

Hypoxia and lung mast cells: influence of disodium cromoglycate

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Mungall, I. P. F. (1976). Thorax, 31, 94–100. Hypoxia and lung mast cells: influence of disodium cromoglycate. Rats kept in 10% O₂ for three or more weeks developed mast cell hyperplasia in the lungs, especially around the alveoli and the small peripheral blood vessels, which become thickened during chronic hypoxia. There was a significant correlation between the degree of right ventricular hypertrophy (RVH) and the numbers of alveolar and small vessel mast cells. However, mast cell hyperplasia developed more slowly than RVH. Daily treatment with disodium cromoglycate failed to prevent RVH in hypoxic conditions but was associated with retardation of growth in both hypoxic and control rats. Neither acute nor chronic hypoxia increased the degree of degranulation in the lung mast cells.

Changes in alveolar oxygen tension control pulmonary vascular tone (von Euler and Liljestrand, 1946; Barer *et al.*, 1969; Abraham *et al.*, 1970). The pulmonary pressor response to hypoxia is not dependent on extrinsic nerves and could be due to a chemical transmitter. Increased levels of histamine have been found in the pulmonary venous blood from hypoxic lungs (Aviado, Samanek, and Folle, 1966; Haas and Bergofsky, 1972) though Brashear, Martin, and Ross (1970) and Dawson *et al.* (1974) were unable to confirm this. The pulmonary pressor response to hypoxia in the rat is inhibited by antihistamines and enhanced by histaminase inhibitors (Hauge, 1968). The mast cell degranulating agent, 48/80, abolishes the hypoxic vascular response in the cat (Barer and McCurrie, 1969; Hauge and Staub, 1969; Dawson *et al.*, 1974) though there is disagreement as to its exact mechanism of action. Lung mast cell granules are a potent source of histamine (Riley and West, 1953; Fawcett, 1954; Riley and West, 1955; Bray and van Arsdell, 1961). Haas and Bergofsky (1972) have shown that suspensions of rat peritoneal mast cells discharge histamine when hypoxic gases are bubbled through them. In view of the relationship between mast cells, histamine, and pulmonary vasoconstriction, it is possible that mast cells may play a functional role in the pulmonary arteriolar and right ventricular hypertrophy (RVH) which develop during chronic hypoxia (Naeye and Bickerman, 1959; Hicken *et al.*, 1965; Hasleton, Heath, and Brewer, 1968; Heath, Brewer, and Hicken, 1968; Abraham *et al.*, 1971; Hunter *et al.*, 1974). Changes

in the population and distribution of mast cells have therefore been sought in chronically hypoxic rat lungs.

If the RVH of chronic hypoxia resulted from pulmonary hypertension caused by sustained histamine release from the mast cells, then the mast cell stabilizing drug, disodium cromoglycate, might prevent this chain of events. Initially disodium cromoglycate was thought to be effective only in IgE mediated antigen/antibody reactions (Altounyan, 1967; Cox, 1967; Assem and Richter, 1971) but it has now been observed to inhibit mast cell degranulation in many situations quite unrelated to reaginic antibody (IgE) reactions (Pepys *et al.*, 1968; Davies, 1968; Orr and Cox, 1969; Altounyan, Cox, and Orr, 1970; Kerr, Govindaraj, and Patel, 1970; de Kock, 1970; Clarke, 1971; Marshall, 1972). It probably acts by stabilizing the mast cell membrane regardless of the stimulus, and it is therefore possible that disodium cromoglycate might prevent hypoxic mast cell degranulation. This hypothesis has been tested by determining whether pretreatment with disodium cromoglycate prevented RVH in chronically hypoxic rats.

In a further series of experiments the degree of mast cell degranulation during hypoxia has been assessed. Haas and Bergofsky (1972) showed increased mast cell degranulation in acutely hypoxic rat lungs, though Kay, Waymire, and Grover (1974), using a histochemical assay, were unable to detect any evidence of histamine depletion from the mast cell during acute hypoxia. In the experiments described

here, mast cell degranulation has been measured in both acutely and chronically hypoxic rat lungs. Different anaesthetics and methods of fixation have been used to determine whether these have any influence on mast cell degranulation. Preliminary accounts of part of this work have already been published (Barer and Mungall, 1974; Mungall and Barer, 1975).

MATERIAL AND METHODS

Male albino Wistar rats were used in all experiments.

