ADAPTIVE FORMATION OF A *VIC* GLYCOL DEHYDROGENASE IN *AEROBACTER AEROGENES**

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SUMMARY

Glycerol is capable of inducing a *vic* glycol dehydrogenase in cells which were originally grown on a glucose or lactate medium. The rate of induced enzyme formation can be measured by using a pyridine nucleotide analogue of DPN, deamino DPN, since the constitutive DPN-linked 1,2-propanediol dehydrogenase is incapable of reacting with this analogue.

Detectable induced enzyme synthesis occurs after a lag of 15 min. The formation of induced enzyme is specifically dependent upon glycerol; other *vic* glycols and dihydroxyacetone are inert as inducers.

The experimentally determined concentration of glycerol required for half maximal enzyme induction is $0.8 \cdot 10^{-2}$ *M*. Other necessary requirements for induced enzyme formation are a nitrogen source, and an energy source.

Induced enzyme formation is inhibited by chloramphenicol, azaserine, potassium cyanide, sodium azide and dinitrophenol. The azaserine inhibition can be partially overcome by the aromatic amino acids but not by aliphatic amino acids, purines, pyrimidines, ribosides and ribotides.

INTRODUCTION

The problem of identifying and separating physically dissimilar proteins poses few problems today. Identification of physically similar proteins generally requires more elegant methodology. Thus, COHN AND TORRIANI[1,2] employed immunochemical methods for the identification and study of the "PZ" and "GZ" proteins originally found in *E. coli*, and SOBER *et al.*[3] have developed special chromatographic techniques for the isolation of several proteins from "homogeneous" serum protein fractions. We have been studying glycol dehydrogenase systems in *Aerobacter aerogenes* which have both physical and functional similarity[4].

When this organism is grown on a medium containing glucose and salts, an enzyme is produced which has glycol dehydrogenase activity with specificity for *vic*

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glycols and for DPN (enzyme B)*, **, ***. If this same organism is grown on a medium containing glycerol plus salts, a *vic* glycol dehydrogenase system is produced which has a more general substrate specificity (enzyme A). It has been possible to observe the interrelationship of these two enzyme systems by use of a "chemical tracer", for enzyme A is active with deamino DPN whereas enzyme B is incapable of manifesting activity when this analogue is substituted for DPN. In the preceding paper, the physico-chemical properties of enzyme A were compared with a partially purified preparation of enzyme B obtained from glucose-grown bacteria. The use of deamino DPN as a "chemical tracer" in studying induced enzyme synthesis is described in this paper as are some of the properties of the induced enzyme formation.

MATERIALS AND METHODS

Materials

Reagent grade glycerol and lactic acid were purchased from the J. T. Baker Chemical Co. 1,2-Propanediol (propylene glycol), technical grade, was purchased from the Matheson Co., Inc. Several strains of *Aerobacter aerogenes* (No. 8724, 884, 8329) and *Acetobacter suboxydans* were obtained from the American Type Culture collection. Pyridine nucleotide analogues of DPN were synthesized according to the general method of KAPLAN AND STOLZENBACH[5] except for the nicotinic acid analogue of DPN which was prepared according to the method of LAMBORG *et al.*[6].

Methods

Because of the multiplicity of enzyme activities measured, we shall refer to glycol dehydrogenase enzyme systems A and B. The enzymes will be operationally defined in terms of their enzyme activities and source as shown in Table I.

TABLE I

DEFINITION OF ENZYME SYSTEMS USED IN THE TEXT

	Growth medium	*Substrate and pyridine nucleotide requirements for enzymic activity*
Enzyme A	Glycerol plus salts	1,2-propanediol plus deamino DPN 2,3-butanediol plus deamino DPN Glycerol plus DPN 1,2-propanediol plus DPN 2,3-butanediol plus DPN
Enzyme B	Glucose plus salts	Glycerol plus DPN 1,2-propanediol plus DPN 2,3-butanediol plus DPN

Enzyme unit of activity

One unit of enzyme is defined as that amount of protein which causes an initial

* The following abbreviations will be employed in this paper: DPN, diphosphopyridine nucleotide; DPNH, reduced form; TPN, triphosphopyridine nucleotide; TPNH, reduced form; RNA, ribose nucleic acid; deDPN (deamino DPN), deaminated form of DPN; AMP, adenylic acid (5'); CMP, cytidilic acid (5'); UMP, uridilic acid (5'); GMP, guanilic acid (5'); IMP, inosinic acid (5').

