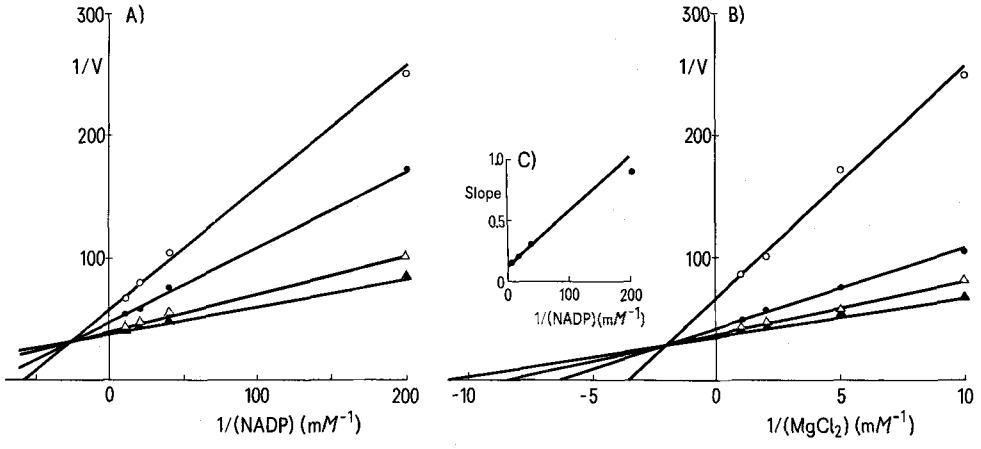

Fig. 3. Effect of the variation of the concentration of NADP and $MgCl_2$ on the velocity of the reaction catalyzed by malic enzyme. The experimental conditions were similar to those described in the legend to Figure 1, with 1 m*M* L-malate and the concentrations of NADP and $MgCl_2$ stated below. A) effect of variable concentrations of NADP, in the presence of 0.1 (○), 0.2 (●), 0.5 (△) or 1.0 (▲) m*M* $MgCl_2$ B) effect of variable concentrations of $MgCl_2$ in the presence of 0.005 (○), 0.025 (●), 0.05 (△) or 0.1 (▲) m*M* NADP. C) secondary plot of the slopes from the lines in Figure B as a function of the reciprocal concentration of NADP.

bind only after NADP is bound to the enzyme, to form the quaternary complex which undergoes reaction[6].

Assuming that the obligatory order of addition of L-malate after NADP also holds for the enzyme from *Pseudomonas*, we can propose that the ternary complex NADP-enzyme-Mg^{2+} reacts with L-malate through the metal ion, which acts as a link between L-malate and the active site. The different effectiveness of Co^{2+}, Mn^{2+} and Mg^{2+} as activators, and the different apparent K_m values for L-malate obtained in the presence of the same activators, could be explained on the basis of a decreasing ability of Co^{2+}, Mn^{2+} and Mg^{2+} to form the quaternary complex according to the Irving-Williams series[4] for the formation of complexes between divalent cations and organic ligands.

Summary. The kinetic order of addition of Mg^{2+} and L-malate to malic enzyme has been determined. Mg^{2+} is the first to bind, and probably acts as a link between the substrate and the active site.

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Conserved Message of *o*-Diphenolase in Wheat Embryos (*Triticum aestivum*)

Our preliminary observations[1] indicated that the stimulation of *o*-diphenolase activity in wheat embryos, excized from germinating grains, is strongly inhibited by cycloheximide but not by actinomycin D. The occurrence of stable messengers capable of supporting general protein synthesis is reported in the ungerminated wheat embryos[2,3]. Other workers, however, observed the early synthesis of mRNA in wheat embryos which is not converted into stable message[4,5]. At present, information is not available about the nature of specific enzyme proteins whose translation is supported by conserved or newly formed messages in wheat embryos. In the present report, we provide evidence for the existence of a long-lived stable messenger of *o*-diphenolase in excized wheat embryos which supports the de novo synthesis of *o*-diphenolase enzyme under conditions of inhibited RNA synthesis.

Materials and methods. Embryos were excized from wheat grains (*Triticum aestivum*, var. Shera) presoaked for 10 h at 4 °C and sterilized with 0.02% mercuric chloride. The excized embryos were germinated in the dark at 25 °C on nutrient medium[6,7] containing 50 µg/ml of chloramphenicol. 40 embryos were homogenized in 0.05 *M* phosphate buffer (pH 6.6) and the homogenate was centrifuged at 10,000 × *g* for 10 min. The supernatant (crude extract) was employed for measuring *o*-diphenolase activity by following the procedure of WONG et al.[8]. One unit of enzyme activity is defined as the amount of enzyme which brings about a change in absorbance of

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Table I. Effect of cycloheximide (CHI) on *o*-diphenolase activity and ^{3}H-leucine incorporation into protein fraction of excized embryos

Additions	*o*-Diphenolase activity		^{3}H-Leucine incorporation	
	Enzyme units	Inhibition (%)	cpm/mg protein	Inhibition (%)
Control	317	—	33,450	—
CHI (5 µg/ml)	51	84	4	99.9

The excized embryos were germinated in the continuous presence of CHI and ^{3}H-L-leucine (1.0 µCi/ml, spec. act. 7600 mCi/mmole) for 48 h in dark at 25 °C.