POPULATION AND DISTRIBUTION OF MAST CELLS IN CHRONIC HYPOXIA The 21-day-old rats were allowed to accommodate to the laboratory for 4–7 days. Littermates were then divided so that half were kept in a low O₂ chamber for varying periods and half were kept in the same room in ambient air. Two chambers (1 and 2) maintained at 10% O₂ were used. In chamber 1 (internal dimensions 86 × 86 × 44 cm; Cryer and Bartley, 1974) CO₂ was absorbed by circulating the contained gases through concentrated KOH; this was changed when its pH, measured daily, began to fall. PCO₂ was not measured in these experiments. Owing to doubt as to the efficiency of CO₂ absorption, the experiments were repeated (with similar results) in a second improved chamber (65 × 65 × 150 cm) where PCO₂ was measured and did not rise above 0.6%. Temperature and humidity were always slightly higher inside than outside the chambers. Rats were given free access to food (Oxoid diet 86) and water. The chamber was opened for about one hour two or three times weekly for cleaning and restocking.

At the end of each experiment the rats from the chamber and their littermate controls were killed with ethyl chloride and 60 mg pentobarbitone intraperitoneally. The lungs were fixed with buffered formol saline either by intratracheal injection of 9 ml or infusion at 1.96 kPa pressure. Transverse sections at 7 μm were made and stained with azure eosin. One complete section, or two sections from different lobes in the early experiments using chamber 1, were scanned by one observer, who was unaware of each section's source. A total mast cell count was made and a note of the position of each mast cell, whether in the bronchi, the pleura, adjacent to large pulmonary vessels (those greater than 125 μm diameter), small pulmonary vessels (less than 125 μm), or in the alveolar septa or ducts. In view of the difficulty of recognition, no attempt was made to differentiate small arteries and arterioles from small veins. In addition, four sections were scanned by a second independent observer to check the degree of observer variation. All counts were expressed in terms of unit area of lung section, measured by planimetry after projection onto graph paper.

The size of the RVs in one experiment was assessed by dissecting the free wall of the RV from the septum and left ventricle (LV). The ventricles were blotted and weighed.

DISODIUM CROMOGLYCATO EXPERIMENTS It was necessary to find a daily period of hypoxia which would cause changes in the heart but not exceed the duration of action of disodium cromoglycate which is estimated to be four hours after intraperitoneal injection. The regime used by Widimsky *et al.* (1972) and McGrath *et al.* (1973) was repeated. Rats were exposed to 8% O₂ for four hours daily. After 20 and 24 exposures there was no change in RV size but after 70 exposures the mean RV weight for five hypoxic rats was 0.213 g (SD ± 0.057) and for six control rats 0.139 g (SD ± 0.039), $P=0.01-0.025$.

For the main experiment, 89-day-old rats were divided into the following groups, each of six rats: (A) hypoxic, injected with disodium cromoglycate; (B) hypoxic, injected with saline; (C) control, injected with disodium cromoglycate; and (D) control, injected with saline. Six animals died in the course of the experiment, one from group A, two from B, and three from D. Each morning the rats were weighed, sedated with ether, and injected intraperitoneally with 10 mg/kg disodium cromoglycate (Intal, Fisons Pharmaceuticals, 1 mg per ml in saline) or an equivalent volume of 0.9% saline. Groups A and B were then placed in the chamber at 8% O₂ for four hours. The maximum interval between injection and exposure was 20 minutes. This regime was carried out for six days a week for 70 exposures. In between exposures the animals were subject to normal atmospheric conditions. After 70 exposures the rats were killed with ethyl chloride and 60 mg pentobarbitone intraperitoneally. The heart and lungs were removed, the ventricles weighed, and the lungs fixed and sectioned, as described above. Blood from the inferior vena cava was taken for haematocrit estimations. One section from each lung was scanned 'blind' and the numbers of mast cells were counted.

DEGRANULATION EXPERIMENTS Rats were anaesthetized with ethyl chloride and 7 mg pentobarbitone intraperitoneally. A small polyethylene cannula was inserted into the trachea. The animals were ventilated with 10% O₂ in N₂ through a T piece connected to the tracheal cannula for either 5, 10, 15 or 30 minutes. Two control rats were ventilated with 100% O₂. The experiment was terminated by injecting 9 ml formol saline into the tracheal cannula. This has the advantage that the lungs are fixed immediately at the time of death, thus preventing postmortem disruption of the mast cells. The lungs were removed and transverse

sections were prepared as above. One complete section from each lung was scanned 'blind' by a single observer. The degree of mast cell degranulation was noted, using the classification of Haas and Bergofsky (1972): grade 1 = intact, 2 = partial degranulation, 3 = complete degranulation. An overall degranulation index, from 1 to 3, was also calculated from this formula:

$$\text{degranulation index} = (x + 2y + 3z)/n,$$

where x = the number of grade 1 mast cells, y = the number of grade 2, z = the number of grade 3, and n = the total number in any section. Thus, the degranulation index for an animal whose mast cells were predominantly intact would tend towards 1, while for an animal with predominantly maximally degranulated mast cells the index would tend towards 3.

