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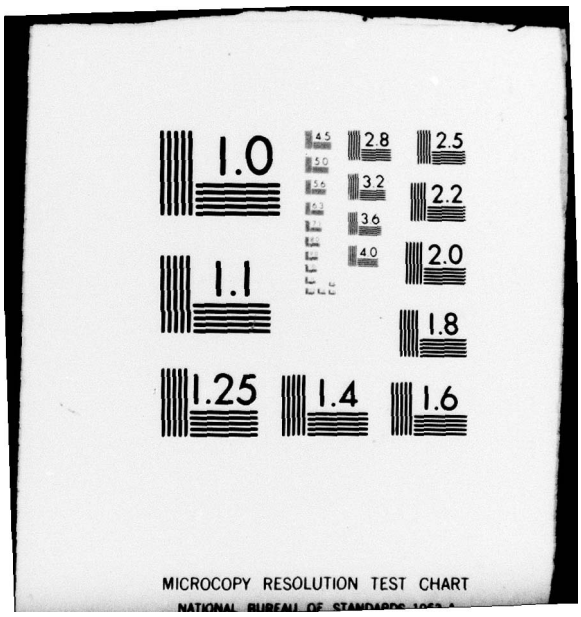
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STUDIES ON PGB_x
ISOLATION OF A PGB_x WITH REDUCED INHIBITOR CONTENT

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PGB_x was separated into two fractions by dialysis against pH 6.5 phosphate buffer. The retentate, which amounted to 90% of the total PGB_x, showed *in vitro* PGB_x effects similar to the unfractionated PGB_x, i.e., activation at low concentration, inhibition at high concentration. The dialysate fraction showed primarily PGB_x activation with only minimal inhibition properties. It is concluded that the activation and inhibition effects of the PGB_x complex are caused by different molecular species in the complex.

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INTRODUCTION

The role of PGB_x* in the *in vitro* recovery of oxidative phosphorylation of degraded aged rat liver mitochondria was first reported by Polis *et al* (1). In a later publication the details of the *in vitro* assay were described fully (2, 6). In both these reports the restoration of oxidative phosphorylation was shown to be directly dependent upon the concentration of PGB_x in the range of 2-10 µg per 4 mg of isolated rat liver mitochondria. In later experiments, Polis (3) showed that at higher concentrations of PGB_x the reversal of oxidative phosphorylation was decreased and eventually reached the level of the control, i.e., no net phosphorylation. Similar results were found by Devlin (4) using a modification of the *in vitro* test system in that the mitochondria were only partially degraded. In this system, high concentrations of PGB_x inhibited the oxidative phosphorylation reactions below that of the control.

This biphasic response of PGB_x on mitochondrial oxidative phosphorylation, i.e., activation at low concentrations and inhibition at high concentrations, suggested a metabolic role for PGB_x in terms of a feedback mechanism that could regulate the rate of formation of biological energy in mitochondria, similar to hormonal control mechanisms. This would imply that the dual *in vitro* effects of PGB_x must be inherent in a single molecular species. Alternatively, these two effects may be associated with different factors making up the PGB_x complex currently being prepared. In view of the known hetereogeneity of the PGB_x complex, it was deemed important to determine if the activating and inhibiting effects of PGB_x were separable. This report describes the separation of PGB_x into fractions of different molecular weights that exhibit the same degree of activation but different degrees of inhibition.

EXPERIMENTAL

PGB_x acid (preparation #27), prepared by the method of Polis *et al* (2), was converted to the sodium salt as described below. PGB_x acid was first dissolved in ethanol and then diluted with water to 60% ethanol. Dilute aqueous NaOH was added to neutralize this solution to pH 7.2-7.4. The solution was frozen at -70° and then lyophylized to yield the dried sodium salt of PGB_x.

The PGB_x *in vitro* effect was assayed by the method described previously (2); however, the system was modified to include the assay for the inhibition effect as well as the activation effect. This modification consisted of testing the effect of PGB_x on oxidative phosphorylation of aged degraded rat liver mitochondria over a concentration range of 2-80 µg PGB_x per 4 mg of mitochondria. Sufficient data points were used to define the rising side of the activation curve (2,4,6,7,10 µg PGB_x) and the falling side (20,30,50,80 µg PGB_x). The degree of activation (K_a) and inhibition (K_1) were quantified as described previously (5).

*Small amounts of PGB_x, for investigative purposes, are available from the authors upon application.

