CHARACTERIZATION OF SELECTED PHYSICAL PROPERTIES OF cGMP - PHOSPHODIESTERASE ISOLATED FROM HUMAN LUNG TISSUE

by

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Abstract

In this study, cyclic 3':5'-monophosphate phosphodiesterase from human lung tissue was investigated. After a brief review of the literature, some physical properties of the enzyme were characterized. A Heat Lability study was done, and it was determined that the enzyme was fairly heat stable, with a critical temperature of 39.9°C. The molecular weight was determined by gel filtration, and reported as 129,500. This value is lower than other literature values. The enzyme was determined to be labile when subjected to pH values below about 7 to 8. This is in accord with the literature. The pI is reported as less than pH 5, which doesn't fit the literature value of pH 5.3.

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I especially want to thank Dr. John B. Moore for putting up with me at times when it seemed I was not only bent on self destruction, but also on his laboratory.

List of Tables and Illustrations

Adenosine 3':5'-monophosphate and guanosine 3':5'-monophosphate (cyclic AMP and cyclic GMP) are cyclic nucleotides found in a great diversity of organisms, and in many tissues of individual organisms. Their biological actions appear to be unique, and in some instances, mutually opposing (1,2). Alterations of the cyclic nucleotide systems have been associated with a variety of pathophysiological states, which indicates a prominent role for these compounds(3). For example, human lung tissue has been implicated as a source of pharmacological mediators in asthma and immediate hypersensitivity reactions(4-6), and studies by Austen(7,8) and Lichtenstein(9) have shown cyclic AMP and cyclic GMP to be closely related to mediator release. If the levels of these cyclic nucleotides could be controlled in vivo, then allergic release of mediators could possibly be controlled to some extent.

Intracellular cyclic nucleotide levels can be controlled in two ways: through regulation of their synthesis via respective cyclases or through degradation via respective phosphodiesterase enzymes(10,11). The present investigation deals with the phosphodiesterase enzymes, and in particular with cyclic GMP-specific phosphodiesterase from human lung tissue.

The footnote format used is taken from the Journal of Biological Chemistry

The study attempts to characterize some of the physical properties of cyclic GMP phosphodiesterase (cGMP-PDE) so that the actions of the enzyme can be better understood.

Literature Review

In looking at the literature, it becomes apparent that cyclic nucleotide phosphodiesterases are inherently different from organism to organism. The molecular weight, activity, and function of the enzymes vary, although the substrates they act upon are one and the same. A good starting point for looking at the literature may be the following: "Most mammalian tissues except human lung, guinea pig lung, and rat erythrocytes possess a cyclic nucleotide phosphodiesterase which is stimulated by a heat-stable activator protein in the presence of Ca^{2+} (12)." In reviewing the literature we will subsequently deal only with human lung phosphodiesterase and guinea pig lung phosphodiesterase.

The first paper we will consider is a paper by Hakan Bergstrand and Britta Lundquist (13). This paper deals with cAMP-PDE and CGMP-PDE isolated from human lung tissue. The purification scheme used by these researchers consisted of:

1. homogenizing the lung tissue in buffer
2. spinning the homogenate at 200000g for 30 min.
3. application of the supernatant from the centrifugation to a DEAE-Sephadex A-50 column
4. application of the various peaks of activity from the DEAE-Sephadex A-50 column to a Sephadex G-200 column
5. reapplication of the activity to DEAE-Sephadex

and/or Sephadex G-200 until reasonably pure

For the purification, they reported that 80-90% of the total phosphodiesterase activity in the crude homogenate was recovered in the supernatant from the centrifugation at 200000g. The DEAE-Sephadex column used in this experiment was reported to have eluted four fractions containing phosphodiesterase activity. Of these fractions, the first was specific for cGMP, the second hydrolyzed cAMP and cGMP equally well, and the last two hydrolyzed cAMP specifically. Further purification steps resulted in heavy losses of enzyme activity, and unless the enzymes were stabilized with bovine serum albumin, heavy losses of activity occurred upon dilution.

The four fractions were tested for thermal inactivation at 37°C, apparent molecular weight, and activation by Ca^{2+}. The molecular weight determinations were carried out on a Sephadex G-200 column. The results are shown in Table 1.

