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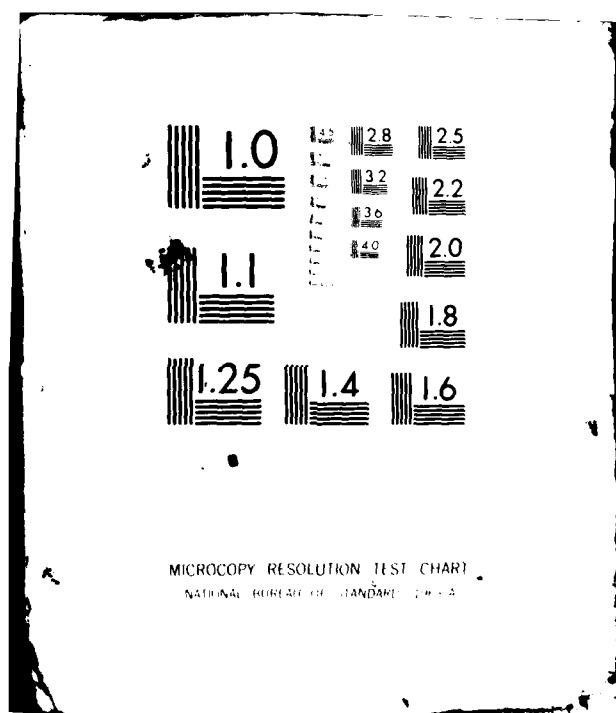
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CHARACTERIZATION OF THE CHEMICAL CONSTITUTION
AND PROFILE OF PHARMACOLOGICAL ACTIVITY OF PGB_x

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Arlington, Virginia 22217

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February 26, 1982

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TECHNICAL REPORT No. 3

Characterization of the Chemical Constitution and
Profile of Pharmacological Activity of PGB_x

by

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26 February 1982

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The Pharmacology of PGBx

Continuing studies have focused on the actions of PGBx as an enhancer of myocardial contractility during periods of ischemia. The preparations used to examine these effects include the isolated blood perfused canine papillary muscle of Endoh and Hashimoto (1) as described in our Technical Report No.2 and the isolated, electrically paced left atrium of guinea pigs.

We had established earlier that PGBx can a) exert a small but statistically significant protective effect on ischemic canine myocardium (2) and b) increase the inotropic vigor of isoproterenol stimulates guinea pig myocardium (3). During the past year we have addressed the following questions: a) To what extent are the actions seen in the canine preparations biologically significant (as opposed to statistically significant), b) How do there effects resemble those produced by other drugs currently used to treat myocardial infarction (e.g. verapamil and propranolol) and c) To what extent do adverse effects manifest themselves when guinea pig preparations are exposed to isoproterenol enhancing doses of PGBx?

Isolated Blood perfused canine papillary muscle. Introduction of PGBx (0.03-3 mg) directly into the anterior septal artery produces a slight, concentration-dependent reduction of force of contraction and automaticity (spontaneous rhythm). The maximum reduction at the high 3 mg dose was about 10%. If comparable doses, on a mg/kg basis, were administered i.v. to the support dog no inhibitory effects could be seen. In fact small doses (~ 0.1 mg/kg) appeared to slightly increase contractile force--but this effect was not statistically significant. Fig 1. illustrates the effects of total occlusion of blood supply to the muscle on force of contraction. Tissues from 20 dogs contributed to these data (along with 10 support dogs). PGBx, in doses of 0 (control), 0.1, 0.5 and 2.5 mg/kg were administered to the support dogs and allowed to recirculate for 15 min. before arterial occlusion was imposed. The black bar from 0 to 30 min. represents the period of complete occlusion. Contractile force dropped precipitously within the first 2-3 min. To everyone's astonishment, recovery occurs with equal rapidity after circulation is reestablished. A 30 min total occlusion is an incredibly long period of deprivation from which to recover and this observation in itself raises some interesting questions, which we are pursuing, but that are peripheral to the central question of PGBx's action.

All control dogs went into standstill (cardiac arrest) within 20 minutes following occlusion. PGBx, 0.5 mg/kg produced statistically significant protection against standstill. Indeed, none of the tissues in this group exhibited standstill. Tissues exposed to the higher dose of PGBx (2.5 mg/kg) did not fare as well,

although only 1 of the tissues in this group ceased to function. Although it is not included in Fig. 1., the control curve is virtually superimposable on the early part of the 0.1 mg/kg curve.

Recovery following reperfusion was approximately equivalent for all treatment groups.

If we examine the behavior of the cardiac tissues before and after occlusion (with and without PGBx treatment), we find that although contractility has returned to normal after reperfusion (it takes about 15 min. post-occlusion for complete recovery), the hearts frequently exhibit subtle changes in their response to stimulation.

Frequency-force diagrams (Figs. 2 and 3), which illustrate the changes in contractile force with changes in frequency of electrical stimulation demonstrate that a) after experiencing a period of ischemia the control hearts have lost the ability to forcefully respond to high (4 Hz) rates of stimulation (Fig 3A); b) tissues that were exposed to 0.1 mg/kg of PGBx show a reduced capacity to respond to intermediate frequencies but do not exhibit any loss of ability to follow high frequency stimulation (Fig. 3B); c) tissues exposed to 0.5 mg/kg of PGBx exhibit a force-frequency curve that is not significantly different from that of the pre-occlusion control (Fig. 3C); d) tissues exposed to 2.5 mg/kg PGBx have also lost "cardiac reserve" and, like the controls in Fig. 3A, cannot follow the 4 Hz stimulation with a strong contraction (Fig. 3D).

Premedication with 0.5 mg/kg of PGBx appears to provide a significant measure of protection against the severest form of cardiac ischemia both in terms of preventing cardiac standstill and reducing the intensity of tension loss during occlusions. This dose also protects papillary muscle from the post-ischemic loss of function reserve normally seen following resumption of blood supply. However, this statistically significant protection must be evaluated in terms of the real biological benefits conferred upon the animal receiving PGBx. The range of efficacious doses is apparently extremely narrow which makes its selection critical. The most effective concentration (0.5 mg/kg) increases the mean systolic tension at 20 min post-occlusion from about 0.1 gram to about 0.2 gram. Although that represents a 100% improvement and is a statistically significant change, it is biologically insignificant insofar as it improves the heart's ability to move blood and maintain circulation. The fact that the presence of PGBx averted the development of standstill is somewhat more impressive and perhaps it is here that real benefit can be realized. On the other hand, in order to exhibit these effects PGBx must be given prophylactically--acute administration produces, if anything, a slight impairment of contractility. Here then, is another limitation restricting the conditions of use. If such limitations in dose and time of administration were to operate in the clinical situation, the likelihood of deriving real benefit from PGBx use would seem small indeed.

Although they operate by different mechanisms, verapamil and propranolol will reduce the work load of the oxygen deprived heart and thereby tend to restore the balance between oxygen demand and oxygen availability. The imposition of calcium transport blockade (with verapamil) or beta-adrenoceptor blockade (with propranolol) clearly diminishes contractility of canine papillary muscle during the early 15 min pre-occlusion period as expected (Figs. 4 and 5). These drugs did not however offer any protection during the ischemic period. On the contrary, the direct effects of these drugs on the blood deprived myocardium promote the rapid loss of contractility and all treated tissues went into standstill (Fig. 4). The direct effect of PGBx, contrasted with those of verapamil and propranolol, is illustrated in Fig. 5. Both in terms of the initial pre-occlusion effects as well as the early occlusion effects the compounds exhibit dissimilar profiles.

Isolated guinea pig left atrium. The spontaneously beating guinea pig right atrial preparation has been employed by us to evaluate the biological activity of PGBx analogues and fragments. The introduction of graded concentrations of isoproterenol into the bath in which the tissue is submerged results in a correlated change in contractile intensity. The maximum contractile intensity (E_{max}) is produced by about 2×10^{-8} M isoproterenol. PGBx, 10 mcg/ml, will increase the E_{max} to about 150% of control, an effect which is impressive (3). PGBx will exert a similar effect on left atria that are electrically paced and primed with isoproterenol. Frequency-force analyses of these tissues reveal, however, that the presence of PGBx slightly reduces the functional reserve of the myocardium (Fig. 6) and in this regard the effect resembles that seen in the canine hearts.

