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It was also noted that some of the variability in ultrastructure, recently reported by others with PYR treatment, might be due to artifacts. Subsequently, all tissues were processed using a 2 hr fixation in Karnovsky's fixative. Three groups of rats (approximately 200g) were used in the ultrastructural study. The control group was injected with buffer, the treated group was injected with 3.6mg PYR/kg, and a group was pretreated with 0.05mg nifedipine/kg 15 min prior to injection with 3.6mg PYR/kg. The consistent component of the

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Pyridostigmine induced alterations in rats: Impact of body weight and nifedipine pretreatment.

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Pyridostigmine induced alterations in rats: Impact of body weight and nifedipine pretreatment. Bowers, W.D.,Jr., Blaha, M., Castro, I., and Daum, P.(1988). Fundam. Appl. Toxicol.

Abstract: When rats were injected with pyridostigmine (PYR) based on a mg/kg dosage, lethality appeared to be greater than anticipated when 500g rats were used. Twenty-four hour survival was determined for groups of large and small rats. These animals received a sc injection of either Mestinon equivalent buffer, 1.8mg or 3.6mg PYR/kg body weight. Serum cholinesterase (ChE) levels were determined for similar doses. With PYR dosage based on mg/kg, small rats were significantly more resistant to PYR than large rats, and also had significantly higher control levels of serum ChE.

It was also noted that some of the variability in ultrastructure, recently reported by others with PYR treatment, might be due to artifacts. Subsequently, all tissues were processed using a 2 hr fixation in Karnovsky's fixative. Three groups of rats (approximately 200g) were used in the ultrastructural study. The control group was injected with buffer, the treated group was injected with 3.6mg PYR/kg, and a group was pretreated with 0.05mg nifedipine/kg 15 min prior to injection with 3.6mg PYR/kg. The consistent component of the early (30 min) focal lesion at neuromuscular junctions (NMJs) was the presence of multiple spherical translucent areas within mitochondria, distinct from swollen mitochondria. All rats treated with 3.6mg PYR/kg showed these alterations in postsynaptic cells, and they were frequently present in presynaptic nerves. Nifedipine, a calcium antagonist, did not improve the lesions. Similar structures have been reported with paraoxon and with monensin, a Na^+ ionophore. The latter may indicate some role for Na^+ influx in addition to that of Ca^{++} in the process of injury.

Pyridostigmine (PYR), a reversible carbamate inhibitor of acetylcholinesterase (AChE), has been shown to reduce the lethality of organophosphates when used as a pretreatment (Gordon et al., 1978; Dirnhuber, et al., 1979; Lennox et al., 1985). Although there were indications that this protection occurs at dosages which do not themselves produce symptoms (McDonough, 1983; Harris et al., 1986; Parker et al., 1986), Hudson et al. (1986) found dose dependent acute focal lesions produced at neuromuscular junctions (NMJs). In another study, designed to compare responses of fast and slow-twitch muscle, and acute versus subacute exposure to PYR, considerable variability in the extent of damage was reported (Hudson et al., 1985). The effects of other antiAChEs had been extensively researched, but ultrastructural data on PYR treatment were lacking. Their PYR studies reveal swollen mitochondria, retracted nerve terminals, Schwann cell processes extending into primary clefts, and disrupted filaments with tissues processed in solutions previously believed to eliminate mitochondrial swelling in exercised skeletal muscle (Gale, 1974; Bowers et al., 1974). Page (1986) reported similar changes, but indicated that several of these, including mitochondrial swelling, also occurred in controls. The work of Laskowski et al. (1977) with paraoxon suggested that fixation artifacts could be avoided by using a fixative similar to Karnovsky's (1965) fixative. Brewer and Lynch (1986) completely avoided aldehyde fixation in their examination of NMJs by using freeze substitution, but they had to discard material which contained freezing artifacts.