Parameter	F-1	F-II	F-III	F-IV
Substrate specificity	cGMP	cGMP/cAMP	cAMP	cAMP
Thermal inactivation at 37°C	slight	slight	profound	profound
Apparent MW	240,000	260,000	110,000	55,000
Ca^{2+} Activation	none	none	none	none

Table 1 - Summary of Some Properties of PDE Fractions Purified from Human Lung

The experiments showed no Ca^{2+} activation for phosphodiesterase from human lung, very slight thermal inactivation of CGMP-PDE at 37°C, profound inactivation of cAMP-PDE at 37°C, and

molecular weight apparents of 240,000 - 260,000 for cGMP-PDE and 55,000 - 110,000 for cAMP-PDE.

A fourth experiment, done on the crude lung supernatant, compared the stability of the phosphodiesterase enzymes at pH 6.0 and pH 8.0. The standard buffer has pH 8.0, and constitutes the control. The enzyme was incubated at pH 6.0, and it was discovered that a major portion of the activity was lost.

We shall now shift our attention to two papers written by C.W. Davis and J.F. Kuo (3,14). These papers deal with cAMP-PDE and cGMP-PDE isolated from guinea pig lung. The emphasis in these papers is on cGMP-PDE. The purification scheme used by Kuo and Davis is as follows:

1. guinea pig lungs were excised and homogenized in buffer for 20s
2. the homogenate was centrifuged at 105,000g for 45 minutes, and the supernatant filtered through glass wool to remove the fat
3. the enzyme was applied to a DEAE-cellulose column (2.5 by 40 cm) and eluted with a .1 to .6M KCl gradient
4. the active fractions were pooled, and a hydroxyapatite gel suspension added which quantitatively absorbed the enzyme
5. the hydroxyapatite was rinsed with buffer, and the enzyme eluted with 100mM potassium phosphate buffer (pH 7)
6. the eluate was concentrated to 3 ml, and subjected to preparatory polyacrylamide gel electrophoresis
7. the enzyme was stabilized with bovine serum albumin since the enzyme was extremely labile without added protein

It was reported that 94% of the cGMP and cAMP activities were present in the supernatant from the centrifugation of the crude homogenate. It was also reported that the DEAE-cellulose chromatography step seperated the cAMP and cGMP activities, with the cGMP activity eluting at 250 mM KCl (85-90% of the recovered cGMP activity) and the cAMP activity eluting at 350 mM KCl (90% of the recovered cAMP activity). Mapping out of the entire procedure for the cGMP-PDE purification is shown in Table 2.

Fraction	Protein (mg)	Total Activity (by 10^{-3})	Specific Activity	Purification	% recovery
Crude extract	1400	150	107	1	100
DEAE-cellulose eluate	55	41	745	7	28
Hydroxyapatite eluate	6.5	23	3538	33	16
Polyacrylamide jel electrophoresis	.23	6.2	26956	251	4

Table 2 - Summary of the Purification of cGMP-PDE Isolated from Guinea Pig Lungs

Most experiments run by Kuo and Davis deal specifically with cGMP-PDE. One experiment dealing with cGMP-PDE was the determination of the enzyme's molecular weight. They reported values of 137000 using density gradient centrifugation and 168,000 using Sephadex G-200 gel filtration.

A second experiment performed by Kuo and Davis was the

effect of temperature on enzyme activity. "When the cyclic GMP-specific enzyme was preincubated at 25-42°, a 25 to 35% loss in activity was observed within 5 min, after which the activity was relatively stable for up to 25 min. At temperatures above 50°. loss in enzyme activity was directly related to length of incubation time, with the half-life of the cyclic GMP enzyme at 50° being 3 min, whereas that at 60° being about 1 min. The effect of temperature on the rate of hydrolysis of cGMP by the cyclic GMP-specific phosphodiesterase was also investigated. The rate of hydrolysis increased between 10 and 42°, with a calculated energy of activation (Ea) of 11.9 kcal/mol."