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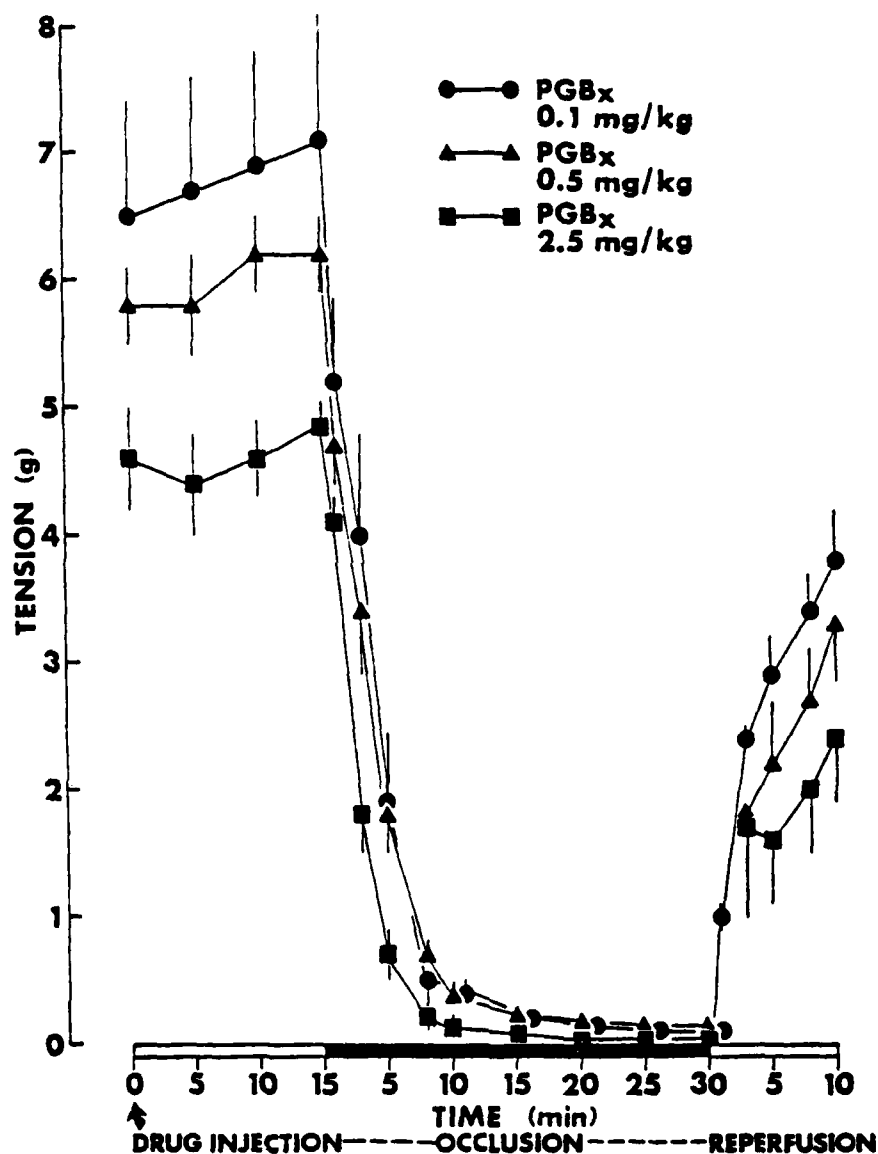
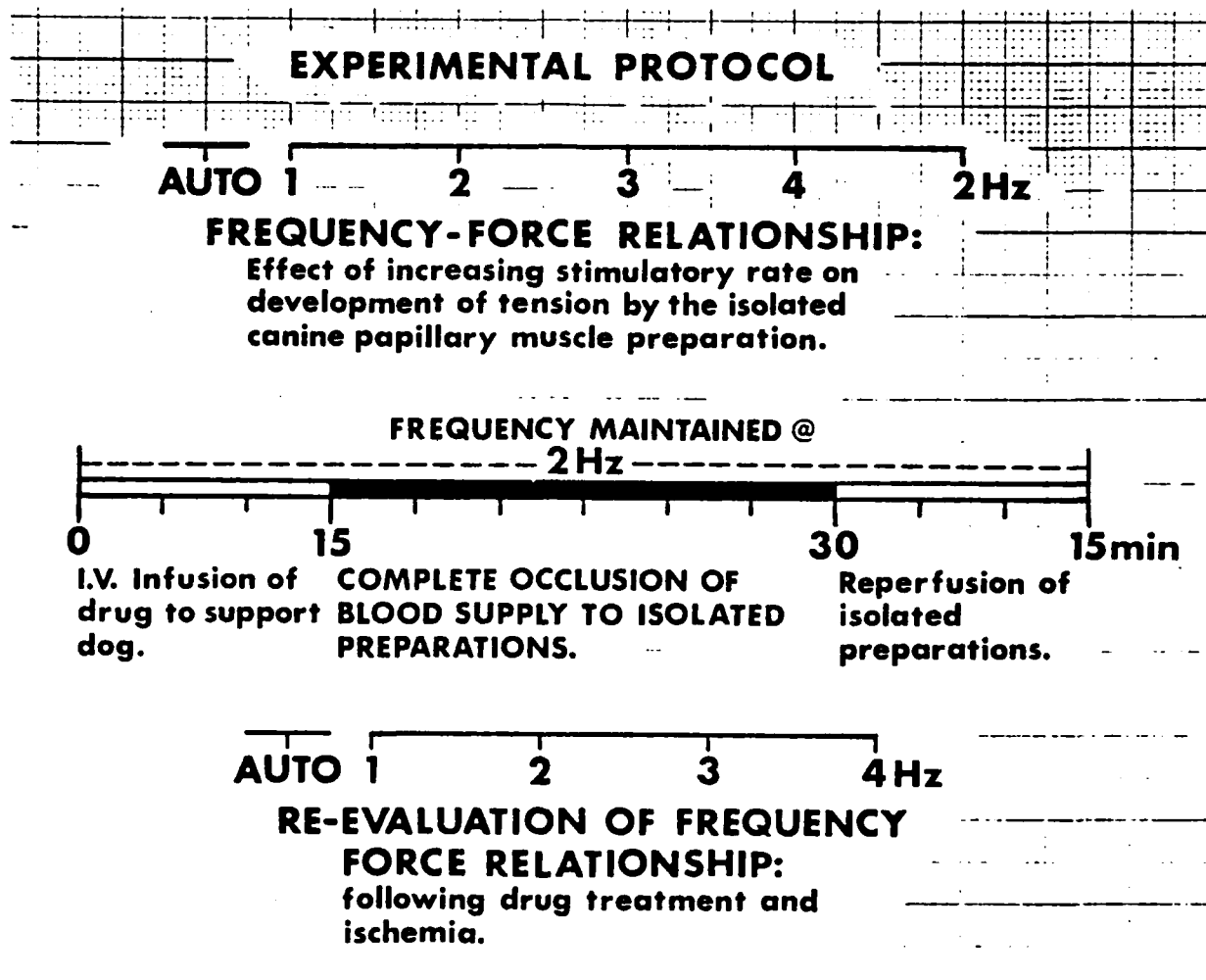


Figure 1.

Influence of PGB_x, administered i.v. into the support dog, on the systolic tension of oxygen deprived, electrically paced (2 Hz) canine papillary muscle. PGB_x was administered 15 min prior to arterial occlusion. The vehicle (control) curve is not illustrated but resembles the early part of the 0.1 mg/kg curve. The control curve, however, terminates at 20 min since all of these preparations went into standstill.



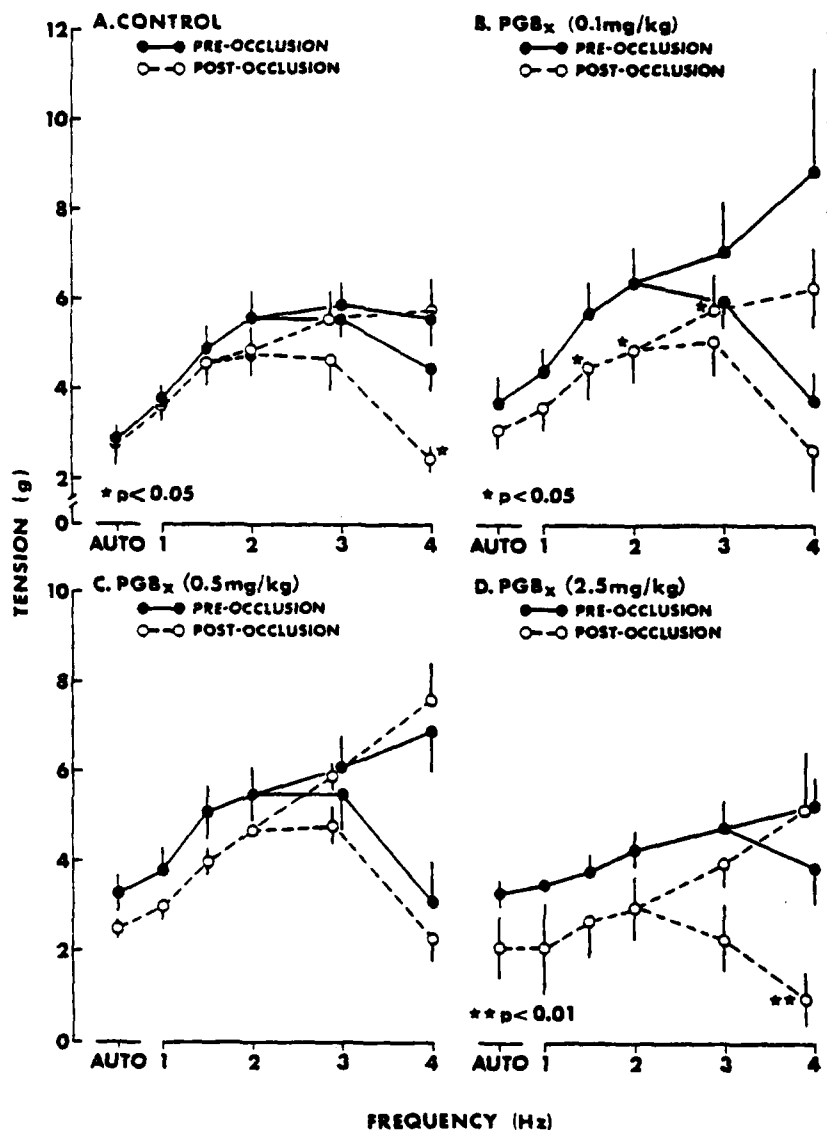


Figure 3.

Frequency-force curves for isolated canine papillary muscle before and after the ischemic period. AUTO represents the developed systolic tension of spontaneously beating tissues. The bifurcated curves that appear at about 2 Hz are a normal phenomena called "pulsus alternans". At higher stimulation frequencies the responses alternate between strong and weak contractions.