Our interest in this research related to indications by Dretchen et al. (1985) that nifedipine, a calcium antagonist, raised the dose of antiAChE required to produce an LD_{50} in mice. If nifedipine decreased susceptability to an antiAChE, it might also affect the ultrastructural lesions produced by PYR

since Leonard and Salpeter (1979) demonstrated the involvement of Ca^{++} influx in the process of agonist-induced myopathy, and since Ca^{++} flux is normally involved in both release of acetylcholine (McComas, 1977) and muscle response. The original intent was to reproduce the ultrastructural lesions described by others and to determine whether nifedipine reduced the damage produced by PYR. When the procedures of Hudson et al. (1986) were reproduced or when our routine procedures were used, swollen mitochondria were detected in some of the controls. This was reduced with Karnovsky's (1965) fixative, as noted by Laskowski et al. (1977). Also, large rats (500g) appeared to be more sensitive to PYR than anticipated.

After preliminary work with processing and dosage, this study (1) compared survival rates for large (mean = 494g/120 days) and small (mean = 219g/56 days) rats, (2) obtained a measure of serum cholinesterase (ChE) levels in large and small rats, and (3) evaluated ultrastructural lesions in rat diaphragms from control rats, from rats treated with 3.6mg PYR/kg body weight, and from rats pretreated with 0.05mg nifedipine/kg body weight prior to PYR treatment. The ultrastructural experiment was conducted under conditions (2 hr fixation with Karnovsky's fixative) similar to those used by Laskowski et al. (1977) where artifacts were reduced in control tissues.

Methods

The experimental design was based on results in which groups of two to five large rats were injected with Mestinon equivalent buffer, 1.0mg PYR/kg or 3.6mg PYR/kg body weight and processed after 30 minutes in our usual fixative (2.5% glutaraldehyde in 0.052M cacodylate buffer pH 7.4 with 6% sucrose), according to Hudson et al. (1986) or with the fixative of Karnovsky (1965). Subsequently, survival, serum ChE and ultrastructure were examined as indicated below.

Survival - large versus small rats

Thirty-five rats in the 475-525g weight range (Group I) and 35 rats in the 175-250g weight range (Group II) were used. Pyridostigmine bromide (Mestinon-Roche Laboratories, Nutley, NJ) or its equivalent carrier buffer was injected sc in the mid-back region (Hudson et al., 1986) according to the following schedule:

Group I (475-525g)

A. Controls (10) - injected with equivalent buffer

B. Treated (10) - injected with 1.8mg PYR/kg

C. Treated (15) - injected with 3.6mg PYR/kg

Group II (175-250g)

D. Controls (10) - injected with equivalent buffer

E. Treated (10) - injected with 1.8mg PYR/kg

F. Treated (15) - injected with 3.6mg PYR/kg

All animals were returned to their individual cages and monitored for 24 hour survival. The Chi-square statistic ($P<.05$) was used to analyze two and three dimensional contingency tables.

Serum Cholinesterase - Large versus small rats

In order to relate survival and lesions to some indicator of the level of inhibition induced by PYR, the Boehringer Mannheim Diagnostics (Indianapolis, IN) Reagent Set for Cholinesterase #124117 was used. A Gilford Stasar III spectrophotometer set for 405nm was used to monitor optical density (O.D.) at 25°C. All reactants were placed in a water bath at this temperature for 10 minutes prior to mixing to allow equilibration. Serum was used because it produced the most consistent results. The chemical blank showed a constant increase in optical density (O.D.) of 0.003 units/minute. Serum (0.08-0.1ml), whether inactivated by heat or inhibited by Eserine (Sigma, St. Louis, MO),

caused an additional constant increase of 0.004 O.D. units or a total of 0.007 O.D. units/minute. The latter value was subtracted from all O.D. readings before conversion to units of enzyme activity. This value would be insignificant for normal serum with 0.02ml sample volume. However, the low level of activity in the PYR inhibited serum necessitated an increase in serum test volume from 0.02ml to 0.1ml. With control serum, 0.08ml was used. This volume was as close as possible to the 0.1ml volume used with inhibited serum, without inducing substrate depletion during the test. The standard formula in the test kit corrected for differences in sample volume.