** See MATERIALS AND METHODS for composition.

*** See Table I for an operational definition of enzyme systems A and B.

rate of change in O.D. (at 340 mμ) of 1.0 per min under the conditions employed. The specific activity is expressed as units/mg of protein.

Enzyme assay

0.5 mmole of 1,2-propanediol or glycerol and 1.2 μmoles of DPN are diluted with 2.7 ml of sodium pyrophosphate (0.1 *M*, pH 9.2). After initially determining the O.D. at 340 mμ, the reaction is started by the addition of approximately 0.2 unit of enzyme.

Growth media, methods of culture, and protein purification are described in the preceding paper.

EXPERIMENTAL

Induced enzyme formation

The results presented in the preceding paper suggest that the increase in enzymic activity observed in the case of glycerol plus DPN and 1,2-propanediol plus deamino DPN may involve the synthesis of induced enzymes. It has been possible to substantiate this viewpoint by measuring the increase in enzymic activity with time.

Procedure

A 4-l culture of a variant of *Aerobacter aerogenes* No. 8724 was grown on glucose and salts according to the method previously described. The cells (approx. $1 \cdot 10^8$/ml) were harvested in a DeLaval electrically driven centrifuge, washed once with cold water, and then centrifuged for 20 min in a Lourdes refrigerated centrifuge at maximum speed (10,000 rev./min, 16,300 × *g*). A heavy suspension of the cells was prepared by suspending the cell paste in a minimum amount of cold water (about 10–20 ml). For measuring induced enzyme formation with time, a number of 1-l Erlenmeyer flasks each containing 200 ml of glycerol media were inoculated with the same volume of cell suspension to give a Klett reading of 150 units (filter 54). This suspension was gently shaken in a water bath (30°). At intervals of time, a flask was removed from the water bath, turbidity was determined, and the suspension was chilled to 5° in a dry ice–acetone bath. The cells were removed by centrifugation, suspended in water to a final volume of 10 ml and disrupted in a Raytheon 10 kc sonic oscillator for 30 min. The cell debris was removed by centrifugation in a refrigerated International centrifuge using the high speed attachment. Protein[7] and enzymic activity were determined on the resulting crude extract.

Synthesis of induced enzyme

The synthesis of induced enzyme is easily demonstrable after a 1-h induction period in the presence of glycerol (see Fig. 1). In the absence of glycerol, no synthesis takes place. Initially, there is a lag phase of short duration before synthesis commences, but the rate of synthesis increases markedly after a 30–45 min induction period. It is also noted that the glycerol plus DPN and the 1,2-propanediol plus deamino DPN activities are elicited at the same rate during induction, suggesting that both activities are catalyzed by the same enzyme.

The constitutive activity (1,2-propanediol plus DPN) also increases during this period. This increase cannot be accounted for in terms of increase in population, for the turbidity usually remains constant during the inducing period (on two occasions

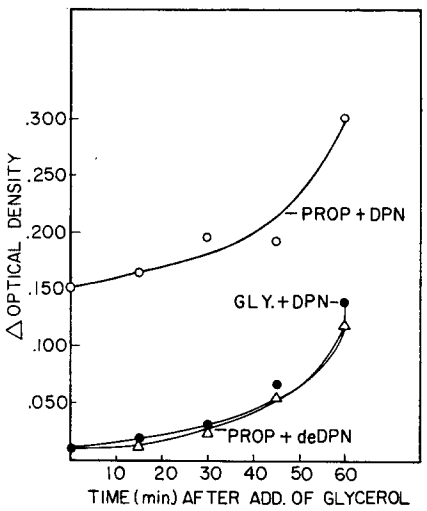


Fig. 1. Synthesis of induced enzyme. Cells were grown in a glucose-salts medium, and transferred to a glycerol-salts medium for induction. At intervals of time, aliquots of the population were removed from the media by centrifugation, suspended in cold water and disrupted by sonic vibration. The concentrations of 1,2-propanediol plus DPN (○, PROP + DPN), 1,2-propanediol plus deamino DPN (△, PROP + deDPN), and glycerol plus DPN (●, GLY + DPN) are the same as those listed in Table II. The activities recorded are the changes in O.D. at 340 mμ for the first 2 min of reaction. See the text for further details.

a 17 % increase in turbidity was noted during the inducing period) while constitutive activity increases 2–3 fold. Since the rate of increase of constitutive enzyme activity parallels the rate of formation of induced enzyme, it is suggested that the additional constitutive enzyme activity, which is manifested during the inducing period, is due to the general specificity of the induced enzyme. The results presented with lactate-grown cells are in agreement with this conclusion.

