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DISCLAIMER

Interactions between Metabolic Intermediates and β-Galactosidase from *Escherichia coli*

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1. 5-Phosphorylribose 1-pyrophosphate, in the presence of β-mercaptoethanol, protected β-galactosidase from heat inactivation. Many other substances, including 3',5'-(cyclic)-AMP, were without effect. 2. The efficiency of *in vitro* complementation of β-galactosidase segments was reduced by 5-phosphorylribose 1-pyrophosphate but not by 3',5'-(cyclic)-AMP. Neither substance affected the activity of the complete enzyme. 3. Some indications as to the possible identity of the catabolite repression effector are presented.

Gest & Mandelstam (1966) suggested that part of the mechanism of catabolite repression might involve interaction between the effector molecule and the nascent enzyme, resulting in some way in a decrease in the rate of the translation process in protein synthesis. Seeking evidence for the existence of "repression recognition sites" on the completed protein molecule, they studied its heat stability in the presence of a variety of metabolic intermediates. Among several

compounds tested, only fructose 1,6-diphosphate, and, to a lesser extent, glucose 6-phosphate, promoted the heat lability of β-galactosidase. More extensive studies by Brewer & Moses (1967) confirmed the efficacy of fructose 1,6-diphosphate, but showed that several other compounds were even more effective. They concluded that the interactions between metabolite molecules and β-galactosidase were non-specific, and were probably not relevant to the phenomenon of catabolite repression.

More recently, interest in possible repression recognition sites on β-galactosidase has been reawakened with the suggestion, on genetic and biochemical grounds, that translational regulation is a significant factor in the mechanism of catabolite repression (Moses & Yudkin, 1968; Yudkin & Moses, 1969). It was of interest to re-examine possible specific interactions between β-galactosidase and metabolites, and to extend the range of the latter to include 3',5'-(cyclic)-AMP and metabolic intermediates whose intracellular concentrations might be affected by the nucleotide.

EXPERIMENTAL

Bacterial strains. The following bacterial strains were used in this study; their relevant genotypes and sources are indicated: 3300 ($\underline{i}^-\underline{o}^+\underline{z}^+\underline{y}^+$) from E. Steers; S908 ($\underline{i}^+\underline{o}^+\underline{z}^-_{908}\underline{y}^-\underline{ade}^-$) and RV/(694)B9 ($\underline{lac}^{del}_{x74}$/F'(B9) $\underline{i}^{del}\underline{o}^{del}\underline{z}^{del}$) from A. Newton; 112-12-A-84 ($\underline{i}^{-sus}\underline{o}^+\underline{z}^+\underline{y}^+$) from B. Müller-Hill.

Growth conditions. Cells were grown with shaking at 37° in minimal medium 63 supplemented with 0.2% (w/v) glycerol (Moses & Prevost, 1966).

Enzyme induction and assay. Strain S908 was grown in the presence of 0.5 mM-IPTG.* Samples for measurement of β-galactosidase activity in cell extracts or in purified preparations were incubated in 0.1 M-phosphate buffer, pH 7.4, containing 0.125 M-NaCl and 2.65 mM-ONPG at the temp. indicated in the text. When a suitable intensity of yellow colour had developed, the reaction was terminated with 0.3 M-Na_2CO_3 and the extinction measured at 420 nm.

In vitro complementation of β-galactosidase segments. Cells of the required strains were grown with or without inducer in glycerol-minimal medium. After harvesting, they were washed, resuspended in buffer and disrupted by ultrasonic vibration. The preparation was centrifuged at 38000 x g_{av} for 2 hr. at 0°. The supernatant solution was used without further purification in complementation experiments.

Aliquots (25-50 µl.) of donor and recipient cell extracts, together with 20 µl. of buffer containing compounds to be tested, were mixed at 0°. The mixtures were then incubated for 90 min. at 30°. Enzyme activity was measured by adding 0.8 ml. of buffer containing ONPG (3.2 mM), and incubation continued at 30° until a suitable density of yellow colour had developed. The reaction was terminated with 0.8 ml. of 0.75 M-Na_2CO_3 and E_{420} measured. The concn. of test compounds was 5 mM during complementation and 0.65 mM during hydrolysis of the substrate. Experimental procedures were taken from Ullmann et al. (1965).