After conditions for assaying rat serum were established, groups similar to those in the survival experiment and similar sc injection procedures were used:

Group III (475-525g)

A. Controls (5) - injected with equivalent buffer

B. Treated (5) - injected with 1.8mg PYR/kg

C. Treated (5) - injected with 3.6mg PYR/kg

Group IV (175-225g)

D. Controls (5) - injected with equivalent buffer

E. Treated (5) - injected with 1.8mg PYR/kg

F. Treated (5) - injected with 3.6mg PYR/kg

The chest cavities of rats that died were immediately opened and approximately 5ml samples of blood were taken from the hearts. Animals that survived were anesthetized with pentobarbital after 25 minutes, and blood was obtained as indicated above after 30 minutes. Blood was allowed to clot and serum was separated after centrifugation. O.D. readings were taken each minute for the first four minutes after mixing serum with assay components. The three values for change in O.D./minute were averaged and each serum sample was run in

triplicate. PYR dissociates from the enzyme in the assay buffer after periods longer than six minutes. Samples were either run on the day of the experiment or frozen for a period not exceeding one week. Two-way analysis of variance and the Tukey multiple comparison test ($P<.05$) were used to determine significance.

Ultrastructure

Rats in these groups were also given mid-back, sc injections. Nifedipine was administered ip. This portion of the study was conducted as follows:

181-239g rats

Group V - Controls (10) - injected with equivalent buffer

Group VI - Treated (10) - injected with 3.6mg PYR/kg

Group VII - Treated (10) - pretreated with 0.05mg nifedipine/kg 15 minutes prior to injection with 3.6mg PYR/kg.

The procedure which proved most acceptable for ultrastructural preservation involved bathing both sides of the diaphragm in situ, with Karnovsky's fixative (1965). This was followed by immediate extraction and continued fixation for two hours at 4°C. Strips approximately one mm thick were stained with Karnovsky's AChE stain (1964) for one hour at room temperature to localize NMJs. Stained strips were examined with an inverted light microscope, and blocks of tissue were cut to obtain specimens which contained NMJs. These blocks were further fixed in cold 1% OsO_4 in 0.052M cacodylate -6% sucrose buffer pH 7.4 and embedded in Epon in the usual manner. Sections were cut with an ultramicrotome, using a diamond knife, and placed on carbon coated grids. Grids were stained with uranyl acetate and lead citrate, and examined with a JEOL 100B electron microscope.

Results and Discussion

Only 20% of the large rats (3 of 15) survived 24 hr after receiving 3.6mg PYR/kg body weight (Group I-C). Seventy-three percent of the small rats (11 of 15) survived (Groups II-F). This difference was statistically significant (Table 1). Although this was a 24-hour survival experiment, all of the rats that died did so in less than 30 min. All surviving rats treated with PYR showed significant indications of improvement by 25 min postinjection. The cholinesterase and ultrastructural experiments were concluded at 30 min postinjection in order to obtain samples not long after the time of maximal effect, and to be consistent with the work of others.

Since (1) all the rats that died in the survival studies did so in less than 30 min, and (2) all survivors showed significant signs of improvement in 15-25 min, survival data from all groups, except for the nifedipine treated group, were combined in Table 2. In the ChE experiment, one of the five large rats which received 1.8mg PYR/kg died. This was the only fatality with this dose, but this one event was enough to preclude a significant difference between B(14/15) and F(20/30). As indicated in the previous table, large rats (C) were significantly less resistant to the 3.6mg PYR/kg than small rats (F).

The control value for serum ChE in samples taken from large rats (Group III-A) 30 min postinjection or immediately after death was significantly lower than that of small rats (Groups IV-D). A comparison of Groups III-B and IV-E from Table 3 with B and E from Table 2 indicates that 92-93% inhibition of serum ChE (1.8mg PYR/kg) corresponds to 93-100% survival. On the other hand, 97% inhibition with 3.6mg PYR/kg (Table 3, Groups III-C and IV-F) relates to 30% survival for large rats Table 2, (C) and 67% survival for small rats (F). This supports indications by others that survival and percent ChE inhibition do not correlate.

