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THE PULMONARY PHAGOCYTES AND PHAGOCYTOSIS

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Presented to the Faculty of Medicine of the University of Ottawa through the Department of Pathology as partial fulfillment of the requirements for the degree of Master of Science.

UNIVERSITAS MEDICINA OTTAVIENSIS

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THE PULMONARY PHAGOCYTES AND PHAGOCYTOSIS

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THE PULMONARY PHAGOCYTES AND PHAGOCYTOSIS

W.J. Wills, M.D.

I INTRODUCTION

Since the early days of tissue study by means of the microscope, students have noted mononucleated cells in the lungs which phagocytize particulate matter, both living and non-living. It is also well known that particulate matter finds its way into phagocytic cells within the pulmonary parenchyma, particularly in the peribronchial and perivascular areas and in the peribronchial and hilar lymph nodes. However, the origin of the alveolar phagocytes as well as the ultimate disposition of these cells has been a controversial subject over the years. In the usual light microscopy tissue sections these cells are seen "free" in the alveolar spaces and in the tracheobronchial tree, as well as "fixed" to the alveolar walls and whether the free phagocytes are merely those which have fulfilled their function and are being released to find their way up the tracheobronchial tree and out of the body or into the lymphatic channels or whether they are mature active functional form of the cell has also been a subject with much division of opinion.

It has also been noted that, in some cases of instantaneous traumatic death, presumably healthy individuals at autopsy show alveolar macrophages containing granules of a pigment with the morphological appearance and staining properties of hemosiderin. Whether this is a normal situation in the human lung, whether it may be due to post mortem activity of the pulmonary phagocytes, or whether

the pigment is some artefact rather than hemosiderin, is not known.

In view of the many controversial and unknown facets of the subject of the pulmonary phagocytes and pulmonary phagocytosis, this study was undertaken in the hope of shedding some light in these controversial and unknown areas. Specifically, it was planned to investigate the general phagocytic responses in the lung especially in respect to timing in the living animal, pulmonary phagocytosis after death of the animal, as well as the phagocytosis and disposal of erythrocytes in the lung.

PART I

EXPERIMENTS WITH NON-LIVING PARTICULATE MATTER (DUST)

1 REVIEW OF PREVIOUS OBSERVATIONS AND CONCLUSIONS REGARDING THE PULMONARY PHAGOCYTES

(a) On the origin of the Alveolar Macrophage

The origin of the alveolar macrophage has been and is still a controversial subject. Some authors have concluded that these cells arise from the alveolar epithelium,[1-4] the existence of which, until recently, has also been a matter of controversy. Others have concluded that they come from mononuclear cells of the interstitial tissue and pulmonary septa [5-10]. Some experimenters have found little or no phagocytosis by the "fixed" phagocytes [11,12], while others have found these cells are actively phagocytic while "fixed" [1,3,4,7,9,13-17]. Mitotic figures are rarely found in these cells except under special circumstances such as early tuberculous infection[18]. Binucleate forms, on the other hand, are not infrequent and this may indicate amitotic division [19]. More recently it has been stated that there is a good possibility that the alveolar macrophage may be derived from extrapulmonary macrophages, particularly those of the spleen and the liver[20]. This conclusion is based mainly on evidence supplied by intravenous injection of particulate matter such as carbon. The author found that this was heavily taken up by the reticule-endothelial cells of the liver, spleen, and bone marrow but very sparsely by the lungs initially. However, in a few days increasing

numbers of macrophages loaded with carbon were noted in the tracheobronchial tree. It was also noted that septal cells in the lung are relatively few in number under normal conditions but that they quickly appear in large numbers under adequate stimulation. The blood histiocyte or monocyte has also been suggested as the source of the pulmonary phagocyte[21]. In summary it has been suggested that the pulmonary macrophages may be derived from alveolar epithelium, pulmonary tissue histiocytes, blood histiocytes or monocytes, extrapulmonary reticulo-endothelial cells, pulmonary vascular endothelium, or even lymphocytes[19]. Although there is some experimental support for many of these origins, there is, as yet, no proof for any of them.

(b) <u>On the effect of Particle size</u>

There has been considerable investigation concerning the relationship of particle size on the retention of particles in various parts of the respiratory apparatus[22,23]. From these experimental investigations it would appear that the maximum respiratory retention of dust increases as the particle size increases but that this increase is mainly due to the retention of the larger particles by the tracheobronchial tree and nasal passages. In the alveoli, retention of dust particles is maximal with particle size of about 1u and retention decreases both above and below this size. Nevertheless, for larger particles, which escape retention in the upper respiratory tract and do enter the alveoli, retention is complete. Depth of penetration of the respiratory system before deposition increases with decreasing particle size, but apparently particles

less than 1u size have a tendency to remain suspended and to retreat out of the respiratory system rather than to be deposited. There is a suggestion that alveolar deposition again rises for particles less than ¼u diameter[24]. These figures on diameter of particles and retention remain remarkably constant for humans and smaller animals, the major difference being that in humans the maximal retention in the upper respiratory passages occurs at 5u and above while smaller animals show maximal upper respiratory retention at somewhat less than 5u. The maximal alveolar retention, however, is at about 1u in both humans and smaller animals[22].

(c) On Clearance of Dusts

Once particles are deposited in the alveoli or upper respiratory apparatus, clearance begins. If the particles have not penetrated beyond the ciliated respiratory epithelium and if the substance induces little inflammation, it is wafted up the tracheobronchial tree, principally by ciliary movement, at a rate of from 0.25 to 1.0 cm/min in the bronchi and approximately 3 cms/min in the trachea[19]. Dusts such as carbon appear to be relatively innocuous in the lung while others such as silica may produce permanent and severe pathological changes in the pulmonary parenchyma. Phagocytosis of carbon begins rather slowly in 14 to 24 hrs after inhalation[3] but after instillation of a liquid suspension phagocytosis begins promptly in 15 to 30 minutes[4,8]. Silica is phagocytized by the alveolar macrophages relatively

early and these cells are stated to move fairly rapidly into the lymphatics and lymphoid tissues where they cause a proliferation of mononuclear cells[19,28]. These macrophages ultimately may degenerate, possibly due to injury by the dust, and release the dust into the tissues where it may be taken up anew by other histocytes. That this movement of macrophages occurs is denied by some workers as previously stated. Dust particles do appear in peribronchial and hilar lymph nodes in phagocytes resembling alveolar macrophages but whether these are indeed alveolar macrophages which have migrated or whether they are local reticuloendothelial phagocytes of the lymph node is not known.

(d) On Disposal of Phagocytes

Dust cells are the principal means for the mobilization and removal of foreign material which has penetrated into the alveoli[19]. It is generally agreed that these pulmonary macrophages migrate into the ductus alveolaris and find their way up the respiratory tree. There are, however, many theories of secondary routes for the ultimate disposal of these cells. Many authors state that particle laden phagocytes do pass into lymphatic channels[25-28]. On the other hand others state that only free particles can enter the lymphatics and not loaded macrophages[29-31]. The late CC Macklin has stated that, in many thousands of lung sections, he has never found unequivocal evidence that alveolar phagocytes enter the interstitial tissue of the lung or the pulmonary lym-

phatics. He also described pulmonary sumps, most abundant in the walls of the respiratory bronchioles, but also found along arterioles, venules, interlobular septa and pleura. Varying numbers of lymphocytes are found in these sumps and, after exposure to dust, particulate matter is found in them as well. The normal superficial alveolar covering is a thin layer of fluid which Macklin believed to be an exocrine type of secretion of the granular pneumocytes (alveolar epithelial cells). This fluid he believed to be constantly draining into the pulmonary sumps and the excess into the bronchial mucous raft to be wafted up the pulmonary escalator. This explains the rich lymphatics present in the lung and is suggested as the route by which particulate matter finds its way into the pulmonary lymphatics and possibly into the interstitial tissues[30]. Evidence has been presented that dust filled phagocytes form layers on alveolar walls, gradually acquiring a network of reticulum fibres around them and eventually, by this layering process, they sink within the pulmonary parenchyma instead of remaining in an intraalveolar position[32].

(e) <u>On "Free" and "Fixed" Alveolar Phagocytes</u>

The more irritating particles produce an acute inflammatory response and phagocytosis by polymorphonuclear leucocytes occurs but with the usual less irritating particles the alveolar macrophage is the cell mainly concerned with phagocytosis. In routine sections prepared for light microscopy, these cells appear "free" in the alveolar spaces and in the tracheobronchial tree as well

as "fixed" to the alveolar walls. From recent work with the electron microscope it has been found that there is a continuous layer of thin attenuated epithelial cells covering the surface of the alveoli and that the "fixed" phagocytes apparently lie on this epithelial plate[33]. Although there may be short processes or pseudopods between the phagocyte and the epithelial cell plate, there is still a clearly defined space between them and so there is some question whether these so-called "fixed" phagocytes are really fixed. However, as the terms "free" and "fixed" are found in most of the literature on pulmonary macrophages prior to the late 1950s, these descriptive terms are retained here.

2 MATERIALS AND METHODS

(a) Animals

Adult male mice, of a strain which sporadically produced pituitary dwarfs, were used in these experiments. Each weighed close to 15 gms and was between 3 and 8 months of age. The only reason for limiting the study to male mice was that the female mice of the colony were being used in another experiment concerning chemical induction of cancer of the uterine cervix.

(b) Dusting Chamber

To expose the mice to suspensions of particulate matter in air, a dusting chamber was devised (fig 1). This consisted of a large glass storage jar covered by a foam rubber pad cemented on a wooden top. A glass tube, appropriately bent, was fitted through this top to the bottom of the chamber where finely divided dusting material was spread. To accomodate the animals a wire cage was

built and mounted on short legs. The bottom of the cage was lined by absorbent paper in an attempt to prevent contamination of the dusting material by urine. The dusting material was suspended by jets of air produced with the aid of a rubber bulb from a blood pressure cuff attached to the glass tube running to the bottom of the chamber. A small venting tube was placed through the top of the chamber to avoid pressure increases from the procedure.

(c) Dusting procedure

A preliminary attempt was made to use carbon in the form of lamp black as a dusting material, but it was found that this was so heavily phagocytized all cellular detail was lost, and it was even difficult to be sure of the number of phagocytes in the field. It was decided to use Carmine S.S. (B.D.H.) as the dusting material as it is insoluble in water, alcohol, and xylol, and shows clearly in sections stained with hematoxylin only. It was necessary to grind the carmine in a mortar and pestle as finely as possible. This was then spread on the floor of the dusting chamber in approximately 50 gram lots. The animals were exposed in the chamber in groups of from 5 to 10 for a single period of 2 hours duration. To keep the dust suspended it was necessary to use the rubber bulb at about 5 to 10 minute intervals. After the dusting period, the mice were sacrificed by exposure to chloroform at times varying from immediate sacrifice to sacrifice after 87 days (table #1). A total of 124 mice were used in the experiment but 10 died

during the period of the experiment and were removed from the study leaving 114 mice.

(d) Autopsy Technique

In order to prevent, if possible, artificial separation of the alveolar phagocytes from the alveolar walls by collapse of the lungs after the death of the animal, it was flayed and the thorax was removed along with the kidneys, spleen, stomach and liver. This flayed portion of the mouse was then fixed in 10% formalin for 48 hours. Following this, the thorax was opened and the contents removed along with the remaining portion of the trachea. The heart was dissected off and the leading anterior edges of the lungs were cut off with scissors to give a reasonably flat plane for sectioning. The lungs were then dehydrated, embedded in paraffin, and sections of a thickness of 5u were prepared and stained with hematoxylin only.

(e) Phagocytosis Evaluation Technique

In each animal phagocytes containing carmine were counted in 8 bronchioles or small bronchi in the left lung, if possible. When the phagocytes were very heavily loaded, it was difficult to distinguish the alveolar macrophages from polymorphonuclear leucocytes and in some cases it was necessary to depend on size of the cell as the main criterion. The number of carmine laden "free" phagocytes in alveoli and the number of carmine laden phagocytes "fixed" to alveolar walls were counted in 10 high power fields (i.e. approximately X 400). The fields were usually chosen in a

linear manner from the apex of the left lung, but if a large blood vessel or a bronchus was encountered that field was omitted. In addition to the number of phagocytes, the amount of free non-phagocytized carmine granules in the same bronchi and bronchioles in which phagocytes were counted, and the amount in the same alveolar fields, was estimated by a system of subjective grading of each from 0 to 4; a grading of ½ represented only a few granules of carmine, and a grading of 4 represented large conglomerate masses of carmine. The results of these counts and estimates in the groups of mice sacrificed at various times after exposure to the carmine dust are appended (table #2). This table was formulated by averaging the varying individual results of the mice sacrificed at the same time interval after completion of dusting (appendix A).

(f) <u>The Electron Microscope</u>

To confirm and possibly to enlarge upon previously published observations[33] on the pulmonary phagocytes, a small additional dust study was done using the Phillips 100B electron microscope. One normal control mouse was used along with one dusted with carmine for 15 minutes and two dusted for 2 hours. Each dusted mouse was sacrificed 3 days after completion of the dusting and small cubes of lung measuring 1mm or less to the slide were immediately fixed in osmic acid in a fume cupboard. Mertha-crylate embedding was used and sections were cut in the usual manner on a Porter-Blum microtome using glass knives. Thick

sections were cut, mounted on glass slides and stained with hot toluidine blue. If examination by light microscope showed carmine-containing phagocytes or other interesting reatures in the block, routine ultra-thin sections were prepared, mounted on the usual copper grids and examined in the electron microscope visually and photographically.

3 OBSERVATIONS

(a) General

In counting the carmine laden phagocytes, certain difficulties were encountered. Generally speaking, the phagocytes in the bronchi and bronchioles were very heavily laden with carmine granules which frequently obscured most of the cellular detail. During the first 24 hours after dusting there was usually a peribronchial infiltrate of polymorphonuclear leucocytes and many of these cells could be found intrabronchially where they also were heavily laden with carmine. When these two types of cell were so heavily laden that cellular detail was completely obscured, it was necessary to rely on size alone to distinguish between them. To a lesser degree this also was the case in the alveolar fields. There was also some difficulty at times in deciding whether a macrophage was "free" or "fixed" to the wall of an alveolus. It was judged "free" if a transparent or translucent line could be seen between the phagocyte and the alveolar wall with the light microscope.

In order to counteract bias on the part of the observer, sections were screened indiscriminately insofar as time of sacrifice of the experimental animal after dusting was concerned. No at-

tempt to compare or to correlate results was made until after the counts and estimates of the whole group of 14 mice were completed. This resulted in a further complication. As the alveolar macrophage was at first considered to be of prime concern, neutrophil polymorphonuclear leucocytes were merely disregarded in these counts. After completion of the initial counts and estimates, it was obvious that the polymorphs were possibly very important in clearance for reasons that will be apparent later. All counts of polymorphs in bronchi and in alveoli were therefore done after the initial phagocyte counts and granule estimates, and do not represent the identical fields or bronchi in which the macrophage counts and the free granule estimates were done.

The grading system of estimating the load of non-phagocytized free carmine granules was developed because it was not possible to count these granules. The granules in the alveoli were estimated at from ½ to 2u in diameter and measurements with an ocular micrometer showed that the majority were in the 1u diameter size range. In the bronchi some larger granules were noted. In both tracheobronchial tree and alveoli there was a tendency to clump into large conglomerates of carmine granules when the loading was heavy. This fact and the large number of carmine granules present made actual counts impossible. The grading of 0 to 4 cannot be related to an actual count in a linear manner as a grading of ½ represents only a few granules while a grading of 4 may represent 500 granules or even more. In totalling the gradings it can be

seen that 8 bronchi with gradings of ½ (i.e. total of 4) might represent some 24 to 100 granules while one grading of 4 (same numerical value) might represent 500 or more granules.