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*Abbreviations: IPTG, isopropyl-β-D-thio-galactopyranoside; 3',5'-(cyclic)-AMP, adenosine 3',5'-(cyclic)-monophosphate; ONPG, o-nitrophenyl-galactopyranoside; PRPP, 5-phosphorylribose-1-pyrophosphate.

In each complementation experiment it was confirmed that excess of the ω recipient was present; the yield of active enzyme was a linear function of the amount of donor extract used.

Effect of heat on β-galactosidase. Purified β-galactosidase, isolated from E. coli strain 3300 by the method of Craven, Steers & Anfinsen (1965), was kindly provided by Drs. D. C. Phillips and L. N. Johnson. Two types of experiment were performed: the combined effect of heat on enzyme stability and activity, and the effect of heat on stability only.

In the former, 1.35 ml. of buffer (0.1 M-KH_2PO_4 plus 0.125 M-NaCl, adjusted to pH 7.4 with KOH) containing ONPG (3.2 mM) and additional substances as required, was mixed with 0.15 ml. of enzyme (30 µg./ml.) in the same buffer. If required, the solutions also contained β-mercaptoethanol (1% v/v). Mixing was performed at 0°. The mixture was then placed in a water bath at the desired temperature. At intervals, 0.2 ml. samples were removed into 1 ml. of 0.75 M-Na_2CO_3, and E_{420} measured in a 1 cm. cuvette. When time-course studies were not required, the vol. of all the reactants was reduced ten-fold, and hydrolysis terminated at a definite time by the addition of 1.0 ml. of Na_2CO_3.

For the second type of experiment, 1.0 ml. of buffer containing 1.5% (v/v) β-mercaptoethanol and test substances as required, was mixed at 0° with 0.25 ml. of enzyme solution. The mixture was placed in a water bath. At intervals, samples (approx. 50 µl.) were added to 0.1 ml. of buffer at 0° in pre-weighed tubes; the tubes were weighed again to determine accurately the amount of sample taken. All samples were subsequently assayed for β-galactosidase activity at 37°.

Chemicals. These were from the following sources: PRPP, fructose 1,6-diphosphate, 3',5'-(cyclic)-AMP, 2',3'-(cyclic)-AMP, 3-phosphoglyceric acid, 3-phosphoglyceraldehyde, ONPG, N-acetyl-D-glucosamine, glucose 6-phosphate, NADP, NADPH, NAD and NADH were from Calbiochem., Los Angeles, Calif.; dihydroxyacetone phosphate was from Sigma Chemical Co., St. Louis, Mo.; glucose 1-phosphate was from Schwartz BioResearch, Inc., Orangeburg, N. Y.; bovine serum albumin was from Armour Pharmaceutical Laboratories, Chicago, Ill.; adenosine 5'-triphosphate was from Mann Research Laboratories, New York, N. Y.

RESULTS

Effect of intermediary metabolites on the heat stability of β-galactosidase. Purified β-galactosidase was heated in the presence of ONPG (3.2 mM) and a variety of metabolites (usually at 4 mM). None of the compounds tested influenced the rate of ONPG hydrolysis at 37°. There was considerable variability between different experiments and the values reported in Table 1 represent the averages of measurements repeated several times.

The effect of heat on β-galactosidase was greatly influenced by the presence in the system of β-mercaptoethanol. Both Gest & Mandelstam (1966), and Brewer & Moses (1967), employed enzyme prepared and used in the absence of the mercaptan (personal communications); for the enzyme used in the present study, β-mercaptoethanol was present throughout the isolation procedure. Craven et al (1965) reported that the presence of reducing agents during isolation affects enzyme activity and heat stability. They observed, furthermore, that enzyme prepared in the presence of reducing agents loses its stabilized properties on removal of the agent, and that this loss cannot be reversed by the subsequent restoration of β-mercaptoethanol.

In the absence of β-mercaptoethanol, several substances accelerated enzyme incativation. The promotion of heat lability in the absence of β-mercaptoethanol is probably a non-specific effect; inclusion of bovine serum albumin (0.25%) in the reaction mixture completely prevented it (Table 2). In the presence of β-mercaptoethanol, only one substance tested, PRPP, was consistently effective in protecting β-galactosidase from heat inactivation; some protection was also afforded by dihydroxyacetone phosphate. Protection of the protein by PRPP was not affected by bovine serum albumin (Table 2), and this interaction appeared to be considerably more specific.