In a second experiment, rats were placed individually in a small perspex chamber ($18 \times 22 \times 9$ cm). They were divided into three groups exposed to either 10% O₂, 12% O₂ or, in the case of the controls, air. For the experimental animals, air was sucked through the chamber initially, and after 30 minutes the gas mixture was changed to either 10% O₂ or 12% O₂ in N₂. After 30, 40, 60 or 90 minutes the experiment was terminated by injecting 30 ml ether through a rubber bung into the chamber. The rat was removed and killed with an intraperitoneal injection of 60 mg pentobarbitone, and the lungs were dissected out, fixed, and sectioned. The whole procedure from removal of the rat to completion of the dissection and fixation took between five and eight minutes (mean six minutes).

The degree of degranulation in a group of rats kept in the large hypoxic chamber (chamber 1) at 10% O₂ for 56 days was also assessed. The rats were killed shortly after removal from the chamber with ethyl chloride and 60 mg pentobarbitone intraperitoneally. The sections were prepared and scanned as described above. Littermates who had been exposed to atmospheric conditions acted as controls.

To ensure that the method was sufficiently sensitive to detect mast cell degranulation, two rats were injected with the mast cell degranulating agent, 48/80; 1 mg/kg was injected intraperitoneally and the animals were killed either two or four hours later with ether and 60 mg pentobarbitone intraperitoneally.

Means and standard deviations are given in the text and tables and were compared by Student's t test for unpaired data. Regressions were calculated by the method of least squares and their significance was tested by the t test.

RESULTS

POPULATION AND DISTRIBUTION OF MAST CELLS IN CHRONIC HYPOXIA In five experiments (duration 21–80 days) there was an increase in total mast cell count (Table I), and in three of these the increase reached statistical significance. This mast cell hyperplasia was apparent in all situations in the lung but was prominent around small peripheral vessels and alveoli, indicated in the right-hand columns of Table I ($P < 0.05$ for all five experiments). In the 14-day and one of the 21-day experiments (g and d) there was no mast cell hyperplasia. There was good correlation between the counts by the two observers ($r = 0.991$, $P < 0.001$) and between counts obtained from different lobes of the same lung (experiment b: $r = 0.809$, $P < 0.001$ for the total count, and $r = 0.579$, $P < 0.01$ for the alveolar and small vessel count).

In experiment e, in which ventricles were weighed there was RVH in the hypoxic group; the mean hypoxic RV weight was 0.263 ± 0.031 g and the control weight 0.148 ± 0.031 g ($P < 0.005$). There was significant correlation ($r = 0.879$, $P < 0.05$) between the hypoxic RV weight and the alveolar and small vessel mast cell count in each rat (Fig. 1).

DISODIUM CROMOGLYCATE EXPERIMENTS The RV weights, expressed in absolute terms, in relation to

TABLE I
EFFECT OF CHRONIC HYPOXIA ON LUNG MAST CELLS

No. of Rats	Days of Exposure to 10% O ₂	Total Mast Cell Count/cm ²			Alveolar and Small Vessel Mast Cell Count/cm ²		
		Hypoxic	Control	P	Hypoxic	Control	P
a 6 ¹	80	447 ± 130	181 ± 65	< 0.001	143 ± 75	43 ± 35	< 0.01
b 10 ¹	70	607 ± 155	237 ± 122	0.001	104 ± 53	14 ± 12	< 0.001
c 8 ¹	56	443 ± 267	389 ± 108	NS	105 ± 89	26 ± 18	< 0.05
d 9 ¹	21	293 ± 79	352 ± 192	NS	58 ± 41	55 ± 66	NS
e 12 ²	21	743 ± 109	478 ± 116	< 0.01	179 ± 78	32 ± 28	< 0.01
f 10 ²	32 or 34	896 ± 431	381 ± 279	NS	150 ± 111	29 ± 13	< 0.05
g 6 ²	14	300 ± 53	299 ± 177	NS	13 ± 7	2 ± 2	NS

¹Chamber 1: Two sections counted from each animal.