(b) Non-Phagocytized Free Granules

The results of the gradings were averaged for each group of mice sacrificed at the same time interval after dusting and these form part of Table #2. These are plotted on linear graph paper (Fig 2 for bronchi-bronchiolar estimates and Fig 3 for alveolar field estimates). As can be seen these curves appear to be exponential decay type curves. If it is assumed that there is a linear relation between the grading and the actual number of granules present, an incorrect assumption as noted above, then the biological half-life of the carmine granules in the pulmonary apparatus is about 24 hours (22 hours in the bronchi-bronchioles and 24 hours in the alveoli). It is certain that, if actual free granule counts had been possible, the curve would have been steeper and the half-life would have been somewhat shorter. There is no doubt, therefore, that ½ of the original load of carmine granules in the bronchi-bronchioles and also in the alveoli is disposed of within the first 24 hours after dusting. Disposal routes of possible importance might be up the tracheobronchial tree as free granules, into the pulmonary lymphatic system possibly by means of the pulmonary sumps, or phagocytosis by macrophages or polymorphonuclear leucocytes. It was not uncommon, especially in the first day or two after dusting, to find free granules in what appeared to be lymphatic spaces in the peri-

bronchial and perivascular areas (Fig 10). In addition there were small numbers of phagocytic cells containing a few carmine granules in the same areas especially in lymphoid tissue in these areas. Morphologically these phagocytic cells resembled alveolar macrophages but in general they were much less heavily loaded with carmine and whether or not they were alveolar macrophages could not be determined. The mediastinal lymph nodes also contained scattered carmine granules at least as early as 2 days after exposure. Much of the carmine in these nodes, indeed all of it in the early stages when only a small amount was present, appeared in the form of two or three granules in one small area and these did not appear to be intracellular. Later, when there was more carmine present, some of the granules were located in phagocytic cells resembling alveolar macrophages or reticulo-endothelial cells of the node. Of interest also is the fact that, even after 60 days a few free granules could be found on the alveolar walls. It could not be determined whether these were part of the original load which had never been phagocytized or whether they represented granules that were phagocytized and subsequently released, possibly due to necrosis of the original alveolar macrophage.

(c) Carmine laden Alveolar Macrophages

The results of the counts of carmine laden alveolar macrophages in the bronchi-bronchioles and those for "free" and "fixed" macrophages in the alveolar fields were averaged for each time period after dusting (Appendix "A") and these averages were plot-

ted on linear graph paper (Figs 4-6 and totals in Fig 7). The counts in the bronchi-bronchioles cannot be directly compared to the counts of the alveolar areas because of the difference in method of counting but the counts of the "free" and "fixed" alveolar macrophages can be compared directly as they are taken from the same fields. It can be seen from the graphs, that these carmine laden macrophages rise in numbers very sharply after exposure to the dust, reaching a peak between 3 and 6 days after the exposure. Following this peak the numbers decrease, but at a much slower rate so that a few are still noted, even after 60 days. It can also be noted from the graphs that "fixed" alveolar macrophages appear in larger numbers than "free" alveolar macrophages but, in spite of this, the general shape of the two curves roughly correspond. There was no direct evidence suggesting that the loaded alveolar macrophages entered the pulmonary parenchyma and proceeded via lymphatic channels to the regional lymph nodes although, as previously noted, some phagocytic cells, usually lightly loaded with carmine, were found in the peribronchiolar, peribronchial and perivascular areas and the lymph nodes of the hilum and mediastinum. In all these areas free granules were also frequently found and these particular phagocytic cells may well have been other than alveolar macrophages.

The carmine laden macrophages could be found in most high power fields in the first few days after dusting, but they tended to be present in largest numbers in the alveolar fields near the

terminal respiratory bronchiole. Possibly for this reason, there was occasionally considerable variation in the counts between animals sacrificed after the same time interval following dusting. A second reason for some variation in counts at the same time intervals after dusting was the fact that not all animals sacrificed at the time interval were members of the same group and therefore they were not dusted under exactly identical conditions. There was variation in dusting conditions for several possible reasons. Among these may have been average particle size; the same dust was used for more than one group exposure and it is assumed that some of the finer particles adhered to the sides of the dusting chamber and the cage more readily than the coarser particles so that there was progressively less exposure to the finer particles. A second factor may well have been the humidity of the air in the dusting chamber; on occasion a second group was dusted immediately after completion of the previous group and the average moisture content of the air in the chamber was definitely higher in this case than if the chamber were allowed to dry overnight.

(d) Carmine laden Neutrophil Polymorphonuclear Leucocytes

Initially the neutrophil polymorphonuclear leucocyte was disregarded as the primary interest of the study was the alveolar macrophage but the observations, that the half-life of free granules in the tracheobronchial tree and the lungs was under 24 hours and that the alveolar phagocytes did not reach maximum numbers un-

til the 3 to 6 day period after dusting, forced subsequent counts of the polymorphs laden with carmine in the bronchi-bronchioles and the alveolar fields. Individual counts of the polymorphs are tabulated in Appendix "A" and average counts are found in Table 2. These averages are plotted on linear graph paper (Fig 7 & 8) and it can be seen that the polymorphs phagocytizing carmine appeared very quickly following dusting, reached maximum numbers between 14 and 18 hours after dusting and then rapidly decreased reaching very small numbers by 4 to 5 days after dusting. They were rarely noted after the first week. They appeared early, in the peri-bronchial and perivascular areas but in this location had no carmine within their cytoplasm (Figs 11 & 12). They were not noted in the lymph nodes either with or without carmine granules. Loaded with carmine, they were found free in the bronchi-bronchioles, free in the alveoli, and apparently attached to the alveolar walls.

(e) <u>The Electron Microscopic Appearance</u>

This part of the study was short due to time limitations. Tissues from one normal and three dusted mice were studied. The alveolar macrophages could be identified fairly readily because they had a structure basically different from the epithelial cells of the alveoli. The major difference was that the epithelial cell possessed numerous round or oval spaces with irregular clean centers as if they had been torn. The macrophage never possessed these structures (Fig 13). It proved somewhat more difficult to

identify carmine granules within the macrophages. There are numerous oval dark fairly regular bodies in these cells and, unless the magnification is quite high or the photograph very clear, the cristae, which identify these as mitochondria, are not seen. The carmine granules appear as very dark, often irregular or angular, patches in the cytoplasm of the macrophages.

As has been noted in previous studies, there is a continuous epithelial layer covering the alveolar surfaces. The epithelial cell contains a nucleus and possesses a moderate amount of cytoplasm around the nucleus. Thin attenuated portions of the cell extend over the surface of the alveolus. From the capillary outwards towards the air surface the following can usually be found; an endothelial cell, basement membrane, tissue space, basement membrane, epithelial cell or attenuated portion of the cell. Beyond the latter there is frequently a small finger-like interdigitations of the alveolar macrophage that seem to fit with similar indentations in the epithelial cell but there is always a visible space separating them. An alveolar macrophage lying directly on a basement membrane was never found. Some of the macrophages were found free in the central part of the alveolus.

4 DISCUSSION

This dusting experiment was designed to study the clearance of dust from the pulmonary apparatus with particular reference to the timing of the phagocytic responses and the relative times of the appearance of maximum numbers of macrophages in the alveoli, "free" and "fixed", and in the tracheobronchial tree.

(a) Clearance of Carmine Granules

Interest centered on the alveolar macrophages and the mechanism of dust disposal in the pulmonary apparatus. The grading system used in assessing the amount of free non-phagocytized carmine present, taking into account the previously discussed non-linearity of the system, reasonably represents an index of the dust load in the alveoli and in the tracheobronchial tree. The decrease of this index in the first 24 hours is striking and it is certain that if actual granule counts had been possible, the curves would have been even steeper and the so-called "biological half-life" of carmine would have been even shorter. The appearance of the curves (Fig 2 & 3) strongly suggests exponential expressions of the decay type and it appears that the biological half-life of carmine under the conditions of this experiment, while somewhat indefinite, is certainly less than 24 hours. While some of this loss of free carmine granules is due to phagocytosis by alveolar macrophages, it is quite obvious that most of it is not due to these cells, as they reach maximum numbers in 3 to 6 days after exposure at which time the biological half-life of the free carmine granules has long passed. The size of the particles is known to influence the rate of clearance of particulate matter from the lung. It has been shown experimentally that, as particle size decreases the time of clearance from the lung increases[34]. It has also been shown that, for a given load of radioactive material, the biological half-life could be considerably shortened by increasing the total

dust load with an inert dust (carbon) and this was correlated with increased phagocytic activity[34]. That a heavier dust load results in larger numbers of macrophages seems reasonable but these present experiments suggest that, at least in the case of carmine, there is another factor operating which appears to be more important in the clearance of the dust in the early hours after dusting.

In the normal lung it has been stated that there is a thin layer of fluid covering the alveolar surfaces[30]. The same source describes lymphoid collections around the respiratory bronchioles, so-called pulmonary sumps, and describes alveolar dust drift in the alveolar fluid to these sumps and then into the pulmonary lymphatics. In these present experiments, even in the early hours, free carmine dust granules were found in perbronchial and perivascular areas believed to be lymphatics. There is a tendency for the carmine granules to clump into larger masses and it may well be that there is considerable increase of the lung fluid and a drift of this along with carmine granules toward the respiratory bronchioles in the early hours. The larger agglomerates are too large to enter the sumps and may pass up the tracheobronchial escalator on a fluid raft but some of the individual granules of dust possibly do enter the sumps and travel into the pulmonary lymphatic channels, accounting for the free carmine granules found in these areas (Fig 10 & 14).

In the first 2 to 3 days after dusting it was not uncommon to find agglomerates of carmine along with polymorphonuclear leu-

cocytes and mononuclear macrophages both loaded with carmine granules in sections of oesophagus. For the first few days the intestinal tract was colored red from carmine. Much of this was swallowed during the dusting period, some came later from swallowing carmine adhering to the fur and some probably came from swallowing carmine granules and loaded phagocytes ejected by the pulmonary escalator. No attempt was made to assess the amount on the skin or in the gastrointestinal tract in relation to that in the lung but it has been shown that during exposure the heaviest concentration was on the skin, next heaviest in the gastrointestinal tract, and least in the lungs; at one day this order of decreasing concentrations changed to gastrointestinal tract, skin, and then lung; at one week after exposure most of the remaining dust was in the lung, with a much smaller concentration in the gastrointestinal tract, and least in the skin[35]. It appears that nearly all the material eliminated from the lung is transported to the gastrointestinal tract and excreted in the feces[35].

(b) Responses of the Alveolar Macrophages

The results show that the alveolar macrophages, both "free" and "fixed" as well as those found in the tracheobronchial tree, reach a maximum number in the time interval of from 3 to 6 days after completion of the dusting. It was presumed that if the "free" forms were the active functional forms of the macrophages, these would increase more rapidly than the "fixed" forms. This, in fact, did not happen. The "fixed" macrophages were always

present in the alveolar field counts in higher numbers than the "free" forms. Once the macrophages are in the tracheobronchial tree, quite obviously they can take no further part in alveolar phagocytosis of dust. This result, therefore, supports the concept that the "fixed" alveolar macrophages are the active functional forms and that the "free" macrophages in the alveoli are, in the main, loaded phagocytes seeking their way into the tracheobronchial tree and out of the pulmonary apparatus.

Brieger and LaBelle, using both dusting and intratracheal injection techniques and doing counts of the phagocytic cells recovered by intratracheal washing, showed that the peak numbers of "free" loaded phagocytes were present during the period of maximal lung clearance and thereafter, as the rate of clearance decreased, so did the number of "free" loaded phagocytes[36]. These authors used carbon and found the biological half-life was 19 hours at which time the loaded phagocytes recovered by wash-out was maximal. The above results are at variance with those of this experiment where the biological half-life of carmine was somewhat under 24 hours, but the maximal numbers of loaded macrophages, either "free" or "fixed" were noted in the period of 3 to 6 days after dusting. The reason for this difference is not obvious. Different materials were used for the particulate matter and the manner of assessing the phagocyte response was quite different. In spite of this, one would expect that the counts by wash-out would be more or less equivalent to the combined counts

of "free" phagocytes in the alveoli and the tracheobronchial tree. Of some interest is the fact that, if one combines the counts of the total numbers of alveolar macrophages and the total numbers of polymorphonuclear leucocytes (Table #2) the resultant combined total reaches a peak at 18 hours after dusting (Fig 9). It is, of course, possible that carbon does not call forth a significant polymorphonuclear leucocyte response in the lung but this suggests that Brieger and LaBelle may have included heavily loaded polymorphs in the macrophage counts.

If there were a direct association between the quantity of alveolar dust and the phagocytic responses one would expect in any group of animals sacrificed at the same time after exposure, that those which had an above average grading of alveolar particle loading should also have an above average phagocytic response. Close checking of the results tabulated in Appendix A show that there is correlation between alveolar loading of granules and "fixed" alveolar macrophages and also between alveolar loading and total alveolar macrophages of approximately 50%. The same correlation can be shown between alveolar loading of granules and alveolar polymorphonuclear response and between bronchial loading of granules and bronchial polymorphonuclear response. A correlation of 50% could be expected between any two variables by chance alone and so there is no proof here that a correlation exists between loading of the lungs with particulate matter and the phagocyte response, although this would appear probable.

(c) Responses of Polymorphonuclear Leucocytes

The earliest response of the polymorphs was an infiltration of these cells in the peribronchial and perivascular areas as well as some margination of leucocytes in the smaller arteries and larger arterioles (Fig 11 & 12). These cells at this stage contain no phagocytized material and the response appears to be one of early inflammation. Later the polymorphs appear in larger numbers in the alveoli and bronchioles and here they contain phagocytized carmine. The numbers of polymorphs containing carmine granules rise very rapidly in the first 24 hours reaching a peak at about 18 hours. After this the numbers drop off again very rapidly so that, after approximately 3 days very few are noted (Fig 15). Disposal of these loaded phagocytes appeared to be entirely by the tracheobronchial route as no polymorph containing carmine was noted in the regional lymph nodes although occasionally one, noted in the peribronchial or perivascular area, appeared to contain a small amount of carmine. Some of the loaded polymorphs could have degenerated and released their load of granules but there was no indication that this did, in fact, happen[39].

Of particular interest is the relationship between the biological half-life of the free carmine granules and the time of appearance of the carmine laden polymorphs in maximal numbers. The biological half-life of the carmine, although somewhat indefinite, is certainly less than 24 hours following dusting and the loaded polymorphs appear in maximal numbers at approximately 18

hours. It appears therefore that this early rapid disposal of free carmine granules is associated with phagocytosis of carmine by polymorphs and clearance of these loaded cells up the tracheobronchial tree. In addition to this mechanism, the rapid phase may be associated with some clearance of granules into the pulmonary sumps and into the lymphatics of the lung as a few granules are noted there early. It is certain that free granules in the ciliated part of the tracheobronchial tree are assisted out of the tree by ciliary movement in this early phase but whether or not there is a drift of alveolar fluid and granules towards the terminal bronchioles is not proven in this experiment. The timing of the polymorph response suggests an acute inflammatory reaction and this may well be accompanied by increased fluid in the alveolar walls and increased lymph flow in the lungs but this also was not evident from the study.

(d) <u>The Electron Microscopic Appearances</u>

The observations here confirm those of Karrer[33]. There is a continuous epithelial covering in the alveoli and the alveolar macrophages, although closely applied to this epithelium, do not appear to be attached and do not lie on a basement membrane but rather on an epithelial cell. Thus the terms "free" and "fixed" alveolar macrophage describe cells which differ only in the amount of space spearating them from the epithelial cell surface. Time limited this part of the study but, in the survey that was done of various areas of the lung parenchyma, no cells were found that re-

sembled the alveolar macrophage closely enough to form a conclusion or even a guess as to the origin of the macrophage.