The stabilizing effect of PRPP was independent of enzyme concentration over an eightfold range (Fig. 1). Fig. 2 indicates a dissociation constant of 1.1 mM for an enzyme-PRPP complex. The kinetics of β-galactosidase decay at 55° in the presence and absence of PRPP, and of substrate, are shown in Figs. 3 and 4. The protective effect of PRPP was not abolished by the inclusion of other metabolites (Table 3).

PRPP, 3',5'-(cyclic)-AMP and in vitro complementation of β-galactosidase segments. In a number of experiments it was found that 5 mM-PRPP, present during the complementation period, invariably reduced the yield of active enzyme as determined in a subsequent measurement of enzyme activity at 37° (Table 4). On some occasions the yield was somewhat higher in the presence of 5 mM-3',5'-(cyclic)-AMP, but this did not always occur. The effects were always in the same direction for a particular compound, though their magnitude was variable. The activity of complete enzyme was affected only slightly by PRPP, and not at all by 3',5'-(cyclic)-AMP (Table 4).

DISCUSSION

The nature of the effector. A translational regulatory component in the catabolite repression of β-galactosidase, for which evidence was presented by Moses & Yudkin (1968), and Yudkin & Moses (1969), probably requires a specific recognition site for the effector molecule on the nascent enzyme protein (Gest & Mandelstam, 1966). Such a site need not necessarily survive in its original conformation on the completed enzyme, though any evidence of its presence there would make more likely its existence on the nascent protein. Investigation of the effects of several metabolites on the heat stability of β-galactosidase, and on the activity of the enzyme formed by *in vitro* complementation of segments, has indicated that such a specific site may indeed exist. Only PRPP, of the substances tested, was very effective in promoting stabilization of the enzyme at high temp., and only this compound reduced the efficiency of complementation. It is not here suggested that PRPP is itself the effector: not only is its affinity for the enzyme low, but it does not act as a repressor when added to cells rendered permeable with EDTA (Moses & Sharp, 1970); however, entry of a substance into the cells may not ensure access to the target molecule. PRPP might nevertheless resemble the effector fairly closely in the manner of its binding to the enzyme, and hence indicate the type of structure to be sought in looking for the effector. Earlier studies (Prevost & Moses, 1967; Moses & Sharp, 1970) have suggested that the effector originates from the pentose phosphate cycle. Once more the cycle is implicated as a source of the effector, for PRPP is one of the products of that metabolic pathway.

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Running title: Effect of metabolites on β-galactosidase

CAPTIONS FOR FIGURES

Fig. 1. Effect of enzyme concn. on heat stability of β-galactosidase. Enzyme was incubated for 20 min. at 55° in the presence of ONPG (3.2 mM) and β-mercaptoethanol (1% v/v). Na_2CO_3 was then added and E_{420} measured. o , control; ● , plus 5 mM-PRPP.

Fig. 2. Lineweaver-Burke plot of heat-stabilizing effect of PRPP on β-galactosidase. Enzyme was incubated for 20 min. at 55° in the presence of ONPG (3.2 mM), β-mercaptoethanol (1% v/v) and various concn. of PRPP. Na_2CO_3 was then added and E_{420} measured. β-Galactosidase activity recorded is calculated as activity in control minus activity in presence of PRPP.

Fig. 3. β-Galactosidase activity at 55°. Enzyme was incubated with ONPG (3.2 mM) and β-mercaptoethanol (1% v/v), with and without PRPP (5 mM). Samples were removed at intervals, the reaction terminated with Na_2CO_3, and E_{420} measured. o,, control; ● , plus PRPP.

Fig. 4. β-Galactosidase stability at 55°. Enzyme was incubated with β-mercaptoethanol (1% v/v), with and without PRPP (5 mM). Samples were removed at invervals, cooled, and enzyme activity was subsequently determined at 37°. o , control; ● , plus PRPP.

Table 1. Effects of temperature on β-galactosidase activity in the presence and absence of β-mercaptoethanol and intermediary metabolites

Purified β-galactosidase was incubated for 20 min. in the presence of 3.2 mM-ONPG and 4 mM-metabolites. The reaction was terminated by the addition of Na_2CO_3 and E_{420} measured. Values are averages of several determinations, expressed as % of control values.