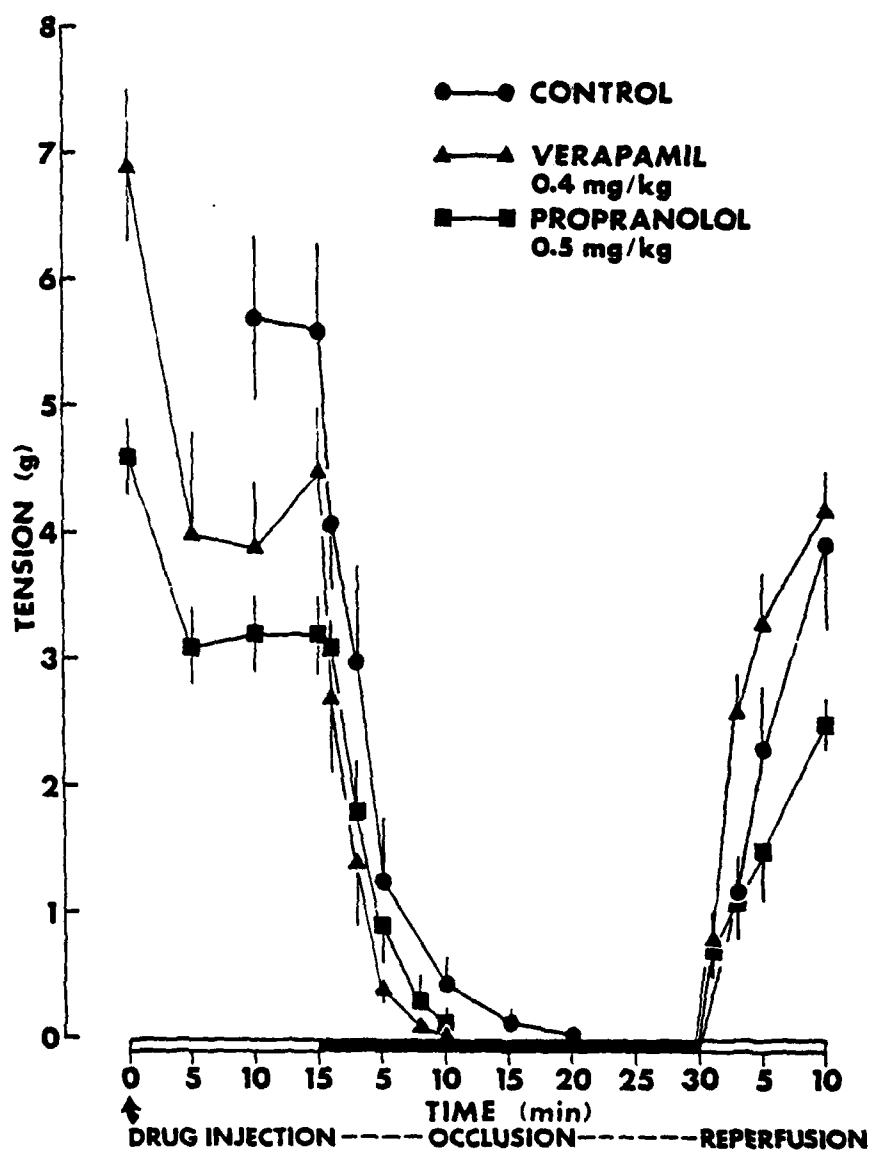


Figure 4.

Influence of verapamil and propranolol, administered i.v. into the support dog, on the systolic tension of oxygen deprived, electrically paced (2 Hz) canine papillary muscle. Drugs were administered 15 min prior to arterial occlusion.

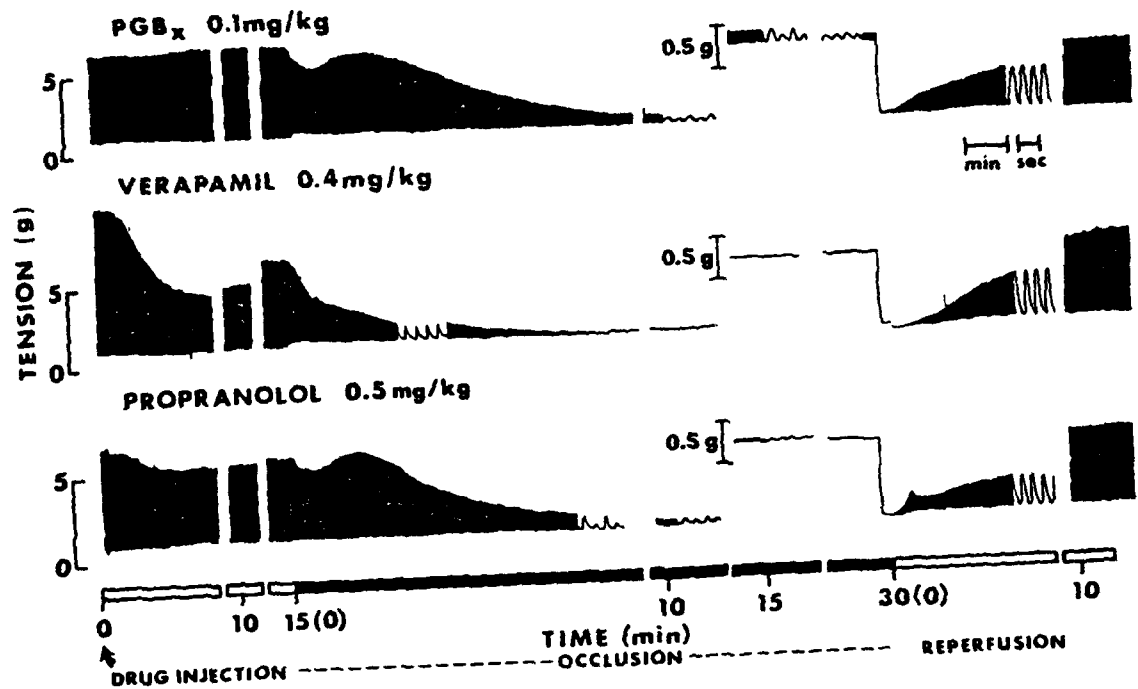


Figure 5.

Oscillographic patterns of contractile tension changes in response to drug administration prior, during and after arterial occlusion of the anterior septal artery of isolated canine papillary muscle. Elevation of baseline during the last 15 min of the occlusion period is the result of a purposeful change in recorder sensitivity.

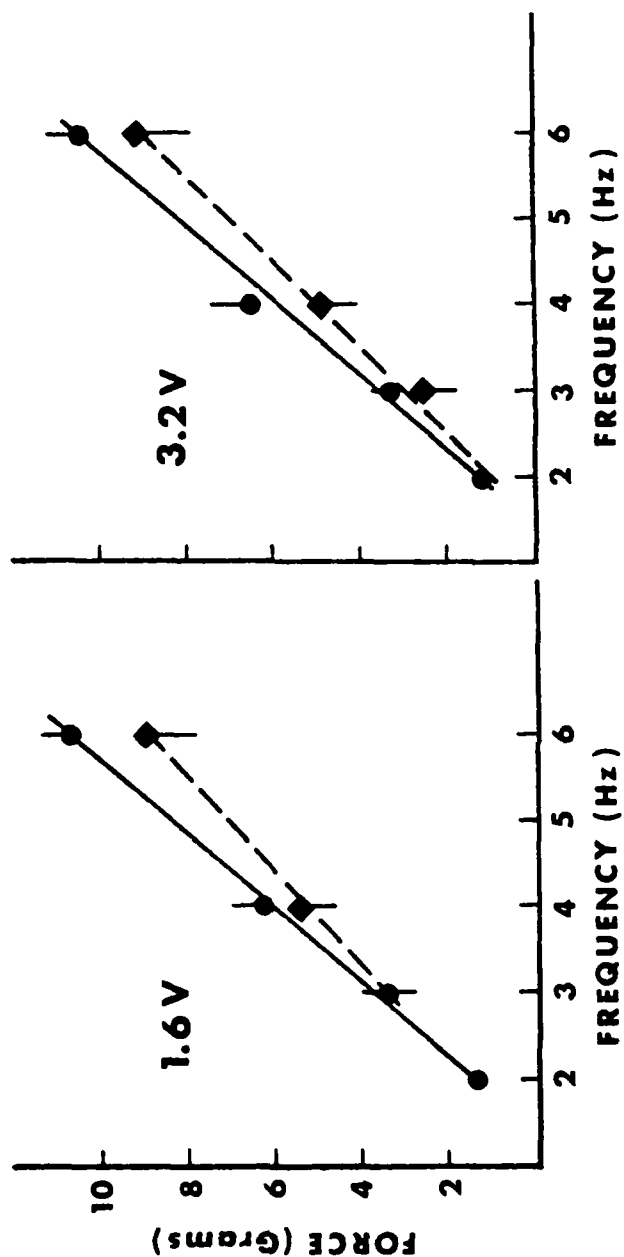


Figure 6.

Frequency-force relationships in paced guinea pig left atria at threshold (1.6 V) and 2 x threshold (3.2 V) stimulus intensities —●— control; —◆— PGBx, 10 mcg/ml bath; N = 5.