The fact that large (older) rats were more sensitive to 3.6mg PYR/kg than small (younger) rats, indicates that the results obtained from PYR studies could depend on the weight (age) of the rats used. The levels of inhibition of serum ChE in this study (93% for 1.8mg PYR/kg and 97% for 3.6mg PYR/kg), as measured with a colorimetric assay, closely correspond to levels reported by Hudson et al. (1986) in whole blood using a radiometric method (90% and 93% inhibition). Shih et al. (1987) described an age related increase in organophosphate toxicity in rats, along with selective decreases in ChE in sections of the brain, but his plasma levels did not change with age. Herscovich and Gershon (1987) found decreases in muscle AChE with age, and decreased choline acetyltransferase; however, these occurred over a period of 26 months. The changes in susceptibility to PYR and decrease in serum ChE reported here occurred in 120 day old rats.

Although diaphragm muscle processed for electron microscopy with our usual fixative resulted in some micrographs of good quality and preservation (Figure 1-A), muscle cells containing swollen mitochondria were frequently observed in cells from control rats. Similar results (Figure 1-B) were obtained with the procedure of Hudson et al. (1986) and the phenomenon appeared to occur regardless to the osmolarity of flushing or fixing solutions. When rats were given 1.0mg PYR/kg in preliminary studies, PYR induced abnormalities were not detected in tissues from some rats (Fig. 2-A), but were present in others (Fig 2-B). Therefore, a 3.6mg PYR/kg dose was subsequently used. The swollen mitochondria in the nerve cell appear similar to swollen mitochondria in Fig. 1-B. With a 2 hr fixation in Karnovsky's fixative (1 hr fixation was inadequate), followed by staining to localize NMJs, and a 2 hr treatment with 1% OsO_4, swollen mitochondria were rare or absent from controls (Fig. 3-A and B). However, better definition and contrast were sacrificed in order to reduce mitochondrial artifacts.

In this study, disrupted filaments were not observed, and retracted nerves were rare. The consistent feature was the appearance of spherical areas of low electron density within muscle cell mitochondria (Fig. 4-A and B). A higher magnification (B), demonstrates that these translucent areas are distinct from those produced by expansion of intracristal space (energized state). Focal mitochondrial lesions were observed in 100% of the specimens from rats treated with 3.6mg PYR/kg (Table 4).

Mitochondrial swelling, as indicated in Fig. 4-B, can be a part of the PYR induced focal lesion, but this was not the characteristic, mitochondrial change associated with an acute 3.6mg PYR/kg dose at 30 min posttreatment. Much of the variability in rarefied areas in the mitochondrial matrix reported by others, particularly with low dosage and acute sc injection, may relate to processing artifacts. Page (1986) came to a similar conclusion, but did not attempt to eliminate artifacts from control tissue, while Laskowski et al. (1977) used a formaldehyde-glutaraldehyde fixation to avoid the problem. Earlier work by Gale (1974) and Bowers et al.(1974) indicated that muscle from exercised animals showed mitochondrial swelling which was dependent on the type of buffer and fixative utilized. In a review of the subject, Gale (1981) indicated that the problem was even more complex. The swelling in presynaptic mitochondria in Fig. 2-B could represent the same phenomenon since artifacts (swollen mitochondria) were frequently present in diaphragms fixed by this method.

Monensin, an antibiotic which acts as a Na^+ ionophore, produces translucent foci in heart muscle mitochondria which are similar to those produced by PYR. Acute exposure to monensin is also reversible, while chronic exposure results in disruption of contractile elements and necrosis (Van Vleet and Ferris, 1984). In this case, intracellular movement of Na^+ is accompanied by Ca^{++} influx. With PYR treatment, excess acetylcholine initiates the influx of Na^+

and Ca^{++}. The latter is believed to be the source of cell injury (Leonard and Salpeter, 1979; Duncan, 1982; Hudson et al., 1985; Gebbers et al., 1986). Meshul et al. (1987) found that diltiazem reduced Z-band damage produced with PYR by preventing acetylcholine induced Ca^{++} flux. This appeared to be consistent with Dretchen's (1985) indication that nifedipine improved survival for mice exposed to antiChE. However, nifedipine (0.05mg/kg) did not improve focal mitochondrial lesions in rat diaphragm (Fig. 6), and in contrast to the work of Dretchen et al. (1985) with diisopropylfluorophosphate (DFP), the survival rates were identical with and without nifedipine. It is possible that the dose of nifedipine was inadequate, but this was the same as the dosage used by Dretchen et al. (1985). It is also possible that different Ca^{++} antagonists such as diltiazem or verapamil might alter the outcome.