5 SUMMARY

This experiment was done to study the phagocytic responses in the lung to airborne particulate matter consisting of finely ground carmine. After a single dusting with carmine, it was found that one half the initial load of granules was disposed of within the first 24 hours. Disposal consisted of elevation up the tracheobronchial tree by the pulmonary escalator and phagocytosis by polymorphs and alveolar macrophages although a small amount of apparently free granules entered the pulmonary lymphatic system.

The maximum numbers of carmine laden polymorphonuclear neutrophils appeared at approximately 18 hours after dusting while the maximum numbers of carmine laden alveolar macrophages appeared at from 3 to 6 days after dusting, both in the bronchi-bronchioles and in the alveoli. The polymorphs almost disappeared from the lungs by 3 days after dusting. It therefore appears that the early clearance of carmine from the lung is primarily a function of the polymorphonuclear leucocytes. The alveolar macrophages clear that carmine not eliminated by the polymorphs, by simple cilial action or by lymph drainage. Only minor clearance of carmine occurs through the pulmonary lymphatics and here the mechanism appears to be by means of free granules entering the pulmonary sumps although this is not entirely certain.

Almost all the carmine deposited with the single dusting is cleared by 60 days after dusting, but it is interesting to note that even at this late date a few intraalveolar free granules could be found. It is probable that these represented granules that had been phagocytized previously and were released when the phagocyte died prematurely before finding its way out of the lung. By direct measurement it was found that most of the granules in the alveoli were close to lu in diameter.

The "free" and "fixed" alveolar macrophages responded in an identical manner chronologically although the "fixed" forms were always present in the alveolar fields in greater numbers than the "free" forms. By means of the electron microscope it was determined that the "fixed" form was not really fixed in the alveolar wall as it rests on the epithelial cell, which forms the lining of the alveoli, rather than on a basement membrane.

PART II

EXPERIMENTS INVOLVING PHAGOCYTOSIS OF ERYTHROCYTES

1 REVIEW OF PREVIOUS OBSERVATIONS

(a) On Phagocytosis of Erythrocytes

There has not been a great deal of work done in this subject. Muir and Niven[37] in 1935, using rats and mice, found that the formation of granular and crystalline pigment from subcutaneously injected erythrocytes was intracellular. They found that formation of hemosiderin was evident from 24 hours onward following subcutaneous injection of erythrocytes and this pigment was both diffuse and granular. Hematoidin, the iron free pigment first appeared about the 7th day and was thought to be from hemosiderin, the iron having been removed by some mechanism. The formation of hematoidin was not noted in rabbits.

(b) On Pulmonary Phagocytosis of Erythrocytes

There are a great many clinical reports of cases of idiopathic pulmonary hemosiderosis but only one experimental study could be found. Magerey[38] used rats and single, as well as repeated, intratracheal instillations of blood in order to cast doubt on the proposition that the size, shape, and distribution of the deposits of hemosiderin laden macrophages in mitral stenosis point to the vessels of the small bronchi as the source of this blood rather than diffuse alveolar capillary hemmorhage. By simple instillation of blood intratracheally, wide, although not general, dispersion was obtained throughout the lungs and yet there was a tendency, eventually, for hemosiderin laden alveolar macrophages

to accumulate in focal intravalveolar aggregates. It was also noted that small amounts of hemosiderin were formed as early as 2 days after the instillation. These aggregates in rat lungs are quite similar to those in human lungs of patients suffering from chronic venous congestion.

2 MATERIALS, METHODS AND OBSERVATIONS

(a) Animals

As a first step in the study of red cell clearance from the lungs of mice, it was decided to follow the production of hemosiderin by means of the Prussian Blue reaction. Routine sections of some of the previously dusted mice stained with Perl's stain all showed greater or lesser numbers of phagocytes staining positive for iron. Therefore, as a preliminary step, a group of 10 mice were sacrificed by exposure to chloroform and, after processing the lungs in the usual manner, routine sections were made and stained with Perl's stain. Of these 10 mice, 5 were older adult mice in the age range of 6 to 9 months, and the other 5 were young mice in the age range of 1 to 3 months. The total number of phagocytes showing a positive stain for iron were counted in the alveoli, in the bronchioles and bronchi, and in the peribronchial and perivascular areas of all lobes in the entire section.

The iron positive macrophage count in the two small groups of young (CGY) and older (CGA) mice can be seen on table #3. Although the average numbers of iron positive macrophages are greater in the older age group (CGA), considerable overlap can be noted between

mouse #4 of the younger group (CGY) and mice #1, 3 and 4 of the older group (CGA). In the older group it was quite common to find iron positive cells in the peribronchial and perivascular areas. Some of these cells were somewhat elongated and may have represented smooth muscle, but many of them are not smooth muscle. They appear to be collections of lymphocytes, plasma cells and histiocytes, the latter of which appear to be the iron-containing cells. It was also common to find iron-containing phagocytic cells in the sinusoids of the lymph nodes in the peribronchial and paratracheal areas. These latter cells are presumed to be part of the reticulo-endothelial system although they are morphologically similar to the pulmonary macrophages and this origin cannot be ruled out completely. In the dusting experiments similar cells were frequently found in the same areas of lymph nodes. In these experiments the cells occasionally contained carmine granules but a few contained a round body that had the morphological appearance of an erythrocyte.

From this small survey it appeared that almost all of the older mice did have phagocytes in the lung containing material which stains positive for iron with Perl stain. The younger group of mice, although also showing some iron positive phagocytes, were much less pronounced in this respect. Using the Student "t" test for significance of differences between averages of the number of iron positive phagocytes in the older and younger groups (table 3B), it can be seen that there are only about 23 chances in 1000 that the averages in the alveoli are chance variations (Probability =

0.0235). In the bronchi and bronchioles and in the peribronchial and perivascular areas there is about 1 chance in 10 that the differences are chance variations. It therefore appears that there is a significant difference in the numbers of alveolar phagocytes containing iron positive material between older mice and young mice, at least in the alveolar areas. For this reason it was decided to use the same mouse colony for these experiments but the litters were segregated on a monthly basis and mice between the ages of 2½ and 4½ months were used.

(b) Autopsy Technique

All mice were killed by exposure to chloroform in a small chamber. The animals were flayed as in the previous dusting experiments but, as there was no necessity to prevent collapse of the lungs and as there was a possible use for mouse livers, the thorax was immediately separated from the abdominal contents by dissection above the liver and severing the spinal cord at this level. Invariably this procedure resulted in perforation of the paper-thin diaphragm and collapse of the lungs. In spite of this it was worhtwhile to retain the technique as the various lobes of the lungs were fixed in the proper position, facilitating preparation of the lungs for section. All tissues were fixed in 10% buffered neutral formalin for 48 hours before being prepared for section as was done in the previous dusting experiment. Most of of the observations and all of the counts were made from routine sections cut at approximately 5u and stained for iron with Perl's

stain. Some sections were also prepared and stained with hemotoxylin alone and others with HPS (hematoxylin, phloxine and safronin).

(c) Evaluation of Hemosiderin Production

All Perl stained preparations were screened under low power (X10 obj.) and if there was concentration of blue staining macrophages in any area of the lungs, 10 high-power fields were counted from that area. Similarly 8 bronchi or bronchioles were selected by choosing those which appeared to be most heavily laden with iron positive macrophages. In other words, the counts were done in the same manner as those of the dusting experiment but, instead of random selection of alveolar fields and bronchi-bronchioles, those fields giving the highest counts were selected. It was felt that this was necessary because the iron positive macrophages were often distributed in a very patchy manner. Wide, generalized distribution was the exception rather than the rule and invariably indicated a high count. In the experiments no note was taken of whether the pulmonary macrophages staining positive for iron with Perl's stain appeared to be "free" or "fixed".

It should be noted that, with the method of counting used, the same count could be found in two mice, one of which had a heavy loading in one lobe only while the other might show the same concentration in all lobes and so have many more iron positive macrophages in the total lung fields. In the observations made on iron containing phagocytes in normal mice, total lung

fields were counted and startling differences were not noted between these total field counts and experimental counts of control mice. The numbers involved however, were quite small and it is not possible to draw conclusions from this.

(d) Procedures and Observations

In the following experiments group A Rh positive human blood was used as a source of erythrocytes.

FIRST EXPERIMENT

(i) In the first experiment 6 mice from 6 to 9 months of age were used. Heparinized human blood was centrifuged and the plasma removed, following which the cells were washed three times in 0.9% saline and the original volume reconstituted with the saline. Under ether anaesthesia 4 mice had 0.05ml of the saline suspension of blood cells instilled intranasally, using a tuberculin syringe and a blunted #24 needle. The other 2 mice had 0.1ml of the saline suspended cells instilled similarly. The mice were then allowed to recover from the anaesthetic and sacrificed at four day intervals as outlined in table 4.

The first experiment was planned as a trial of method and technique. The results of counts of iron positive phagocytes as shown in table 4 suggested that hemosiderin was produced as early as the fourth day and that the maximum production was probably about 8 to 12 days after instillation. The counts also suggested that instillation of 0.1ml of blood give considerably higher numbers of iron positive phagocytes than instillation of 0.05ml.

It was felt that the higher counts would be of assistance in distinguishing between the amount of iron "normally" found and that resulting from the breakdown of instilled erythrocytes, and so it was decided to use 0.1ml of blood in the larger third experiment. Unfortunately the mouse sacrificed at four days after the 0.1ml instillation (mouse 4-2-4) developed a marked bronchopneumonia. At this time it was felt that the instillation was probably not the prime cause of the pneumonia and so it was decided to go ahead with the larger third experiment. It was also decided at this time that plasma might aid phagocytosis and so the third experiment was planned to utilize heparinized human blood rather than washed saline reconstituted red blood cells.

SECOND EXPERIMENT

(ii) In the second experiment an attempt was made to inject blood into the lung directly but blindly through the chest wall. Eight mice were anaesthetized with ether and 0.1ml of heparinized human blood was injected into the left chest from the mid-axillary line near the mid chest in a slightly dorsal direction. It was thought that this would probably hit the left lung satisfactorily. One mouse died approximately one hour after the procedure but the rest recovered and they were sacrificed at 6 hours, 1 day, 2 days, and 3 days after injection.

It was thought that, by direct injection of blood into the lung, there would be a well defined area of trauma along with the blood and this would allow the rate of hemosiderin formation to be determined with greater accuracy. This proved to be dif-

ficult in practice. The mouse that died approximately one hour after the operative procedure was a victim of mediastinal haemorrhage, the needle having damaged one of the mediastinal blood vessels. Of these 8 mice, there was no gross evidence of a definite nature to indicate an area of trauma in the left lung. The mice were killed in pairs at the times noted previously. One mouse killed at 1 day after injection showed a haematoma of the left chest wall where the needle entered. One mouse killed at 2 days after injection showed congestion of the left lung but the companion mouse showed a similar appearance in the right lung lobes. Those mice killed at 3 days after injection showed congestion bilaterally in the anterior and lower lung areas. Microscopic examination of sections stained with hematoxylin and with Perl's stain failed to reveal a definite area of the left lung traumatized or containing many alveolar phagocytes containing iron positibe material or containing many alveolar erythrocytes.

THIRD EXPERIMENT

(iii) In the third experiment 48 mice were used along with 22 control mice. The experimental animals all had 0.1ml of heparinized human blood instilled into the nostril while anaesthetized as outlined in the first of these experiments. It was necessary to do this rather slowly as it was comparatively easy to block the airway and cause the death of the animal. The controls were anaesthetized along with the experimental animals, but had no instillation of material in the tracheal bronchial tree. All

animals were allowed to recover and then sacrificed at 2, 6, 12, 18 hours, 1, 2, 3, 5, 7, 9, 15, and 24 days after instillation. There were 4 experimental mice and 2 controls sacrificed at each time interval after instillation except at 15 and 24 days when only one control mouse was available. Each group of experimental and control mice averaged either 3 or 4 months of age. This is all outlined on table 5.

This third experiment was planned as a large scale follow-up of the first experiment. It will be noted on table 5 that there was considerable scatter to the counts but the averages show a peaking around the seventh day after the instillation of blood. Following this there is a gradual decrease of iron positive macrophages but even after 24 days following instillation there is still a moderately high count in the alveolar field. In the bronchi-bronchioles there is a similar, although less marked, increase in iron positive phagocytes at least from 9 to 15 days after instillation. Very few mice were without any iron positive cells resembling pulmonary macrophages in the paratracheal lymph nodes if these were included in the section. The control mice did not differ in this respect from the experimental mice. None of the mice in this experiment showed any visible adverse effect from the experimental procedure except mouse #3 in the group killed one day after instillation, in which there was a light patchy bronchopneumonia. There was a tendency for the areas of maximal iron positive macrophage counts to be located peripherally near the pleural

surface, although this was not universal. No initial polymorph response was noted nor were there any polymorphs, staining positive for iron with Perl's stain, noted later. This was in contrast to the polymorph response noted in the dusting experiments with carmine.

3 DISCUSSION

(a) The Student "t" Test

Considerable use has been made of this test in an effort to test the statistical significance of the results obtained in these experiments. This test is designed to test the significance of differences between the means (averages) in two groups. The assumption is made that the two groups being averaged separately are part of an identical larger population and the results of the test (i.e. value of "t") can be translated to probably that the two smaller groups are part of the same larger population by mathematical tables. The formula for "t" is as follows:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{s^2}{n_1} + \frac{s^2}{n_2}}}$$

Where $\bar{X}_1$ = mean of the first group

$\bar{X}_2$ = mean of the second group

n_1 = number of separate items in first group

n_2 = number of separate items in second group

and $$s^2 = \frac{\text{Sum } (X_1 - \bar{X}_1)^2 + \text{Sum } (X_2 - \bar{X}_2)^2}{n_1 + n_2 - 2}$$

where X_1 and X_2 = individual values of items in first and second groups respectively.

It can not be proven statistically that the two groups involved are not part of the same general population but, the probability that they are part of the same population can be shown mathematically. A probability level must be selected at which the experimenter is willing to accept the fact that the chance, of the two groups averaged being from the same population, is remote enough to indicate that the original proposition is incorrect and that the difference of averages is significant. This probability level may be set as high as one wishes but it is usual to accept a P value of 0.05 as a reasonable level. This means that the chance that the two groups averaged are random variations of a single larger group is less than 1 in 20. This value has been used in assigning significance to the results in these experiments.

(b) Erythrocyte Phagocytosis and Hemosiderin Production

Insofar as the initial control groups of 5 young and 5 adult mice are concerned, the counts outlined in table 3 suggest that phagocytic cells staining positive for iron with Perl's stain are present in most mice and the counts also suggest that older mice possess these cells in larger numbers than younger mice. A sta-

tistical comparison of these two groups (CGA & CGY) and their means, using the student "t" test, is shown on table 3B. As can be seen there is a fair chance that the results obtained for iron positive phagocytes in the peribronchial and perivascular areas are from the same general population (P = 0.1 or chance is 1 in 10) but there is only a slight chance that the alveolar averages are from the same population (P = 0.0235 or chance is about 1 in 50). It was assumed from this that there is an increase in the number of alveolar phagocytes staining positive for iron with Perl's stain as mice get older.

(i) For the first experiment the results of the total counts of iron positive phagocytes in the sections of these lungs, noted on table 4, showed a definite increase in the numbers of these cells by 8 days and in addition, suggested that the 4 day counts were also probably increased. It also appeared that instillation of 0.1ml rather 0.05ml of blood resulted in higher counts as can be seen on the table (i.e. the difference between mouse 4-1-8 and mouse 4-2-8). For these reasons the third experiment was planned to cover the period of 2 hours to 24 hours after instillation of 0.1ml of blood.

(ii) For the second of these experiments there were no results obtained on which conclusions could be based. It is quite possible that, in most of these mice, the blood was injected into the pleural cavity instead of into the lung parenchyma. It was not repeated but, in retrospect, it is thought that the in-

jection area could have been determined easily if some inert particulate matter had been used with the blood (e.g. sterile carmine or carbon). It would then have been possible to see whether this particulate matter was in the pleural cavity or the lung and to localize it.