The induction period chosen for most experiments was 1 h, minimizing the possibility of spurious side effects due to contamination (sterile conditions were not maintained from the onset of induction until completion of the experiment). One relatively long-term inducing experiment (4 h) was carried out. During the course of this experiment the turbidity of the cell suspension increased 19 %, constitutive enzyme activity (1,2-propanediol plus DPN) quadrupled, and there was a 14-fold increase in the induced enzyme activity (1,2-propanediol plus deamino DPN).

Requirement for a nitrogen source during induction

Attempts were made to induce enzyme formation in resting cell suspensions (in the absence of a nitrogen source). After a 1-h induction period no induced enzyme was formed, a 64 % decrease in constitutive enzyme activity ensued, and no change in the turbidity was noted. If one assumes that the induced enzyme is being formed *de novo*, the lack of induction in the absence of a nitrogen source is not surprising since *A. aerogenes* is reputed to have no pool of free amino acids[8].

Specificity of inducing agent

The following compounds were substituted for glycerol in the inducing medium (1.5 % used in each case): *n*-propanol, glucose, 2,3-butanediol, 1,3-propanediol, 1,2-propanediol, and dihydroxyacetone. No induced enzyme was formed under these circumstances. In contrast with the inducer specificity of the β-galactosidase system[9], this enzyme induction has a strict requirement for glycerol which could not be replaced by 1,2-propanediol (the other substrate utilized by the induced enzyme) or dihydroxyacetone (the product of the reversible DPN-linked oxidation of glycerol).

2,3-Dimercapto-1-propanol did not act as inducer and did not inhibit glycerol induction.

Effect of sodium lactate on induced enzyme formation

Glycerol must serve as energy source as well as induce protein synthesis when it is used as the sole carbon source during induced enzyme formation. In studying the quantitative requirement of inducer during induction an additional carbon source was added to spare the glycerol requirement for growth (see MONOD's "gratuitous conditions"[10]). As NEIDHARDT AND MAGASANIK[11] have previously reported, we found that the addition of glucose to the inducing medium completely inhibited induced enzyme formation. When lactic acid (adjusted to pH 7.0 with NaOH) was used instead of glucose, no inhibition of induced enzyme formation was observed and when lactic acid was used as the sole carbon source in the growth media no *vic* glycol dehydrogenases are formed. The lactate growth media had the advantage over the glucose growth media in that no constitutive *vic* glycol dehydrogenase activity was present prior to induction (Table II, part B). Using lactate-grown cells one can causally relate *vic* glycol dehydrogenase activity with the addition of inducer. Table II also compares the capacity of glucose- and lactate-grown cells to induce enzyme formation when placed in an inducing medium (glycerol plus salts). It was noted that less enzyme was formed in cells grown on glucose (Table II, part A).

After induction of lactate-grown cells, each of the enzymic activities increased 8–9 fold, (*i.e.*, 1,2-propanediol plus DPN, 1,2-propanediol plus deamino DPN and glycerol plus DPN). The same relationship was found for the glucose-grown bacteria

TABLE II

EFFECT OF GROWTH MEDIA ON INDUCED ENZYME FORMATION

	Inducer	*Enzyme activity (units)**		
		Enzyme B	*Enzyme A*	
		1,2-propanediol plus DPN	*1,2-propanediol plus deamino DPN*	*Glycerol plus DPN*
A. *Cells grown on glucose*				
1. Before induction	—	0.255	0.017	0.015
2. After induction	Glycerol	0.350	0.070	0.074
Increase in enzymic activity after induction	(2/1)**	5.6***	4.1	4.9
B. *Cells grown on lactate*				
3. Before induction	—	0.039	0.029	0.057
4. After induction	Glycerol	0.327	0.262	0.471
Increase in enzymic activity after induction	(4/3)	8.4	9.0	8.3

* 500 μmoles of glycerol or 1,2-propanediol and 1 μmole of DPN or deamino DPN were diluted to a final volume of 3.0 ml with sodium pyrophosphate buffer (0.1 *M*, pH 9.3). The reaction was started with 1 unit of enzyme. The activity is recorded in terms of change in O.D. at 340 mμ per unit time.

** Increase in enzymic activity after induced enzyme formation was determined by dividing the enzymic activity after induction by the activity measured before induction except as noted in footnote ***.