There is little doubt that elevated levels of Ca^{++} are ultimately involved in the production of lesions (Leonard and Salpeter, 1979), but the initial event appears to be focal mitochondrial changes observed at 30 min rather than Ca^{++} activated Z-band destruction. The latter may result as a progression from the disruption of myofibrillar organization in a small number of sarcomeres at 30 min with PYR (Hudson et al., 1985) to dissolution of Z-bands in 2 hr with DFP (Leonard and Salpeter, 1979). Unlike DFP treatment, surviving PYR treated rats showed rapid recovery. The similarity between spherical areas of low electron density in mitochondria reported here, and those produced with monensin, along with the absence of any nifedipine effect, may suggest participation of Na^{+} influx in localized changes in membrane permeability early (less than 30 min) in the process, as initially suggested by Laskowski et al. (1977). This sequence of events is consistent with the hypothesis of Trump et al. (1981 and 1987) which indicates that increased permeability to Na^{+} leads to elevated intracellular Na^{+} and reduced Na/Ca exchange with subsequent increases in intracellular Ca^{++}.

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TABLE 1

Twenty-four Hour Survival after Pyridostigmine Treatment.

Group	I		II	
Weight	475-525g		175-250g	
Treatment subgroup		Surviving/Total	Treatment subgroup	Surviving/Total
A. Control		10/10 (C)	D. Control	10/10 (C)
B. 1.8mg PYR/kg		10/10 (C)	E. 1.8mg/kg	10/10 (C)
C. 3.6mg PYR/kg		3/15 (A,B,D,E,F)	F. 3.6mg/kg	11/15 (C)

Letters in parentheses indicate significantly different subgroups, (Chi-square P<.0

TABLE 2

Combined survival data for all rats excluding nifedipine treated group assuming those surviving 30 minutes would not die from the pyridostigmine treatment.

Weight	475-525g		175-250g	
Treatment subgroups		Surviving/Total	Treatment subgroup	Surviving/Total
A. Control		15/15 (C,F)	D. Control	25/25 (C,F)
B. 1.8mg PYR/kg		14/15 (C)	E. 1.8mg PYR/kg	15/15 (C,F)
C. 3.6mg PYR/kg		6/20 (A,B,D,E,F)	F. 3.6mg PYR/kg	20/30 (A,C,D,E)

Letters in parentheses indicate significantly different subgroups, (Chi-square P<.05

TABLE 3

Serum cholinesterase levels, mu/ml of serum at 25°C for large and small rats 30 minutes after injection of buffer or pyridostigmine.

GROUP	III		IV	
WEIGHT	475-525g		175-225g	
	ESTERASE		ESTERASE	
	MEAN± S.E.M.	% INHIBITION	MEAN±S.E.M.	% INHIBITION
A. CONTROL	359±19 (B,C,D,E,F)	0%	D. CONTROL	421±18(A,B,C,E,F)	0%
B. 1.8mg PYR/kg	29±13 (A,D)	91.9%	E. 1.8mg PYR/kg	30±3 (A,D)	92.9%
C. 3.6mg PYR/kg	12±6 (A,D)	96.7%	F. 3.6mg PYR/kg	12±4 (A,D)	97.2%

Letters in parentheses indicate significantly different subgroups, (Tukey multiple comparison test P<.05).

TABLE 4

Effects of nifedipine pretreatment on pyridostigmine induced ultrastructural lesions at NMJ and lethality in rats 30 minutes postinjection.