A third experiment performed was a dose-dependent study of activation of the cyclic GMP enzyme by divalent ions. "Activity of the enzyme in the absence of added metal ions was about 6% of the maximal activity stimulated by Mg^{2+}. The strict requirement for divalent cations for activity was found to be more apparent with increasing purity of the enzyme. In addition to Mg^{2+}, Mn^{2+} or Co^{2+} was found to stimulate enzyme activity, while Zn^{2+} or Cu^{2+} was inactive." "Unexpectedly, Co^{2+}, in contrast to Mg^{2+} and Mn^{2+} disproportionately stimulated cyclic AMP hydrolysis by the cyclic GMP enzyme. The effect of combinations of the cations were also studied. Stimulation of enzyme activity caused by Mg^{2+} and Mn^{2+} was almost additive, while Co^{2+}, in combination with either Mg^{2+} or Mn^{2+}, appeared to inhibit the stimulatory effect of either metal ion."

A fourth experiment performed was a pH profile of cGMP-

PDE. This profile showed a maximal activity between pH 7.5 and 8.0. Isoelectric focusing revealed a major enzyme activity peak with pI of pH 5.3 and a minor enzyme activity peak at pH 7.4.

A fifth experiment by Kuo and Davis, this one using both cAMP-PDE and cGMP-PDE, was a study of the effects of ethanol and methanol (both 13 per cent v/v) on the activities of these two enzymes. "Ethanol and methanol were found to stimulate cyclic GMP-PDE 80 and 115 per cent, respectively, while conversly inhibiting cyclic AMP-PDE 75 and 50 per cent respectively. Systematic analysis of a series of aliphatic alcohols revealed that other alcohols were unable to stimulate cyclic GMP-PDE. Activation of the cyclic GMP-enzyme by either ethanol or methanol was due to increases in the V_{max} without altering the K_m for cyclic GMP." The latter sentence states that the rate is changed, not the binding specificity of the enzyme.

Personal Studies

Materials and Methods

Cyclic GMP (8-^{3}H, 8.8 Ci/mmole, 1 mCi/ 2 ml) was purchased from Schwarz-Mann, Orangeburg, New York. Snake venom (Crotalus adamanteus) was purchased from Sigma Chemical Co. Bio-Rad Laboratories (Richmond, California) furnished the AG1-X2 ion exchange resin (chloride form, 200-400 mesh). Guanosine was obtained from Eastman Chemical Co.

A modification of the two-step method by Thompson and Appleman (15) was used in assaying the cGMP-PDE isolated from

human lung tissue. Briefly, tritium labelled cGMP (1uM final concentration) and 100ul of suitably diluted enzyme were mixed in a total volume of 0.4 ml and allowed to react for 30 min at room temperature. Linear reaction kinetics were observed with the conditions used (i.e., length of incubation and amount of enzyme). This reaction converts some of the cGMP into 5'-GMP, the amount converted depending on the activity of the enzyme used. The reaction is ceased by boiling for 1 min at 100°C. The reaction mixture is cooled, and 100ul of snake venom (1mg/ml) is added and allowed to incubate at room temperature for 10 minutes. This step converts all the 5'-GMP into the corresponding guanosine nucleoside, which is uncharged. At the end of the incubation period, 1 ml of resin (Bio. Rad, 1:3 v/v) was added. The resin binds negative species in solution (i.e., unreacted cGMP) and leaves the neutral species in solution (i.e., the guanosine nucleoside). This mixture was centrifuged in a table top centrifuge on high for 10 minutes, and 200ul removed from each sample to count in a Beckman LS 7000 scintillation counter. The two reactions involved in the assay are shown in Figure 1, which is on the next page.

Purification of cGMP-PDE from Human Lung

The enzyme used in these experiments was purified by employing basically a three step process. Frozen human lung tissue was trimmed of excess fat and connective tissue, and homogenized for 3 minutes with 3 volumes of buffer A (.05M Tris, pH 8.0, 5mM $MgCl_2$, 3.75mM 2-mercaptoethanol) in a Waring blender. The subsequent homogenate was centrifuged for 30 min