Substance added	55° + mercapto-ethanol (1% v/v)	50° without mercaptoethanol
None	100	100
N-acetylglucosamine	102	108
AMP	68	95
ADP	92	72
ATP	58	58
2',3'-(cyclic)-AMP	92	115
3',5'-(cyclic)-AMP	122	109
Dihydroxyacetone phosphate	160	34
Fructose	101	67
Fructose 6-phosphate	85	18
Fructose 1,6-diphosphate	103	67
Fucose	92	93
Galactose	91	126
Na Gluconate	77	84
Glucose	91	100
Glucose 1-phosphate	92	88
Glucose 6-phosphate	93	71
NAD^+	105	156
NADH	97	73
$NADP^+$	107	45
NADPH	125	10
3-Phosphoglyceraldehyde	109	55
3-Phosphoglyceric acid	105	138
PRPP	228	99
Ribose	59	68

Table 2. Effect of bovine serum albumin on β-galactosidase activity at high temperatures, in the presence of intermediary metabolites

Experimental details as for Table 1. Bovine serum albumin was used at a final concn. of 1 mg./ml.; it had no effect on the control samples.

Substance added	55° + mercapto-ethanol (1% v/v)	50° without mercaptoethanol
No bovine serum albumin		
None	100	100
Fructose 1,6-diphosphate	94	64
NADPH	113	21
PRPP	221	101
With bovine serum albumin		
None	100	100
Fructose 1,6-diphosphate	138	115
NADPH	137	93
PRPP	294	136

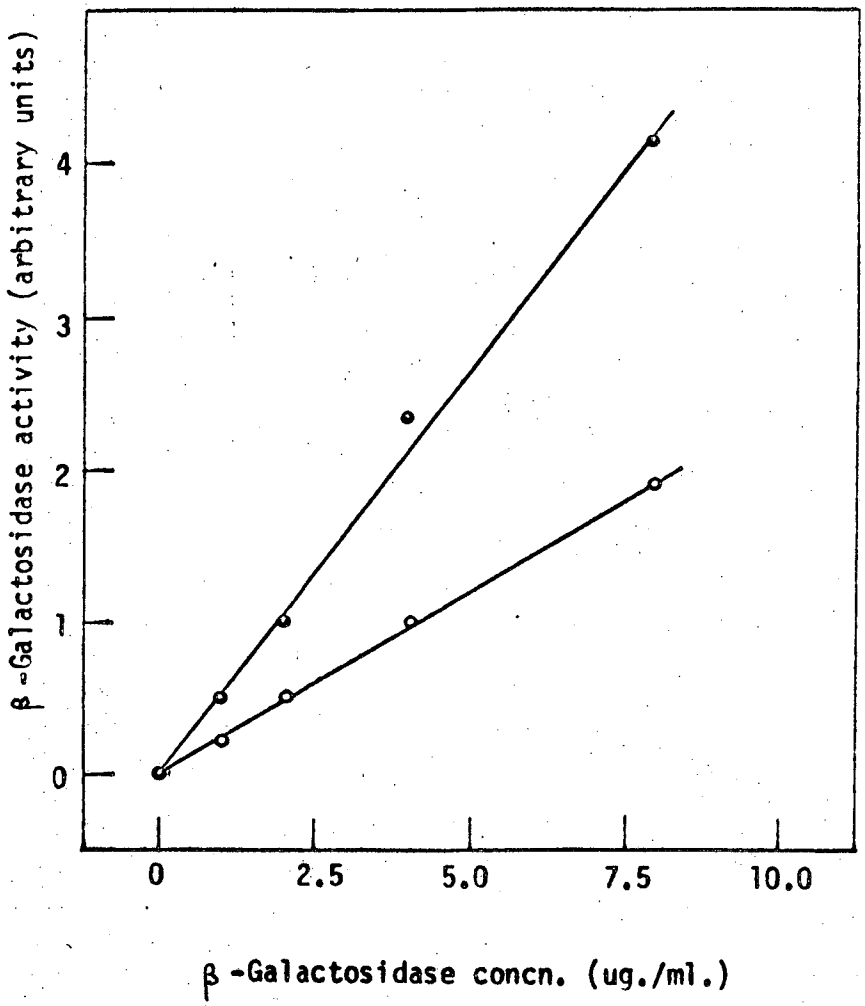
Table 3. β-Galactosidase activity at high temperatures in the presence of PRPP together with other intermediary metabolites

Substance added	Enzyme activity
None	100
PRPP	185
PRPP + 2',3'-(cyclic)-AMP	192
PRPP + 3',5'-(cyclic)-AMP	205
PRPP + dihydroxyacetone phosphate	226
PRPP + fructose 1,6-diphosphate	160
PRPP + glucose 1-phosphate	221
PRPP + 3-phosphoglyceraldehyde	203

