CALCIUM ACCUMULATION IN LARVAL ECHINOCOCCUS MULTILOCULARIS

With Some Observations on the Leucocyte Counts in Infected Rodents

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by

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ABSTRACT

1. Morphological examination of the calcareous corpuscles from the cysts of Echinococcus multilocularis has shown that the size of these corpuscles does not depend on the age of the cysts, though their numbers increase with progressing age.
2. In both the rat strain and the mouse strain, the cyst calcium accumulates gradually. For example, the 2 month rat strain cysts contain 49 mg./% calcium (almost 5 times the value for blood of a normal mouse i.e. 10 mg./%) and rises to a maximum of 240 mg./% calcium in the 8 month cysts. (Here the concentration has risen to 24 times that of blood).
3. The extended study of the rat strain cysts in mice has shown that the calcium concentration in cysts over 8 months old gradually decreases, falling to a level of 164 mg./% calcium. (This value is still over 16 times that of the blood).
4. The cyst size generally increases up to the age of 8 months when a plateau is reached. The subcutaneous cysts often decrease slightly after this age.
5. Mice infected subcutaneously develop a pronounced leucocytosis (with total counts as high as 12,000 W.B.C. per c. mm. of blood) in the course of the first month of infection.

6. The absolute numbers of neutrophils increase 5 times, that of monocytes 3 times, and eosinophils 6 times; the peak of the eosinophilia is attained much later than that of the neutrophilia.

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INTRODUCTION

INTRODUCTION

Perhaps the most prominant feature which prompted the present research was the fact that the cysts of Echinococcus multilocularis differ widely both macroscopically and microscopically from those of other cestodes. When mature they are filled with brood capsules to such an extent that the lumen of the cysts themselves is almost completely obliterated. The germinative layer of the brood capsules and to a lesser extent the tissues of the scoleces are filled with innumerable calcereous corpuscles which impart to the cysts a chalky appearance. The specific gravity of such cysts may be as high as 1.2.

These facts, as well as the occurrance of tetany in experimentally infected gerbils and cotton rats, has prompted an examination of the process of calcium accumulation in the cysts of Echinococcus multilocularis. In addition, the long term observations necessary for this kind of work provided an excellent opportunity for a haematological study of the infected animals.

Thus the enormous accumulation of calcareous corpuscles in the older cysts, and the cases of tetany in experimental rodents, were the two salient facts which prompted the selection of this research problem.

HISTORICAL REVIEW

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A HISTORY OF RESEARCH ON ECHINOCOCCUS MULTILOCULARIS

Alveolar echinococcus was first observed in the mid 19th century, in sourthern Germany. R. Virchow (26) in 1856 reported that these "malignant tumors" of the liver, which had been previously regarded as alveolar colloids, or colloid carcinomas, were actually parasitic infestations. He called them "multilocular ulcerous growths caused by echinococci". The characteristics which distinguish this from the common hydatid form of Echinococcus, namely the microvesicular and multivesicular structure, exogenous proliferation, and the infiltrating and tissue destroying growth, which makes the alveolar echinococcus the most dangerous worm parasite found in man, have given rise to a large number of investigations by pathologists and clinicians.

From Virchow's time up to the last decade, there has been considerable controversy on the nature of the above disease and to the species to which the multilocular echinococcus belongs. The workers in this field held two distinct views; some as the Innsbruck surgeon, Posselt (21), maintained a dualist view and believed that Echinococcus multilocularis was a separate species of tapeworm distinct from the common Echinococcus granulosus.

The unicist theory was upheld chiefly by the helminthologists Déve (3) and Dew (4). They believed that there was only one species of echinococcus and considered the alveolar echinococcus as an aberrant type of common Echinococcus granulosus, resulting from atypical larval development. Déve stated that there existed transitional intermediate forms between the alveolar and the hydatid echinococcus.

The dualists based their view on: (1) the fact that the geographical distributions of the alveolar echinococcus differs from that of the hydatid echinococcus, (2) on differences in the structure, proliferation, and pathogenicity of the two larval forms, (3) differences in the shape of the hooklets of the larval scolices. The hooklets of the multilocularis are more slender and possess a longer manubrium than those of the hydatid echinococcus. There are also some peculiarities in the adult tapeworms. Since it seemed that the problem could be clarified by studying the adult parasite, attempts were made to raise the parasites to the adult stage by feeding parts of the alveolar echinococci from human cases to dogs. Most of these experiments resulted in failure, but some, however, were successful.

The first such successful experiment was carried

out by Klemm (9) in Munich in 1882. Several thousand echinococcus tapeworms were obtained from the intestine of an experimental dog 9 weeks after being fed material of human origin. The worm had a double row of 24-29 hooklets and 3-4 proglittids. In the terminal proglottis the uterus had no lateral sacculations and the eggs were gathered in an oval clump. Klemm compared his experimentally obtained tapeworms with those from naturally infected dogs and felt that he was able to separate the multilocular from the hydatid echinococcus.

Another successful experiment was made by Mangold (18) in 1891. He fed echinococcus material from a deceased woman to two dogs. On the 56th day he found three echinococcus tapeworms in the intestine of one dog and one in the intestine of the other. He published some data on the form of the uterus, eggs, and hooklets. This experiment is particularly interesting in that Mangold was successful in obtaining two juvenile alveolar echinococci of the liver by feeding the parasitized intestine of one of the experimental dogs to a 12 week old pig. After four months of growth these two echinococcus cysts attained the size of a hazelnut.