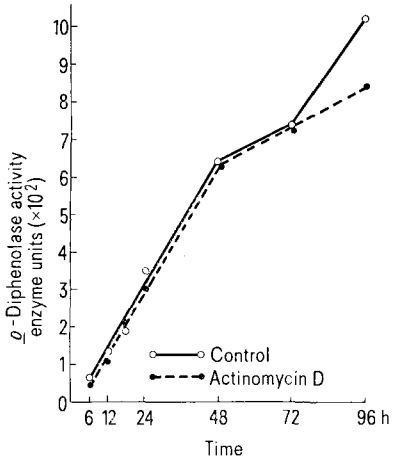

Fig. 1. Effect of actinomycin D (100 µg/ml) on *o*-diphenolase activity during early germination of excized wheat embryos cultured in dark at 25 °C.

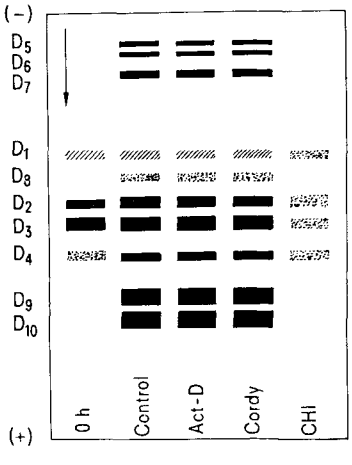

Fig. 2. Effect of actinomycin D (Act-D, 100 µg/ml), cordycepin (Cordy, 3×10^{-4} *M*) and cycloheximide (CHI, 5 µg/ml) on the isoenzymes of *o*-diphenolase fractionated on acrylamide gels. The excized embryos were germinated for 48 h in the continuous presence of inhibitors. The 0 h represents gel pattern of isoenzymes at zero h germination of excized embryos. ■, high activity; ▨, medium activity; ▒, low activity.

0.01/min/mg protein at 430 nm. The *o*-diphenolase isoenzymes were fractionated on acrylamide gel electrophoresis as described earlier[1]. Slightly modified procedure of SHANNON et al.[9] was adopted for demonstrating de novo synthesis of *o*-diphenolase isoenzymes.

Results and discussion. Excized wheat embryos cultured on nutrient medium (48 h) exhibited a 13-fold stimulation of *o*-diphenolase activity. The enhanced *o*-diphenolase activity is not dependent on fresh transcription, while protein synthesis seems obligatory. Cycloheximide (CHI) caused a strong inhibition of *o*-diphenolase activity (84%) and ^{3}H-leucine incorporation into protein fraction (99%) (Table I). This suggested that the enzyme stimulation is probably dependent on de novo protein synthesis. Treatment of excized embryos with actinomycin D (Act-D) showed no significant retardation of *o*-diphenolase activity (Figure 1). The negative response of Act-D cannot be attributed to its lack of penetration or ineffectiveness in excized embryos. Studies with ^{14}C-uracil incorporation clearly indicated that Act-D was an effective inhibitor of RNA synthesis in germinating excized wheat embryos. Act-D caused 76% inhibition of RNA synthesis, while the enzyme activity showed a negligible (12%) decrease (Table II). Similarly, Act-D showed no inhibitory effect on ^{3}H-leucine incorporation into protein fraction. In order to further examine the role of fresh transcription, if any, in the stimulation of enzyme activity, the effect of cordycepin (3′-deoxyadenosine) was tested on *o*-diphenolase activity. Cordycepin too failed to inhibit *o*-diphenolase activity (Table III). Thus the results obtained with Act-D and cordycepin strongly favour the existence of conserved message of *o*-diphenolase in ungerminated wheat embryos which is capable of stimulating enzyme activity at the time of germination. The occurence of stable message is also reported in sea urchins[10], cotton embroys[11], rice embroys[12], seeds of

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Table II. Effect of actinomycin D (Act-D) on *o*-diphenolase activity and ^{14}C-uracil incorporation into RNA fraction in excized embryos cultured for 48 h in dark at 25 °C

Additions	*o*-Diphenolase activity		^{14}C-Uracil incorporation	
	Enzyme units	Control (%)	cpm/mg protein	Inhibition (%)
Control (acetone)	510	100	5,260	—
Act-D (50 µg/ml)	510	100	3,167	38
Control (acetone)	510	100	5,085	—
Act D (125 µg/ml)	450	88	1,220	76

The excized embryos were cultured in the continuous presence of Act-D and ^{14}C-uracil (0.5 µCi/ml, spec. act. 49.3 mCi/mmole). Act-D (1.0 mg) was dissolved in a small aliquot (0.02 ml) of 79% acetone and the desired concentration was prepared by the addition of sterile nutrient medium.