*** In this case the absolute increase in activity is 0.095, which is manifested as a result of induced enzyme formation. If one assumes that the activity in the absence of inducer would be approximately 0.017 (assuming that the constitutive enzyme was not present), then 0.095/0.017 equals 5.6.

if one corrected for the initial "constitutive" enzyme activity which was present before the cells were induced (for further explanation and calculations see footnote *** of Table II).

Determination of the amount of glycerol needed for induced enzyme formation

It was not necessary to remove the lactate from the media in order to observe induced enzyme formation. In determining the quantitative measurement of glycerol needed for induction, cells grown in lactate were harvested by centrifugation in the cold and equally divided among several flasks each of which contained salts, lactate (1.5 %), and varying amounts of glycerol. Growth was measured before and after induction. After the 1 h induction period cells from each of the flasks were removed by centrifugation, resuspended in 10 ml of distilled water and disrupted by sonic vibration. The estimation of glycol dehydrogenase activity was made on the crude, clear sonicate after removing cell debris by high speed centrifugation. The results of this study are presented in Fig. 2. It can be seen that appreciable enzyme was induced when the concentration of glycerol exceeded $0.16 \cdot 10^{-2}$ *M*. In addition, the glycerol concentration required for half maximal enzyme induction was the same for the three activities measured (approx. $0.8 \cdot 10^{-2}$ *M*).

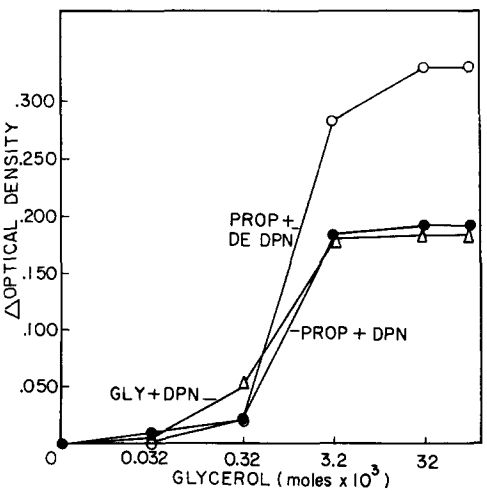


Fig. 2. Amount of glycerol needed for induced enzyme formation. Induction and assay conditions are the same as those outlined for Fig. 1 except that the growth medium contained sodium lactate as the sole carbon source for growth. See the text for other details.

Inhibition of induced enzyme formation

In addition to the inhibition of induced enzyme formation by glucose, a variety of chemical compounds was tested in an effort to learn some of the requirements for this synthesis. The effects of some of these compounds are summarized in Table III. The cells used for this study were grown in glucose-salts media and transferred to the glycerol media for induction. Expt. 1 of Table III shows the normal enzymic activity for cells derived from this source. There was a DPN-linked 1,2-propanediol dehydrogenase activity and very little activity using other glycols. Expt. 2 of this table is chosen as the control. These cells were induced for 1 h in the glycerol-salts media in the absence of glucose or any other inhibitors. Expts. 3, 4, and 5 show the results of cells which were induced in the glycerol-salts media plus various known inhibitors of purine or pyrimidine synthesis. Azaserine*, an anti-purine compound (Expt. 5) completely inhibited induced enzyme formation, and the other two compounds (thioguanine and 6-mercaptopurine) gave only slight inhibition at the concentration employed. No inhibition of the "constitutive" enzyme was noted in any of

* Azaserine was kindly provided by Dr. MOORE of Parke-Davis and Co.

TABLE III

INHIBITION OF INDUCED ENZYME FORMATION

Expt.	Inhibitor	Growth (%)*	Enzyme activity (units)**					
			1,2-propanediol plus DPN		1,2-propanediol plus deamino DPN		Glycerol plus DPN	
			Units	% Inhibition***	Units	% Inhibition	Units	% Inhibition
1§	—	123	0.197	—	0.012	—	0.021	—
2§	—	100	0.346	—	0.073	—	0.158	—
3	Thioguanine (80 μg/ml)	96	0.365	0	0.054	30	0.134	15
4	6-mercaptopurine (65 μg/ml)	91	0.335	7	0.054	30	0.123	25
5	Azaserine (65 μg/ml)	80	0.224	82	0.010	100	0.013	100
6	Chloramphenicol (30 μg/ml)	86	0.180	100	0.015	95	0.048	80
7	KCN ($1 \cdot 10^{-3}$ M)	92	0.194	100	0.022	85	0.033	93
8	Dinitrophenol ($1 \cdot 10^{-3}$ M)	94	0.178	100	0.008	100	0.015	100

* Growth is measured turbidometrically using the Klett-Summerson colorimeter (No. 54 filter).