Posselt, in 1901 conducted an extremely successful experiment. The intestine of an experimental dog which

had died on the 49th day after being fed the cysts, was heavily infested with mature echinococcus tapeworms. In his later papers, Posselt stressed the peculiarities of the shape of the hooks and of the arrangement of the eggs in the uterus. On the basis of these characteristics he established the sub-species Taenia echinococcus alveolaris. It was not until half a century later that additional feeding experiments of this type proved successful. The dispute over the nature of the alveolar echinococcus would probably have been settled 50 years ago if the sometimes very numerous tapeworms obtained experimentally had been examined by someone familiar with the anatomy and classification of the cestodes. However, the descriptions of that time were incomplete. The sex organs of the middle segment, which present day knowledge has demonstrated to be very important for the characterization of the species, were disregarded completely. The investigators did agree on two characteristics; first, the arrangement of the eggs in the uterus, either in a rounded or in an oval clump, in most cases in the anterior portion of the terminal proglottis, and secondly, on the shape of the hooklets.

The interpretation of Mangold, Posselt, and others who regarded these two characteristics as sufficient

criteria for the establishment of a new tapeworm species was generally not accepted. Déve observed ova in rounded clumps gathered in the anterior part of the uterus in many specimens of Echinococcus granulosus. He admitted that there was differences in the shape of the hooks, but he thought that this was only of minor importance in view of the recognized variability in the shape of echinococcus hooks. Déve moreover, thought it inadmissible to establish a separate tapeworm species merely on the basis of hook shape alone.

The major weakness in the unitarian theory was the fact that its supporters were unable to specify the factors, in their opinion which determined the development of a single echinococcus species into a hydatid echinococcus in one patient and into a multilocular echinococcus in another. A second weakness inherent in this view was that it offered no explanation for the differences in the geographical distributions. For example, in Switzerland the human cases of alveolar echinococcus are much more frequent than those of the hydatid, whereas the former is completely absent, or extremely rare in regions of frequent occurrence of hydatid echinococcus, e.g. Yugoslavia.

Thus both views persisted side by side as hypoth-

eses for decades. Because of the authority of Déve and Dew in the field of echinococcus research the unicist doctorine found a large number of adherents until quite recently. In 1951 a new phase of study on alveolar echinococcus was begun by Rauch and Schiller (22). In a number of articles they reported an echinococcus species which is prevalent on St. Lawrence Island in the Bering Sea. Polar foxes and sled dogs are the hosts of the tapeworm in the adult stage and species of Microtus are the natural intermediate hosts of the area. In its adult stages this species differed from Echinococcus granulosus and other recorded species in a number of characteristics. Rauch and Schiller have described this species as Echinococcus sibiricensis. Since the larval form in the liver of the field mouse was multilocular in structure, and since sporadic cases of alveolar echinococcus were reported in man on St. Lawrence Island, Rauch assumed that this parasite of the polar region was identical to the European alveolar echinococcus.

Stimulated by the interesting observations of Rauch and Schiller, Vogel, (27) began in 1954 the study of South German wildlife for echinococcus stages. This study was confined to a region where the incidence of human infection was high (South Western Germany, adjacent to the Swiss border). Vogel found echinococcus tapeworms

in the intestines of wild foxes. He fed the eggs of these parasites to several species of mice, other rodents and to monkeys. This resulted in the development of microvesicular and multivesicular echinococcus cysts in their livers. After a few months the cysts contained numerous scolèces. Cavities caused by central necrosis later appeared in large clusters of vesicles in one monkey and in ~~those of~~ two muskrats. Thus the pathological picture was very similar to human alveolar echinococcus. Vogel fed the liver of an experimentally infected field mouse to a dog, and tapeworms of the same type as those found in wild foxes were obtained from it. The parasite strains isolated from the naturally infected field mice and from wild foxes were maintained in the laboratory animals by serial transfers through rodents as intermediate hosts and through dogs, cats and foxes as final hosts. Careful morphological studies were made on these parasites, and a number of specific differences from Echinococcus granulosus were found. These consisted of differences in body length, absolute and relative size of gravid terminal proglottids, number of segments, shape and size of hooks, position of genital pores, number and arrangement of testes, and the structure of both the ovaries and the gravid uterus.

Despite the fact that this species of echinococcus of animals originated from a region where human cases of alveolar echinococcus had been observed, and that the larval stages of the animal and human strain showed a striking similarity, a careful study of all the factors involved still left some doubt as to the identity of the wild animal parasite with the alveolar echinococcus of man. These doubts could have been removed by a comparison between the echinococcus tapeworms from wild foxes and those obtained as a result of feeding alveolar echinococcus of man to experimental animals.

Vogel appeared to have the opportunity in 1955, when two portions of the intestine of a dog which had been infected experimentally by Posselt in 1901, were found in the collection of the Pathological Institute, Innsbruck. They were entered in the catalogue as follows: "November 15, 1901 - small intestine of a dog with Taenia echinococcus alveolaris, (experimentally produced; Professor Posselt)."

Although stored for 53 years, they still were readily stainable and had complete crowns of hooks. This material, however, was not entirely satisfactory since the tapeworms had contracted greatly on fixing. This made the study of the genital organs difficult and the

comparisons of measurements worthless. The need for new material was therefore urgent.

Vogel and his colleagues obtained portions of alveolar echinococcus from surgically operated patients and from autopsies. This material was fed to cats, dogs and foxes and there were no difficulties encountered in raising the adult tapeworm in the latter two animals. With eggs obtained from these definitive hosts both normal and albino voles were infected. The cysts which developed were identical to those found in naturally infected wild rodents. The strain of human origin was then passed through the definitive and intermediate hosts three times in succession.