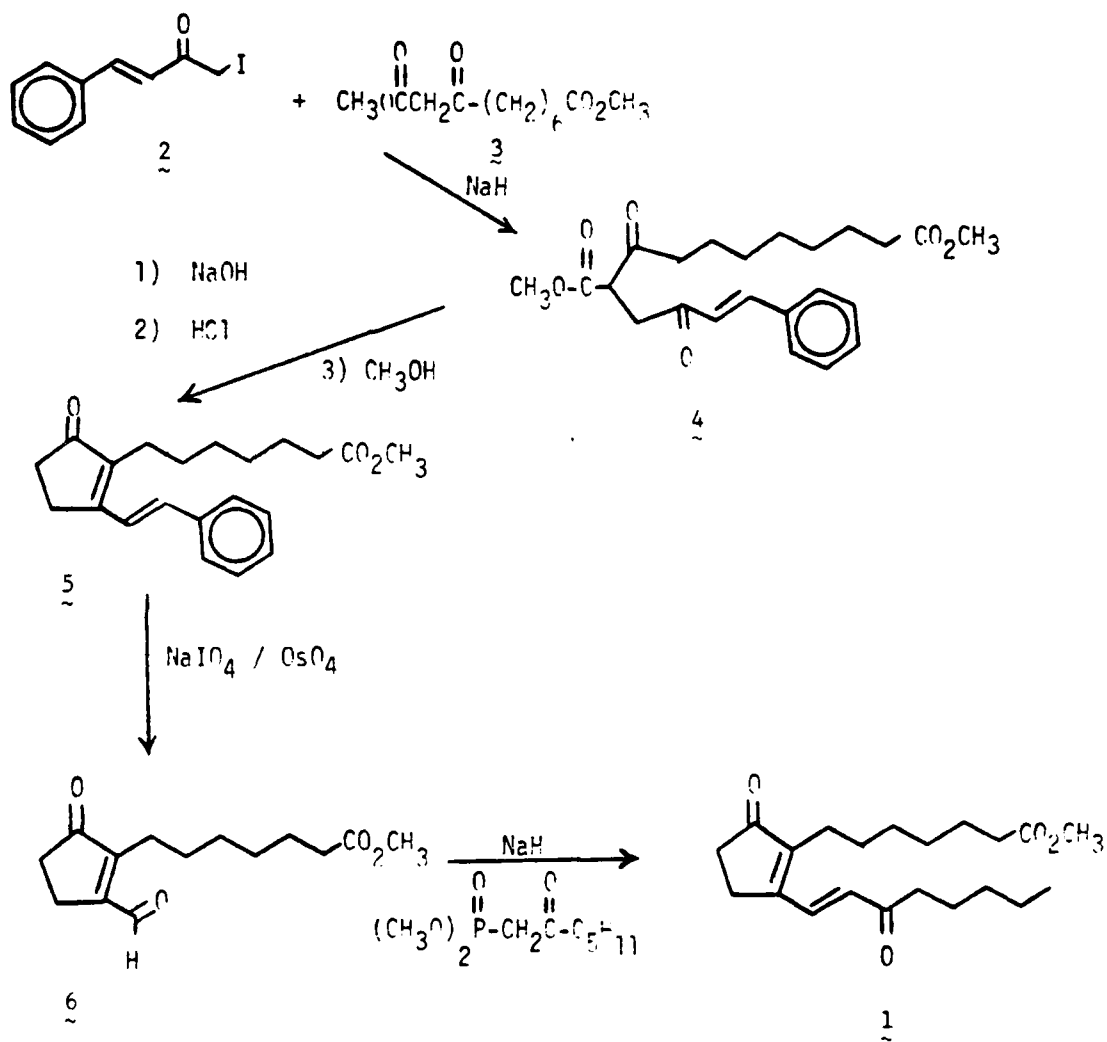
Synthesis PGB_x

The synthesis of PGB_x involves the treatment of the methyl ester of 15 keto-PGB₁ (1) with potassium hydroxide in aqueous ethanol.¹ Since there is a lack of a good economical source of the starting material, (1), we have directed our efforts towards finding a shortened synthetic sequence that would provide 1 on a large scale. In our effort to find such a sequence we also wanted such a synthetic scheme to provide intermediates that could be converted to analogs of 15-keto PGB₁ that in turn could be converted to PGB_x congeners. These PGB_x congeners could aid in the structural identification of PGB_x and provide insight into what structural features are needed for the observed biological activities of PGB_x.

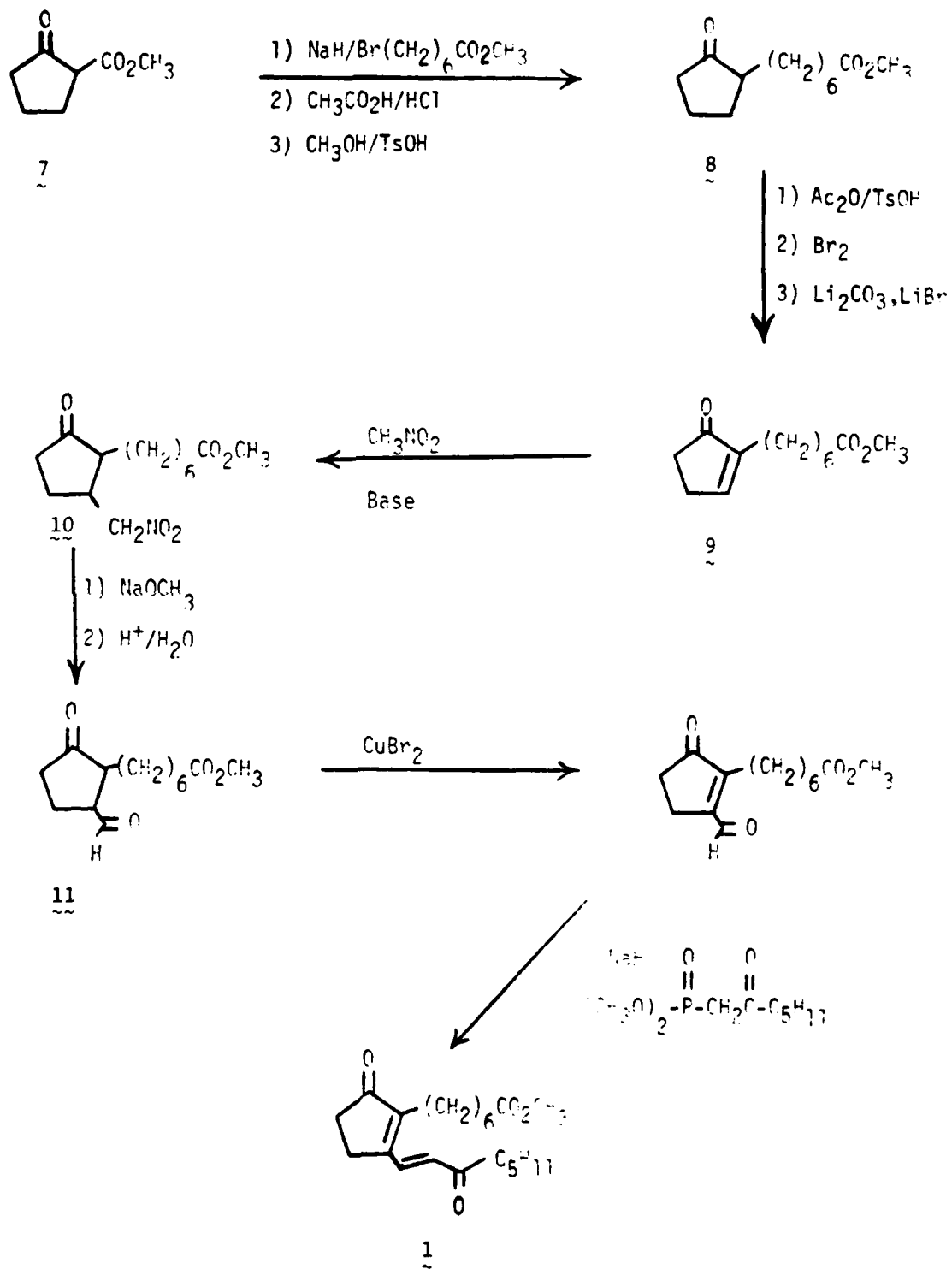
Initially, we developed a new synthesis of 15-keto-PGB₁ shown in Scheme 1. This scheme utilizes two known starting materials, 1-iodo-4-phenyl-3-buten-2-one (2)² and dimethyl 3-oxoundecan-1, 11-dioate (3)³. Although the initial alkylation of 3 with 2 in the presence of sodium hydride gives good yields of 4 we have had considerable difficulty in the cyclization step to give the substituted cyclopentenone 5. We could obtain small yields of 5 and it could in turn be converted to the desired methyl ester of 15-keto PGB₁ (1) as outlined. However, because of the difficulty with the cyclization step and after a number of attempted modification we turned our attention to a second synthetic sequence shown in Scheme 2.

The new synthetic sequence (Scheme 2) utilizes commercially available methyl 2-oxocyclopentanone carboxylate (7) and methyl 7-bromoheptanoate, prepared according to the procedure of Ames, Bowman and Mason,⁴ as starting materials. The formation of cyclopentanone 8 was carried out following the procedure of Bernady and co-workers.⁵ Condensation of the sodium salt of the methyl 2-oxocyclopentane carboxylate (7) with methyl 7-bromoheptanoate in glyme

SCHEME 1



SCHEME 2



provided a ketodiester and treatment of the diester with a mixture of 25% hydrochloric acid in glacial acetic acid followed by esterification with methanol in the presence of p-toluenesulfonic acid provided the monoester 8 in 71% yield. The procedure of Bernady and co-workers⁵ was found to be satisfactory for converting the substituted cyclopentanone 8 to the cyclopentenone 9. This procedure consists of refluxing ester 8 with acetic anhydride in the presence of p-toluenesulfonic acid to give an enol acetate that was brominated and the resulting α -bromoketone was treated with LiBr/Li₂CO₃ in dimethyl formamide to give crude 9 in 45% yield. The crude product was found to be contaminated with a small amount of an exocyclic olefin as reported by Bernady et al.⁵ We have found recently that treatment of 8 with cupric bromide in chloroform and ethyl acetate provided a better yield of cyclopentenone 9 in a single reaction step. Following the procedure of Barco and co-workers⁶ the cyclopentenone 9 was converted to the nitro compound 10 by Michael addition of nitromethane to 9 in the presence of 1,1,3,3-tetramethylguanidine. The nitroketone 10 was then converted to the sodium salt of the corresponding nitronic acid on treatment with an equimolar amount of sodium metabolite in methanol and hydrolyzed by dropwise addition to ice cold dilute sulfuric acid to yield keto aldehyde 11. Alternatively the nitroketone could be treated with titanium trichloride⁷ in glyme at room temperature to give 11 in 37% yield. The keto aldehyde 11 was allowed to react with a suspension of Cupric bromide in chloroform-ethyl acetate to give unsaturated aldehyde 12 in 40% yield. The reaction of compound 9 with dimethyl (2-oxoheptyl) phosphonate in the presence of sodium hydride gave the desired methyl ester of 15-keto PGB₁. We are now using this synthetic scheme to provide gram quantities of 1 for conversion to PGB_x.