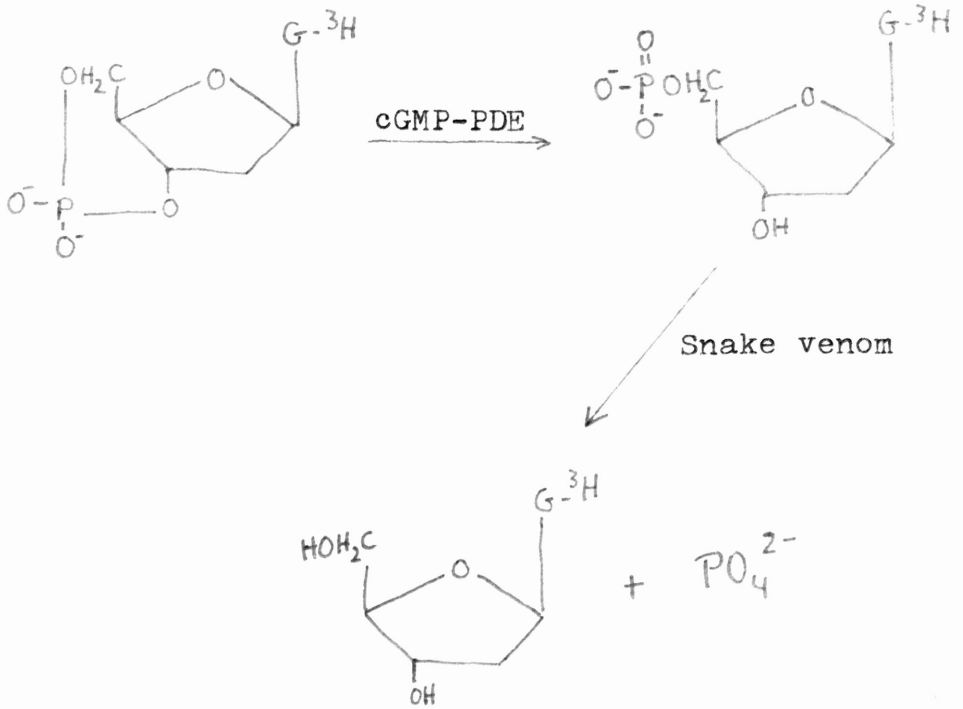

Figure 1 - Reactions involved in the Assay

at 16,000 rpm in a Beckman J-21 ultracentrifuge. The supernatant was saved, and the pellet resuspended in 20 ml of buffer A, recentrifuged, and the two supernatants combined. This constitutes what is referred to as the crude supernatant. The crude supernatant was subjected to 20% saturation with ammonium sulfate, and recentrifuged at 16,000 rpm for 30 min. The supernatant from this centrifugation was retrieved, and the pellet discarded. The resulting supernatant was brought to 45% saturation with ammonium sulfate, and centrifuged for 30 min at 16,000 rpm. In this step, approximately 85% of the cGMP-PDE activity is recovered in the pellet, whereas the majority of the cAMP-PDE activity (approximately 65%) remains in the soluble portion. The supernatant was discarded, and

the pellet resuspended in 20-25 ml of buffer A. The resulting enzyme solution was dialyzed overnight vs. one liter of buffer A, and applied to a DEAE-cellulose column (2.8 by 20 cm) equilibrated with buffer A. The resulting eluate contained no activity, and when trapped proteins were eluted with a NaCl gradient from 0 to .5M (in buffer A), active fractions were obtained, pooled, and concentrated via vacuum dialysis to 4 ml. The enzyme was subsequently applied to an ACA-34 gel filtration column, and the active fractions pooled, concentrated, aliquoted, and frozen for future use. The entire purification procedure took place in a cold room at @ 4°C.

Experimental Phase

The experimental phase was broken down into three main experiments: a heat lability study, a molecular weight determination, and a study of pH effects on the enzyme. The experiments will be discussed in this order.

Heat lability study

In the heat lability study of cGMP-PDE, the purified enzyme was incubated at temperatures ranging from 35-47°C for varying amounts of time (every five minute interval with a maximum incubation time of 60 min). The resulting sets of data were plotted as activity vs. length of incubation at a fixed temperature, and the time at which 75% of the activity remained extrapolated from each graph. This point was chosen since the 50% death point was not obtained for the majority of the data sets. The $t_{75\%}$ values were then plotted vs. the temperature at which they were incubated. Figure 2 shows this graph.