On the basis of careful and extensive morphological studies, Vogel was able to show beyond any doubt that the wild life strains and the strains of human origin were identical and belonged to the same species, Echinococcus multilocularis Leuckart (1863).

As material for the present study hydatid cysts of a vegetatively propagated strain of larval Echinococcus multilocularis were used. Two substrains were employed; a cotton rat strain and a mouse strain. The cotton rat strain was established by Dr. Lubinsky (14) at the Institute of Parasitology, Macdonald College, McGill

University, in December 1958. This strain was initiated by injecting intraperitoneally, primary cysts aspirated from the liver of a cotton rat which had been infected with Echinococcus multilocularis eggs into a batch of animals of the same strain. The strain was maintained by serial intraperitoneal transfers through cotton rats at 2 to 4 month intervals and is presently in its 22 transfer. The intraperitoneal cysts grow quickly and reach average weights of 19 - 25 gms. in 2-2½ months (15).

The mouse strain is derived from the third transfer of the cotton rat strain. White mice of the mouse colony of the Institute of Parasitology (McGill University) were injected subcutaneously with intraperitoneal cysts of the third transfer of the cotton rat strain. The mouse strain has been maintained since that time by serial subcutaneous transfers in mice of the same colony and was later propagated in a black hybrid mice ~~strain~~ (A/Jax♀ x C57Bl♂). It grows slowly and is being transferred every 5-6 months. The morphological peculiarities of the scolices of these strains were discussed in a recent paper. (16)

The vegetatively propagated strain accumulates a considerable amount of calcium in the form of calcareous corpuscles. (14-Fig.5) In his earlier experiments

Dr. Lubinsky observed several cases of tetany in both cotton rats and gerbils which had large clusters of cysts of E. multilocularis (17). These observations suggest that the tetany may be a result of hypocalcemia caused by withdrawal of calcium from the host and its accumulation in the cysts of the parasite.

A HISTORY OF RESEARCH ON THE CALCAREOUS CORPUSCLES OF CESTODES

The calcareous corpuscles of tapeworms were first reported late in the eighteenth century by Pallas (20) and by Goeze (6). They were the first to investigate the microscopic structure of tapeworms.

The subsequent history of research on the calcareous corpuscles is odd, in that already by 1863, a considerable amount of information concerning these structures had already been accumulated. From this early date to the present however, surprisingly little interest and almost no research was devoted to the morphology and function of the calcareous corpuscles.

When comparing the current knowledge of these corpuscles to that of 100 years ago, it is amazing how little progress has been made. In order to fully appreciate the extent of knowledge on this subject by 1863 it may be

appropriate to quote directly from Leuckart's book.(10)

"The middle layer of the body of cestodes whose external boundary is marked off by a light margin, contains the sexual organs as well as the large longitudinal vessels. The cortical layer is predominantly of a muscular nature and in the majority of cases contains a considerable number of hard, spherical, layered concrements. These are usually designated as calcareous corpuscles and consist predominantly of calcareous salts."

"The presence of these calcareous corpuscles is not strictly limited to the cortical layer as they are also found in the middle layers, at least in the larger segments containing sexual organs in taenias. Before the genitals develop however, the number of calcareous corpuscles in the middle layer is large. (In Bothriocaphalus cordatus and other forms, the calcareous corpuscles are evenly distributed throughout the entire perenchyma.)"

"Though the calcareous corpuscles occur in considerable numbers in the great majority of the larger tapeworms, there are certain cases where they are almost completely absent. To this group belongs the species in which we are especially interested, Bothriocephalus latus,

whose calcareous corpuscles are so scanty that they may be completely overlooked."

"Küchenmeister feels that in this animal one must regard the large compressed ball-shaped bodies (the ventral and dorsal granules of Eschricht) as agglomerations of calcareous corpuscles. He even pointed out that these will dissolve and effervesce upon the addition of acetic acid."

"Recently it became probable that this system of vessels (excretory ducts) has some relation to the previously mentioned calcareous corpuscles."

"The existence of these calcareous corpuscles and their widespread prevalence in tapeworms is known from the time of Pallas and Goeze, when the first attempts were made to investigate the microscopic structure of tapeworms. It is virtually impossible to overlook these corpuscles in process of microscopical examination, especially of the larger taenias. In each section one sees thousands of these corpuscles closely packed together and filling the spaces between the longitudinal and radial fibrils. Often the corpuscles are found between the fibrils, especially centrally located, where they are less numerous and arranged in linear series. They are smaller and larger bodies, mostly spherical or

disc shaped, resembling the well known starch corpuscles both in their hardness, refractility, and concentric structure."

"The history of research on the calcareous corpuscles is filled with errors and erroneous interpretations. The older observers have identified these as eggs; (especially in the bladder worms) others have regarded them as blood or lymph corpuscles. V. Siebold thought it possible to regard these as a dermal skeleton. This last conception seems to be closest to the truth because it stresses the previously (up to Doyere's time) neglected calcium content which can be detected either microchemically or with the aid of elementary chemical analysis."

"(According to the investigations of Dr. Naumann made in this University laboratory at my suggestion, the fresh body of Taenia marginata contains, when dried, almost 21% salts (1*)-predominantly calcium salts, with small quantities of magnesium, iron oxide, sodium and potassium, which are combined with carbonic, phosphoric, hydrochloric and sulphuric acids.)"