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Studies on the Separation of PGB_x

The studies reported herein were performed by Dr. G. P. Dhareshwar who is on research leave from a pharmaceutical firm in Bombay and holds the Research Associate appointment. After attempting a variety of separations some of which were previously indicated by the work of Dr. S. F. El-Naggar (the first postdoctoral appointee on the project) a reexamination of the use of Sephadex LH-20 was made. This technique was adopted by the original investigators of PGB_x and we were aware of their results. The PGB_x material when placed on a column of Sephadex LH-20 in methanol is eluted as a broad peak which contains the activity and can be arbitrarily cut into fractions. We repeated this procedure and obtained the previously reported results. There were some differences in activities of the fractions; for example, the six arbitrarily formed fractions had activities in the restorations of oxidative phosphorylation in aged mitochondria of 71, 65, 141, 134, 76 and 31% of the original crude PGB_x (taken at 100%). Rechromatography of the Sephadex LH-20 fractions under the same conditions (methanol as eluant) took a considerable amount of time and effort but it was felt that there was a chance a purification that could be obtained by this relatively mild technique. However, monitoring these fractions by thin layer chromatography on silica gel G with the solvent system of methanol--ethyl acetate--acetonitrile--0.1N ammonium hydroxide (2:15:5:5 , upper phase) and visualization by ultra-violet light (adsorbent contained a fluorescent indicator which was quenched by PGB_x constituents causing zones to appear as dark regions on a fluorescing background) showed scarcely a difference between fractions. Clearly, this method was not going to provide us unique fractions.

Although Sephadex LH-20 is most generally thought of as a molecular seive or molecular exclusion material which effects a separation on the basis of molecular size (and shape), experience in our laboratory in another study showed that it has unusual adsorptive properties. Application of PGB_x to a Sephadex LH-20 column poured in chloroform and followed by elution with chloroform and chloroform containing successive increments of methanol gave separation into several discrete peaks (Figure 1). In this experiment, 230 mg of PGB_x was separated on 115 gm of the adsorbent. To determine how much material could be effectively separated, three experiments on the same column were performed utilizing 450 mg, 950 mg and 1.2 g of PGB_x . The 450 mg sample could still be resolved but beyond that too much overlapping occurs. In following the separation, the effluent was monitored by ultraviolet light absorption at 310 nm. Although this value is beyond the absorption maximum of PGB_x (about 240 nm) the large quantities of material used in the separation necessitated a shift to the higher wavelengths, if the analytical spectrophotometer was to be effective. A thin layer chromatographic analysis of the nine fractions of the separation in Figure 1 now showed a definite separation was occurring (Figure 2).

The separations studies reported above were with PGB_x preparations obtained from Professor D. D. Miller and his co-workers, and up to this point we had no basis to compare these products with the PGB_x material obtained from Dr. H. W. Schmuckler. A separation of the three fractions (samples #28:Fr. 1, Fr. 2 and Fr. 4) on the Sephadex LH-20 column with the chloroform--methanol solvent system showed it was possible to resolve this purified fractions further (Figure 3). The column subfractions made in the manner indicated in the figure were submitted for testing in the restoration of

oxidative phosphorylation by aged mitochondria, with the results reported in Tables 1 and 2. Of the two end points in the test (restoration of activity and inhibition of phosphorylation) which measure the biphasic nature of the crude PGB_x , the column fractions show no separation of the two activities. There is apparently some differences in specific activities, but not one fraction was obtained that was completely inactive. Consequently, the problem reduces itself to not simply seeking out one or several active constituents but looking for the most potent. Since the materials are very closely related, the differences in specific activity will probably not vary in orders of magnitude. Also, it is desirable to obtain the constituent with the lowest inhibitory activity.

A preparation equivalent to Fraction 7 of the separation shown in Figure 1 but on a larger scale was rechromatographed (Figure 4) and the Fraction A of that separation again chromatographed (Figure 5). This procedure was repeated four more times (Figures 6, 7, 8 and 9) and the largest peak of the last three separations was assayed in the oxidative phosphorylation test (Table 3). This study demonstrated that rechromatography although very time consuming could yield PGB_x fractions that formed discrete peaks in spite of the fact that their physical properties differ little. A thin layer chromatographic separation (Figure 10) showed at least three closely related spots of approximately the same size at $R_f \sim 0.45$. Thus, the single peak is still a mixture, but compared to the original material a much cleaner fraction. The bioassay suggests that the largest peak is possibly losing the inhibitory activity while still retaining the protective potency.

Application of this tedious and very time consuming rechromatography to other fractions from the first separation (as in Figure 1) resulted in

similar multipeak patterns. The many elution diagrams obtained are not included in this report as they would add little to the example already illustrated. However, a summary as a flow chart (Figure 11) of the repeat chromatographic separations will show the extent of the work performed in this regard. Spectral examination (^1H nmr mass) of the purest appearing peaks showed little differences from other fractions. We concluded that spectral studies were premature as the cleanest fractions were still mixtures and no useful purpose would be served in collecting this data.

This past year's work has given us a clearer picture of the PGB_x problem. In summary, they are as follows: First, the activity of PGB_x (crude) does not appear to reside in a single or several very closely related substances. Second, separation of the mixture into clearly separated fractions has been obtained for the first time on a preparative scale. Third, restoration of the oxidative phosphorylation in aged mitochondria activity can be separated from the inhibitory activity, but as yet not completely. It cannot be said whether the two activities can be entirely separated or that both can reside with the same molecule as single material fractions have not been isolated. Fourth, a method (Sephadex LH-20 with chloroform--methanol) is available to fractionate the crude PGB_x into fractions that could be further purified by other methods. It will, however, take a large quantity of crude PGB_x and much column chromatography to get homogeneous material in sufficient amounts for pharmacological, spectral and chemical analyses. Unfortunately, the project has been terminated and we will not be able to accomplish this goal. It is hoped that the results reported here could be of use to anyone following up this study.

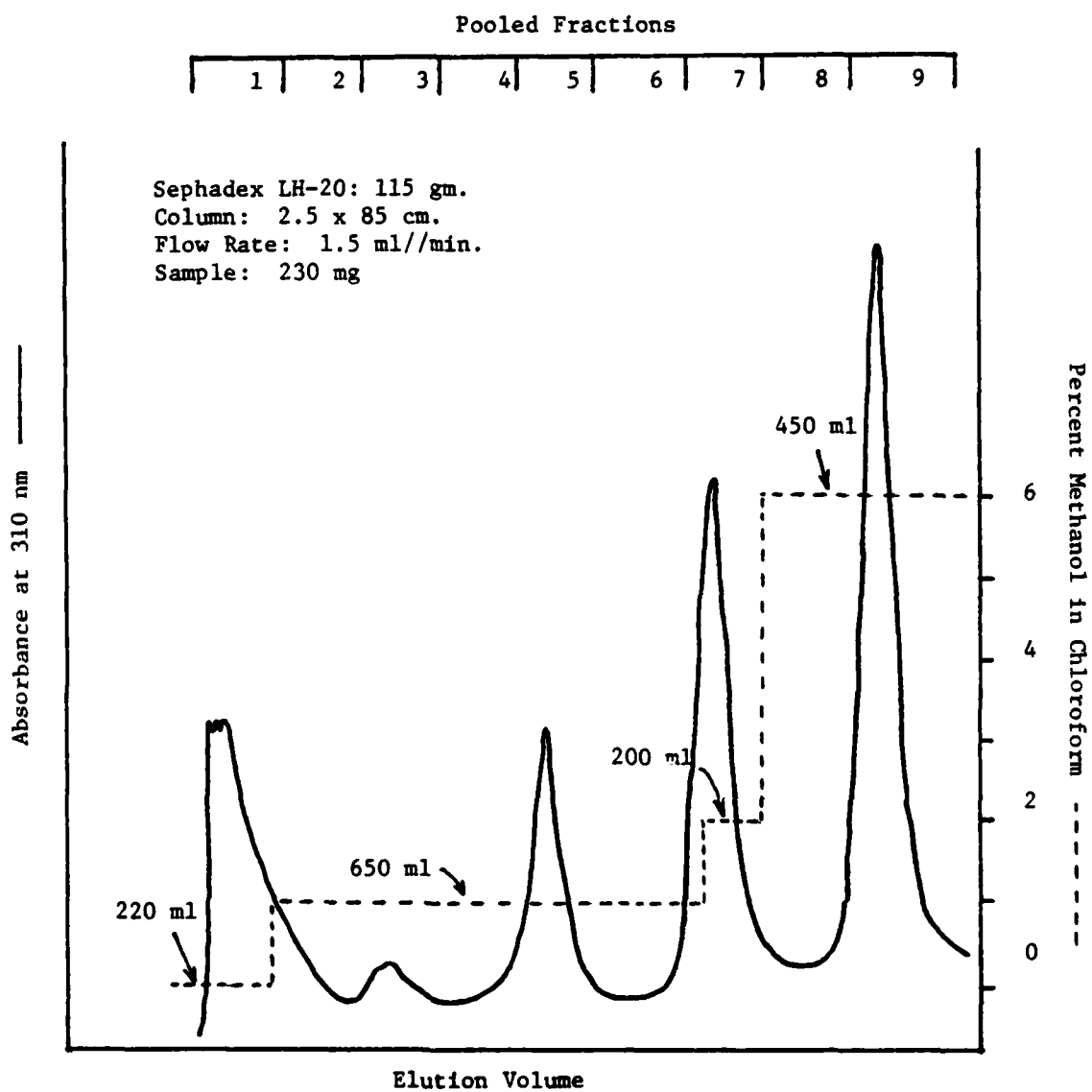


Figure 1: Chromatography of PGB_x on Sephadex LH-20 with Chloroform--Methanol Mixtures