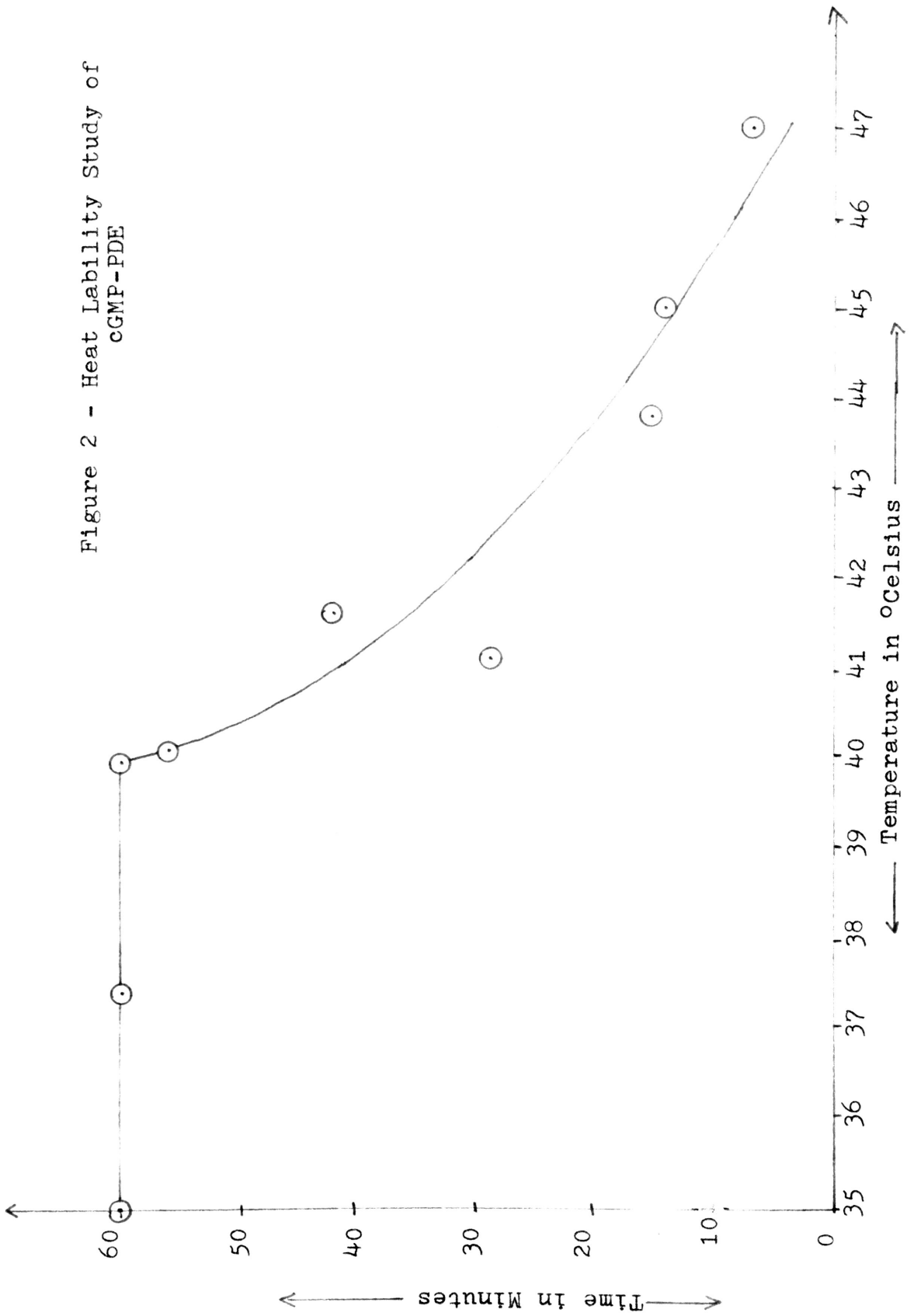

Figure 2 - Heat Lability Study of cGMP-PDE

From the graph it can be seen that cGMP-PDE from human lung is fairly heat stable up to approximately 39.9°C, after which activity falls of exponentially. The enzyme is fairly hardy when temperature is the parameter, and this makes it nice to work with at room temperatures (25°C). This experiment shows quite nicely why the cGMP-PDE isolated by Bergstrand and Lundquist showed only slight loss of activity when treated with 37°C.

