Proc. Nat. Acad. Sci. USA
Vol. 72, No. 8, pp. 2920–2924, August 1975
Biochemistry

Interaction of Enzyme I of the phospho*enol*pyruvate:sugar phosphotransferase system with adenylate cyclase of *Escherichia coli*

(cyclic AMP/glucose inhibition/phosphorylation-dephosphorylation)

ALAN PETERKOFSKY AND CELIA GAZDAR

Laboratory of Biochemical Genetics, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014

Communicated by E. R. Stadtman, May 9, 1975

ABSTRACT **Transient repression by glucose of induced enzyme synthesis involves lowering of intracellular cAMP levels. This glucose effect is partially explained by a glucose inhibition of adenylate cyclase [EC 4.6.1.1; ATP pyrophosphate-lyase(cyclizing)]. Since the phospho*enol*pyruvate:sugar phosphotransferase system has been implicated in repression phenomena, an investigation was made of adenylate cyclase activity in mutants of that transport system. The results suggest that glucose phosphorylation is not necessary for inhibition of adenylate cyclase since an HPr mutant retained sensitivity to glucose inhibition. The results also suggest that adenylate cyclase activity requires the presence of Enzyme I in a phosphorylated form and that adenylate cyclase activity may be regulated by a phosphorylation-dephosphorylation mechanism.**

Previous studies have indicated that the effect of glucose in lowering cAMP levels is mainly due to inhibition of adenylate cyclase [EC 4.6.1.1; ATP pyrophosphate-lyase(cyclizing)] (1). Various other sugars can lead to adenylate cyclase inhibition provided the transport systems for those sugars are induced (2). The involvement of transport systems with regulation of adenylate cyclase activity has led us to examine the properties of some mutants in the phospho*enol*pyruvate:sugar phosphotransferase (PTS) system.

Measurements of cAMP levels of actively growing cells indicated that, while an HPr mutant had a cAMP concentration comparable to wild-type, a leaky Enzyme I mutant had severely depressed cAMP levels. These low cAMP levels in the Enzyme I mutant were correlated with an adenylate cyclase activity in permeabilized cells that was substantially lower than in the parent strain. In contrast to carbon-source-supplemented conditions, when the Enzyme I mutant was starved for carbon source it contained cellular cAMP at a concentration higher than the parent. Measurements of adenylate cyclase in intact cells under carbon-source-starvation conditions indicated that the Enzyme I mutant had higher than parental levels of enzyme activity; therefore, carbon-source-dependent variations in the cellular cAMP levels in the Enzyme I mutant were due to changes in the activity of the adenylate cyclase. The addition of phospho*enol*pyruvate (PEP) to permeabilized cell preparations of the Enzyme I mutant converted the low activity adenylate cyclase to the high activity form. On the basis of these results, we propose that adenylate cyclase interacts with Enzyme I and that the PEP-dependent phosphorylation of Enzyme I leads to an activation of adenylate cyclase.

MATERIALS AND METHODS

Cells of the various strains of *Escherichia coli* were grown in a New Brunswick gyrotory shaker at 37° in the salts medium of Vogel and Bonner (3) supplemented with the designated carbon sources. Strains 1100 (a β-gl$^+$ mutant of Hfr 3000), 1101 (a mutant in the HPr protein of the phosphotransferase system), and 1103 (a leaky mutant in Enzyme I of the PTS system) were originally described by Fox and Wilson (4) and were kindly provided by Dr. Ira Pastan. All assay methods have been previously described. Adenylate cyclase was measured in intact cells by the method of Peterkofsky and Gazdar (5) and in toluene-treated cells by the procedure of Harwood and Peterkofsky (6). Cellular cAMP levels, including a correction for contaminating extracellular cAMP, were determined by the method of Peterkofsky and Gazdar (1). The potassium salt of phospho*enol*pyruvate (PEP) was from Calbiochem.

Abbreviations: PEP, phospho*enol*pyruvate; PTS system, phospho*enol*pyruvate:sugar phosphotransferase system.