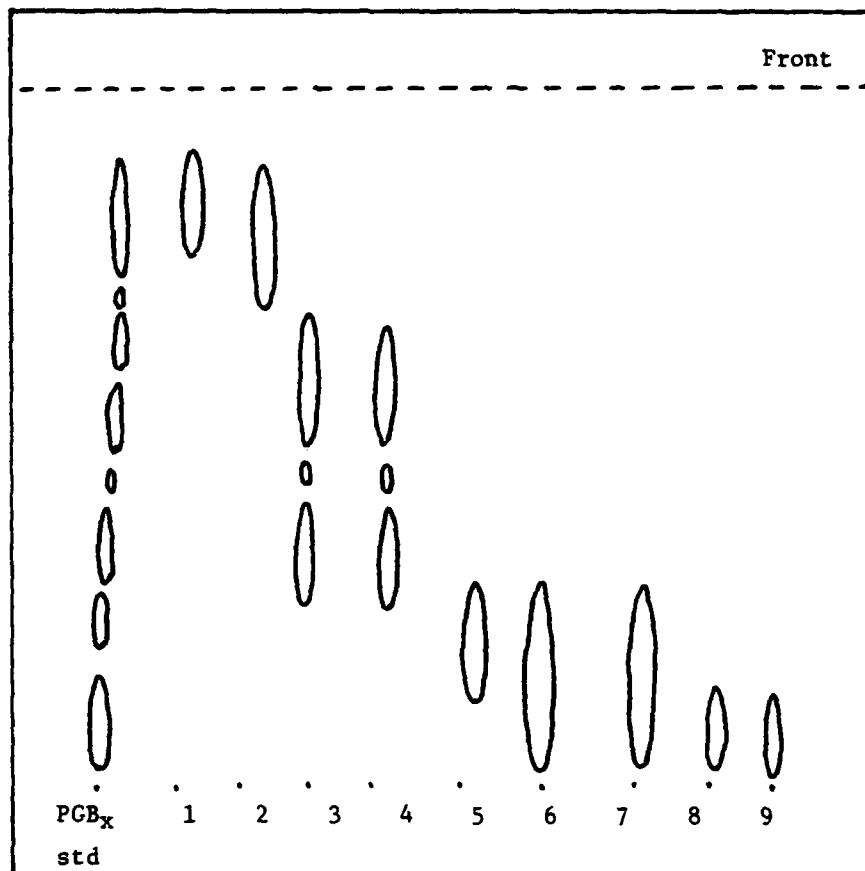


Figure 2: Thin Layer Chromatography of PCB_x Fractions from Column Separation Shown in Figure 1.

Silica Gel - Kieselguhr Plates

Solvent System: MeOH--EtOAc--MeCN--0.1NNH₄OH
(2:15:5:5, upper phase)

Detection: UV absorption

Pooled Fractions

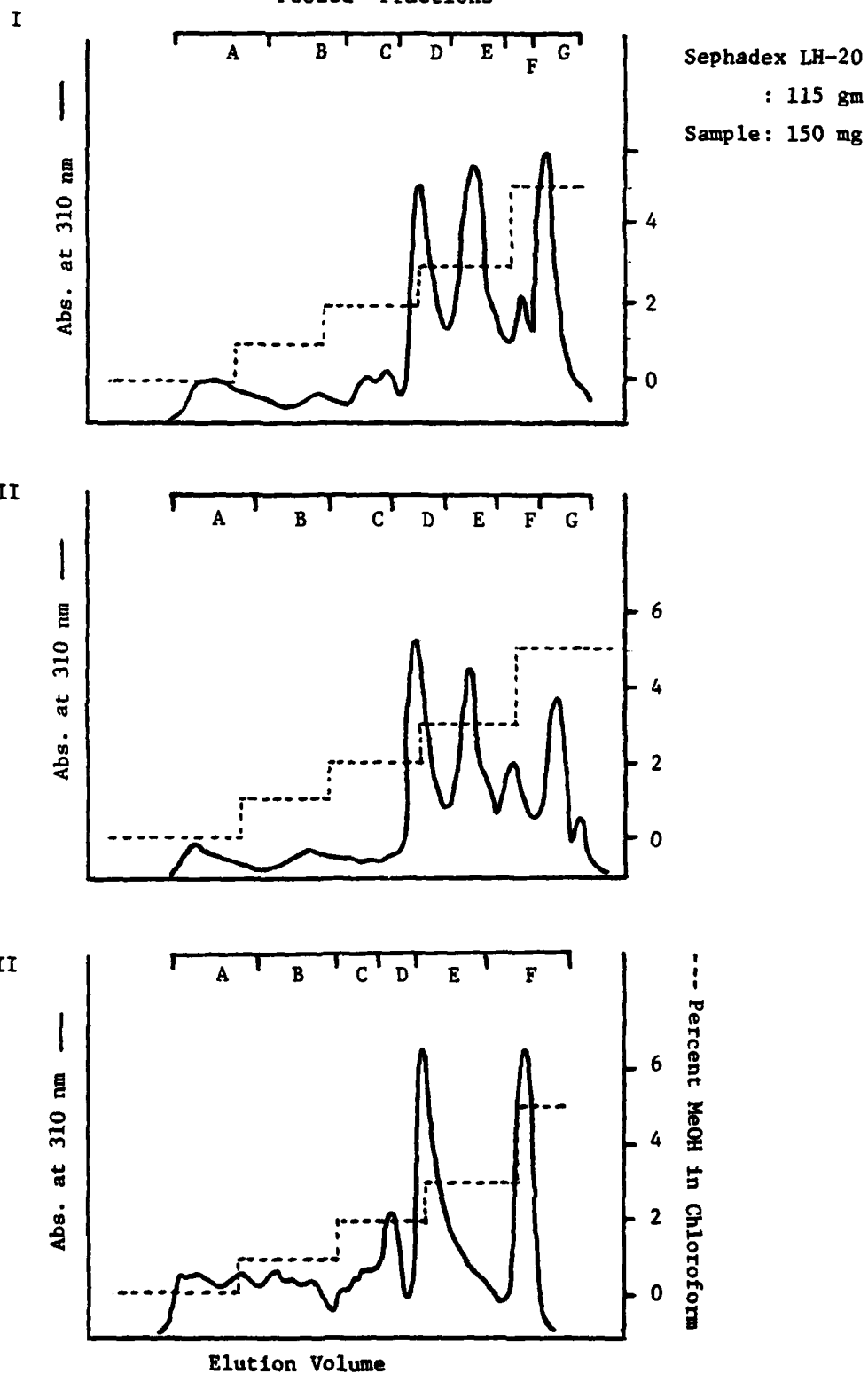


Figure 3: Chromatography of PCB_x Fractions from Dr. Schmuckler; I, Sample #28 Fr.1; II, #28 Fr.2; and III #28 Fr.4.

Table 1. Evaluation of PGB_x Fractions from Sephadex LH-20 Chromatography
in Restoration of Oxidative Phosphorylation

Sample	Protection of Oxidative Phosphorylation		Inhibition of Oxidative Phosphorylation
	% PGB _x Std. Activity 10 µg	20 µg	% PGB _x
#28, Fr. 1 Std.	125	100	60
1A	70	90	60
1B	20	30	40
1C	25	20	20
1D	25	20	35
1E	25	20	60
1F	40	60	90
1G	15	15	90
#28, Fr. 2 Std.	60	80	100
2A	50	50	30
2B	80	90	~30
2C	110	90	~30
2D	120	125	60
2E	110	100	90
2F	135	140	95
2G	75	105	95

Table 2. Evaluation of PGB_x Fractions from Sephadex LH-20 Chromatography
in Restoration of Oxidative Phosphorylation

Sample	Protection of Oxidative Phosphorylation*		Inhibition of Oxidative Phosphorylation**
	% PGB _x Std. Activity		% PGB _x
#28, Fr. 4 Std.	90		70
4A	45		60
4B	45		85
4C	45		100
4D	70		80
4E	100		110
4F	140		100

* Protection of Oxidative Phosphorylation with Mitochondria Preincubated at 30°C; samples tested at 10, 20 and 60 µg/2.8 ml.