(iii) In the third and largest experiment in this series, the maximum number of iron positive alveolar macrophages was noted at approximately 7 days (table 5). The control mice were of two groups differing in their average age, the first control group being from 2½ to 3½ months old and the second control group being 3½ to 4½ months old. All mice, both experimental and control, killed at the same time after the experiment started, belonged to one of these age groups only and were not mixed. The student "t" test was applied to the two age groups of control mice and the results of this can be seen on table 5B. It will be noted that the chance that these two groups were merely part of the same population was fairly high in respect to the iron positive macrophages in bronchi (P=0.175) and in alveolar areas (P=0.296)(i.e. approximately 2 in 10 and 3 in 10 respectively). Nevertheless, as the previous study had shown that there was probably a significant difference in this respect when mice with greater difference in age were used, the experimental mice were compared with the control group of corresponding age with the student "t" test. The results of this can be seen on table 5C and it will

be noted that the first alveolar result which is significant (i.e. P = 0.05 or less) is at 2 days and the results from 7 to 24 days all appear significant. Insofar as the bronchi-bronchioli are concerned, it can be seen that only the counts at 2 hrs, 5, 9, and 15 days show a significant difference from the control group.

It can not be proven statistically that any of these averages really are significant of experimental appearance of hemosiderin in the macrophages but at 7 days there is only 1 chance in 100 that the alveolar count in the experimental group is a chance variation of the same larger group containing the controls. This count at 7 days also happens to be the highest average count obtained. In view of this, as it is not probable that hemosiderin appeared in maximal quantity suddenly, it is also probable that some of the earlier counts are significant although most do not appear statistically significant.

The prime method of disposal of erythrocytes injected subcutaneously is phagocytosis by tissue histiocytes and hemosiderin is often present amounts detectable by the Prussian blue reaction as early as 24 hours after the injection[37]. This suggests that the statistically significant alveolar count at 2 days is correct and that there is a slow rise from instillation to 7 days and then a gradual slow fall in numbers of iron positive macrophages. If this is true, then the bronchial counts from at least 5 days and possibly from 3 days as well are likely significant.

No extracellular hemosiderin was noted during this study and

the evidence, partly statistical, supports the concept that the disposal of erythrocytes free in the alveoli is by phagocytosis by the alveolar macrophages in which cells hemosiderin is produced from the breakdown of the red blood cells. It would appear from this study and from the results of the previous dust study that most of these phagocytes find their way up the tracheobronchial tree and out of the lung. Whether or not the iron in these cells is lost to the animal would depend upon whether the sputum is swallowed or expectorated. In the mouse there does not appear to be much of a cough reflex or expectoration and so it is probably swallowed and not lost to the animal. In the human this might well account for loss of iron from the body in conditions such as mitral stenosis.

5 SUMMARY

Most of the mice in the colony used for these experiments had alveolar macrophages which stain positive for iron with Perl's stain and the counts suggested that the numbers of these increase with increasing age of the mouse. For this reason direct evidence of the first appearance of hemosiderin after instillation of blood into the trachea is lacking. However, by statistical means, it was shown that the increased numbers of iron positive macrophages in the alveoli at 2 days after instillation was likely significant and that the maximum count of iron positive macrophages in the alveoli which occurred at 7 days was almost certainly significant (only 1 chance in 100 that it is not significant).

It is likely, therefore, that alveolar disposition of erythrocytes is similar in timing to subcutaneous disposition and that, if these lungs normally contained no iron positive alveolar macrophages, one would probably find traces of hemosiderin as early as 24 hours after instillation[37]. It also appears that the production of hemosiderin is intracellular and that many, if not all, of these iron positive macrophages are cleared up the tracheobronchial tree.

PART III

POST MORTEM PHAGOCYTOSIS OF CARMINE BY ALVEOLAR MACROPHAGES

1 REVIEW OF LITERATURE

During this study nothing was found on this subject although there is considerable experimentation on phagocytosis in tissue culture. This is not quite the same condition as in the lung after the death of the animal and so a short experiment was planned.

2 MATERIAL AND METHODS

(a) Animals

Adult mice of the same colony and age range as used in the dusting experiments were used in this experiment.

(b) Experimental Procedure

A group of 7 mice were killed by cerebral trauma and the bodies placed in a water bath at 38° C. A suspension of carmine was prepared in advance by mixing approximately 2ccs of ground carmine in approximately 15ml of 0.9% saline and allowing 30 minutes for the larger particles to settle. When these animals had been dead for 5, 10, 20, 30, 40, 50, and 60 minutes each was taken from the bath, the trachea was exposed and tied off, and 1ml of the saline-carmine suspension was injected into the lower tracheobronchial tree following which the animal was returned to the water bath for 1 hour.

It was felt that use of serum rather than saline might encourage phagocytosis of the carmine particles and so a second short experiment was carried out. A suspension of carmine in

serum was prepared by using 1cc of ground carmine in 5ml of mouse serum, mixing and centrifuging at 1000cps for 5 minutes, thus allowing the larger particles to precipitate. The supernatant suspension was injected into the trachea as previously described and under the same experimental conditions. The injections were made into the tracheas of mice that had been dead 0, 5, 10, 20, and 30 minutes. As before, the bodies were then returned to the water bath for 1 hour.

(c) Autopsy Technique

The technique of autopsy was essentially the same as that described in the dusting experiment. Sections were stained with hematoxylin only.

(d) Evaluation

These sections were merely screened for alveolar macrophages containing carmine granules. No actual count of the number of macrophages loaded with carmine was made.

3 OBSERVATIONS

(a) General

The carmine granules in these experiments had a tendency to concentrate in the periphery of the lungs near the pleural surface rather than around the alveolar ducts and respiratory bronchioles..

(b) Phagocytosis of Carmine

There was essentially no difference between the experiment using a saline suspension of carmine and that using a serum sus-

pension, although there may have been a bit heavier uptake of carmine in the latter case. It was comparatively easy to find carmine laden macrophages in the lungs of mice receiving the suspension up to ½ hour after death and most of these were of the so-called "free" type. After this time, however, these carmine laden macrophages were found with increasing difficulty. It was very difficult to find a macrophage containing carmine in the lungs of mice that received the instillation one hour after death. Even at 1 hour, however, there were alveolar phagocytes which appeared to contain carmine granules but, when the microscope was focussed through the thickness of the cell, the granules could be seen adhering to the periphery of the cell rather than inside of the cell.

4 DISCUSSION

(a) Distribution of Fluid Suspension in Lungs

It has been previously noted that fluid material entering the lower respiratory tract tends to flow directly to the terminal parts of the lung[19]. This is different than the distribution of particulate matter suspended in air and it appears to depend on fluid viscosity, body position and other less understood factors. These short experiments confirm previous observations on the difference in distribution of air-borne and fluid-borne particles.

(b) Post Mortem Phagocytosis of Carmine

That there would be phagocytosis by alveolar macrophages af-

ter death of the animal seemed probable, as it is well known that the state of clinical death is not synonymous with the simultaneous death of all cells of an organism. These experiments showed that the alveolar phagocytes remain functionally active to some extent for at least ½ hour following clinical death of the animal and are able to phagocytize carmine granules. The accumulation of carmine granules around the cellular membrane of cells exposed after this ½ hour period may be evidence of some further functional activity or it may be merely the result of changed surface tension of the cell membrane on the death of the cell. From part II of this study it appears that the formation of hemosiderin from erythrocytes and macrophages takes 24 hours or more and so post mortem production of hemosiderin appears to be very unlikely or impossible.

GENERAL SUMMARY

Experiments were performed to study (in the lung) phagocytosis of air-borne particulate matter, fluid-borne red blood cells, and fluid-borne particulate matter after death of the animal.

In the experiments with air-borne particulate matter in the form of carmine it was found that more than ½ of the original load of inhaled particles was disposed of within the first 24 hours. During this time the polymorphonuclear leucocytes were numerous and very active phagocytes while the alveolar macrophages, although showing increased numbers containing phagocitized particulate matter, were not the predominate cells. Alveolar macrophages reached their maximum numbers between 3 and 6 days after dusting and by this time polymorphonuclear leucocytes had almost disappeared. It therefore appeared that the very early removal of air-borne carmine granules from the pulmonary apparatus was a concern of the polymorphonuclear leucocytes, supported by ciliary action in the tracheobronchial tree and, possibly increase in fluid movement on the surfaces of the alveoli and bronchi. After the early hours, removal of foreign particles was increasingly the concern of the alveolar macrophages. The main, and possibly only, route of clearance of these loaded macrophages out of the alveoli was up the tracheobronchial tree although it could not be absolutely proven that none entered the pulmonary lymphatic system or the pulmonary parenchyma. It was reasonably certain that free granules of particulate matter entered the pulmonary lymphatics,

possibly by means of the so-called pulmonary sumps.

By direct measurements it was found that the granules of particulate matter which reached the alveoli were mainly in the 1u diameter size range. Electron microscopy confirmed the fact that the alveolar macrophage was not really "fixed", as seen in the standard light microscope, but rested on the epithelial cell of the alveolar wall.

Most of the mice studied possessed alveolar macrophages which stained positive for iron with Perl's stain. For this reason statistics had to be used to indicate probable time of breakdown of hemosiderin from erythrocytes. It was shown that, two days after instillation of erythrocytes into the lungs, there was a significant rise in the number of alveolar macrophages staining positive for iron. It was also shown that the highest number of iron positive alveolar macrophages occurred at 7 days after instillation of blood. It was concluded that the disposal of erythrocytes in the alveoli was likely similar in timing to the disposal of erythrocytes in the subcutaneous tissues i.e. the earliest appearance of the breakdown of hemoglobin to hemosiderin at about 24 hours and the maximum amount appearing at from 3 to 7 days[37]. The production of hemosiderin appeared to be within the macrophages and the main and possibly only route of clearance of these cells was up the tracheobronchial tree.

After the death of the animal the alveolar macrophages remained phagocytically active for at least ½ hour. After this

period there was a suggestion that the phagocytic cell might still be active to a slight extent but only enough to hold particles to the surface and not phagocytize them. There was no indication that these cells could remain active for 24 hours which is about the minimum time necessary for the formation of hemosiderin from erythrocytes and so post mortem formation of hemosiderin was not considered possible.

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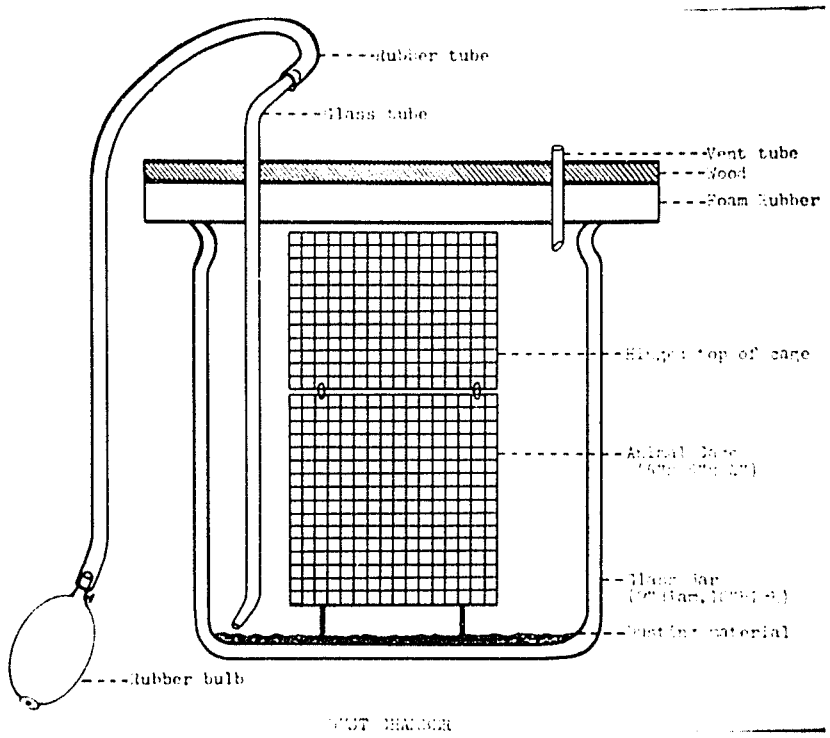

Figure 1 - Dusting Chamber

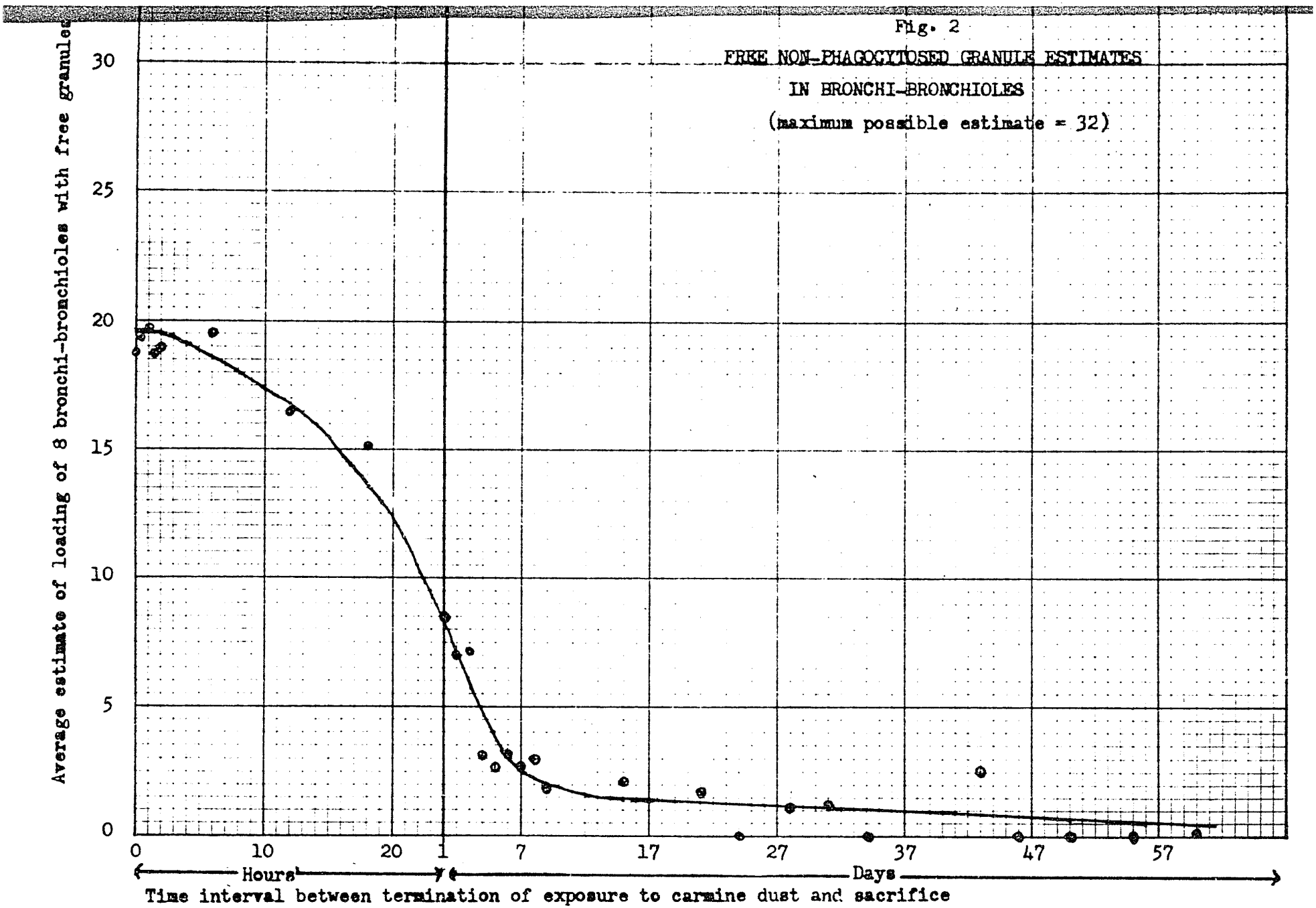
Fig. 2
FREE NON-PHAGOCYTOSED GRANULE ESTIMATES
IN BRONCHI-BRONCHIOLES
(maximum possible estimate = 32)
Average estimate of loading of 8 bronchi-bronchioles with free granules
30
25
20
15
10
5
0
0
10
20
1
7
17
27
37
47
57
Hours
Days
Time interval between termination of exposure to carmine dust and sacrifice