"Such calcareous corpuscles are found however not only in the cestodes, but also in the trematodes. Claparede (2**) has produced evidence that these corpuscles

do not occur freely in the parenchyma of the body as we previously believed, but are enclosed in the sack-shaped expansions of the terminal branches of this system of vessels. The suggestion that these are similar to the calcareous corpuscles of the cestodes was completely corroborated by the research of Leuckart and Pagenstecher on an oceanic form Echinobothrium parasitizing ray fishes. In larger taenias the amount of calcareous corpuscles is too great and they are too crowded, thus unsuitable for the solution of this problem. I feel however, that I was able to confirm these data in examining young transparent specimans of Taenia cucumerina. This discovery also explains van Benedin's observation that the vessel system of Taenia serrata becomes filled with carbonic acid bubbles when acetic acid is added (3***)."

1* As one can predict the calcium content in the cestodes is highly variable. Thus according to the research of Naumann, a Taenia solium which was preserved for a longer period in alcohol contained only 4.9% calcium. It might be asked in this case whether some of the salts were extracted by the alcohol diluted with water.

2** Zeitchr, fur Wissensch Bd. lx S99.

3*** Memoire ect. P. 182 (van Benedin).

"The effervescence of the calcareous corpuscles upon the addition of stronger acids in cestodes is not a rule without exceptions. In some cases they dissolve without the formation of carbonic acid, but after some swelling, which indicates the presence of another chemical compound. Probably in these cases the carbonic acid is replaced by phosphoric acid; thus by an acid which is not completely absent in taenias with calcareous corpuscles containing carbonic acid."

"The dissolution of the calcareous salts does not result however in the complete disappearance of the calcareous corpuscles. Organic remnants which still preserve the shape remain and prove thus that we are here concerned not with simple inorganic substances (e.g. calcium albuminate)."

"On the basis of these facts it was conjectured that the organic substance of the calcareous corpuscles becomes calcified only later, after they have already attained their final size. The calcareous corpuscles were even regarded as calcified connective tissue cells (Virchow). However in my opinion it is easy to become convinced in the wrongness of this supposition by a direct examination of the material. This is easy to do in observing young bladder worms at the time of scolex

formation soon after the appearance of the vessel system (excretory Auct.), in which the calcareous corpuscles appear as extremely small rounded or ovoidal granules. From their very beginning they possess all the optical and chemical properties of well developed corpuscles and increase in size as a result of the growth of their crust, i.e. by deposition of new layers."

"The form and shape of the calcareous corpuscles is quite variable, but these variations are seldom characteristic enough to be of some use for the differentiation of the species. These variations can be observed even within the body of the same animal. In Taenia solium for example, one can find simultaneously with round and ovoidal calcareous corpuscles irregular angular bodies. Besides those with regular concentric layers, there exist also corpuscles which are almost homogenous. They vary in size, the larger being 0.019 m.m., the smallest 0.0015 m.m. in diameter. Some of these variations can be explained by the unequal age of these bodies. Their numbers increase with the age of the worm and the production of proglottids. New corpuscles are being constantly added. The formation of new calcareous corpuscles is accompanied by a dissolution of the older ones. This is proved by the fact that the young segments of Taenia serrata and of some other

cestodes contain numerous calcareous corpuscles in the middle layer of their body, thus in a place in which after the development of the sex organs they are almost completely absent."

"To understand the relationship of calcareous corpuscles to the system of excretory vessels and also from the physiological standpoint, one must remember that the urinary organs ("Harnwekzeuge") of various lower animals excrete calcareous salts as well as salts of uric acid."

"It is known e.g. that the malpighian tubules of various caterpillars (among these Bombyx quercus) contain crystals of calcium oxalate. In the kidneys of Pinna and of some other bivalves, (known as Bojanus organs), are found massive deposits of calcium phosphate, (according to Schlossberger). We must stress the importance of the latter deposits because they are similar to the calcareous corpuscles of cestodes both in their form and concentric structure."

After Leuckart's somewhat lengthy review of the current knowledge on this subject, no significant research on the calcareous corpuscles of cestodes was published for over 80 years.

Starcoff, (25) in 1939 stated that our knowledge of the nature and significance of the calcareous corpuscles

in cestodes was at best very obscure. He thought that the calcification process in cestodes was not simply the result of regressive and degenerative changes but involved complex aspects of mineral metabolism not yet completely understood.

Starcoff studied the morphology of the calcareous corpuscles using calcium specific stains. He used the purpurine stain of Grandis and Mainini and also the silver impregnation technique of Kossa. He stained sections of proglottids of various cestodes and studied morphological features such as shape and size, numbers, distribution and structure of the calcareous corpuscles which are a normal constituent of the tissues of both larval and adult cestodes.

His interpretations of these observations are interesting if not quite accurate. Starcoff concluded:

1. "The so called calcareous corpuscles are not simple conglomerations of calcium salts. They possess a definite structure and probably a complex function.
2. Their microscopic structure varies with the degree of calcification which the worm has undergone. The calcification begins in the form of a central or slightly eccentric nucleus, which becomes larger as the calcification progresses. When the calcification

is complete the primary nucleus becomes enlarged to the point of occupying the entire calcareous corpuscle, transforming it to a solid block of secondary calcification of concentric or radial structure. The diameter of the nucleus varies inversely with the thickness of the peripheral stratified zone and is therefore an exponent of the degree of calcification which the worm has undergone.

3. There was no difference in structure between the calcareous corpuscles of larva and those of the adult cestode."

Starcoff's conclusions are rather obscure and even questionable. His description of the corpuscles developing around a primary calcareous nucleus are not correlated with any cellular processes and therefore are confusing. It is remarkable how little Starcoff's research added to the knowledge already existing in Leuckart's time.

Logachov (11) in 1951 published a most interesting and informative paper on the structure and development of calcareous corpuscles in cestodes.