** Inhibition of Oxidative Phosphorylation at 80 µg/2.8 ml.

Fig. 4

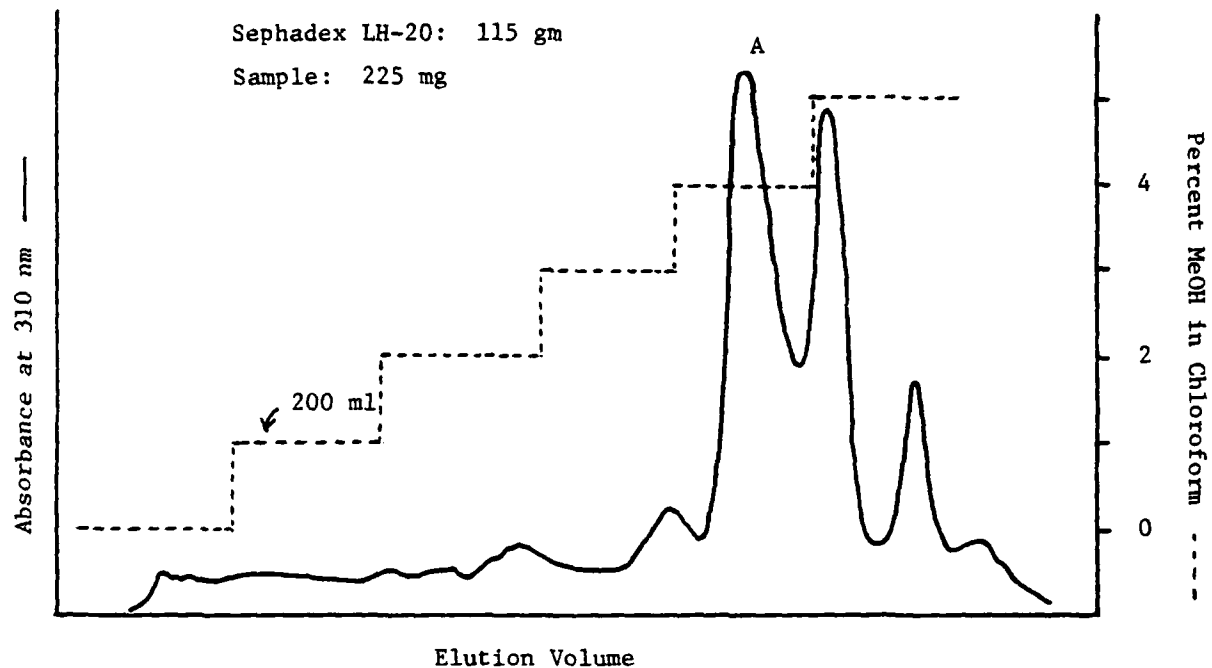


Fig. 5

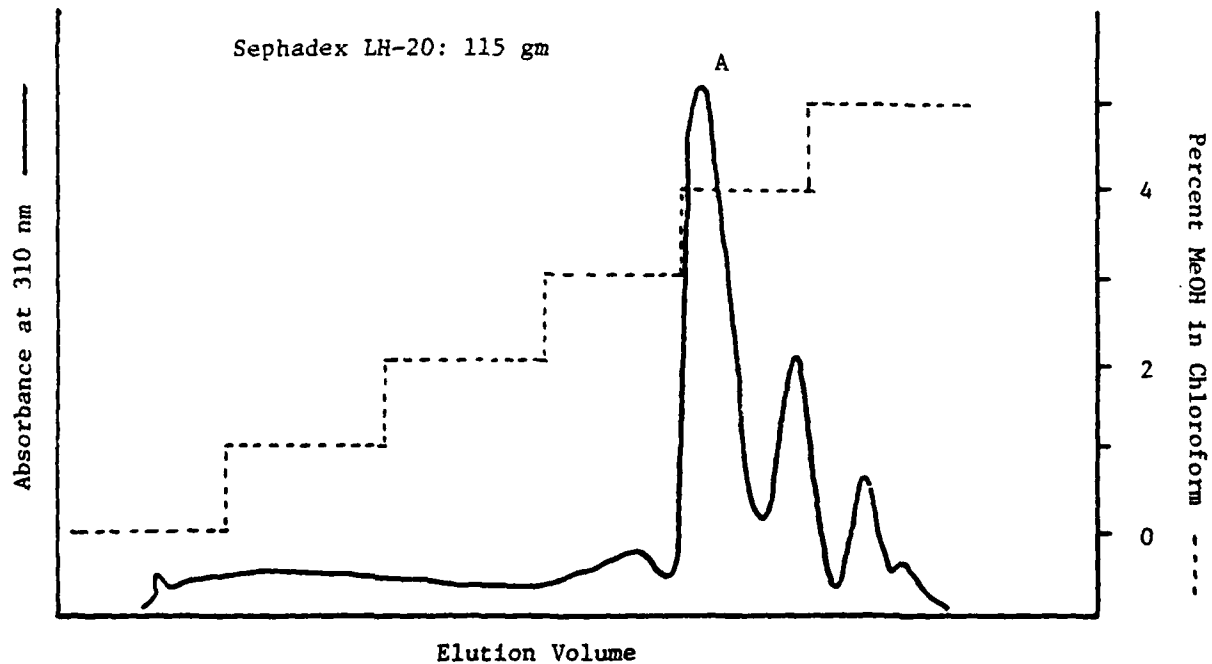


Figure 4: Rechromatography of FR. #7 of Figure 1 Type Separation

Figure 5: Rechromatography of Peak A of Figure 4 Separation

Fig. 6

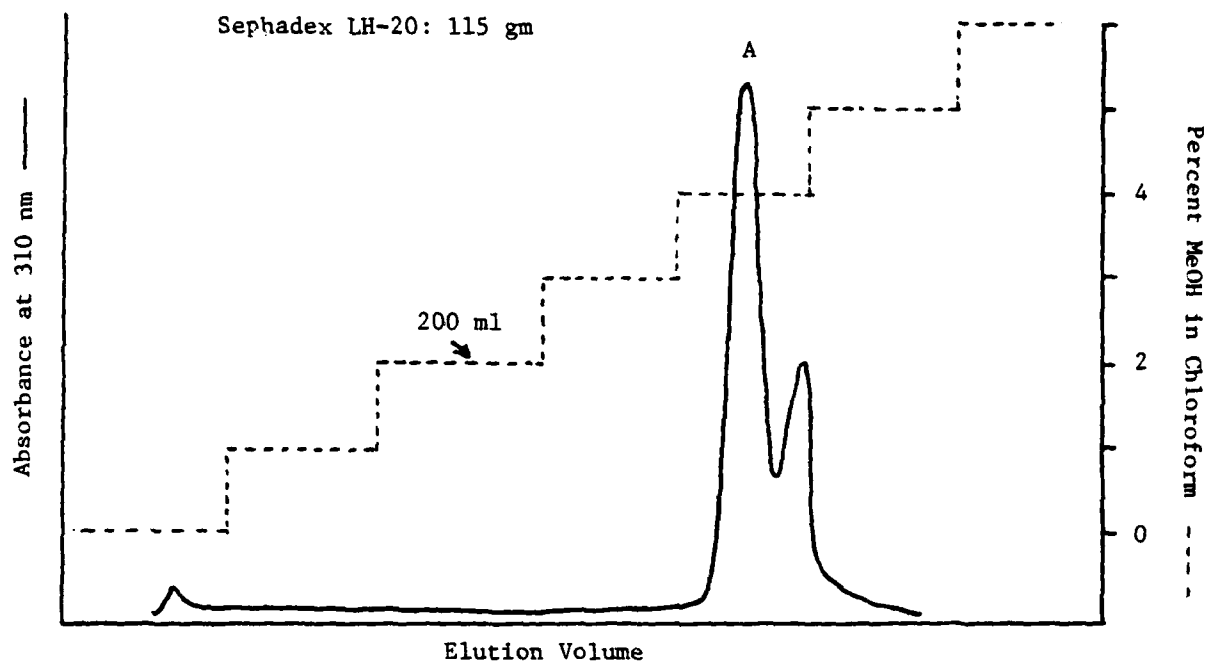


Fig. 7

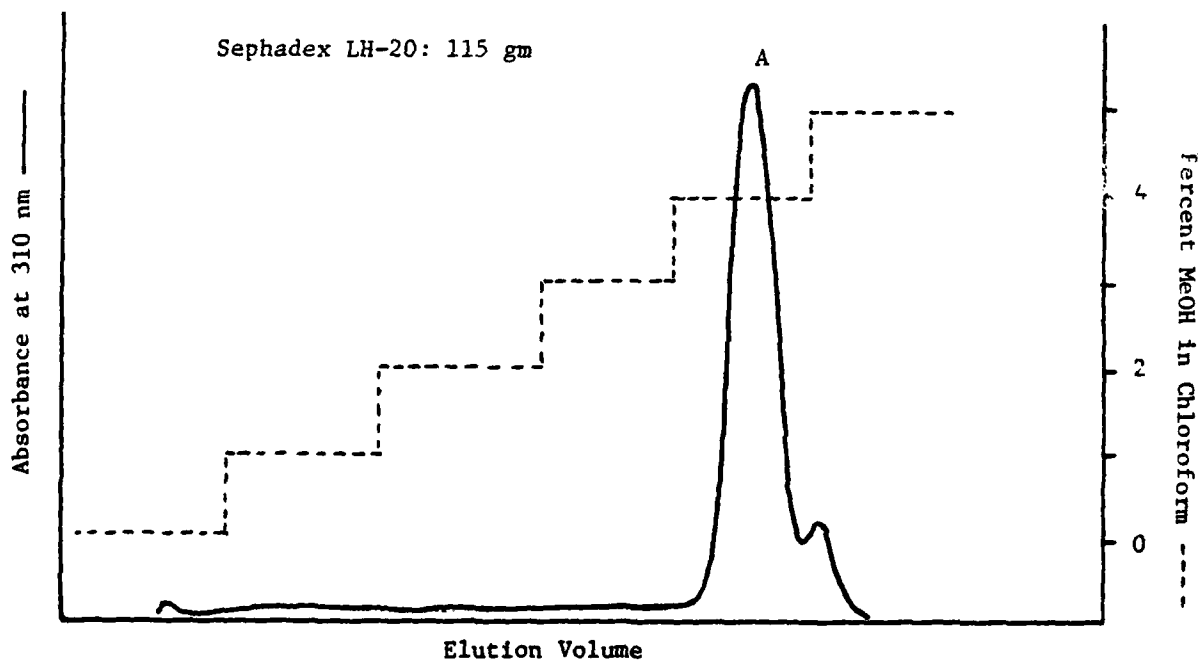


Figure 6: Rechromatography of Peak A of Figure 5 Separation

Figure 7: Rechromatography of Peak A of Figure 6 Separation

Fig. 8

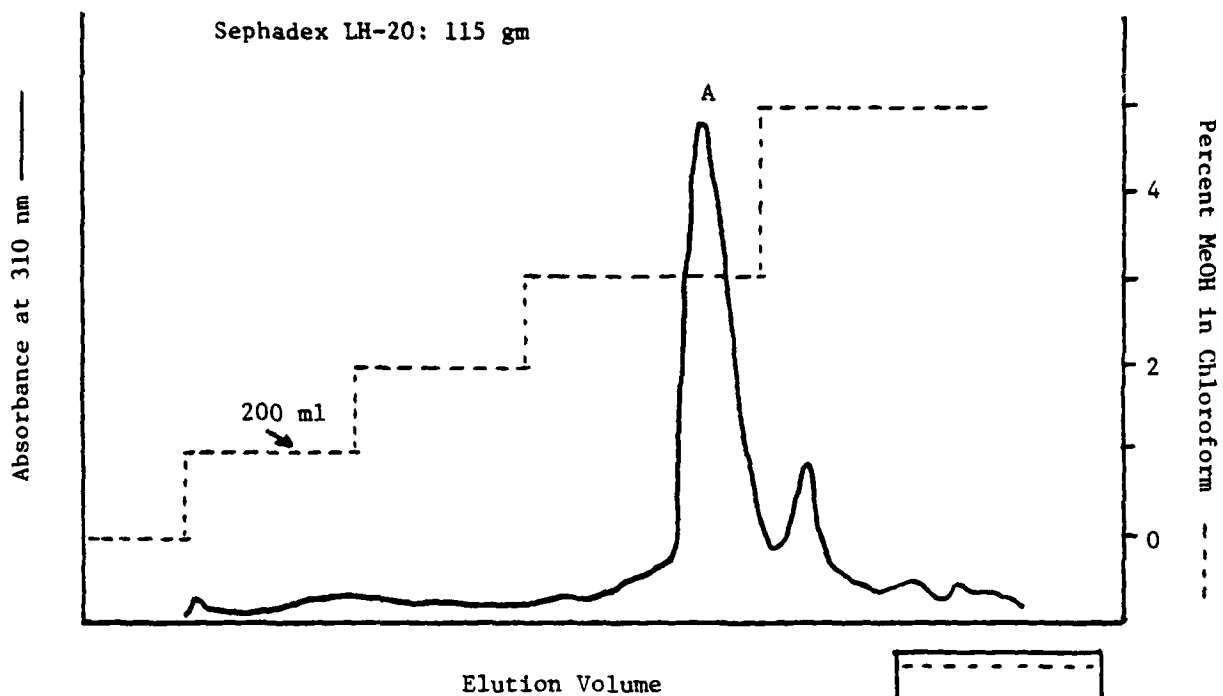


Fig. 9

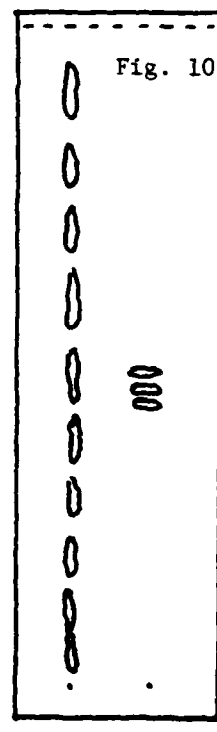
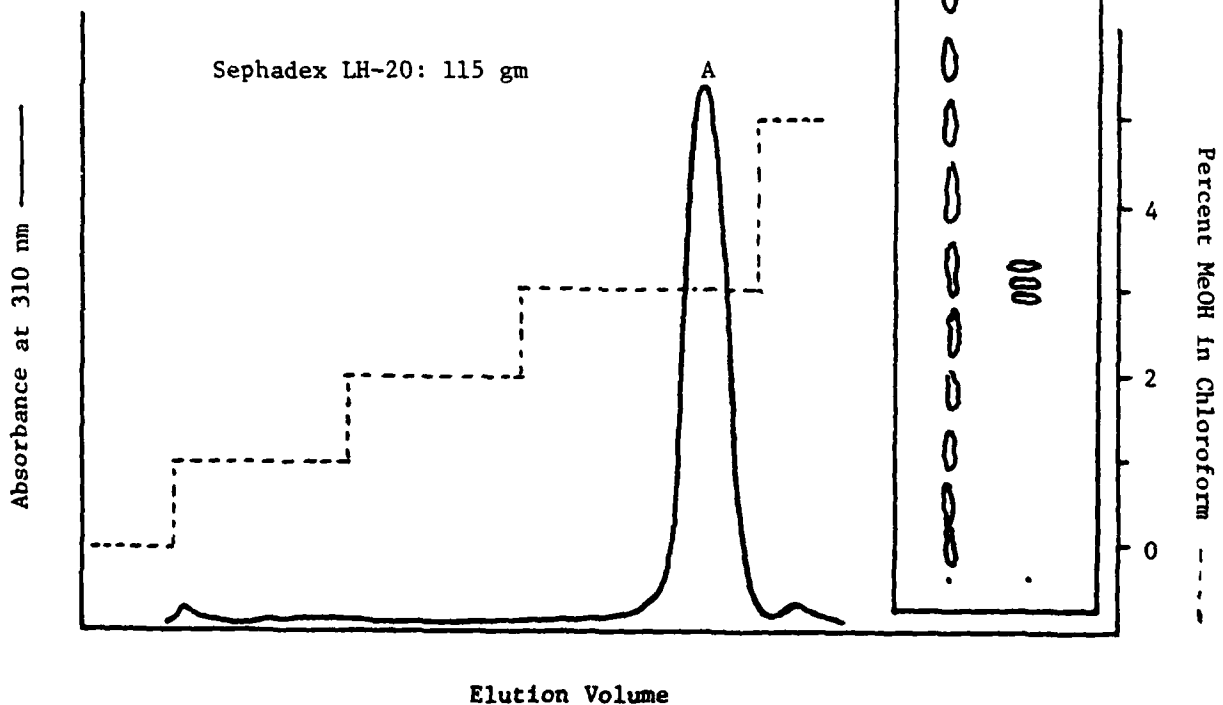


Figure 8: Rechromatography of Peak A of Figure 7 Separation

Figure 9: Rechromatography of Peak A of Figure 8 Separation