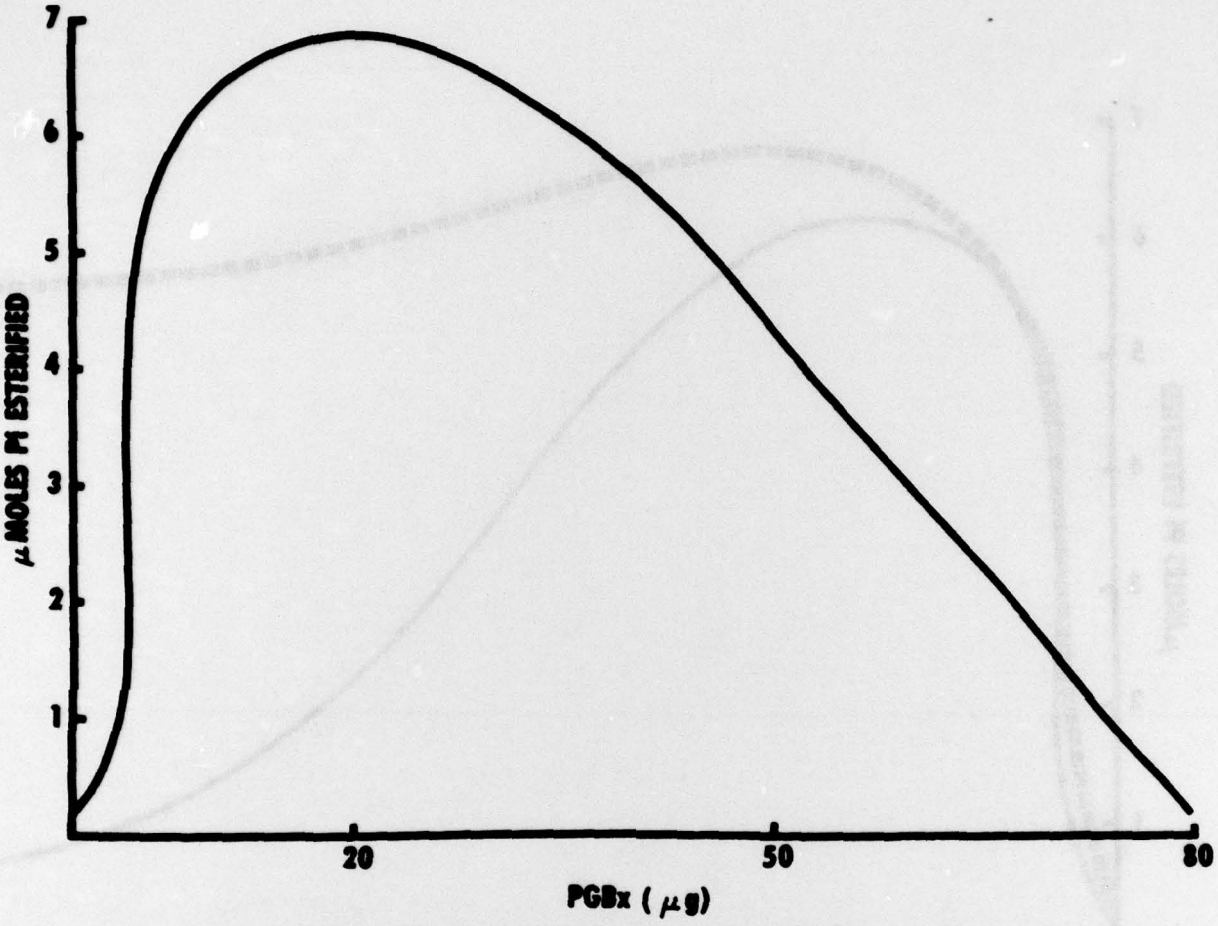
The dialysis experiments were carried out by dissolving the sodium PGB_X in the minimum amount of dialysis buffer (< 5 ml) needed to dissolve the salt. The PGB_X solution was then placed inside a dialyzer tube, which was secured at both ends and then immersed into 1000 ml of buffer at 4°. By continually stirring the external buffer solution, dialysis equilibrium was attained more rapidly. After 24 hours, the dialysate was removed and fresh buffer added. Dialysis was allowed to proceed an additional 24 hours. The dialysates were then combined, acidified to pH 3.0 with dilute perchloric acid and the PGB_X acid extracted into isobutanol. The isobutanol layer was washed 3 times with water, and the PGB_X acid in the isobutanol layer was dried by flash evaporation at 50°. The residue was dissolved in ethanol and stored at 3° until used. The fraction of PGB_X that did not pass through the dialysis tubing also was extracted, dried, and stored as described above.