He stated that they are peculiar morphological structures and arise in cells adapted for the binding of carbon dioxide produced in the course of anaerobic metabolism; also that they probably serve for the neutralization

of intestinal acids, or are places where the final products of metabolism are deposited.

Logachov stated that the peculiar nature of the corpuscles and the absence of data on their morphological structure and development have led him to study these problems. Mature segments of the common anoplocephalid cestode parasitizing sheep, Thysaniezia ovilla, were fixed with neutral formalin to prevent decalcification and sections 10-14μ thick prepared using the freezing microtome. These sections were impregnated by Kossa's method specific for calcium salts.

Excellent results were obtained using Achukarro's method. The argyrophilic fibres took up the stain, whereas the calcareous corpuscles as well as the cells containing them stained different shades from dark yellow to brown, depending on their calcium content.

The calcareous corpuscles did not represent a homogenous group. Some were in their earlier stages of formation and contained only a small amount of calcium salts whereas others contained considerable amounts of calcium. A third group included large cells with enormous calcareous concretions with a thin layer of cytoplasm surrounding them. Finally there were also naked corpuscles without any traces of surrounding cytoplasm. This

last group consisted of completely formed naked calcareous corpuscles which have already completed their development. The first type Logachov called "calcareous cells" - because the calcium deposits in these are still included in the cytoplasm of the almost normal cells.

Logachov concluded that the calcareous cells originated from the motile cells of the parenchyma - the macrophage amoebocytes. These were situated largely in the peripheral portions of the cortical layers of the body, and often almost immediately beneath the subcuticular layer of cells. The amoebocytes were spherical or ovoidal and possessed a vesicular nucleus with a clearly visible light nucleolus. (Figure 1).

Some of these macrophages migrated into the deeper layers of the parenchyma and underwent a series of peculiar changes consisting of an accumulation of calcium salts in the protoplasm. The deposition of calcium salts appeared to start in the nucleus. It became darker and a layer of calcareous salts appeared around the nucleolus (Figure 1б).

The next stage in the development of the calcareous corpuscle consisted of the deposition of concentric layers of calcium in the nucleus, which gradually became completely filled (Figure 1. DEJ). The concretions

increased in size as a result of the deposition of further concentric layers of calcium salts and finally reached a condition in which the petrified nucleus in the center became indistinguishable and the large calcareous corpuscle was surrounded by only a thin layer of cytoplasm. (Figure 1Z).

Logachov felt that the peripheral protoplasm of the calcareous cells was able to change its shape; to invaginate and contract its separate portions. Cells with pseudopodia like projections (Fig.) were observed quite frequently.

Logachov thought that the calcareous concretions in the cells later became impregnated with organic substances, because they were stained homogeneous black by Kossa's method. (Figure 2) (It is a well known fact that Kossa's reaction is very intensive in the presence of organic substances).

Further changes in the calcareous cells occurred within the cytoplasmic layer surrounding the calcareous corpuscle. (Figure 2a) Here calcareous salts appeared as granules of various sizes from almost invisible ones to comparatively large particles. (Figure 2БВГД) Sometimes they tended to be deposited concentrically. After the cytoplasmic layer became completely filled with granules (Figure 2В) it began to disintegrate and finally

disappeared leaving a naked calcareous corpuscle.

Logachov described the following stages in the formation of the calcareous corpuscles:

1. Formation of a macrophage from the cambial elements.
2. Petrification of the nucleus of the macrophage.
3. Deposition of calcium salts in the cytoplasm and the production of the "calcareous cell".
4. Increase in the size of the cell as a result of the deposition of concentric layers of calcium salts.
5. Petrification of the peripheral cytoplasm of the calcareous cell.
6. Disintegration of the cytoplasmic layer and the liberation of the naked corpuscle.

Logachov concluded by stating that the macrophages of the parenchyma may develop in one of two directions:- some differentiate in the direction of adaptation to trophic function - the granular amoebocytes - whereas others developed in the direction of specialization towards respiratory function - the calcareous cells.

The research on the nature of the calcareous corpuscles was limited almost exclusively to their morphology. Almost no data on the process of calcium accumulation in the tissues of the cestodes were reported.

This fact provided the major impetus for the present research project.

Рис. 1. Последовательные стадии формирования известковой клетки. *А* — макрофаг; *Б, В, Г* — стадии петрификации ядра; *Д, Е, Ж* — отложение солей кальция в протоплазме вокруг ядра; *З* — сформированная известковая клетка. Окраска по способу Ачукарро и эозином. ×1000

FIGURE 1. (From Logachov).

Рис. 2. Превращение известковой клетки в известковое тельце. *А* — известковая клетка; *Б, В, Г, Д, Е* — последовательные стадии накопления известковых включений в ободке протоплазмы; *Ж* — распад ободка протоплазмы; *З* — «голое» известковое тельце. Окраска по способу Косса и эозином. × 1000

FIGURE 2. (From Logachov).