Figure 10: TLC of Peak A of Figure 9 and PGB_x Crude

Table 3. Evaluation of PGB_x Fractions from Repeated
Chromatography of Fraction 7 of Figure.

Sample	Protection of Oxidative Phosphorylation* PGB _x = 100%	Inhibition of Oxidative Phosphorylation** PGB _x = 100%
A of Figure 7	80%	115%
A of Figure 8	110%	55%
A of Figure 9	110%	30%

* Samples tested at 30 and 60 µg/2.7 ml for protection of preincubated mitochondria; results in comparison to PGB_x

** Samples tested at 80 µg/2.7 ml for uncoupling of mitochondria not preincubated; results in comparison to PGB_x

Crude PCB_x

(450 mg)

Sephadex LH-20

MeOH-Chloroform

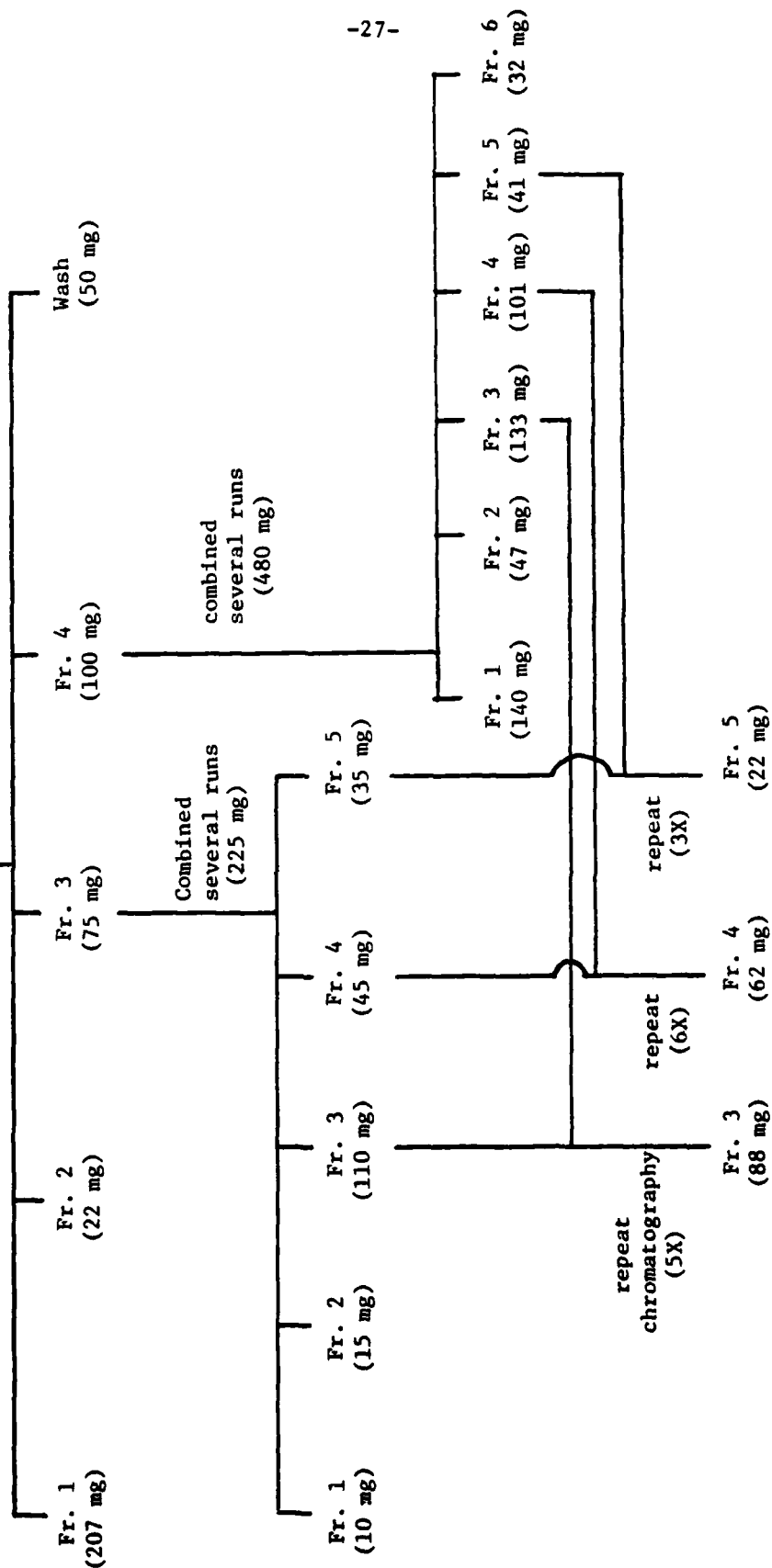


Figure 11: Summary of Separation of PCB_x (2.6 gm) on Sephadex LH-20 with Methanol--Chloroform Mixtures.