RESULTS

Abnormal cAMP metabolism in an Enzyme I mutant

An examination was made of the activities of adenylate cyclase in permeabilized cells of various strains of *E. coli* with lesions in the PTS system. Both strains 1101 (a mutant in the HPr protein) and strain 1103 (a mutant in Enzyme I) were derived from the same parent (strain 1100). The various strains were grown to mid-logarithmic phase on several carbon sources, then tested for adenylate cyclase activity in the presence and absence of glucose. The data of Table 1 show several points of interest. As we previously showed in *E. coli* B (2), the adenylate cyclase activity can vary depending on the carbon source used for growth. For example, cells of strain 1100 grown on glycerol and glucose-6-phosphate have roughly three times as much activity as cells grown on nutrient broth. Both the wild-type and the HPr mutant show enzyme activities that are inhibited by glucose (1). On the other hand, the Enzyme I mutant shows unusual behavior in two ways. First, irrespective of the carbon source, the activity is substantially lower than that of the other two strains. Second, the activity that is present is not inhibited by glucose.

In order to further define the anomalous pattern of adenylate cyclase activity in the Enzyme I mutant, another parameter in cAMP metabolism was measured. Cultures of the three strains were grown in nutrient broth medium to mid-logarithmic phase, at which point duplicate samples were taken for determination of intracellular and extracellular cAMP levels. Table 2 shows that the Enzyme I mutant can again be distinguished from the other strains by its low cAMP levels (five to seven times lower intracellular cAMP levels than the other members of the isogenic set).

Normal cAMP metabolism of an Enzyme I mutant under carbon-source-starvation conditions

A further exploration of the properties of the Enzyme I mutant revealed that the defect in cAMP levels could be re-

Table 1. Effect of culture conditions on adenylate cyclase activity in toluene-treated cells of phosphotransferase mutants

		Adenylate cyclase activity*					
		Carbon source					
		Nutrient broth		Glycerol		Glucose 6-phosphate	
Strain	Genotype	Control	+Glucose	Control	+Glucose	Control	+Glucose
1100	Wild-type	372	89	989	55	1097	117
1101	HPr$^-$	682	171	717	153	1538	147
1103	Enzyme I$^-$	161	155	102	67	493	505

Cells of the various strains were grown in salts medium (3) supplemented with thiamine (5 μg/ml) and the carbon sources, as indicated (nutrient broth, 1%; glycerol, 0.2%; or glucose 6-phosphate, 0.2%). At mid-logarithmic phase, cell suspensions were processed for assay of adenylate cyclase in toluene-treated cells (6). The designated incubation mixtures contained glucose (1 mM). Rates were calculated from the best line drawn through four experimental points of samples incubated for 10, 20, 30, and 40 min.

* Activity is expressed as pmol of cAMP formed/mg of protein per hr in the toluene-treated cells.

versed (see Fig. 1). Cells were grown in nutrient broth medium, then washed free of carbon source and suspended in minimal salts medium at 30°. In marked contrast to the data of Table 2, this study shows that *both* the HPr and Enzyme I mutant have higher cellular cAMP levels than does the parent strain. Under these carbon-source-starvation conditions, addition of glucose leads to a rapid lowering of cellular cAMP levels in all three strains.

It is unlikely that the increase in cAMP levels in the Enzyme I mutant that resulted from washing the cells free of carbon source (Fig. 1) is an artifact of centrifugation, temperature change, or manipulation of the cells. Simply depositing a sample of a suspension of actively growing cells on a Millipore filter followed by a 10-ml wash with fresh medium devoid of carbon source (at 37°) led to an approximately 100-fold increase in cellular cAMP in the Enzyme I mutant (before washing, cAMP = 0.6×10^{-6} M; after washing, cAMP = 62.4×10^{-6} M). The wild-type cells showed only a small effect of similarly washing cells on a membrane filter (before washing, cAMP = 7.0×10^{-6} M; after washing, cAMP = 11.6×10^{-6} M).

Additional support for the idea that the unusual behavior of the Enzyme I mutant was restricted to carbon source supplementation conditions was obtained from the experiment shown in Fig. 2. Cells were grown to mid-logarithmic phase in nutrient broth medium, then washed and suspended in minimal salts medium at 30°. After temperature equilibration was obtained, the cell suspensions were assayed for adenylate cyclase in intact cells by our previously described method involving pulse-labeling with [^{3}H]adenosine (5). The data show that under those conditions, all three strains have high levels of adenylate cyclase activity which is essentially completely inhibited in the presence of glucose.