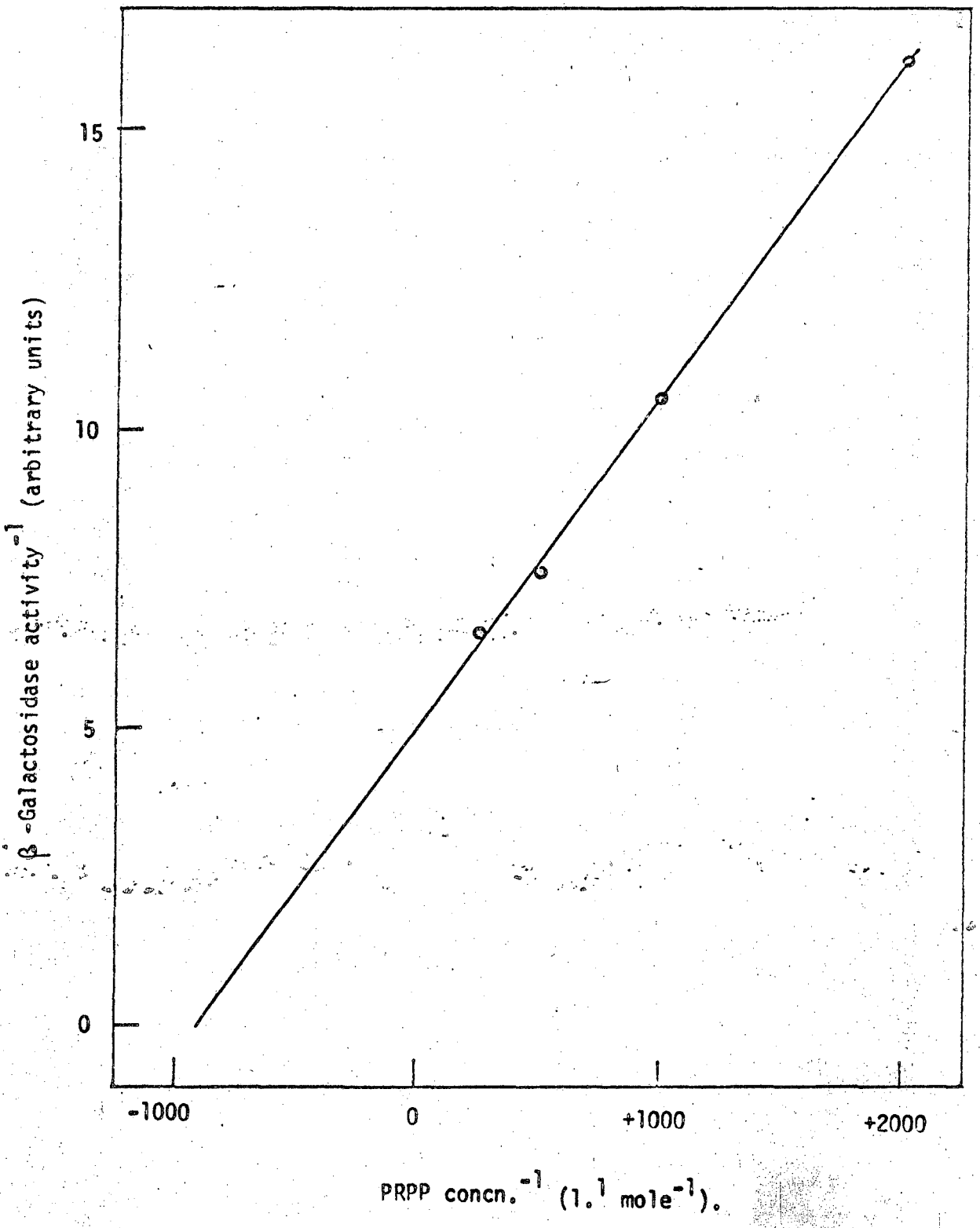
Table 4. Effects of 3',5'-(cyclic)-AMP and PRPP on in vitro complementation of β-galactosidase segments

