{14}C] (0.5 μCi/ml, sp act 49.3 mCi/mmol) or adenine-[8-^{14}C] (0.5 μCi/ml, sp act 12.4 mCi/mmol) to excised embryos throughout the period of germination. Embryos (40) were homogenized with 7 ml of chilled perchloric acid (PCA) and centrifuged at 3000 *g* for 10 min in cold. Supernatant was discarded and the pellet was washed (×4) with 7 ml of chilled PCA. The pellet was suspended in 2 ml of 0.3N KOH and centrifuged at 10000 *g* to remove undissolved cell debris. Supernatant was incubated for 18 hr at 37° for the hydrolysis of RNA, while the DNA and protein were precipitated with PCA (60%). Samples were centrifuged and the supernatant adjusted to pH 7 with 6N KOH. An aliquot (200 μl) of hydrolyzed RNA was plated on Whatman paper No. 4 (18 × 18mm square) and radioactivity measured using toluene–PPO–POPOP mixture (1 litre: 5g:0.3g).

Determination of free pool of uracil-[^{14}C] and adenine-[^{14}C] in cultured embryos. Excised embryos were cultured on a medium containing Act D + uracil-[2-^{14}C] (0.5 μCi/ml, sp act 49.3 mCi/mmol) and cordycepin + adenine-[8-^{14}C] (0.5 μCi/ml, sp act 12.4 mCi/mmol). Embryos (40) were rinsed (×6) with sterile H_2O and homogenized in cold PCA (2%, 6 ml). Homogenate was centrifuged and the supernatant was saved. The pellet was washed (×4) with a 6 ml aliquot of PCA (2%). Supernatant fractions were pooled for determining the free pool of uracil-[^{14}C] and adenine-[^{14}C] in excised embryos. The pH of the supernatant was adjusted to pH 7 with 10N KOH. An aliquot (1 ml) of this fraction was mixed with 15 ml of Bray's soln [33] for measuring radioactivity.

L-Leucine-[^{3}H] incorporation into protein fraction. L-Leucine-[^{3}H] (1 μCi/ml, sp act 7600 mCi/mmol) incorporation into protein fractions was measured in excised embryos germinated in presence of inhibitors of RNA (Act D, cordycepin, 6-MeP) and protein (CHI) synthesis. Embryos (40) were homogenized with 7 ml of TCA (5%). Homogenate was centrifuged at 3000 *g* for 5 min and the supernatant discarded. The pellet was washed (×4) with TCA (5%) to remove the free pool of labelled leucine. The pellet was suspended in 7 ml of TCA (5%), maintained at 80° for 30 min and washed once with TCA (5%). The pellet was suspended twice in a mixture of EtOH–Et_2O (1:1) and maintained at 50° (10 min) to remove the lipid fraction. This was followed by extraction with Et_2O at room temp. The protein fraction was eluted by suspending the pellet in 1N KOH (2 ml) and the clear supernatant obtained after centrifugation was precipitated with 60% PCA. The pellet was redissolved in 2 ml of 1.5N ammonia soln and an aliquot (200 μl) plated on Whatman paper No. 4 (18 × 18mm square) and dried at 70° to remove ammonia. Radioactivity was measured in a liquid scintillation counter.

Acrylamide gel electrophoresis. Multiple forms of *o*-diphenolase were fractionated on acrylamide gel electrophoresis as described earlier [1].

Effect of cycloheximide on L-Leucine-[^{3}H] incorporation and enzyme activity in fast migrating multiple forms of o-diphenolase. Embryos (100) cultured on nutrient medium containing L-leucine-[^{3}H] (2 μCi/ml, sp act 7600 mCi/mmol) with and without CHI (2 and 4 μg/ml) were homogenized in 10 ml of Pi buffer (50mM, pH 6.6) and centrifuged for 15 min at 20000 *g*. The supernatant (*crude extract*) was dialyzed exhaustively in order to remove the free pool of leucine-[^{3}H]. The dialyzed extract (300 μg protein/gel column) was fractionated by acrylamide gel electrophoresis. The *o*-diphenolase multiple forms were developed by smearing gels with cotton swab soaked in DL-DOPA (2 mg/ml). The region of the two fast moving activity bands (4 mm thick) was eluted (for 24 hr) with 1 ml of Pi buffer (50mM, pH 6.6). Bray's soln was added to each vial for determining the radioactivity of leucine-[^{3}H] present in the region of multiple forms. Three gel pieces were excised from the region of fast moving multiple forms from parallel unstained gels and eluted with 2.5 ml of Pi buffer (50mM, pH 6.6). The *o*-diphenolase activity was measured in the *eluted fraction* (0.5 ml) using 3.5 ml catechol soln (100 μmol/ml). Extract prepared from blank gels along with substrate served as control.

Protein estimation. Protein was estimated following the procedure of ref [34].

Acknowledgements—One of us (SRT) is thankful to the Council of Scientific and Industrial Research for the award of Senior Research Fellowship. Actinomycin D was a generous gift from Merck Sharp and Dohme, U.S.A.

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