** Assay conditions are the same as those listed in Table II. Units represent the enzymic activities listed and are defined in Table II.

*** Represents inhibition of the adapted enzyme formation.

§ Experiment 1 is the uninduced control, Experiment 2 is the induced control (no inhibitors added).

TABLE IV

ANTAGONISTS OF AZASERINE INHIBITION

Assay conditions and enzymic activities measured are listed in Table II. The general media to which the supplements were added is recorded in MATERIALS AND METHODS.

	Activity (units)		
	1,2-propanediol plus DPN	1,2-propanediol plus deamino DPN	Glycerol plus DPN
Control	0.379	0.317	0.384
+ Azaserine (10 μg/ml)	0.223	0.017	0.007
+ Azaserine + amino acids*	0.274	0.185	0.234
+ Azaserine + phenylalanine	0.252	0.124	0.213
+ Azaserine + tyrosine	0.236	0.133	0.208
+ Azaserine + tryptophane	0.178	0.039	0.082
+ Azaserine + glycine	0.230	0.019	0.013
+ Azaserine + lysine	0.230	0.016	0.014
+ Azaserine + arginine	0.202	0.018	0.011
+ Azaserine + AMP, CMP, GMP, UMP	0.212	0.026	0.005
+ Azaserine + AMP, CMP, GMP, UMP (80 μg/ml)	0.121	0.006	0.012
+ Azaserine + IMP	0.230	0.031	0.009
+ Azaserine + nicotinamide	0.235	0.021	0.018
+ Azaserine + histidine	0.146	0.014	0.016
+ Azaserine + glutamine	0.187	0.016	0.016
+ Azaserine + serine	0.228	0.020	0.018

* The amino acids mixture contained 10 μg/ml of each of the amino acids listed individually. The concentration of each of the separate amino acids and other compounds listed was also 10 μg/ml (except as indicated).

these experiments. Chloramphenicol, potassium cyanide, and dinitrophenol were also capable of inhibiting induced enzyme formation without affecting the constitutive enzyme. Table IV shows that the azaserine inhibition can be partially overcome by the aromatic amino acids, but not by aliphatic amino acids or ribotides. Pyrimidines, purines and ribosides are also incapable of relieving the azaserine inhibition.

DISCUSSION

In comparing the synthetic ability of glucose- and lactate-grown cells, it is found that less induced enzyme is formed in cells originally grown on the glucose-salts media (see Expt. 2, Table II). This inhibition could be due to a difference in the rate of formation of induced enzyme. That is, the inhibition might be the reflection of a difference in the capacity of glucose- and lactate-grown cells to synthesize induced enzyme. It is also possible that the inhibition is due to a small amount of glucose (or a catabolic product of glucose) remaining within the cell which is capable of partially inhibiting induced enzyme formation.

The inhibitor experiments detailed earlier in this section provide important information on the characteristics and requirements for induced enzyme formation. All of the chemicals are found to inhibit selectively only induced enzyme activities (the constitutive enzyme activity is unaffected). Inhibition by azaserine suggests that nucleic acid, or precursors of nucleic acid are somehow involved in the induced enzyme formation. The inhibition may be effected by the synthesis of "abnormal" nucleic acid or by the inhibition of normal nucleic acid synthesis (or its necessary precursors). Aromatic amino acids are capable of antagonizing the azaserine inhibition. A similar antagonism of azaserine inhibition in the synthesis of 5-aminoimidazole ribotide has been reported by GOTS AND GOLLUB[12], and HALVORSON[13] reported that leucine could relieve the azaserine inhibition of induced enzyme formation in yeast. The mechanism of this antagonism is not understood. GALE AND FOLKES have reported that the bacteriostatic effect of chloramphenicol is due to an inhibition of protein synthesis[14]. The complete inhibition caused by the higher amount of chloramphenicol suggests that the protein associated with the induced activities is synthesized *de novo* from free amino acids. Since cyanide, and dinitrophenol are known to inhibit the synthesis of high-energy compounds it is presumed that high-energy intermediates required for many synthetic reactions are also required for the formation of induced enzymes. In summary then, the requirements for induced enzyme synthesis are: the synthesis or presence of normal nucleic acid (or its precursors), amino acids (or their precursors), high energy-intermediates, and the inducer.