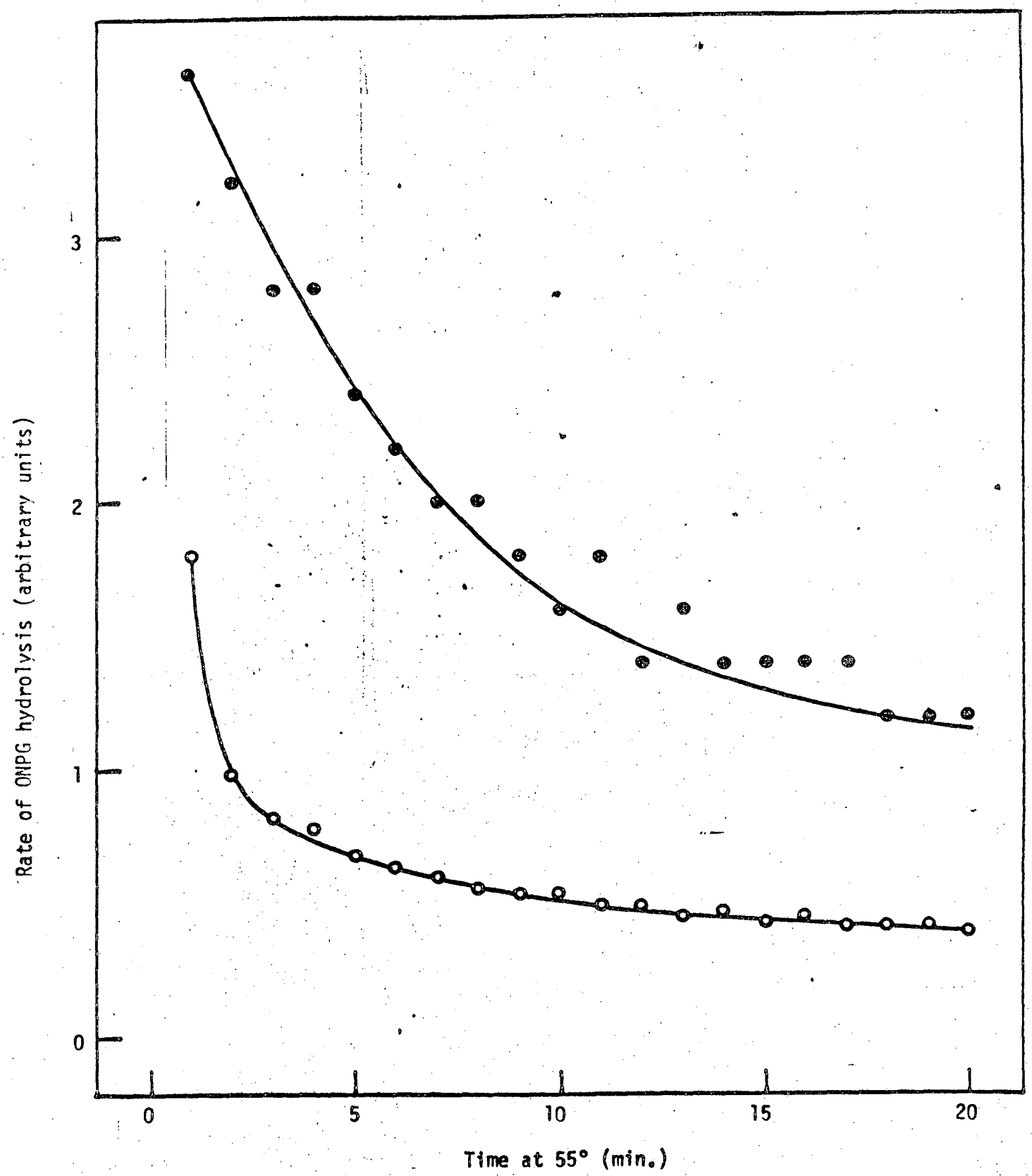
Extracts of strains S908 (ω-acceptor) and RV/(694)B9 (ω-donor) were incubated for 90 min. at 30° in the presence and absence of 5 mM-3',5'-(cyclic)-AMP and 5 mM-PRPP. The reaction mixtures were then diluted with buffer containing ONPG, and enzyme activity measured as E_{420} after 90 min. at 30°. The effects of the metabolites on native enzyme were determined using an extract of strain 112-12-A-84. Enzyme activities are reported in arbitrary units.