Table III. Effect of cordycepin on *o*-diphenolase activity

Additions	*o*-Diphenolase activity	
	Enzyme units	Control (%)
Control	600	100
Cordycepin a) 10^{-5} *M*	670	112
b) 10^{-4} *M*	580	97
c) 3×10^{-4} *M*	620	103

The enzyme activity was measured in crude extract prepared from excized embryos germinated (48 h) in continuous presence of cordycepin.

Table IV. Effect of cycloheximide (CHI) on the *o*-diphenolase activity and ^{3}H-leucine incorporation in the eluted fraction

Additions	*o*-Diphenolase activity in eluted fraction		^{3}H-leucine incorporation in eluted fraction	
	Enzyme units	Inhibition (%)	cpm/mg protein	Inhibition (%)
Control	30	—	1,120	—
CHI, 2 µg/ml	18	40	486	57
CHI, 4 µg/ml	0	100	68	94

The dialyzed crude extract was fractionated on acrylamide gels and the region of 2 fast moving isoenzymes of *o*-diphenolase was eluted with 0.05 *M* phosphate buffer (pH 6.6) and designated as eluted fraction.

Pisum[13] and soybean[14]. In sea urchin eggs, Act-D failed to inhibit the activation of protein synthesis, although it retarded mRNA formation[10]. Also, in germinating cotton cotyledons[11], the induction of protease and isocitratase is supported by pre-existing mRNA and is not inhibited by Act-D.

Fractionation of crude extract on acrylamide gels revealed 4 *o*-diphenolase isoenzymes at zero h germination (D_1–D_4 in Figure 2). After 48 h germination, 6 new isoenzymes (D_5–D_{10}) were formed (Figure 2). Embryos cultured in presence of CHI (5 µg/ml) showed a virtual disappearance of 6 newly formed isoenzymes (D_5–D_{10}). Furthermore, CHI (2 and 4 µg/ml) caused a concomitant decrease in enzyme activity and ^{3}H-leucine incorporation in the eluted fraction obtained from the fast moving isoenzyme bands (D_9–D_{10}) of *o*-diphenolase (Table IV). This indicated that the stimulation of *o*-diphenolase represents de novo enzyme synthesis. Unlike CHI, Act-D and cordycepin failed to abolish the activity of newly formed *o*-diphenolase isoenzymes (Figure 2). Briefly then, the stimulation of *o*-diphenolase activity and its isoenzyme formation during early germination of wheat embryos is supported by conserved message already present in ungerminated embryo. Thus the stimulation of *o*-diphenolase enzyme seems to be regulated at the translational level.

Zusammenfassung. Nachweis, dass die Stimulation der *o*-Diphenolase-Aktivität und der Isoenzymbildung während des frühen Keimungsstadiums von Weizenembryonen von einem konservierten und bereits im ungekeimten Samen vorgebildeten Informationsträger abhängig ist.

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Renal Glucose Utilization in Genetically Diabetic Microangiopathy

The common feature of diabetic microangiopathy is associated with thickening of the capillary basement membrane[1]. A detailed chemical analysis of human diabetic basement membrane reveals the fact that the content of hexose and hydroxylysine increased[2,3]. In alloxan diabetic rat kidney, an elevation of post-ribosomal glucosyltransferase activity[3] is found which may be responsible for the increased amount of glomerular basement membrane[4]. However, it is yet unknown whether glucosyltransferase or/and other factors are involved in the genetically transmitted diabetic microangiopathy.

A human type glomerulosclerosis (diffuse, exudative and nodular) associated with protein urea and increased blood urea nitrogen levels was described in the non-obese genetically diabetic KK mice[5]. Thus KK mice are the ideal model for studying the biochemical change during the development of genetically transmitted diabetic microangiopathy.

Since glucose content was increased in the hydroxylysine-linked disaccharide unit[2], present investigation was to study glucose utilization by renal tissue of KK mice during the process of microangiopathy development. Moreover, the glucosyltransferase which incorporated glucose from UDPG (uridine diphosphoglucose) to β-D-galactose-hydroxylysine basement membrane was also examined in KK mice.

Materials and methods. The KK mice were maintained under constant laboratory temperature and regular mouse chow diet, containing 11% fat was given.

D-glucose-^{14}C (U), and UDPG-^{14}C (U) were purchased from New England Nuclear, Company. Calf skin collagen was the product of Sigma Company. NCS Tissue solubilizer was purchased from Amersham/Searle. All other chemicals were of reagent grade.

Non-fasted KK mice and Swiss albino mice were sacrificed by stunning. Kidneys were removed and dryed by Kimex. The tissue was weighed, cut and placed in 1.5 ml of Krebs-Ringer-bicarbonate solution (KRB) for 15 min. The preincubated tissue was then transferred to a flask

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