Table 2. Cellular cAMP levels of phosphotransferase mutants

Strain	Genotype	Intracellular cAMP (μM)
1100	Wild-type	4.86
1101	HPr$^-$	3.65
1103	Enzyme I$^-$	0.71

Cultures of the different strains were grown in salts medium supplemented with 1% nutrient broth. At mid-logarithmic phase, 10-ml aliquots of cell suspension were filtered on Millipore membranes and processed for cAMP determinations in cells. Corrections were made for contaminating extracellular cAMP as described (1).

Phospho*enol*pyruvate corrects the abnormal cAMP metabolism of the Enzyme I mutant

The observation that the Enzyme I mutant had low adenylate cyclase activity in the presence of carbon source but high activity in the absence of carbon source suggested that the cells contained enzyme in a form that was subject to some form of regulation. A search for a procedure for unmasking the potentially high adenylate cyclase activity in the Enzyme I mutant revealed that PEP produces a profound stimulation of the activity in toluene-treated cells (Table 3). While inclusion of PEP in the assay produces essentially no effect on the enzyme activity in either the wild type or HPr mutant, the activity of the Enzyme I mutant could be stimulated as much as 70-fold. This high activity produced in the presence of PEP is inhibited by glucose. Thus, in the presence of PEP, the adenylate cyclase of the Enzyme I mutant

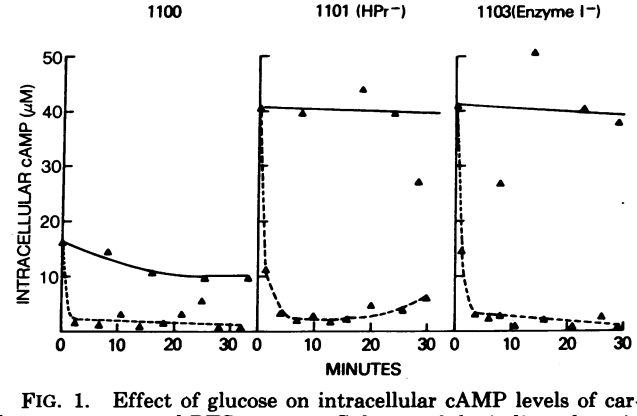

FIG. 1. Effect of glucose on intracellular cAMP levels of carbon-source-starved PTS mutants. Cultures of the indicated strain were grown in salts medium (3) supplemented with nutrient broth (1%). When the OD_{650} reached 0.6–0.8, aliquots of the cell suspensions were centrifuged, washed once with 50 mM Tris·HCl, pH 7.5, then diluted to their original volume in fresh minimal medium (3) without trace elements. The cell suspension was divided into two aliquots, one of which received glucose (1 mM); both aliquots were incubated with shaking at 30°. At the indicated times, aliquots (10 ml) were removed and the cells were collected on Millipore filters with no washing. Intracellular cAMP levels were determined as described (1). A correction was made for contaminating cAMP in the culture filtrate (1). Zero time corresponds to the time after the washed cells had been equilibrated at 30° for at least 5 min, at which time glucose was added to the designated cultures. ▲—▲, Control; △– –△, 1 mM glucose.

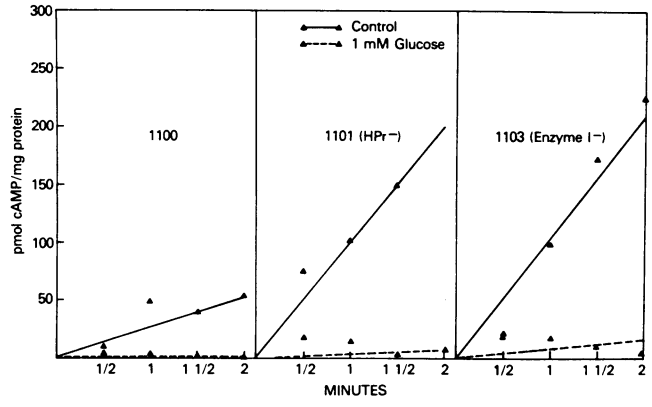

FIG. 2. Effect of glucose on adenylate cyclase activity in carbon-source-starved PTS mutants. Cultures of the indicated strains were grown as described for Fig. 1. Washed cell suspensions (10-ml aliquots) in minimal medium were equilibrated with shaking at 30° and assayed for adenylate cyclase in intact cells as described (5). Where indicated, glucose (1 mM) was added to cell suspensions. At the designated times, incubations were terminated with HCOOH (5).

behaves like the parent with respect to level of activity and sensitivity to glucose inhibition. It is noteworthy that Ide (7) reported a slight stimulation by PEP of adenylate cyclase activity in extracts of a cAMP-phosphodiesterase negative strain of *E. coli*.