²Chamber 2: One section counted from each animal.

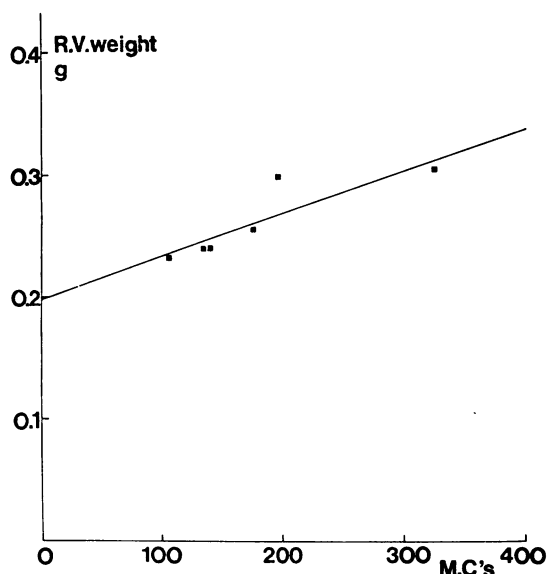


FIG. 1. Relationship between RV weight and alveolar and small vessel mast cell count (M.C.) Experiment e, Table I. Six rats, 21 days' exposure to 10% O₂. Regression line ($r = 0.879$, $P < 0.05$) is plotted.

body weight and as a ratio of left ventricle+septal weights are shown in Table II. There was no significant difference between the absolute RV weights in the two hypoxic groups (A and B). As expected, there was a clear increase in the RV weight of the hypoxic saline animals (B) compared with their controls (D). However, there was also RVH in the hypoxic disodium cromoglycate group (A) compared with their controls (C). The difference between RV weights in C and D was probably due to differences in body weight, as it was not apparent when the RV weights were normalized for body weight.

The ratio of RV weight to LV+septal weight is probably the most sensitive indicator of RV hypertrophy. From these ratios it can be seen that there was RVH in both the hypoxic groups (A and B) and that disodium cromoglycate had no protective action on the right ventricle.

Haematocrits, changes in body weight, and mast cell counts are also listed in Table II. Haematocrits were higher in the hypoxic groups compared with the controls; the reason for the slight but significant depression of haematocrit in the hypoxic disodium cromoglycate group (A) compared with B is unclear. As expected, the hypoxic animals failed to gain weight as rapidly as the controls. However, disodium cromoglycate also appeared to have a depressant effect on growth, in both the hypoxic animals and the controls, and in the hypoxic disodium cromoglycate group (A) the animals actually lost weight. There was no significant difference in the mast cell counts between any of the four groups, with regard to either the total count or the alveolar and small vessel count.

DEGRANULATION EXPERIMENTS The degranulation grades and overall index for the three experiments are shown in Table III. On comparing both the individual degranulation grades and the overall indices, there was no significant difference either between the hypoxic animals and the controls or between the individual experiments. The degree of degranulation in experiment i for all animals was slightly lower than in the other two experiments but the difference did not reach statistical significance.

The degree of degranulation in the perivascular mast cells might be expected to be greater than in the lung as a whole. This question was investigated in experiment ii. The perivascular mast cell degranulation index was 1.40, 1.36, and 1.67 for 10% O₂,

TABLE II

EFFECT OF CHRONIC INTERMITTENT HYPOXIA AND/OR DISODIUM CROMOGLYCAT (DSCG) ON BODY WEIGHT, RV WEIGHT, HAEMATOCRIT, AND LUNG MAST CELL COUNT