RESULTS AND DISCUSSION

In a previous study (5) it was found that the dialysis of PGB_X against neutral aqueous phosphate buffers separated the PGB_X into two fractions: dialysate and rententate. On the basis of the manufacturer's specifications for the molecular weight separations obtainable with this dialysis tubing, the dialysate was assigned a molecular weight of less than 12000 daltons and the rententate a value of more than 12000 daltons. In contrast, the molecular weight measurements by vapor phase osmometry of the retentate fraction dissolved in methanol was 2500. This discrepancy between the values for the molecular weight of the retentate fraction, as measured by vapor pressure osmometry and by the dialysis experiments, cannot be explained at this time.

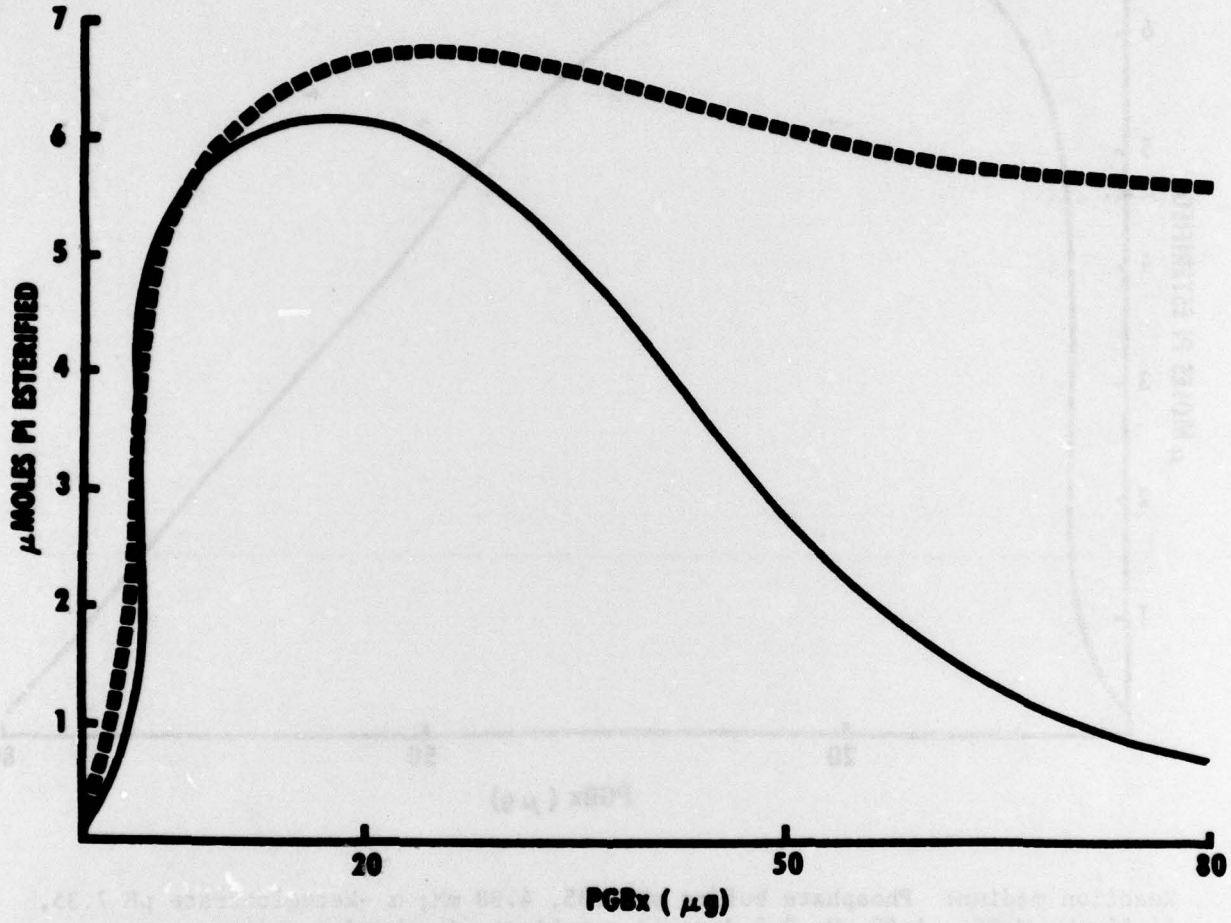
In the dialysis study previously reported (5), it was shown that the PGB_X activation effect of the dialysate and retentate were almost identical regardless of the pH of the dialysis separation. In this study, the PGB_X assay was extended to include the inhibition effect as well. Figure 1 shows the PGB_X assay as a function of the concentration of added PGB_X. At low PGB_X concentrations (2-10 µg) the amount of phosphorylation increases with increasing amounts of PGB_X. At medium PGB_X concentrations (13-20 µg) the phosphorylation is constant with increase in PGB_X. In contrast, at high PGB_X concentrations (20-80 µg) the phosphorylation decreased with increasing amounts of PGB_X. The fractions from the dialysis experiments at pH 7.8, pH 7.2 and pH 6.5 (5) were assayed for both effects. In general, the dialysates and retentates from the pH 7.8 and pH 7.2 gave PGB_X effects quite comparable to the standard PGB_X, so that at these pH's the dialysis apparently separated PGB_X into fractions according to molecular size with no apparent separation of the activation and inhibition effects. However, after dialysis at pH 6.5 the dialysate had a markedly reduced amount of inhibitor content, while the activator content of both fractions were almost identical.