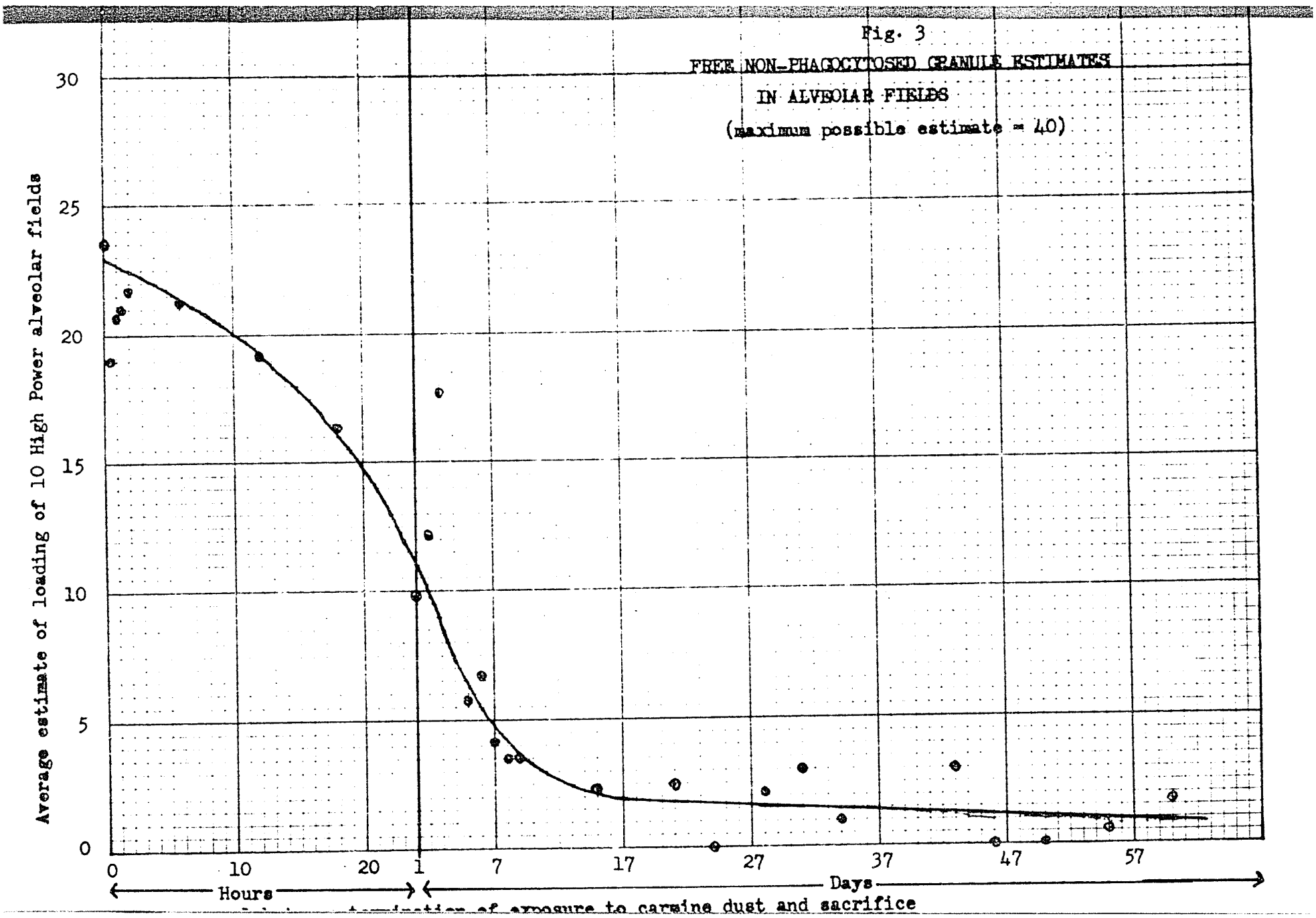
Fig. 3
FREE NON-PHAGOCYTOSED GRANULE ESTIMATES
IN ALVEOLAR FIELDS
(maximum possible estimate = 40)
Average estimate of loading of 10 High Power alveolar fields
30
25
20
15
10
5
0
0
10
20
1
7
17
27
37
47
57
Hours
Days

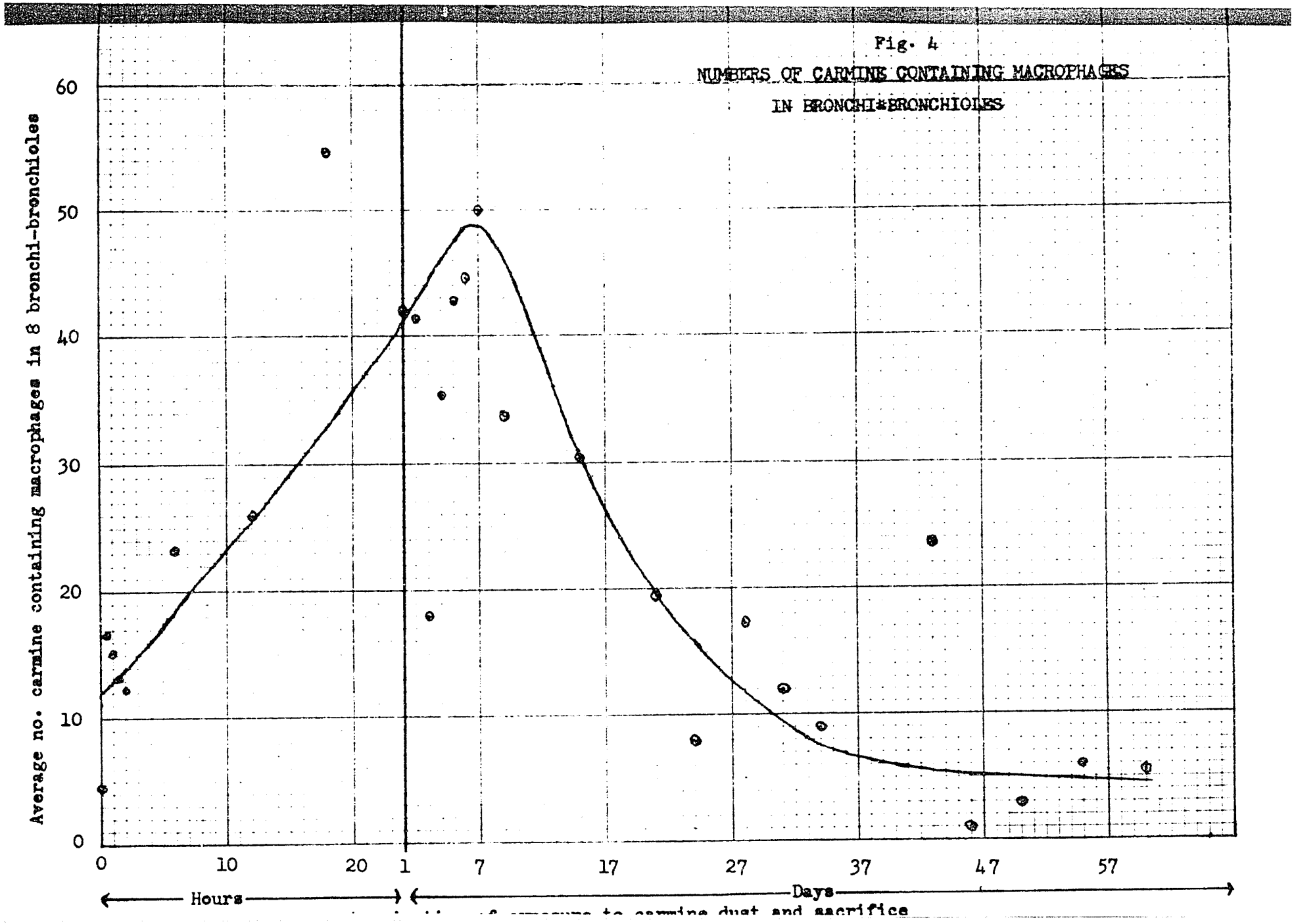
Fig. 4
NUMBERS OF CARMINE CONTAINING MACROPHAGES
IN BRONCHI*BRONCHIOLES
Average no. carmine containing macrophages in 8 bronchi-bronchioles
60
50
40
30
20
10
0
0
10
20
1
7
17
27
37
47
57
Hours
Days

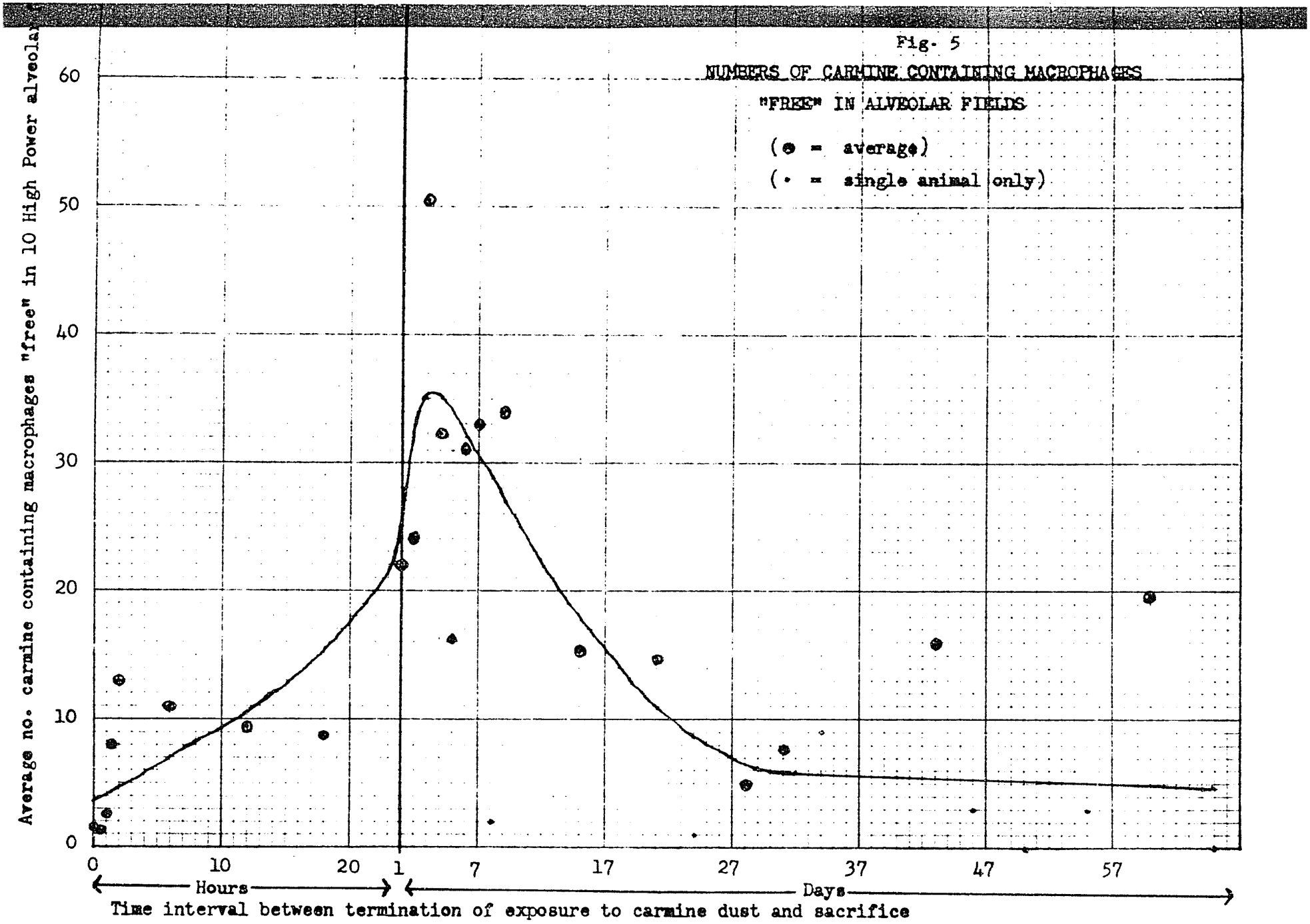
Fig. 5
NUMBERS OF CARMINE CONTAINING MACROPHAGES
"FREE" IN ALVEOLAR FIELDS
(● = average)
(• = single animal only)
Average no. carmine containing macrophages "free" in 10 High Power alveolar
60
50
40
30
20
10
0
0
10
20
1
7
17
27
37
47
57
Hours
Days
Time interval between termination of exposure to carmine dust and sacrifice

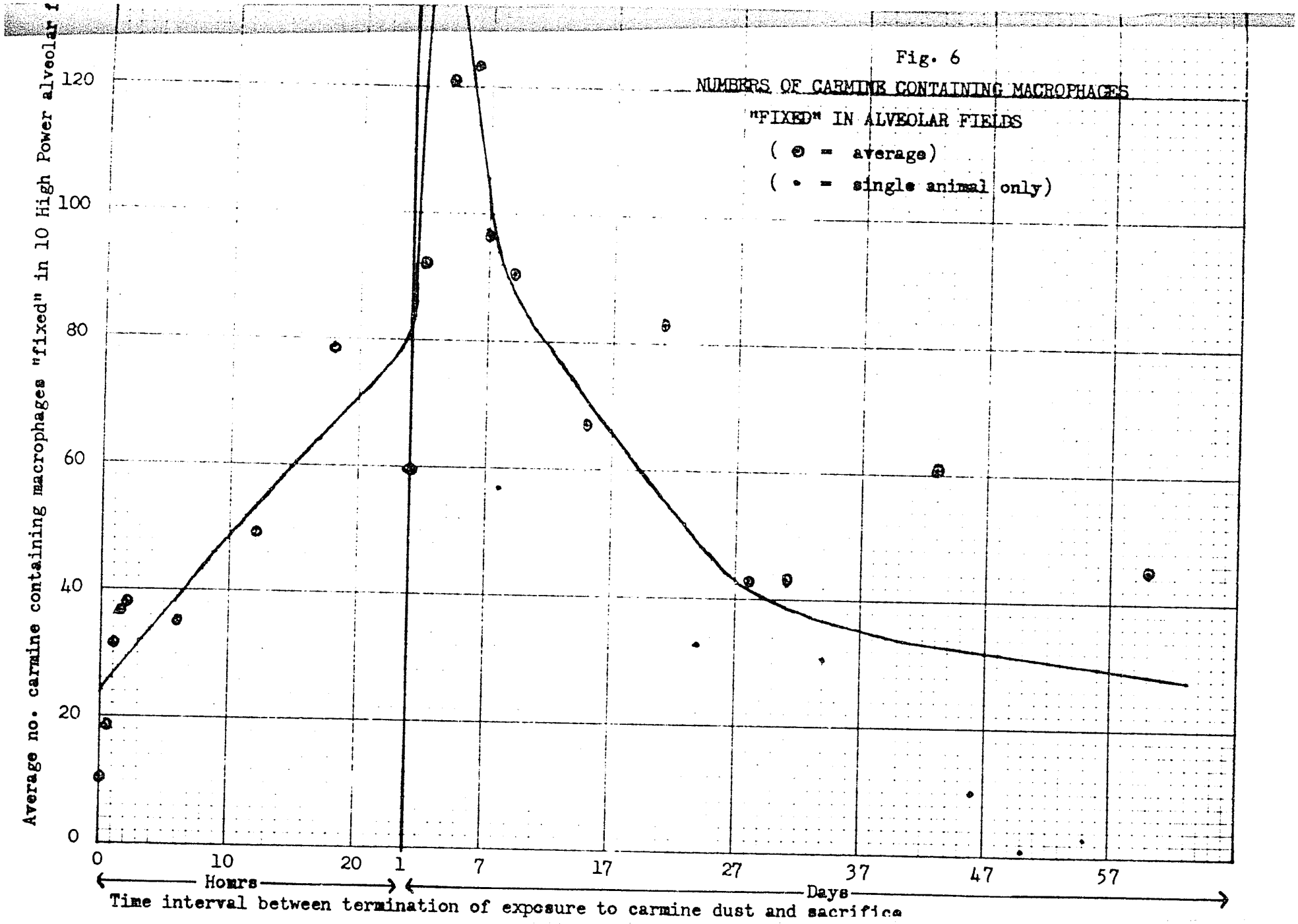
Fig. 6
NUMBERS OF CARMINE CONTAINING MACROPHAGES
"FIXED" IN ALVEOLAR FIELDS
(⊙ = average)
(• = single animal only)
Average no. carmine containing macrophages "fixed" in 10 High Power alveolar f
0
20
40
60
80
100
120
0
10
20
1
7
17
27
37
47
57
Hours
Days
Time interval between termination of exposure to carmine dust and sacrifice