Weight 181-239g

Groups	Treatment	Rats with Lesions at NMJ/Total	30 Min. Survival/Total
V	Control	0/10 (VI, VII)	10/10
VI	3.6mg PYR/kg	10/10 (V)	6/10
VII	0.05mg nifedipine 3.6mg PYR/kg	10/10 (V)	6/10

Numbers in parentheses indicate significantly different groups, (Chi-square $P<.05$). With the 3.6mg PYR/kg, all of the junctions observed contained similar lesions.

FIGURE 1. A. Control rat diaphragm fixed by perfusion with 2.5% glutaraldehyde in 0.052M cacodylate-6% sucrose, pH 7.4. Mitochondria are usually normal as indicated here (X34,000). B. Control rat diaphragm fixed by perfusion with 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4 (X15,000). Mitochondria may be swollen as indicated by arrows or normal as indicated in 1-A.

FIGURE 2. A. Diaphragm from rat treated with 1.0mg PYR/kg body weight and processed as indicated in 1-A (X30,000). Mitochondria may appear normal as indicated here. With this dosage, lesions were not always present. B. Diaphragm from rat treated in the same manner as in 2-A (X15,000). Arrows indicate altered mitochondria, but the swollen mitochondria (*) could be artifacts (X20,000).

FIGURE 3. A. Control rat diaphragm fixed for two hours in Karnovsky's fixative (X26,000). Some definition and contrast are lost; however, mitochondrial artifacts were reduced or eliminated. B. Control rat diaphragm fixed for two hours in Karnovsky's fixative (X20,000).

FIGURE 4. A. Diaphragm from rat treated with 3.6mg PYR/kg and fixed two hours in Karnovsky's fixative. Spherical areas of low electron density were always present in muscle cell mitochondria near the NMJ for that cell. Similar changes were often, but less frequently, present in the nerve (X15,000). B. Diaphragm from rat treated with 3.6mg PYR/kg and fixed two hours in Karnovsky's fixative (X15,000). Swollen or disrupted mitochondria (*), and those showing spherical areas of low electron density (arrow) are present even when artifacts are unlikely.

FIGURE 5. Diaphragm from rat treated with 3.6mg PYR/kg fixed in Karnovsky's fixative. Note that spherical areas of low electron density (arrows) are unrelated to changes associated with the condensed configuration (X51,000).

FIGURE 6. Diaphragm from rat treated with 0.05mg nifedipine/kg prior to treatment with 3.6mg PYR/kg fixed two hours in Karnovsky's fixative. PYR induced changes were not improved (X15,000).