	(A) Hypoxic DSCG (5)	(B) Hypoxic Saline (4)	(C) Control DSCG (6)	(D) Control Saline (3)	P	
Initial body weight (g)	359.5 ± 31.7	368.5 ± 39.3	338.7 ± 15.7	397.0 ± 1.7		
Final body weight (g)	347.0 ± 40.5	394.5 ± 48.8	351.0 ± 21.5	485.7 ± 23.6		
Change in body weight %	-4.0 ± 4.7	+7.0 ± 6.0	+3.6 ± 3.6	+22.3 ± 5.5	A/B = < 0.01 B/D = < 0.01 A/B = < 0.05 B/D = < 0.005	A/C = < 0.01 C/D = < 0.005 A/C = < 0.005 C/D = NS
Haematocrit %	67 ± 2	70 ± 2	46 ± 3	43 ± 4		
Total mast cell count/cm ³	369 ± 180	224 ± 84	444 ± 184	334 ± 236		
Alveolar and small vessel count/cm ³	27 ± 26	33 ± 20	26 ± 20	46 ± 55	NS NS	
Mean RV weight (g)	0.211 ± 0.026	0.204 ± 0.022	0.142 ± 0.013	0.171 ± 0.010	A/B = NS B/D = < 0.05 A/B = < 0.025 B/D = < 0.01	A/C = < 0.005 C/D = < 0.01 A/C = < 0.005 C/D = NS
RV/body weight (mg/g)	0.608 ± 0.049	0.520 ± 0.070	0.398 ± 0.039	0.350 ± 0.036		
RV/LV + septum	0.30 ± 0.02	0.30 ± 0.02	0.21 ± 0.03	0.22 ± 0.02	A/B = NS B/D = < 0.005	A/C = < 0.005 C/D = NS

*Number of rats in each group.

TABLE III
EFFECT OF ACUTE AND CHRONIC HYPOXIA ON LUNG MAST CELL DEGRANULATION

Experiment	No. of Rats		Hypoxic		Controls
			10% O ₂	12% O ₂	Air
i Acute hypoxia-anaesthetized	7	Grade 1% Grade 2% Grade 3% Overall index	63.7 ± 10.4 31.8 ± 9.9 4.5 ± 3.1 1.41 ± 0.12		65.4 ± 14.3 29.2 ± 11.7 5.5 ± 2.7 1.40 ± 0.17
ii Acute hypoxia-unanaesthetized	9	Grade 1% Grade 2% Grade 3% Overall index	47.9 ± 23.6 43.5 ± 17.3 8.6 ± 6.5 1.61 ± 0.30	57.2 ± 15.0 33.7 ± 10.1 9.2 ± 9.5 1.52 ± 0.23	44.8 ± 8.9 41.0 ± 1.5 14.1 ± 7.5 1.69 ± 0.16
iii Chronic hypoxia	11	Grade 1% Grade 2% Grade 3% Overall index	48.7 ± 16.3 42.5 ± 12.1 9.6 ± 5.0 1.60 ± 0.21		50.2 ± 13.6 41.0 ± 11.3 8.8 ± 3.5 1.59 ± 0.16

12% O₂, and air respectively, while the degranulation index for the whole lung was 1.61, 1.52, and 1.69 respectively (not significant).

The degree of degranulation was similar in experiment i where ethyl chloride and pentobarbitone were used and in experiment ii where ether and pentobarbitone were used. Also, there was no difference in degranulation between experiment i, where the lung was fixed during acute hypoxia, and experiment ii, where there was an interval before fixation. There was no evidence of increased degranulation during either acute or chronic hypoxia.

In the 48/80 experiment the overall degranulation index for the two rats was 2.09 and the grading was 1 = 22.2%, 2 = 47.0%, and 3 = 30.8%. These results suggest that the technique was sufficiently sensitive to detect mast cell degranulation.

DISCUSSION

POPULATION AND DISTRIBUTION OF MAST CELLS
Chronic exposure to hypoxia was characterized by an increase in the numbers of lung mast cells, especially around the alveoli and small vessels (Fig. 2). It is in these situations that the mast cells are exposed to alveolar gases and it is the small peripheral vessels that hypertrophy in chronic hypoxia (Hunter *et al.*, 1974; Barer and Mungall, 1974). In experiment e, there was a significant linear relationship between the alveolar and small vessel mast cell count and RV weight (Fig. 1). The hypothesis that mast cell hyperplasia leads to pulmonary hypertension and consequent RV hypertrophy seems particularly attractive in this context. Since completing this study, Kay *et al.* (1974) have demonstrated mast cell hyperplasia in the lungs of rats kept in a hypobaric chamber (equivalent to 10% O₂) for 21 days. They correlated log RV weight with log mast cell count for both their experimental and control animals.

In one of the 21-day experiments (d) and in the

14-day experiment (g) there was no increase in mast cell count. It is likely that 21 days is approximately the threshold for mast cell hyperplasia. However, it is known that only 14 days' exposure to 10% O₂ is necessary to produce RV hypertrophy (Hunter *et al.*, 1974). This anomaly is difficult to reconcile with a functional role for mast cells in hypoxic pulmonary vascular disease. Possibly mast cell hyperactivity precedes hyperplasia.