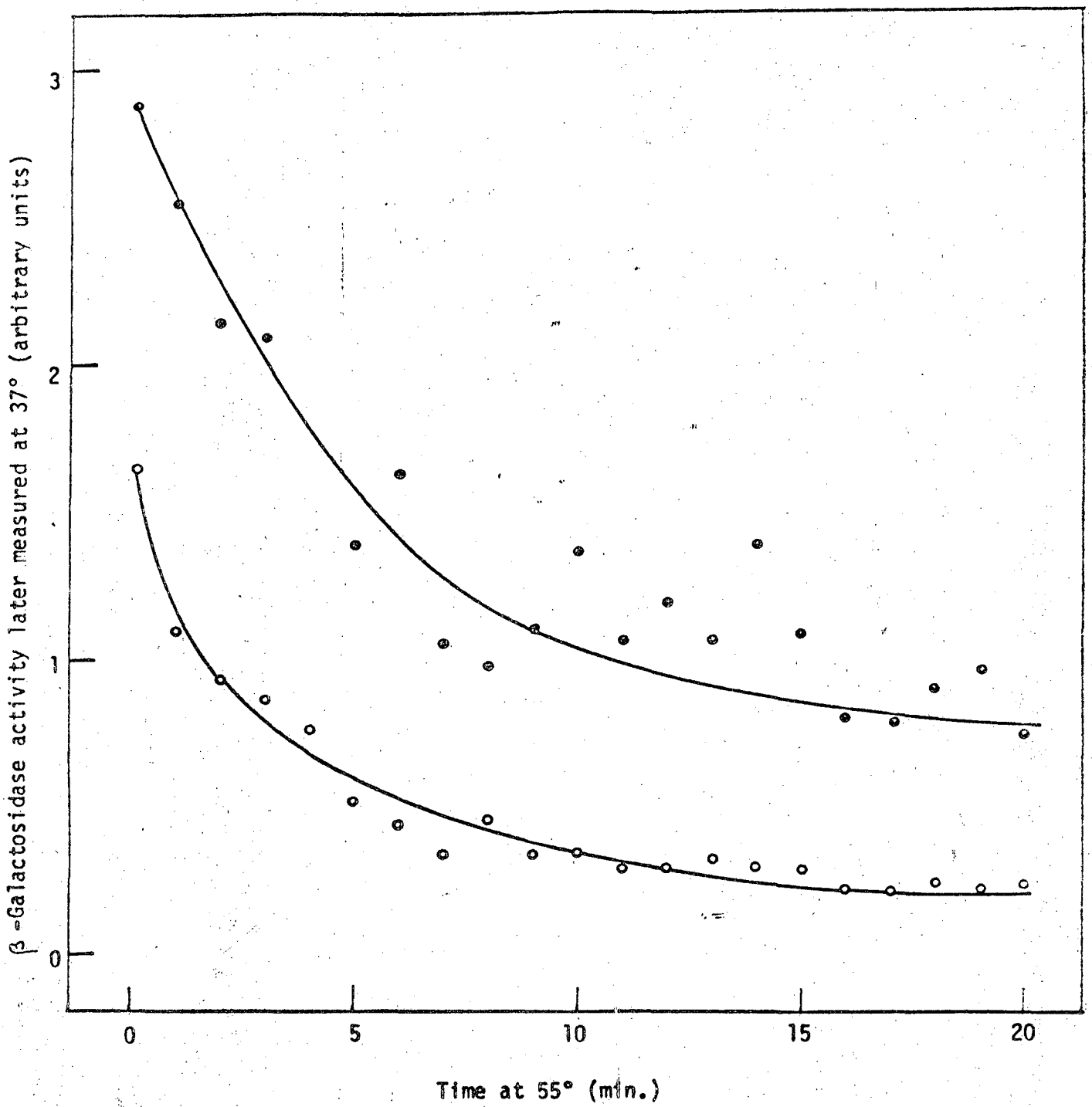
Additions	None	3',5'-(cyclic)-AMP	PRPP
Extract:			
S908	1.15	1.04	1.02
RV/(694)B9	1.00	1.22	1.04
S908 + RV/(694)B9	14.2	15.2	3.49
112-12-A-84	1.00	0.99	0.83


β-Galactosidase activity (arbitrary units)
0
1
2
3
4
0
2.5
5.0
7.5
10.0
β-Galactosidase concn. (ug./ml.)

Moses & Sharp. Fig. 2.




Rate of ONPG hydrolysis (arbitrary units)
0
1
2
3
0
5
10
15
20
Time at 55° (min.)


β-Galactosidase activity later measured at 37° (arbitrary units)
3
2
1
0
0
5
10
15
20
Time at 55° (min.)

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