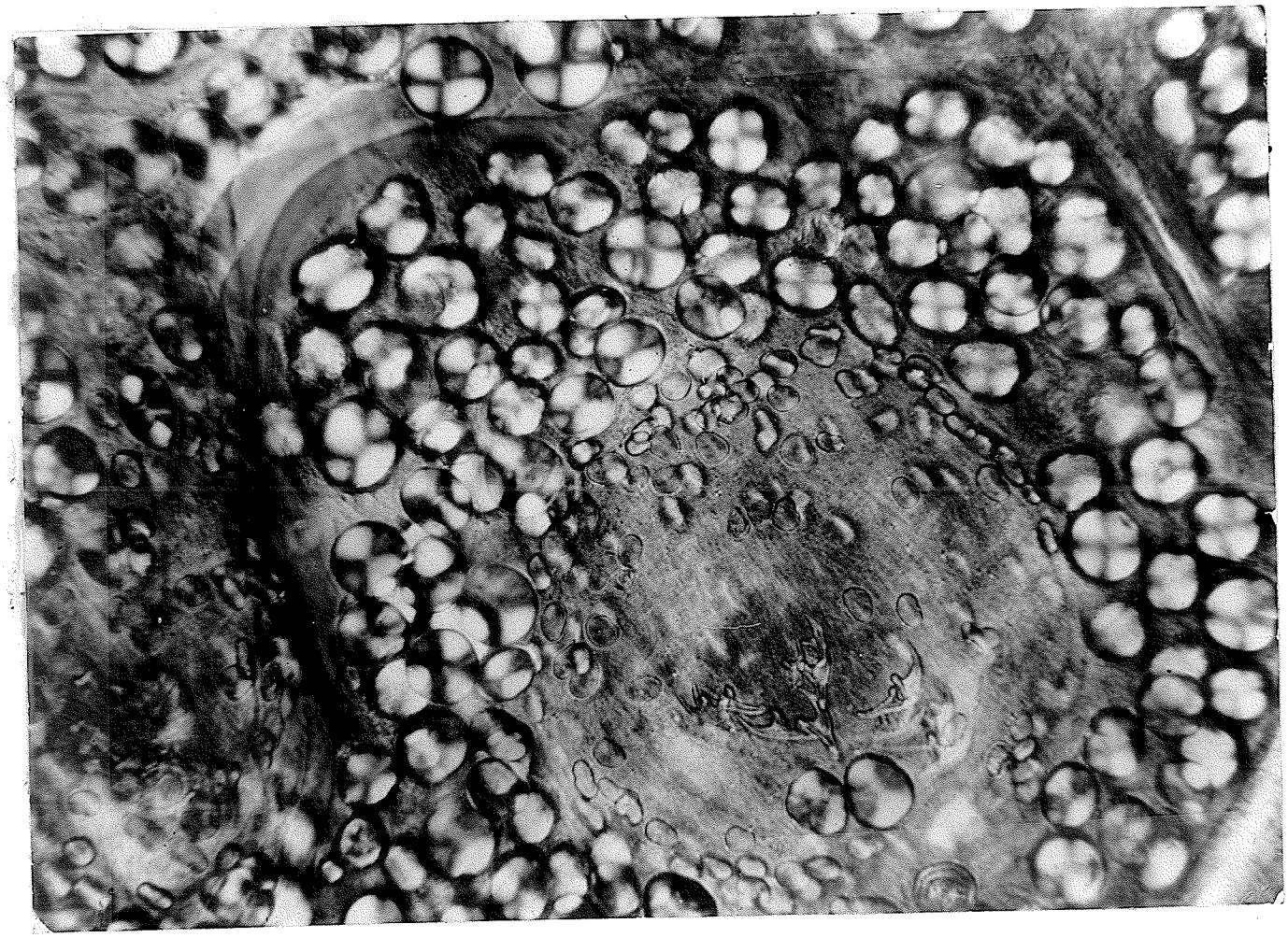
FIGURE 3. Photomicrograph in polarized light of a squash of Echinococcus multilocularis cyst showing numerous sub-spherical calcareous corpuscles and a scolex with the crown of hooks and smaller calcareous concretions.

Zeiss planachromat 40X

Zeiss eyepiece K.P.L. 10X

Attachment camera 35 m.m.

Crossed polarizers and compensator plate Red 1 order x 400.



MATERIALS AND METHODS

MATERIALS AND METHODS

As material for the examination of calcium metabolism, a vegetatively propagated strain of larval Echinococcus multilocularis was used which was established in December, 1958, and maintained by serial transplants from rodent to rodent without passage through any definite host.(14)

Cyst material from two sub-strains were used: that of the cotton rat was maintained by intraperitoneal transfers in cotton rats (Sigmodon hispidus) and that of a mouse sub-strain established in white mice (A/Jax) early in 1959 at the Institute of Parasitology of McGill University. This strain has been transferred and maintained since 1961 in the A/Jax♀x C57Bl♂ mice by serial subcutaneous transfers. Male hybrids of uniform age were used in this experiment. Two lots of 20 A/Jax♀ x C57Bl♂ mice were injected subcutaneously with Echinococcus multilocularis: 20 mice with rat strain and 20 with mouse strain.

TECHNIQUE OF SERIAL TRANSFER

An intraperitoneally infected cotton rat was killed with chloroform and the cyst material aseptically removed from the abdominal cavity. Care was taken in removing the cysts to avoid contamination by perforating

the intestine. The removed cyst material was placed in a petri-dish containing physiological saline and some penicillin. Next, some material was minced through a sterile wire mesh into a beaker containing physiological saline and again a small amount of penicillin. One c.c. of the sterile saline containing the infective scolices was injected subcutaneously into the left hind quarter of half of (20) the black hybrid mice.

In transferring the mouse strain the same general procedure was followed. In this case, the cyst material was taken from a subcutaneously infected mouse and injected into the other 20 black hybrids.