The PGB_X activity vs concentration curves of the dialysate and rententate shown in figure 2 demonstrate the separation of PGB_X into fractions of different molecular weights, with similar amounts of PGB_X activator and different amounts of inhibitor factor. Clearly the pH 6.5 dialysate yields a PGB_X fraction with a low molecular weight and a marked reduction in the PGB_X inhibitor content.



Reaction medium: Phosphate buffer pH 7.35, 4.98 mM; α -ketoglutarate pH 7.35, 14.93 mM; $MgSO_4$, 4.98 mM; 3-5 day old rat liver mitochondria, 4 mg protein (containing sucrose and EDTA to yield 5.97 mM and 0.010 mM respectively); and water to 2.01 ml. When PGB_x was added, the water was reduced an equivalent amount. The mixture was shaken in covered beakers maintained at 27°. At the end of 8 minutes AMP, ADP, KCl and serum albumin were added to a final concentration of 2.27 mM, 2.27 mM, 45.45 mM and 0.68 mg/ml respectively. The reaction was allowed to proceed for 20 minutes and stopped by the addition of 0.5 ml of 31% $HClO_4$. Esterified phosphate was determined as described previously (2).

Figure 1 - The Effect of Standard PGB_x on the <u>in vitro</u> Oxidative Phosphorylation of Degraded Aged Rat Liver Mitochondria



Solid curve, retentate; dashed curve, dialysate. Assay conditions are the same as figure 1.

Figure 2 - The Effect of Additions of PGB_x Fractions Separated by Dialysis at pH 6.5 on the <u>in vitro</u> Oxidative Phosphorylation of Degraded Aged Rat Liver Mitochondria

These results are summarized in table I, which shows the distribution of the weight in the K_a and the K_i for the PGB_x fractions separated by dialysis.

TABLE I

The Analysis of PGB_x Fractions
Separated by Dialysis at pH 6.5

		In Vitro PGB_x Effect			
		Activation		Inhibition	
Fraction	Weight (mg)	K_a	Total K_a	K_i	Total K_i
PGB_x	116.4	1.0	116.4	1.0	116.4
Rententate	105.3	0.99	104.2	1.063	111.6
Dialysate	9.5	1.08	10.3	0.25	2.4
% Recovery	98.6		98.4		97.9

The dialysis technique used in this study is a relatively simple and rapid method. However, it does not separate quantitatively the PGB_x activator from the PGB_x inhibitor, since only a small portion (5) of the total activator is obtained relatively "free" of the inhibitor factor. Conceivably the activator and inhibitor factors exist as hetereogeneous mixtures of different molecular weight substances which make up the PGB_x complex. The activator factor range of molecular weights falls within the molecular size separable by dialysis. On the other hand the inhibitor factor molecular weight range is above this cut off point and consequently is not dialyzable. Because of the wide range in molecular weights of both factors,the separation is not sharp,and some of the inhibitor factor does pass through the membrane, as evidenced by the drop in the phosphorylation level at the high concentrations of the dialyzed PGB_x (figure 2).

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