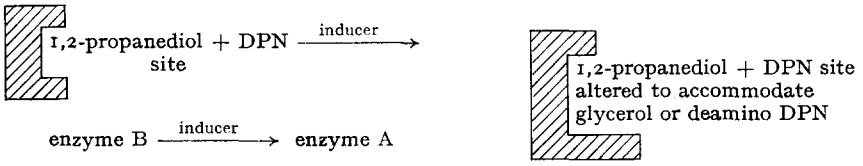
It is suggested that inducing lactate-grown cells of *A. aerogenes* with glycerol leads to the activation of a single enzyme which is capable of the oxidation of 1,2-propanediol mediated by DPN or deamino DPN and the oxidation of glycerol mediated by DPN (the same enzyme can be obtained by growing the bacterium solely on glycerol). In support of this suggestion one may site the following facts. (a) A simultaneous activation of all three enzymic activities is effected by the same concentration of glycerol. (b) The rate of formation of each of the enzymic activities is the same. (c) Chloramphenicol, azaserine, etc., inhibit the appearance of all three activities to the same extent. (d) The ratio of activities remains constant during the course of purification.

The concentration of glycerol required in this system exceeds the "saturating" concentration of inducer required for the maximal rate of production of β-galactosidase[10] (measured during the first 10 min after induction by methyl-β-D-galactoside). Since glycerol, the inducer, is metabolized by the induced enzyme, one can safely conclude that if a non-metabolizable inducer was available, the concentration required for half maximal enzyme formation would be considerably less.

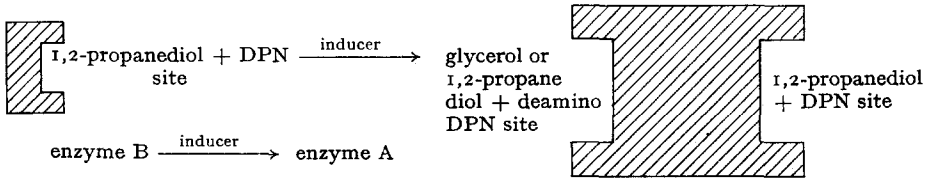
Several mechanisms can be suggested to explain the induction phenomenon resulting from the exposure of glucose grown bacteria to glycerol. It may be that enzyme B is really a partially inactive form of enzyme A which is inhibited by glucose or a catabolic product of glucose and that this metabolic inhibition is somehow relieved when the bacteria are transferred to glycerol. If this hypothesis is correct then the inhibition should be relieved when glucose grown bacteria are transferred to a medium containing glycerol plus chloramphenicol. The experimental observations contradict this hypothesis. Three alternative hypotheses may be proposed which are in agreement with the experimental results. Briefly stated these possibilities are:

1. *De novo* protein synthesis
   - amino acids $\xrightarrow{\text{inducer}}$ enzyme A
   - precursor + amino acids $\xrightarrow{\text{inducer}}$ enzyme A

2. Alteration of the active site resulting from the addition of inducer. In some way, the inducer initiates a change in the physical or chemical composition of the enzymically active site of the enzyme protein; this results in a glycol dehydrogenase of more general substrate and pyridine nucleotide specificity.



3. Synthesis (or activation) of a new site on the same protein molecule, resulting in a protein with more than one active site.



The later two possibilities can be ruled out by the chloramphenicol and immunochemical experiments (presented in the preceding paper), only if it is assumed that alteration or activation of proteins does not involve some type of *de novo* protein synthesis. BRACHET and others[15–18] have presented a great deal of circumstantial evidence in favor of the hypothesis that RNA synthesis is a necessary requirement for protein synthesis. Assuming that the BRACHET hypothesis is correct, the inhibition of induced enzyme formation by azaserine supports the conclusion that the enzyme formation studied herein takes place via some form of *de novo* protein synthesis.

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A CHLOROMERCURIBENZOATE RESIN FOR THE SELECTIVE BINDING OF NONPROTEIN SULFHYDRYL COMPOUNDS

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SUMMARY

An insoluble reagent formed by the binding of sodium *p*-chloromercuribenzoate to Dowex-2 resin has been used for the selective removal of thiols from solutions or from tissue homogenates by reverse dialysis. It was possible to liberate the bound thiol from the reagent by exchange with other sulfhydryl compounds.

INTRODUCTION

Many enzyme systems require the presence of sulfhydryl groups for their activation, and often incorporate a sulfhydryl reservoir such as glutathione. Presumably,

Abbreviations: *p*CMB, *p*-chloromercuribenzoate; EDTA, tetrasodium ethylenediamine tetraacetate.