Molecular weight determination

In this experiment, the apparent molecular weight was determined using an ACA-34 gel filtration column (2.6 by 85 cm). The column was calibrated with:

1. pepsin, MW=34,500 (16)
2. ovalbumin, MW=45,000 (17)
3. hemoglobin, MW=64,500 (18)
4. yeast alcohol dehydrogenase, MW=150,000 (18)
5. fumarase, MW=194,000 (18)

A plot was made of the logMW vs. the elution fraction, and linear regression analysis was employed. The apparent molecular weight of cGMP-PDE was extrapolated from the graph, and was 129,500. This graph is shown in Figure 3 on the following page. The value obtained is lower than any previously reported value (Bergstrand and Lundquist reporting values of 240,000 to 260,000, and Kuo and Davis reporting values of 137,000 to 168,000), but not too much lower than the value of 137,000 reported by Kuo and Davis for enzyme purified from guinea pig lung.

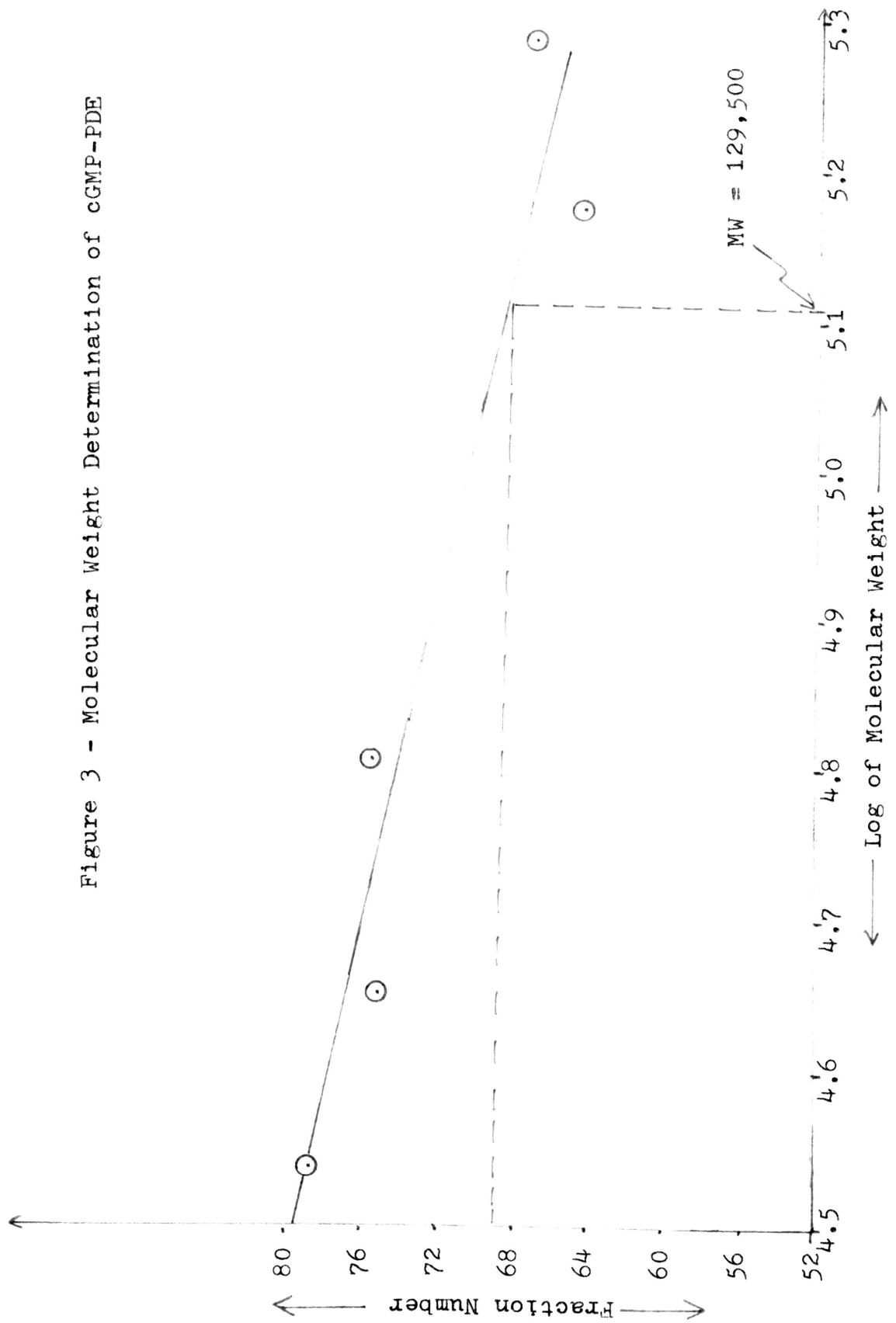

Figure 3 - Molecular Weight Determination of cGMP-PDE

pH behavior of cGMP-PDE

This experiment is divided into three parts: isoelectric focusing gels, two DEAE-cellulose columns, and the effects of treatment with various pH's on the activity of the enzyme.

In part one of this experiment, samples of the purified enzyme were subjected to reverse isoelectric focusing electrophoresis on 7.5% acrylamide gels with pH 3.5-10 ampholines. The samples were electrophoresed for 3 hours at 100V. Some gels were removed and stained, see Figure 4 below, while others were sliced into .5 cm slices, ground, left in buffer A overnight, and assayed for activity. No activity was found, and

Figure 4 - Isoelectric Focusing Gels

it was concluded that the enzyme had perished. The stained gels showed that the enzyme was still relatively impure.

In part two of this experiment, two small DEAE-cellulose columns were poured (2 by 3 cm). One was equilibrated with .05M KH_2PO_4-NaOH buffer (pH 6.5, 5mM $MgCl_2$, 3.75 mM 2-mercaptoethanol), while the other was equilibrated with .05M acetic acid-sodium acetate buffer (pH 5.0, 5mM $MgCl_2$, 3.75 mM 2-mercaptoethanol). After dialysis vs. these buffers for an hour, the enzyme was applied to the columns, diluted with these buffers, and allowed to enter the columns. The enzyme stuck