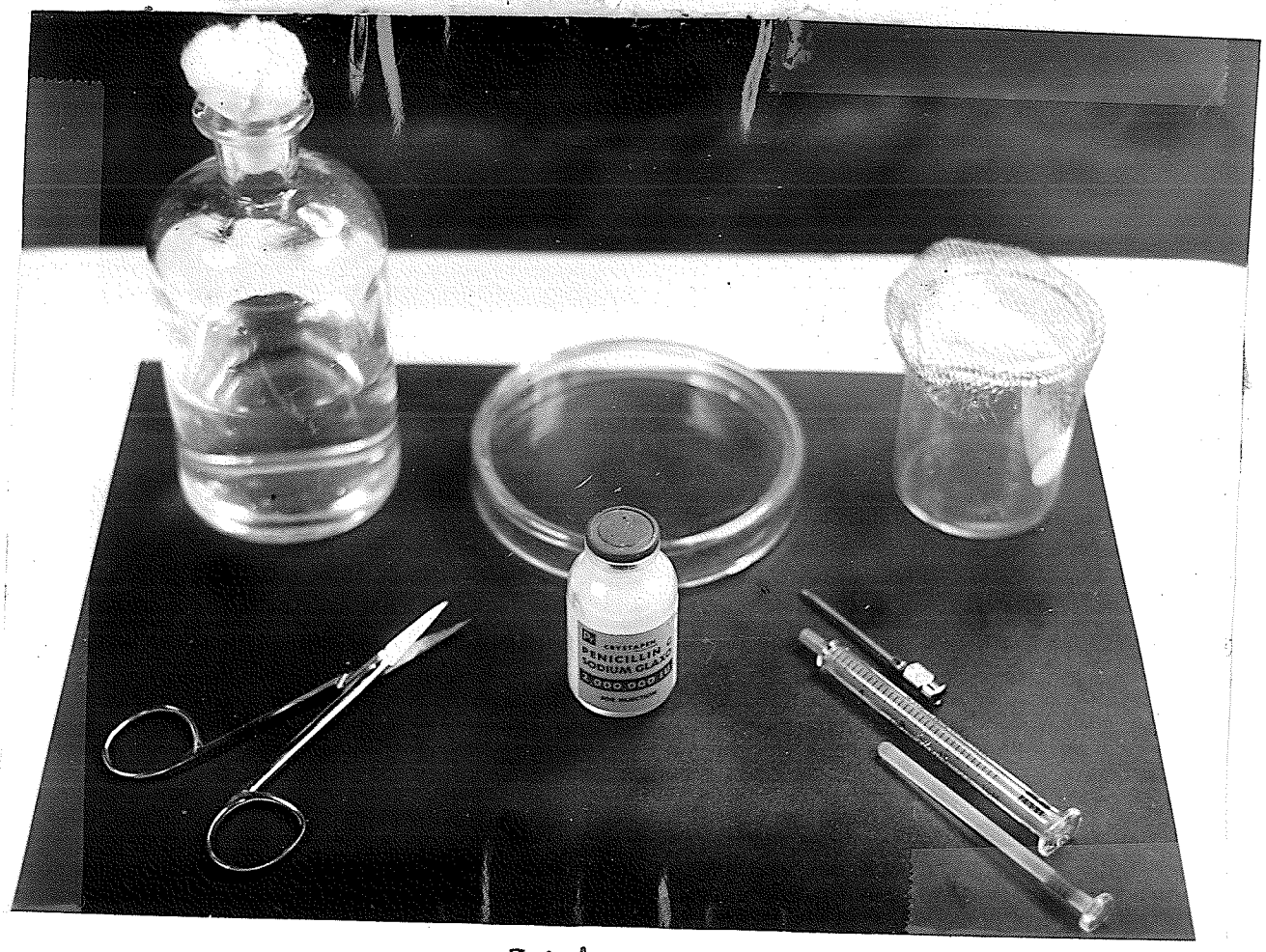


FIG. 4

In the 40 infected animals there was no mortality due to peritonitis or any other cause during the course of the experiment. Every experimental animal developed Echinococcus cysts, however, these varied in size and rate of growth.

Preparation of Cysts for Analysis

Four mice were killed at two month intervals. Chloroform was found to be much more lethal than ether. The animals were placed in a small jar containing cotton soaked in chloroform. They died in no more than 30 seconds.

The removed cysts were weighed on a torsion balance, placed in clean glass vials and frozen immediately at the temperature of -6°C.

This procedure proved useful as the analysis could be conducted at my convenience.

The extent and rate of growth of the cysts vary with the host and the point of injection. Cotton rats, for example, when intraperitoneally infected are excellent hosts and the cyst growth is rapid and extensive. (Figure 5). When mice, on the other hand, are infected intraperitoneally, only a small percentage show this extensive cyst development. Thus in many infected mice, there may be relatively little cyst growth. The great

variability in the growth rate of the intraperitoneal cysts in mice rendered them unsuitable to work with in this experiment.

The chemical analysis of the total calcium in these intraperitoneal cysts would be a much more tedious task, involving considerably more time and apparatus. In order to arrive at a correct estimation of the total calcium in these cysts, all the cyst material must be removed from the peritoneal cavity and homogenized before analysis. This would be necessary since the numerous individual cysts in the animal are in various stages of development, some containing much more calcium than others. The removal of all the intraperitoneal cyst material is difficult and the preparation of the material for analysis is time consuming.

The subcutaneous cysts, on the other hand, were much more convenient to work with since they could readily be removed in their entirety. Rarely, even in the most advanced stages of the infection, did these cysts weigh more than 2 g. (Figure 6). Thus the subcutaneous cysts were more suitable for this work because of their relatively uniform and predictable growth rate, convenience of handling and storage, and finally, their comparitive simplicity of preparation for analysis.

FIGURE 5.