Whereas PEP has no significant effect on the level of enzyme activity in either the wild type or HPr mutant, it has an effect on the glucose sensitivity of the adenylate cyclase in the parent strain (Table 3). In the presence of PEP, glucose inhibition is partially relieved. This release of glucose inhibition by PEP is not seen in either of the mutants.

Table 3. Effect of phospho*enol*pyruvate on adenylate cyclase activity in toluene-treated cells of phosphotransferase mutants

Additions	Strain 1100 (Wild type)	1101 (HPr⁻)	1103 (Enzyme I⁻)
	Adenylate cyclase activity*		
None	814	2055	52
Glucose	71	138	26
PEP (1 mM)	1108	2305	2500
PEP (1 mM) + glucose	395	166	39
PEP (10 mM)	724	2333	3736
PEP (10 mM) + glucose	296	138	39
Pyruvate (1 mM)	595	541	50
Pyruvate (1 mM) + glucose	23	166	50
Pyruvate (10 mM)	23	83	50
Pyruvate (10 mM) + glucose	47	27	50
Creatine-*P* (10 mM)	1047	2166	50
Creatine-*P* (10 mM) + glucose	71	194	50

Cells were grown in salts medium (3) supplemented with 1% nutrient broth. At mid-logarithmic phase, cell suspensions were processed for assay of adenylate cyclase in toluene-treated cells with the designated additions. Rates were calculated from the best line drawn through four experimental points for samples incubated for 10, 20, 30, and 40 min. The amounts of protein used for each experimental point were: strain 1100, 0.084 mg; strain 1101, 0.072 mg; strain 1103, 0.076 mg. Where indicated, glucose was added at a concentration of 1 mM.

* pmol of cAMP/mg of protein per hr in toluene-treated cells.

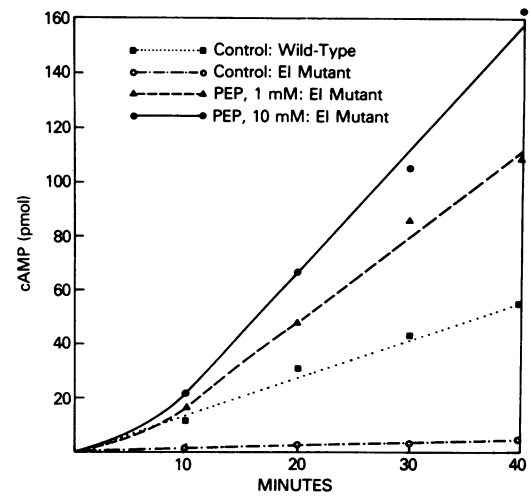

FIG. 3. Kinetics of phospho*enol*pyruvate activation of adenylate cyclase in an Enzyme I mutant. Strains 1100 and 1103 (Enzyme I⁻) were grown in salts medium supplemented with nutrient broth (1%). At mid-logarithmic phase, permeabilized cells were prepared and assayed for adenylate cyclase activity (6) in the absence or presence of PEP (concentrations as indicated). Strain 1100 contained 0.09 mg of protein and strain 1103 contained 0.082 mg of protein per assay point.

A test of some other compounds related to PEP suggests that the effect of PEP is specific. Creatine phosphate, another high energy phosphate compound, has no effect on either adenylate cyclase activity or sensitivity of the enzyme to glucose inhibition in any of the bacterial strains tested (Table 3). Pyruvate does not promote the activity in the Enzyme I mutant as does PEP; rather it leads to inhibition of the adenylate cyclase activity in the wild type and HPr mutant (Table 3). This is in agreement with the previous studies of Tao and Huberman (8), who showed that adenylate cyclase in *E. coli* extracts was inhibited by pyruvate. At a pyruvate concentration (1 mM) that produces only partial inhibition, the residual activity is further inhibited by glucose. The effect of PEP cannot be attributed to its possible property of regenerating ATP. Substrate levels of ATP are present in the assay for adenylate cyclase. A determination of ATP concentrations (5) in adenylate cyclase assays of strains 1100 and 1103 showed no significant difference in the rate of ATP utilization by permeabilized cells of either strain (data not shown).