in both cases, being eluted readily with buffer A made .5M in NaCl. This indicates that the enzyme has an isoelectric point (pI) of less than pH 5. This does not agree with the reported pI of 5.3 reported by Kuo and Davis for cGMP-PDE from guinea pig lung.

The third part of this experiment deals with the stability of the enzyme at various pH values. In this experiment, .5 ml of enzyme was dialyzed against each of four buffers for 21 hours (pH values of 8.0, 7.0, 6.5, and 6.0), and subsequently back dialyzed for 23.5 hours against buffer A (pH 8.0). The buffers used were buffer A, and various .05M KH_2PO_4-NaOH buffers adjusted to the proper pH and containing 5mM $MgCl_2$ and 3.75mM 2-mercaptoethanol. The results of the experiment showed relative activities (compared to the control at pH 8.0) of:

1. pH 8.0 - 100%
2. pH 7.0 - 79%
3. pH 6.5 - 60%
4. pH 6.0 - 46%

The experiment shows that below about pH 7-8 the enzyme dies off fairly rapidly. This concurs with the literature reports.

Conclusions

Several conclusions can be drawn from the experimentation. The cGMP-PDE from human lung appears fairly heat stable, with a critical temperature of 39.9°C, but fairly labile when subjected to pH alteration. The isoelectric focusing gels showed that the enzyme preparation is still impure, and needs further purification. The reported molecular weights show no consistency, and

this needs to be clarified. Experiments such as those by Kuo and Davis on guinea pig lung cGMP-PDE need to be carried out on human lung cGMP-PDE (i.e., check requirement for divalent cations, check to see if Co^{2+} stimulates cAMP hydrolysis by cGMP-PDE, and check to see if cGMP-PDE from human lung is activated by ethanol and methanol). The exact pI needs to be determined, and the isoionic point also. These and many other studies need to be done before we can fully understand the actions of cGMP-PDE from human lung.

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