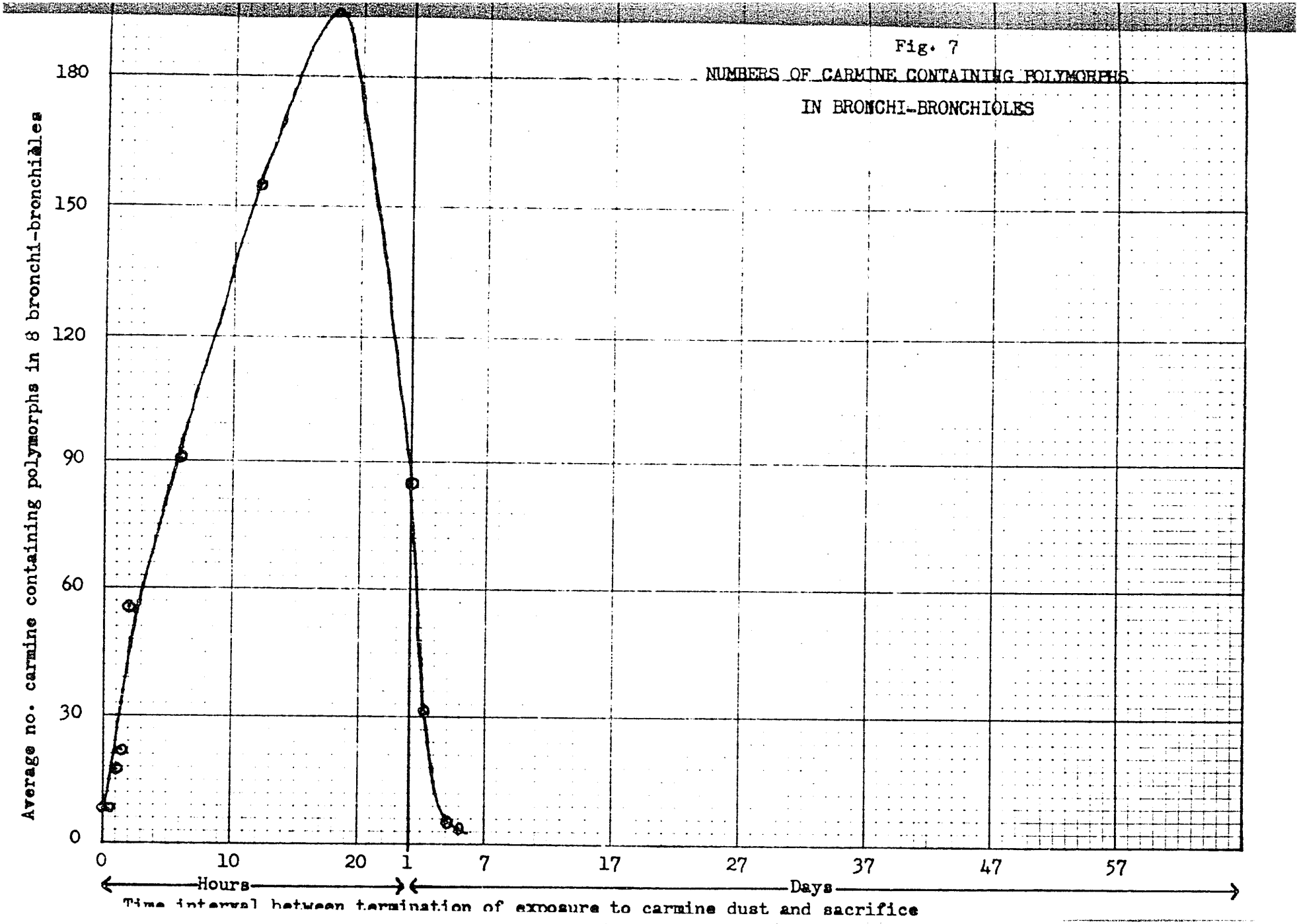
Fig. 7
NUMBERS OF CARMINE CONTAINING POLYMORPHS
IN BRONCHI-BRONCHIOLES
Average no. carmine containing polymorphs in 8 bronchi-bronchioles
180
150
120
90
60
30
0
0
10
20
1
7
17
27
37
47
57
Hours
Days
Time interval between termination of exposure to carmine dust and sacrifice

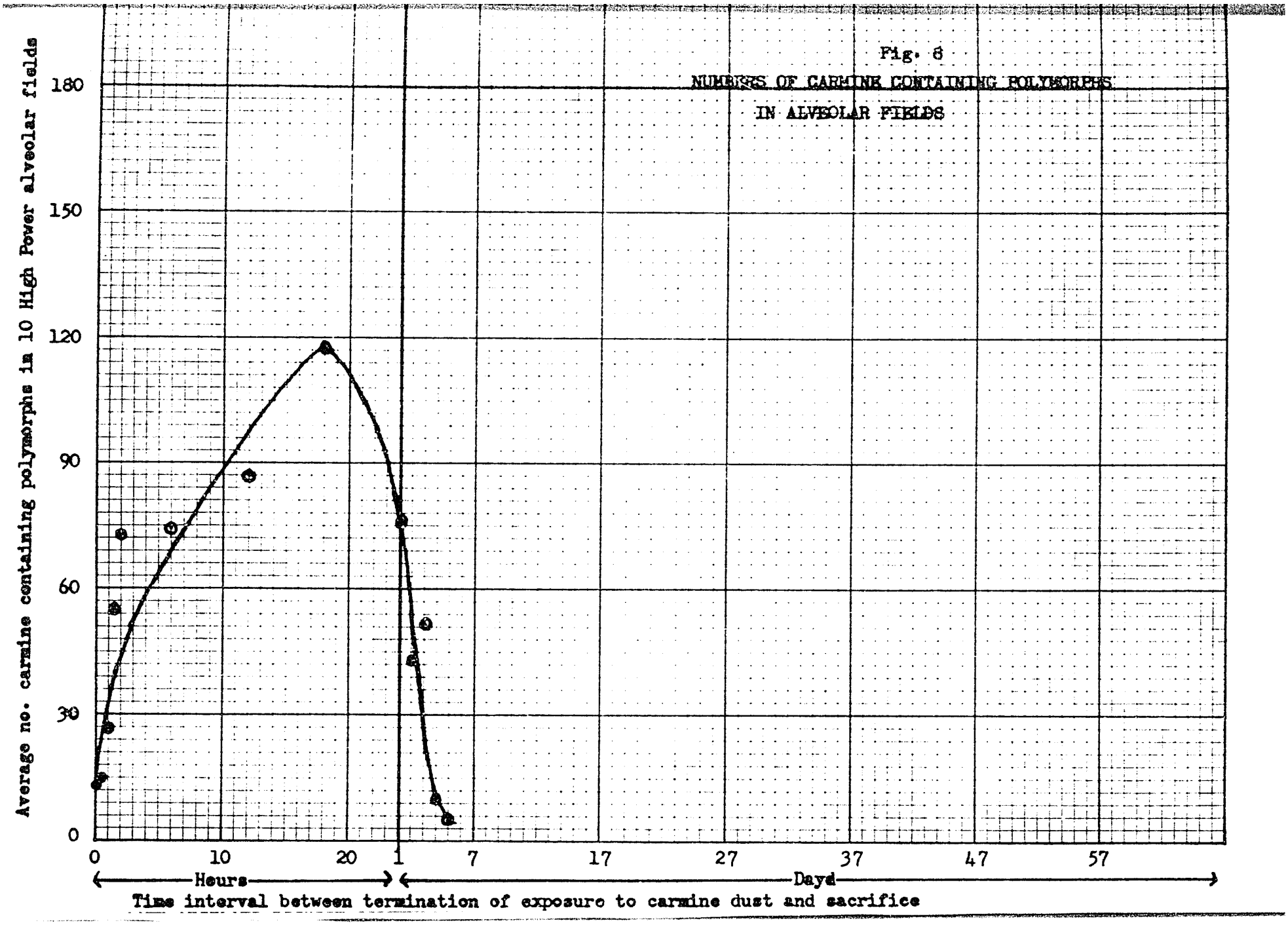
Fig. 6
NUMBERS OF CARMINE CONTAINING POLYMORPHS
IN ALVEOLAR FIELDS
Average no. carmine containing polymorphs in 10 High Power alveolar fields
180
150
120
90
60
30
0
0
10
20
1
7
17
27
37
47
57
Hours
Days
Time interval between termination of exposure to carmine dust and sacrifice

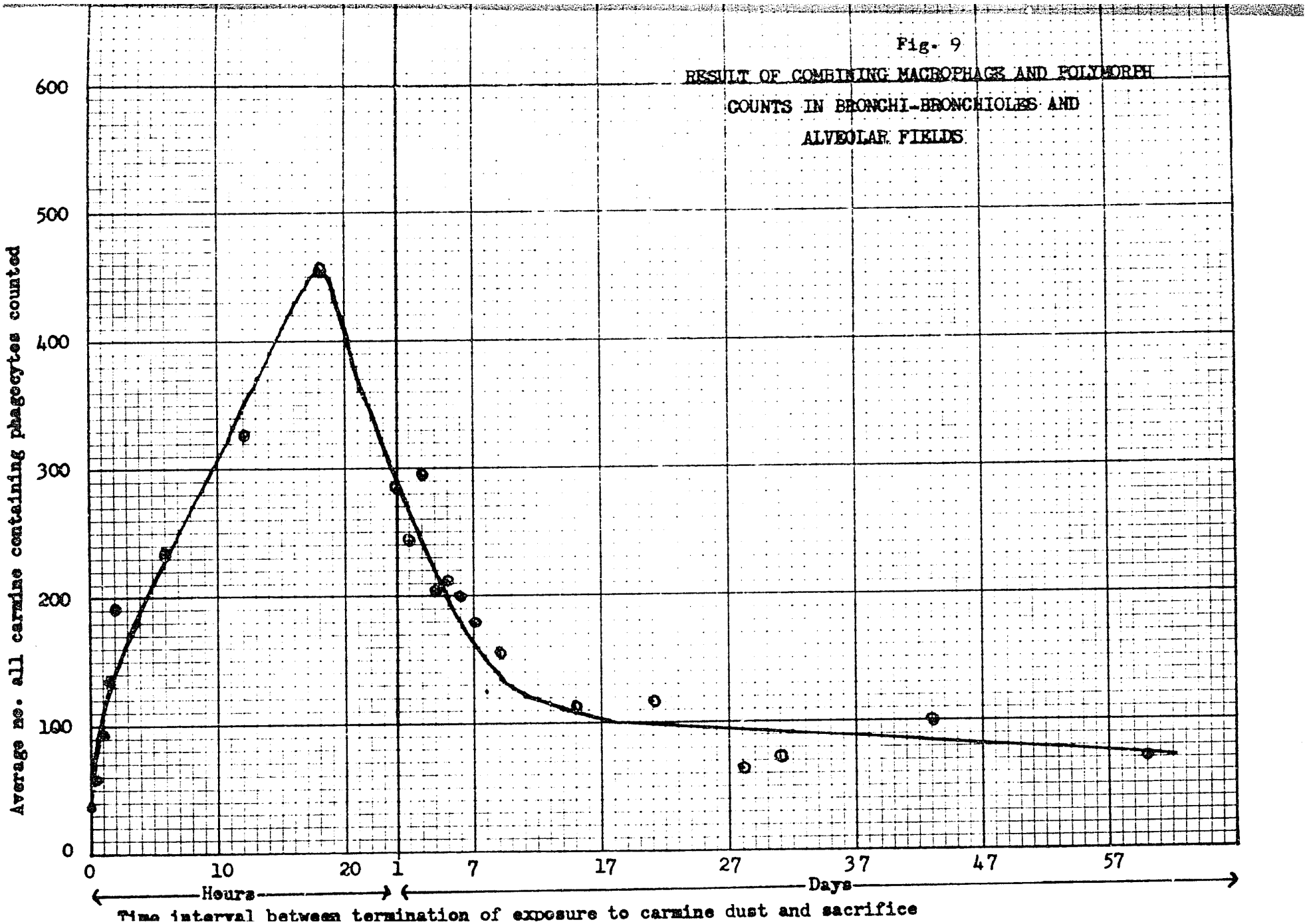
Fig. 9
RESULT OF COMBINING MACROPHAGE AND POLYMORPH COUNTS IN BRONCHI-BRONCHIOLES AND ALVEOLAR FIELDS
Average no. all carmine containing phagocytes counted
600
500
400
300
200
100
0
0
10
20
1
7
17
27
37
47
57
Hours
Days
Time interval between termination of exposure to carmine dust and sacrifice

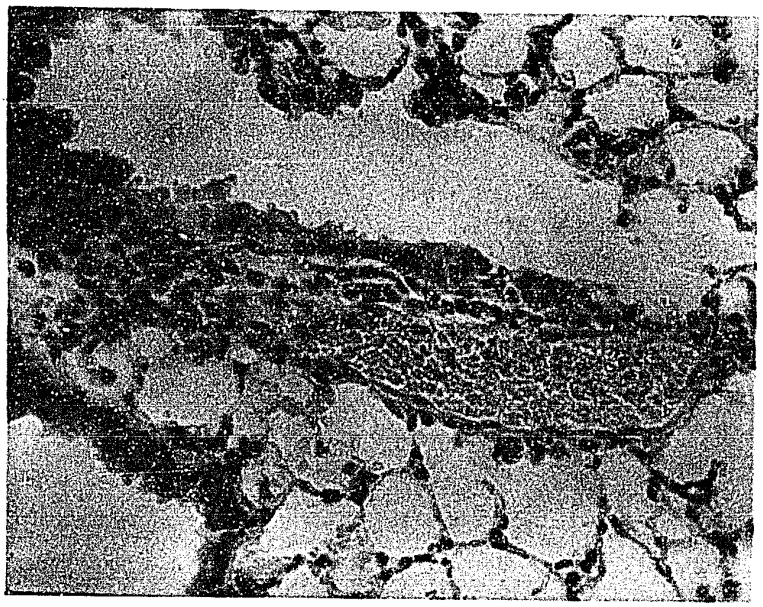

Figure 10 - (MP1-5-5) Peribronchiolar and perivascular area of mouse lung 5 days after dusting with carmine. Note "free" and "fixed" alveolar macrophages containing carmine. Also note a few granules of carmine in lymphoid tissue between the blood vessel and bronchiole (pulmonary sump) and that these do not appear to be intracellular. (Hematoxylin only X 350)