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Fig 1-a

Fig. 1-b

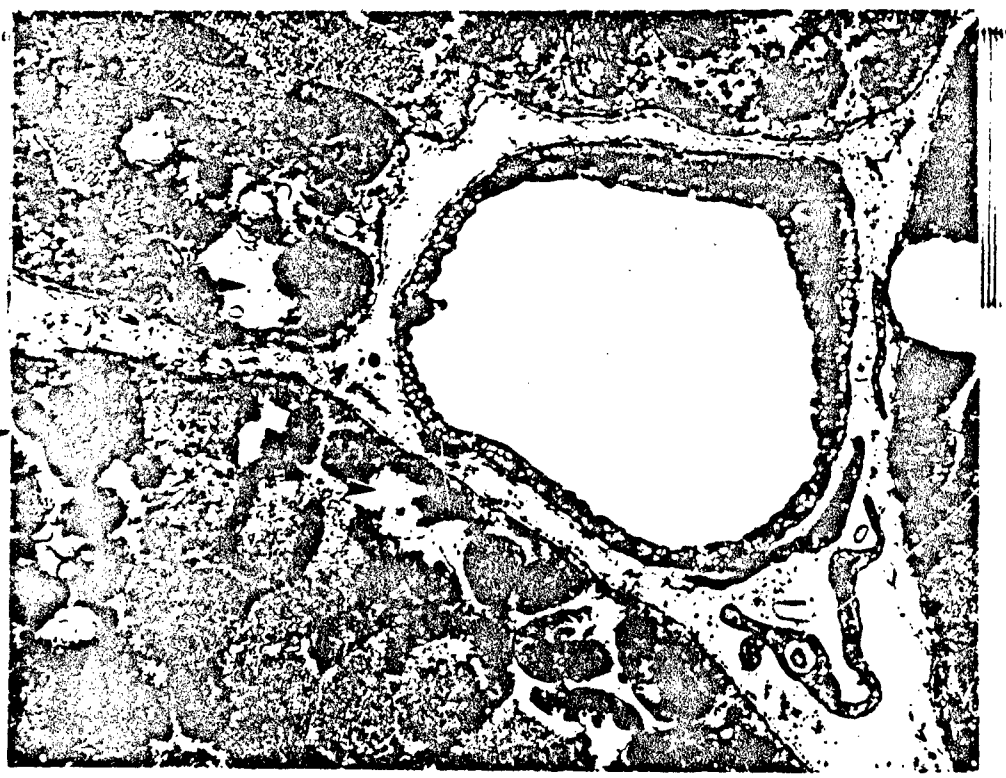

Fig. 2-a

Fig 2-b

Fig 3.a

Fig 3b

Fig. 4.a

Fig. 4.b

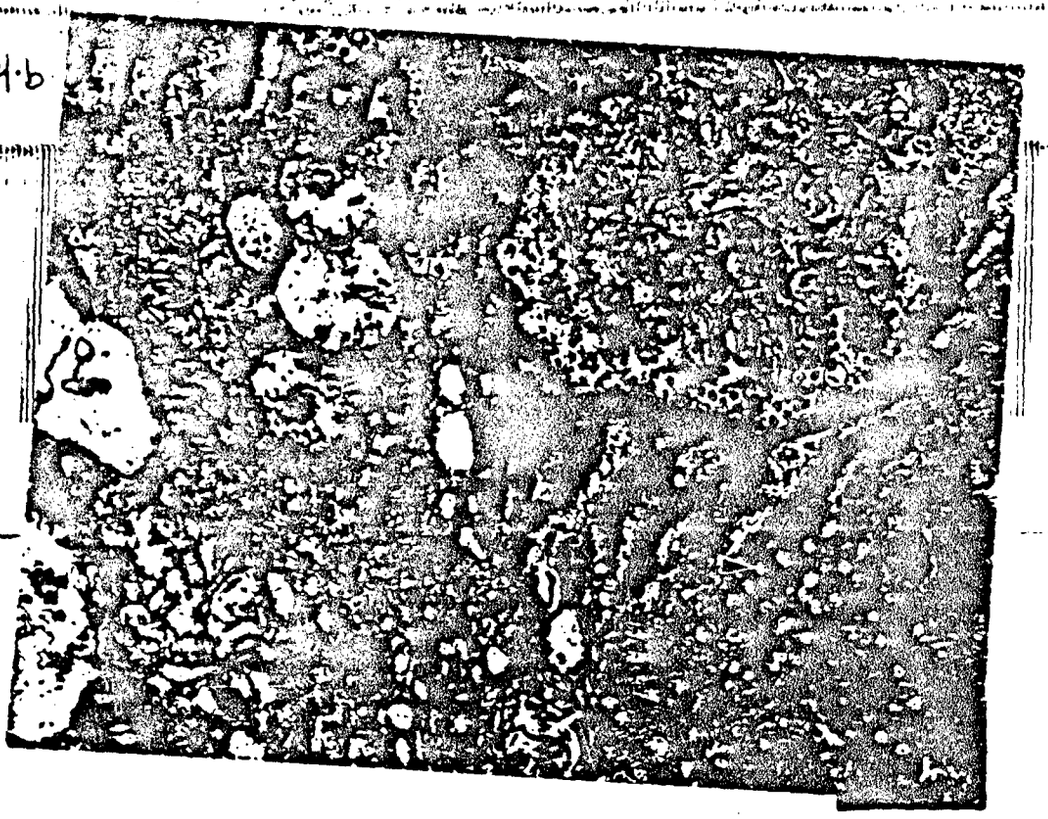

Fig. 5

Fig. 6

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