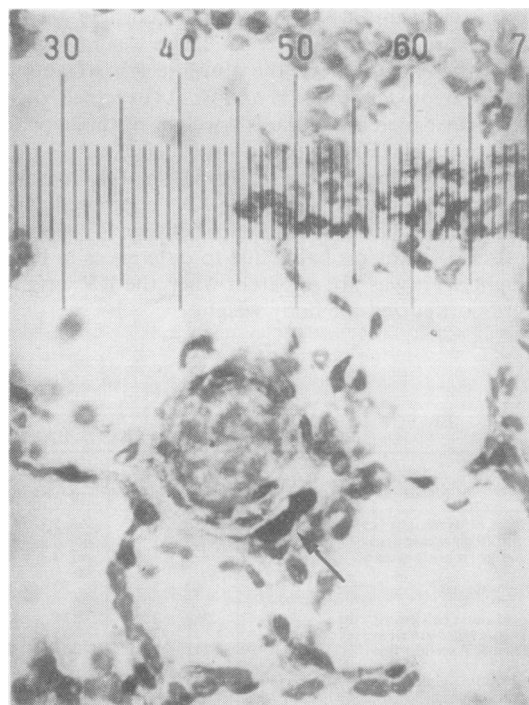


FIG. 2. Mast cell (arrowed) by a small peripheral pulmonary vessel. Experiment a, Table I. Vessel diameter = 32.5 μ m (each graticule division = 2.5 μ m). Azure eosin \times 400.

Mast cells have other functions beside the storage and release of vasoactive amines. They are associated with the laying down of connective tissue and oedema formation (Riley, 1959). It is possible that in chronic hypoxia the mast cells are related to these functions rather than to vasoconstriction. However, there was no microscopic evidence of pulmonary oedema in the hypoxic animals.

Lung mast cell hyperplasia has previously been reported in association with the pulmonary hypertension of mitral stenosis (Heath, Trueman, and Sukonthamarn, 1969) and Crotalaria poisoning (Takeova, Angevine, and Lalich, 1962; Kay, Gillund, and Heath, 1967). In these studies, the mast cell hyperplasia correlated better with chronic exudation of fluid and the formation of new fibrous tissue than with RVH.

DISODIUM CROMOGLYCATe EXPERIMENTS The failure of disodium cromoglycate to prevent RVH infers that mast cells do not have a functional role in the development of RVH in chronic hypoxia. However, other possibilities should be considered. Although there is good circumstantial evidence that disodium cromoglycate stabilizes the mast cell regardless of the stimulus, there is no definite proof that it is effective in hypoxic degranulation. Also the acute hypoxic experiments have failed to confirm Haas and Bergofsky's original observation that hypoxia does indeed cause mast cell degranulation (Haas and Bergofsky, 1972). Kay and Grover (1975) showed that disodium cromoglycate inhibited the pulmonary pressor response to acute hypoxia in three out of eight anaesthetized dogs, though Howard *et al.* (1975) were unable to confirm this.

Inadequate dosage of disodium cromoglycate is a possible explanation. However, the dose used is known to stabilize the mast cell in sensitivity reactions, and the intraperitoneal route of administration should have ensured effective levels for the duration of each hypoxic exposure. A further anomaly is the demonstration of RVH in both hypoxic groups (A and B) without an increase in mast cell numbers. Possibly four hours' hypoxia daily was insufficient stimulus for mast cell hyperplasia, or possibly, as with the continuous exposure experiments, RVH predates mast cell hyperplasia.

The depressant action of disodium cromoglycate on body weight has not previously been reported and is unexplained. From Table II it can be seen that the low body weights in group A actually made the normalized RV weights significantly heavier than in group B.

DEGRANULATION EXPERIMENTS There was no significant difference between the degree of degranula-

tion, either between the hypoxic and control animals or between the three experiments. The reason for the discrepancy between these results and Haas and Bergofsky's (1972) demonstration of hypoxic mast cell degranulation is not clear. Haas and Bergofsky only measured degranulation in perivascular mast cells though this is unlikely to be the explanation as in experiment ii (Table III) the degree of degranulation in the perivascular mast cells and in the lung as a whole was similar. The demonstration of mast cell degranulation in over one-third of the control animals is in agreement with Haas and Bergofsky's observations. The reason for this degranulation has not been resolved; possibly the trauma of dissection and tissue processing is responsible.

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