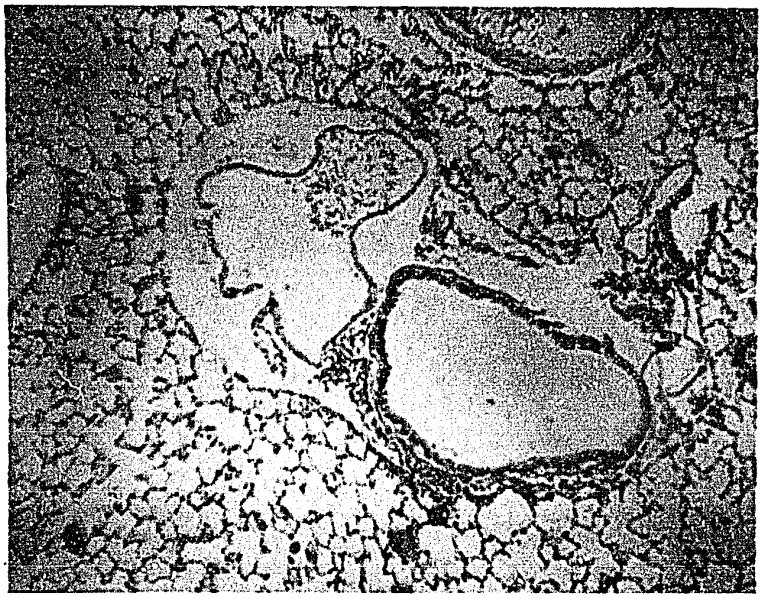

Figure 11 - (MP-CG-Y3) Area of lung of untreated control mouse showing perivascular and peribronchial clear areas. (Hematoxylin only X 72)

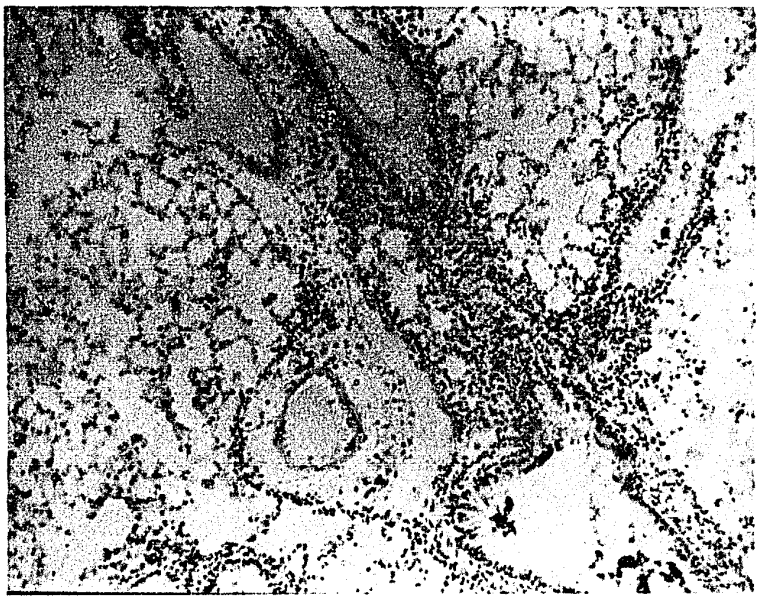

Figure 12 - (MP1-13-0-4) Area of lung of mouse sacrificed immediately after 2 hours dusting with carmine. Note infiltration of perivascular and peribronchial clear areas by polymorphs and cells resembling plasma cells. (Hematoxylin only X 72)

Figure 13 - Electron microphotograph of "fixed" alveolar macrophage, wall of an alveolus, along with a capillary channel. Note that there is a thin attenuated cell situated between the macrophage and the basement membrane. Dark angular bodies at 3 and 10 o'clock in cytoplasm of macrophage are probably carmine granules but most of the oval dark bodies are mitochondria. (X 9,000)

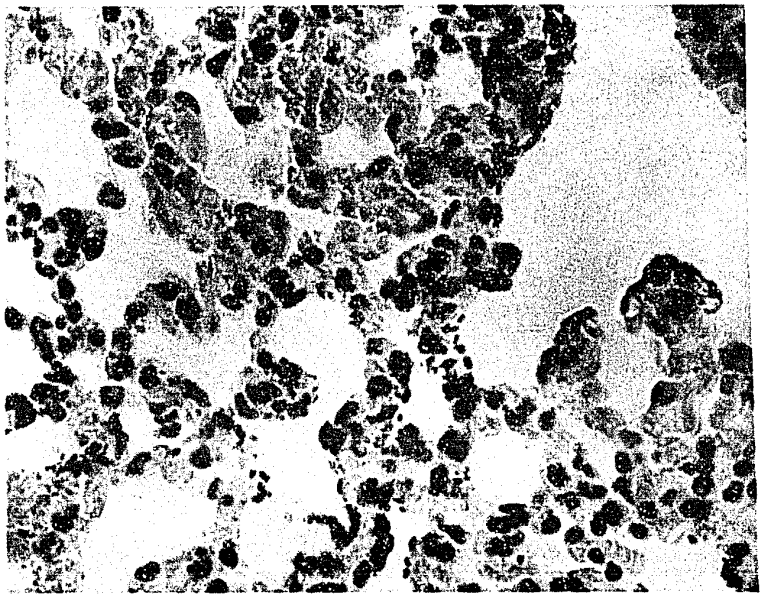

Figure 14 - (MP1-13-0-2) Mouse sacrificed immediately after 2 hour dusting with carmine. Alveolar area shows that most of the carmine granules are free and non-phagocytized although concentrated into clumps. (Hematoxylin only X 500)

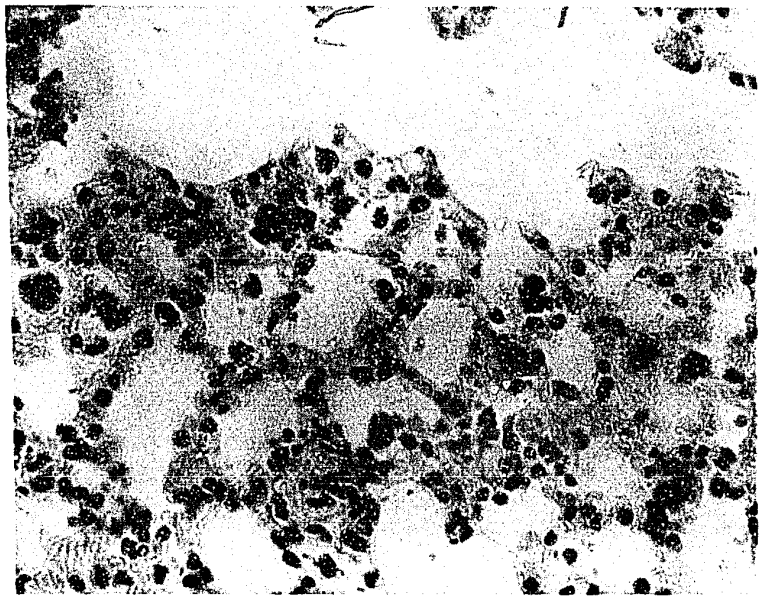

Figure 15 - (MP1-12-3-1) Mouse sacrificed 3 days after 2 hour dusting with carmine. Alveolar area shows most carmine granules now within "free" and "fixed" alveolar macrophages. (Hematoxylin only X 450)

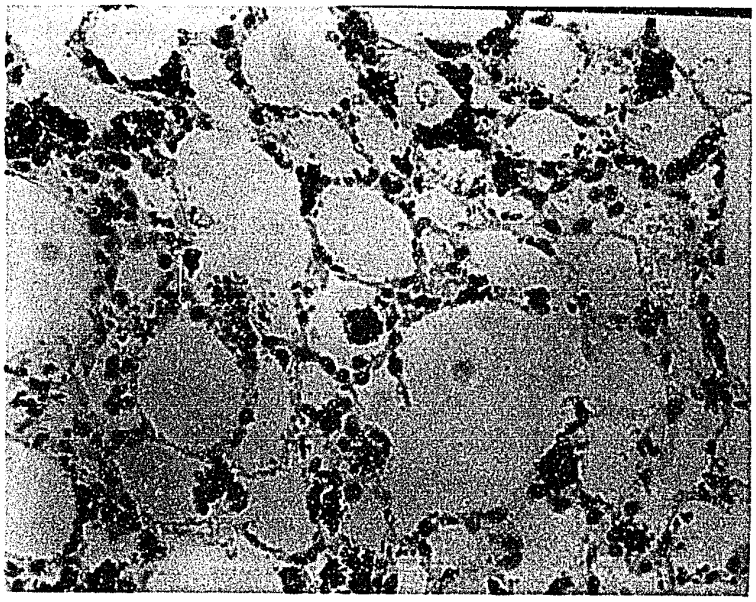

Figure 16 - (MP5-2-30) Mouse sacrificed and 30 minutes later serum suspended carmine granules were instilled into the tracheobronchial tree through the trachea. This alveolar field of the lung shows carmine granules within alveolar macrophages and also apparently accumulating on the surface of the same type of cell. (Hematoxylin only X 350)

TABLE 1

MICE DUSTED FOR 2 HOURS WITH CARMINE

Group Number *(1)	Number of mice in group	Number of mice died *(2)	Number of mice sacrificed after indicated time interval following exposure																									
			0 mi	30 mi	60 mi	90 mi	2 hr	6 hr	12 hr	18 hr	1 dy	2 dy	3 dy	4 dy	5 dy	6 dy	7 dy	8 dy	9 dy	15 dy	21 dy	24 dy	28 dy	31 dy	34 dy	43 dy	46 dy	50 dy
	5												1	1	1	1	1											
	5		1								1	1						1	1									
*(3)	5	1																			1							
*(4)	5	3																										
*(5)	5																			1						1		1
	5																					1	1	1	1		1	
	8	2																								3		
	8	1																					3	4				
	8																		4		4							
	8																4			4								
	8	1													4	3												
	8	1											3	4														
	8		4									4																
	8			5							3																	
	10				5					5																		
	10					5	5																					
	10	1						4	5																			
otal	124	10	5	5	5	5	5	4	5	5	4	5	4	5	5	4	5	1	5	5	5	1	4	5	1	4	1	1

*(1) All mice in one group exposed at the same time
*(2) Mice dying during the experiment removed from the experiment
*(3) One mouse also available from this group at 80 and 87 days
*(4) One mouse also available from this group at 65 and 76 days
*(5) One mouse also available from this group at 71 days

TABLE 2

AVERAGE OF FREE GRANULE ESTIMATES AND PHAGOCYTE COUNTS AT VARYING POST EXPOSURE TIMES

Time lapse exposure to sacrifice	Number Mice Averaged	Free granules non-phagocytized		Carmine laden macrophages			Total carmine laden macrophages all fields	Number mice averaged	Carmine laden polymorphs		
		BRONCHI	ALVEOLI	BRONCHI	ALVEOLI "free"	ALVEOLI "fixed"			BRONCHI	ALVEOLI	TOTAL
0min	5	18.8	23.5	4.5	1.6	10.6	16.8	3	8	13.3	21.3
30min	5	19.4	19	16.6	1.4	18.6	36.6	3	7.3	15	22.3
60min	5	19.7	20.7	15.2	2.6	31.8	49.6	3	17.3	27	44.3
90min	5	18.6	21	13.2	8	36.6	58	3	21.6	55.6	77.2
2hrs	5	18.9	21.7	12.2	13	38	63.2	4	55	72.7	127.7
6hrs	4	19.5	21.3	23.2	11	35.2	69.5	3	90.3	74	164.3
12hrs	5	16 5	19.2	26	9.4	49.4	84.8	4	155	87	242
18hrs	5	15.1	16.4	54.8	8.8	78.6	143.6	5	195.6	117.6	313.2
1day	4	8.5	9.8	42	22	59.5	123.5	3	85.3	76.3	161.6
2dys	5	7	12.2	41.4	24	92.2	157.8	3	42.3	42.6	84.9
3dys	4	7.2	17.7	18	50.5	156.7	227.7	3	14.6	52.3	66.9
4dys	5	3.2	7.3	35.4	32.4	121	188.8	3	5	10.3	15.3
5dys	5	2.7	5.8	42.8	16.2	143.8	202.8	3	3.3	5.3	8.6
6dys	4	3.2	6.7	44.7	31	123.4	199.2				
7dys	5	2.7	4.2	50	33	96.6	179.6				
8dys	1	3	3.5	67	2	57	126				
9dys	5	1.9	3.5	33.6	34	90.2	157.8				
15dys	5	2.1	2.3	30.2	15.4	67.2	112.8				
21dys	5	1.8	2.5	19.4	14.8	83.4	117.6				
24dys	1	0	0	8	1	33	42				
28dys	4	1.1	2.1	17.2	5	43	65.2				
31dys	5	1.2	3	12	7.8	43.4	73.2				
34dys	1	0	1	9	9	31	49				
43dys	4	2.5	3	23.7	16	61	100.7				
46dys	1	0	0	1	3	10	14				
50dys	1	0	0	3	0	1	4				
55dys	1	0	0.5	6	3	3	12				
60dys	4	0.12	1.7	5.5	19.7	45.2	70.5				
65dys	1	0	0	0	0	0	0				
71dys	1	0	0	0	0	rare	rare				
76dys	1	0	0	0	0	0	0				
80dys	1	0	0	0	0	rare	rare				
87dys	1	0	0	0	0	rare	rare				

TABLE 3A

CONTROL MICE - ROUTINE LUNG SECTIONS - PERL'S STAIN

Group	Mouse Number	Alveolar macrophages positive for iron	Bronchial macrophages positive for iron	Total	Peribronchial and Perivascular iron positive cells
CGA - (adult 6 to 9 months old)					
	1	25	0	25	31
	2	85	3	88	4
	3	14	1	15	28
	4	33	1	34	52
	5	112	4	116	31
	Sum	269	9	278	146
	Average	53.8	1.8	55.6	29.2
CGY - (young 1 to 3 months old)					
	1	0	0	0	0
	2	0	0	0	0
	3	3	2	5	20
	4	46	1	47	0
	5	1	0	1	0
	Sum	50	3	53	20
	Average	10	0.6	10.6	4

TABLE 3B

Application of Student "t" test for significance of differences between averages of iron positive phagocytes in control mice in 1 - 3 months age group and 6 - 9 months age group.