A study of the kinetics of the adenylate cyclase reaction in toluene-treated cells of the Enzyme I mutant (Fig. 3) indicated that the activating effect of PEP was time-dependent. While the low level "basal" activity of the Enzyme I mutant and the normal control activity in the wild-type cells gave linear rates that converged to the origin of the plot, the rates in the Enzyme I⁻ cells in the presence of PEP were not linear. It took about 5 min for PEP to achieve full activation of the adenylate cyclase in the Enzyme I⁻ cells.

DISCUSSION

Our previous studies have indicated that inhibition of adenylate cyclase by a sugar requires the presence of a transport system for that sugar (2). There is a good correlation with the inhibition effect of sugars on adenylate cyclase and the effect of those sugars in establishing transient repression. Pastan and Perlman (9), using the same mutants as used in this study, concluded that transient repression was due to lowered cAMP levels and that phosphorylation of a sugar

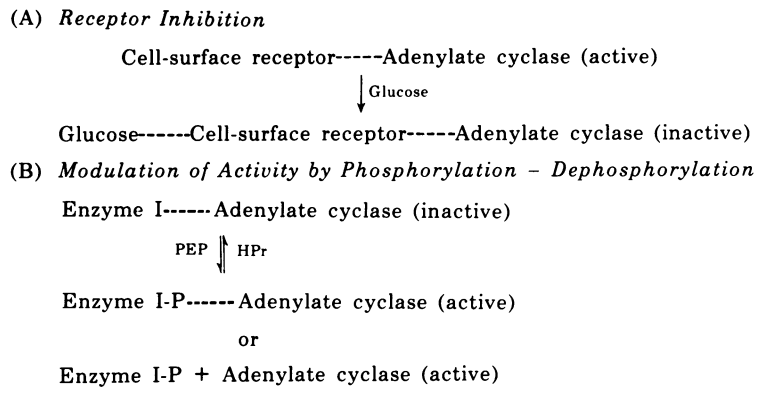

FIG. 4. Two possible mechanisms for regulation of adenylate cyclase.

was not necessary for repression. Tyler and Magasanik (10) also concluded that metabolism of a sugar was not necessary for transient repression and suggested that repression was due to passage of a sugar through the membrane. Other studies from this laboratory, consistent with this conclusion, have shown that, whereas glucose inhibits adenylate cyclase, glucose-6-phosphate does not (6). Thus, there is strong evidence that the molecular explanation for transient repression by a sugar is the PTS-mediated inhibition by that sugar of adenylate cyclase.

The mechanism of the phospho*enol*pyruvate:sugar phosphotransferase system (PTS) may be represented by the following series of reactions:

(1) PEP + Enzyme I ⟶ Enzyme I-P + Pyruvate
(2) Enzyme I-P + HPr ⟶ P-HPr + Enzyme I
(3) P-HPr + Enzyme II (sugar-specific) ⟶ Enzyme II-P + HPr
(4) Enzyme II-P + sugar ⟶ sugar-P + Enzyme II

The evidence for such a pathway has been discussed by Roseman (11). Evidence for reaction 1, the phosphorylation of Enzyme I by PEP, has recently been presented by Stein *et al.* (12).

Other workers have implicated Enzyme I of the PTS system uniquely in the repression of enzyme synthesis. Fox and Wilson (4) suggested that a low level of Enzyme I may be required for optimal growth on a number of carbohydrates that are not phosphorylated by the PTS system and suggested that Enzyme I may play a role in addition to its PTS function. The studies of Saier *et al.* (13, 14) and Pastan and Perlman (9) suggested that Enzyme I mutants are hypersensitive to catabolite repression, while those of Tyler and Maganasik (10) implicated Enzyme I as a necessary component for transient repression. Lo *et al.* (15) showed that an Enzyme I mutant grew on succinate in the presence of cAMP but not in its absence, suggesting that Enzyme I mutants have low cAMP levels.