1 For phagocytes in the alveolar fields

$$t = \frac{53.8 - 10}{\sqrt{\frac{621.25}{5} + \frac{621.25}{5}}} = \frac{43.8}{15.7} = 2.79$$

For (n-2) 8 degrees of freedom

$p = 0.0235$

2 For phagocytes in bronchi and bronchioles

$$t = \frac{1.8 - 0.6}{\sqrt{\frac{1.186}{5} + \frac{1.186}{5}}} = \frac{1.2}{0.65} = 1.841$$

For (n-2) 8 degrees of freedom

$p = 0.1$

3 For phagocytes in peribronchial and perivascular areas

$$t = \frac{29.2 - 4}{\sqrt{\frac{467.5}{5} + \frac{467.5}{5}}} = \frac{25.2}{13.7} = 1.839$$

For (n-2) degrees of freedom

$p = 0.1$

TABLE 4

SUMMARY OF PRELIMINARY EXPERIMENT ON PHAGOCYTOSIS OF ERYTHROCYTES

Mouse #	Time lapse instillation to sacrifice	Amount blood instilled	Macrophages positive for Iron (Perl's Stain) Bronchi and Bronchioli	Alveolar areas	Peribronchial Perivascular
4-1-4	4 days	0.05ml	27	67	10
4-1-8	8 days	0.05ml	35	170	47
4-1-12	12 days	0.05ml	8	169	48
4-1-16	16 days	0.05ml	38	204	55
4-2-4*	4 days	0.1ml	10	137	32
4-2-8	8 days	0.1ml	14	404	83

*mouse also showed a moderately extensive acute bronchopneumonia

RESULTS OF IRON POSITIVE MACROPHAGE COUNTS AFTER INSTILLATION OF ERYTHROCYTES

xperimental mice												Control mice			
		No. of macrophages positive for iron with Perl's stain										No. of macrophages positive for iron with Perl's stain			
		in 8 bronchi or bronchioles in mouse					in 10 HP alveolar fields in mouse					in 8 bronchi-bronchioles in mouse		in 10 HP alveolar fields in mouse	
roup No.	Time lapse instil-lation to sacrifice	#1	#2	#3	#4	Average	#1	#2	#3	#4	Average	#1	#2	#1	#2
1	2 hrs	2	1	1	0	1	64	11	77	22	43.5	5	4	78	82
2 *	6 hrs	3	6	0	1	2.5	51	37	6	7	25.2	4	0	3	7
3	12 hrs	14	19	7	5	11.2	41	388	45	72	136.5	10	18	44	129
4 *	18 hrs	21	9	21	7	14.5	154	13	87	37	72.7	2	23	19	62
5 *	1 day	4	12	3	1	5	41	24	24	16	26.2	2	4	31	12
6	2 days	28	0	14	8	12.5	242	167	83	48	135	2	2	10	4
7	3 days	17	15	21	11	16	60	111	64	146	95.2	24	7	30	33
8	5 days	9	18	21	32	20	41	41	58	81	55.2	3	14	5	113
9 *	7 days	3	14	36	5	14.5	8	757	374	299	359.5	1	2	53	64
10	9 days	21	5	83	2	27.7	145	7	725	144	255.2	5	7	26	1
11	15 days	8	36	43	17	26	161	247	73	188	167.2	12		131	
12	24 days	13	5	11	28	14.2	20	42	185	350	149.2	18		24	

*Groups 2,4,5, and 9 contain mice 2½ to 3½ months old. All other groups contain mice 3½ to 4½ months old.

NB - All counts taken from areas showing heaviest loading of macrophages containing iron positive material with Perl's stain.

* Average 3 Mos Gps	4.7	31.4
Average 4 Mos Gps	9.3	50.7
Average all groups	7.6	43.7

TABLE 5B

Application of the Student "t" Test for significance of the difference of average numbers of iron positive phagocytes in the control groups of 8 mice of 3 months average age and 14 mice of 4 months average age.

1 For phagocytes in bronchi and bronchioles

$$t = \frac{\frac{131}{14} - \frac{38}{8}}{\sqrt{\frac{50.64}{8} + \frac{50.64}{14}}} = \frac{4.61}{3.163} = 1.461$$

For 20 degrees of freedom

P = 0.175

2 For phagocytes in alveolar fields

$$t = \frac{\frac{710}{14} - \frac{251}{8}}{\sqrt{\frac{1654.8}{8} + \frac{1654.8}{14}}} = \frac{19.34}{18.02} = 1.073$$

For 20 degrees of freedom

P = 0.296

TABLE 5C

Application of the Student "t" Test for significance of the differences between the means of the control and experimental mice treating the 3 and 4 month groups separately.

Time lapse between instillation of blood and sacrifice	Bronchi		Alveoli	
	"t"	Probability	"t"	Probability
2 hrs	2.387	0.026 *	.2798	0.79
6 hrs	.5593	0.6	.4140	0.68
12 hrs	.4509	0.63	1.795	0.1
18 hrs	2.133	0.052	1.685	0.13
1 day	.0672	0.95	.3875	0.7
2 days	.7074	0.5	2.622	0.019 *
3 days	1.851	0.085	1.670	0.115
5 days	2.560	0.02 *	.1524	0.875
7 days	1.541	0.17	3.147	0.01 *
9 days	2.140	0.05 *	2.507	0.024 *
15 days	3.156	0.0065 *	3.901	0.0033 *
24 days	1.163	0.265	2.208	0.044 *

* indicates prabability of 0.05 or less (i.e. less than 1 chance in 20 that the experimental animals are a chance variation of the normal control group concerned).

APPENDIX "A"

TABULATED RESULTS OF FREE GRANULE ESTIMATES AND PHAGOCYTE COUNTS

Time lapse Dusting to sacrifice	Mouse	FREE GRANULES Bronchi	FREE GRANULES Alveoli	LOADED MACROPHAGES Bronchi	LOADED MACROPHAGES "free" Alveoli	LOADED MACROPHAGES "fixed" Alveoli	LOADED MACROPHAGES Total	POLYMORPHS Bronchi	POLYMORPHS Alveoli
0 hours	1-2-0	15.5	23	0	1	18	19		
	1-13-0-1	20	21.5	5	1	6	12	7	9
	1-13-0-2	18.5	24.5	9	3	7	19	9	17
	1-13-0-3	20	25	2	2	9	13	8	14
	1-13-0-4	20	23.5	7	1	13	21		
	Sum	94	117.5	23	8	53	84	24	40
	Average	18.8	23.5	4.5	1.6	10.6	16.8	8	13.3
½ hours	1-14-30M-1	21	19	26	0	33	59	7	17
	1-14-30M-2	21	18.5	25	1	14	40	12	10
	1-14-30M-3	19	18	17	5	20	42	3	18
	1-14-30M-4	18.5	19.5	10	1	14	25		
	1-14-30M-5	17.5	20	5	0	12	17		
	Sum	97	95	83	7	93	183	22	45
	Average	19.4	19	16.6	1.4	18.6	36.6	7.3	15
1 hour	1-15-60M-1	19.5	21	33	0	29	62	32	24
	1-15-60M-2	20	19	12	0	31	43	4	33
	1-15-60M-3	23.5	22	6	5	32	43	16	24
	1-15-60M-4	18.5	20	8	6	38	52		
	1-15-60M-5	17	21.5	17	2	29	48		
	Sum	98.5	103.5	76	13	159	248	52	81
	Average	19.7	20.7	15.2	2.6	31.8	49.6	17.3	27
1½ hours	1-16-90M-1	23.5	18	22	10	26	58	15	55
	1-16-90M-2	19.5	21	10	7	26	43	21	55
	1-16-90M-3	17.5	22	16	10	54	80	29	57
	1-16-90M-4	14.5	20	11	4	35	50		
	1-16-90M-5	18	24	7	[illegible]	43	59		
	Sum	93	105	66	40	184	290	65	167
	Average	18.6	21	13.2	8	36.8	58	21.6	55.[illegible]

Time lapse Dusting to sacrifice	Mouse	FREE GRANULES Bronchi	FREE GRANULES Alveoli	LOADED MACROPHAGES Bronchi	LOADED MACROPHAGES "free" Alveoli	LOADED MACROPHAGES "fixed" Alveoli	LOADED MACROPHAGES Total	POLYMORPHS Bronchi	POLYMORPHS Alveoli
2 hours	1-16-2H-1	21.5	19	22	16	48	86	171	96
	1-16-2H-2	19.5	23	11	8	35	54	26	44
	1-16-2H-3	19.5	23.5	8	14	27	49	8	64
	1-16-2H-4	17.5	24	12	12	46	70	15	87
	1-16-2H-5	16.5	19	8	15	34	57		
	Sum	94.5	108.5	61	65	190	316	220	291
	Average	18.9	21.7	12.2	13	38	63.2	55	72.7
6 hours	1-17-6H-1	17.5	24.5	41	5	48	94	63	80
	1-17-6H-2	20.5	20	14	8	33	55	98	85
	1-17-6H-3	19.5	22	20	17	29	66	110	57
	1-17-6H-4	20.5	19	18	14	31	63		
	Sum	78	85.5	93	44	141	278	271	222
	Average	19.5	21.3	23.2	11	35.2	69.5	90.3	74
12 hours	1-17-12H-1	16.5	17.5	31	7	52	90	137	74
	1-17-12H-2	14.5	15.5	17	14	47	78	133	83
	1-17-12H-3	17.5	22.5	38	5	57	100	224	118
	1-17-12H-4	14.5	18	22	9	53	84	126	73
	1-17-12H-5	19.5	22.5	22	12	38	72		
	Sum	82.5	96	130	47	247	424	620	348
	Average	16.5	19.2	26	9.4	49.4	84.8	155	87
18 hours	1-15-18H-1	16.5	15.5	60	9	92	161	249	115
	1-15-18H-2	11	19.5	82	12	110	204	230	218
	1-15-18H-3	18	18	39	10	72	121	219	108
	1-15-18H-4	14.5	12.5	67	7	70	144	169	67
	1-15-18H-5	15.5	16.5	26	13	49	88	111	80
	Sum	75.5	82	274	51	393	718	978	588
	Average	15.1	16.4	54.8	10.2	78.6	143.6	195.6	117.6
1 day	1-2-1	7	6	20	9	36	65		
	1-14-1-1	14.5	15.5	34	21	65	120	69	66
	1-14-1-2	7.5	13	17	36	81	134	55	87
	1-14-1-3	7	5	97	22	56	175	132	76
	Sum	34	39.5	168	88	238	494	256	229
	Average	[illegible]							

Time lapse Dusting to sacrifice	Mouse	FREE GRANULES		LOADED MACROPHAGES				POLYMORPHS	
		Bronchi	Alveoli	Bronchi	"free" Alveoli	"fixed" Alveoli	Total	Bronchi	Alveoli
2 days	1-2-2	5.5	8	29	4	38	71		
	1-13-2-1	2	8	13	42	62	117		
	1-13-2-2	8	12	53	12	106	171	31	55
	1-13-2-3	6.5	15.5	47	26	115	188	50	33
	1-13-2-4	13	17.5	65	36	141	242	46	40
	Sum	35	61	207	120	462	789	127	128
	Average	7	12.2	41.4	24	92.2	157.8	42.3	42.6
3 days	1-1-3	10.5	22.5	30	9	131	170		
	1-12-3-1	5	15.5	12	81	164	257	23	60
	1-12-3-2	6	20	9	71	225	305	0	74
	1-12-3-3	7.5	13	31	41	107	179	21	23
	Sum	29	71	72	202	627	911	44	157
	Average	7.2	17.7	18	50.5	156.7	227.7	14.6	52.3
4 days	1-1-4	2.5	5.5	13	32	46	91		
	1-12-4-1	3	8	31	24	146	201	12	10
	1-12-4-2	2	9.5	39	34	148	221	3	12
	1-12-4-3	4.5	10.5	38	44	164	246	0	9
	1-12-4-4	4	3	56	28	101	185		
	Sum	16	36.5	177	162	605	944	15	31
	Average	3.2	7.3	35.4	32.4	121	188.8	5	10.3
5 days	1-1-5	1	8	7	19	59	85		
	1-11-5-1	2.5	6	31	11	172	214	2	4
	1-11-5-2	3.5	6	7	17	131	155	4	11
	1-11-5-3	1.5	4	45	13	160	218	4	1
	1-11-5-4	5	5	124	21	197	342		
	Sum	13.5	29	214	81	719	1014	10	16
	Average	2.7	5.8	42.8	16.2	143.8	202.8	3.3	5.3
6 days	1-1-6	4.5	14.5	43	34	128	205		
	1-11-6-1	2	4.5	28	44	168	240		
	1-11-6-2	3	2.5	52	18	109	169		
	1-11-6-3	3.5	5.5	56	28	89	173		
	Sum	13	27	179	124	494	797		
	Average	3.2	6.7	44.7	31	123.4	199.2		

Time lapse Dusting to sacrifice	Mouse	FREE GRANULES Bronchi	FREE GRANULES Alveoli	LOADED MACROPHAGES Bronchi	LOADED MACROPHAGES "free" Alveoli	LOADED MACROPHAGES "fixed" Alveoli	LOADED MACROPHAGES Total	POLYMORPHS Bronchi	POLYMORPHS Alveoli
7 days	1-1-7	2.5	3.5	37	6	88	131		
	1-10-7-1	4.5	4.5	66	50	84	200		
	1-10-7-2	1.5	4.5	43	31	73	147		
	1-10-7-3	3	5.5	63	26	124	213		
	1-10-7-4	2	3	41	52	114	207		
	Sum	13.5	21	250	165	483	898		
	Average	2.7	4.2	50	33	96.6	179.6		
8 days	1-2-8	3	3.5	67	2	57	126		
9 days	1-2-9	0	0	8	4	10	22		
	1-9-9-1	4	4.5	51	12	86	149		
	1-9-9-2	0	3	26	59	181	266		
	1-9-9-3	4	5.5	36	54	59	149		
	1-9-9-4	1.5	4.5	47	41	115	203		
	Sum	9.5	17.5	168	170	451	789		
	Average	1.9	3.5	33.6	34	90.2	157.8		
15 days	1-5-15	2	1	28	0	17	45		
	1-10-15-1	2	2	30	16	89	135		
	1-10-15-2	4.5	4	44	21	96	161		
	1-10-15-3	1	3	26	29	69	124		
	1-10-15-4	1	1.5	23	11	65	99		
	Sum	10.5	11.5	151	77	336	564		
	Average	2.1	2.3	30.2	15.4	67.2	112.8		
21 days	1-3-21	0	0	4	1	5	10		
	1-9-21-1	2.5	3	11	22	63	96		
	1-9-21-2	0	1.5	20	9	85	114		
	1-9-21-3	2.5	4	21	34	126	181		
	1-9-21-4	4	4	41	8	138	187		
	Sum	9	12.5	97	74	417	588		
	Average	1.8	2.5	19.4	14.8	83.4	117.6		

Time lapse [illegible]ing to [illegible]crifice	Mouse	FREE GRANULES Bronchi	FREE GRANULES Alveoli	LOADED MACROPHAGES Bronchi	LOADED MACROPHAGES "free" Alveoli	LOADED MACROPHAGES "fixed" Alveoli	LOADED MACROPHAGES Total
[illegible] days	1-6-24	0	0	8	1	33	42
[illegible] days	1-6-28	0	2	2	0	23	25
	1-8-28-1	2.5	3.5	26	7	33	66
	1-8-28-2	1.5	1.5	23	8	59	90
	1-8-28-3	1.5	1.5	18	5	57	80
	Sum	5.5	8.5	69	20	172	261
	Average	1.1	2.1	17.2	5	43	65.2
1 days	1-6-31	0	1	19	8	50	77
	1-8-31-1	1	4.5	6	2	31	39
	1-8-31-2	1	1.5	7	11	52	70
	1-8-31-3	1.5	3.5	16	7	50	73
	1-8-31-4	2.5	4.5	12	11	84	107
	Sum	6	15	60	39	217	366
	Average	1.2	3	12	7.8	43.4	73.2
[illegible]4 days	1-6-34	0	1	9	9	31	49
[illegible]3 days	1-7-43-1	4.5	2	47	16	65	128
	1-7-43-2	5.5	5.5	29	17	90	136
	1-7-43-3	0	4.5	19	31	88	138
	1-5-43	0	0	0	0	1	1
	Sum	10	12	95	64	244	403
	Average	2.5	3	23.7	16	61	100.7
[illegible]6 days	1-6-46	0	0	1	3	10	14
[illegible]5 days	1-3-55	0	0.5	6	3	3	12
[illegible] days	1-5-60	0	0	0	0	0	0
	1-7-60-1	0.5	2.5	4	23	51	78
	1-7-60-2	0	2	12	12	55	79
	1-7-60-3	0	2.5	6	44	75	125
	Sum	0.5	7	22	79	181	282
	Average	0.12	1.7	5.5	19.7	45.2	70.5

Time lapse Dusting to sacrifice	Mouse	FREE GRANULES		LOADED MACROPHAGES			
		Bronchi	Alveoli	Bronchi	"free" Alveoli	"fixed" Alveoli	Total
65 days	1-4-65	0	0	0	0	0	0
71 days	1-5-71	0	0	0	0	rare	rare
76 days	1-4-76	0	0	0	0	0	0
80 days	1-3-80	0	0	0	0	rare	rare
87 days	1-3-87	0	0	0	rare	rare	rare