The observation that an Enzyme I mutant (strain 1103) has reduced adenylate cyclase activity suggests that Enzyme I may interact with adenylate cyclase. The activation by PEP of adenylate cyclase in strain 1103 suggests that the phosphorylated form of Enzyme I may confer a high activity on adenylate cyclase. A model involving interaction of Enzyme I with adenylate cyclase which is compatible with all the available data can be entertained (see Fig. 4B). We suggest that strain 1103 is a mutant with a "leaky" Enzyme I which can be only slowly phosphorylated by PEP. Previous studies (4, 11) have indicated that strain 1103 has from 1 to 5% of the wild-type Enzyme I activity. In the presence of carbon sources, when the PTS system is transporting substrate, the "leaky" Enzyme I may be completely dephosphorylated. We postulate that in the presence of dephospho-Enzyme I, the adenylate cyclase is essentially inactive (see Fig. 4B). The type of data observed in Tables 1 and 2 would be examples of this condition. Under conditions of carbon source starvation, when the PTS system is not operating, we suppose that Enzyme I can slowly be converted to the phospho-form, resulting in activation of adenylate cyclase (see Figs. 1, 2, nd 3). The addition of glucose to carbon-source-starved cultures of strain 1103 could lead to inhibition of adenylate cyclase by interaction with a cell surface receptor (Fig. 4A) and also by conversion of Enzyme I to the dephospho-form (Fig. 4B) in this "leaky" mutant. The hypersensitivity of Enzyme I mutants to catabolite repression (10, 11) may be partially explained by this double mode of inhibition; also, it has been reported (14) that Enzyme I mutants have a higher concentration of surface glucose receptor.

The idea that glucose inhibition of adenylate cyclase is partially explained by dephosphorylation of Enzyme I is additionally supported by the antagonistic effects of glucose and PEP in the wild-type strain. The partial reversal of glucose inhibition by PEP is compatible with the idea that whereas glucose inactivates adenylate cyclase by Enzyme I dephosphorylation (by phosphate transfer through the PTS system), PEP activates cyclase by an Enzyme I phosphorylation.

The inhibition by glucose of adenylate cyclase in the HPr mutant can be explained in two ways. If strain 1101 does not possess the capacity to participate in phosphate transfer through the PTS system (i.e., the mutation is complete), then the glucose inhibition of adenylate cyclase in this mutant is probably exclusively through interaction of glucose with the cell-surface receptor (Fig. 4A). On the other hand, if the HPr mutant has a limited capacity to participate in active or abortive reactions with Enzyme I-P (i.e., the mutation is leaky), then glucose inhibition of adenylate cyclase may involve the Enzyme I-P dephosphorylation mechanism (Fig. 4B). In this case, glucose inhibition of adenylate cyclase need not involve the receptor mechanism (Fig. 4A), which does not require penetration of glucose into the cells. A further study of other HPr mutants, including deletions, should help to resolve the question of whether mechanism A plays a role in catabolite repression. Another observation suggests that a mechanism not involving Enzyme I may operate in adenylate cyclase regulation. *E. coli*, induced to grow on lactose, show a lactose-dependent inhibition of adenylate cyclase, even though lactose is transported into cells via a PTS-independent mechanism (2); in this case, mechanism A may op-

erate for adenylate cyclase regulation. In any case, the data presented here strongly support mechanism B as an important, if not the sole, element in glucose regulation of adenylate cyclase. Clearly, however, a definitive proof of this working model will require a demonstration *in vitro* that adenylate cyclase forms complexes with different forms of Enzyme I.

There is some precedent for the idea that adenylate cyclase may be regulated by phosphorylation. Constantopoulos and Najjar (15) proposed that activation by fluoride of the adenylate cyclase of polymorphonuclear leukocytes and platelet membranes was due to conversion of a phospho-(inhibited) form of the enzyme to a dephospho-(activated) form. Our model suggests that *E. coli* adenylate cyclase associated with Enzyme I-P is active while the dephospho-form is inactive. Whereas, the effect of most hormones on mammalian adenylate cyclase leads to an activation reaction, effectors of *E. coli* adenylate cyclase (like glucose) lead to an inhibited reaction. Thus, mammalian and bacterial adenylate cyclases may use the same fundamental mechanisms for opposite types of regulation.

We thank Dr. Ira Pastan for providing the strains of *E. coli* originally described by Fox and Wilson (4) which were used on this study. We are indebted to Mary Ellen Miller for her help in preparing the manuscript.

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