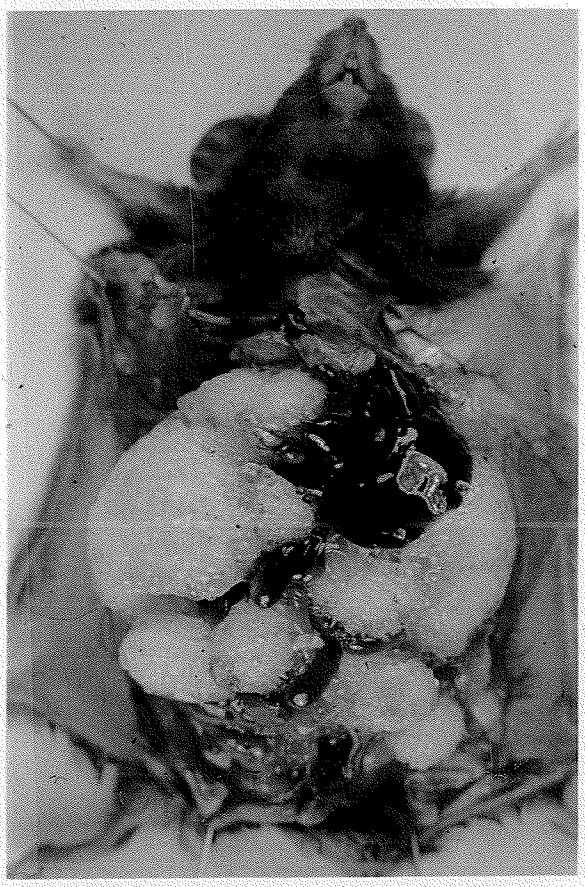
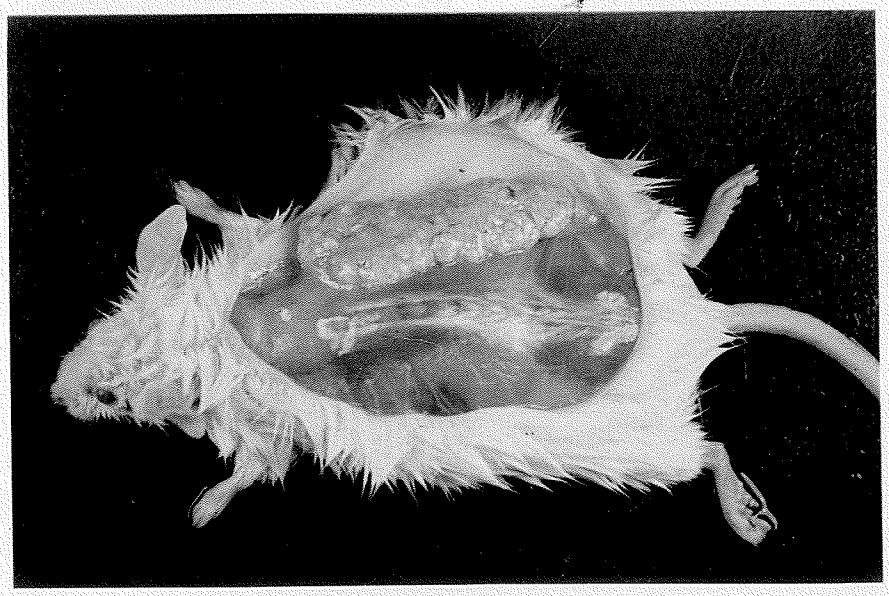


FIGURE 6.



HAEMATOLOGICAL TECHNIQUES

Periodically, at one month intervals over an eight month period, both total and differential W.B.C. counts were made. Four mice were examined during each examination period.

Blood samples were obtained by holding the mouse securely and severing the tip of the tail. Blood was induced to run freely without the use of excessive pressure.

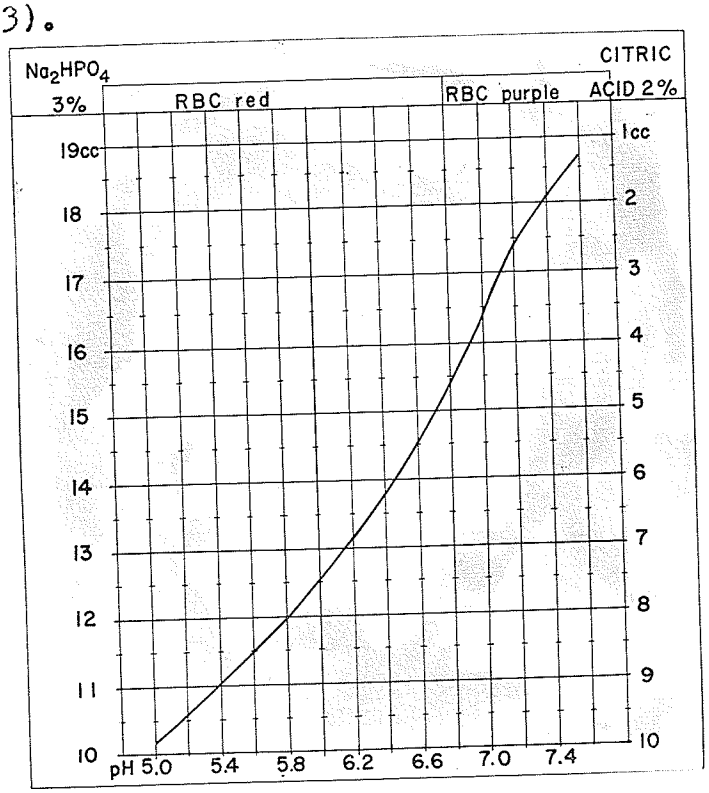
In counting the white cells the blood was drawn with a standard certified pipette, and diluted 1:20 with a diluting solution (10 ml. glacial acetic acid, 10 mg. crystal violet brought up to 100 mls. with distilled water). The diluted blood was introduced into the "improved Neubauer ruling" haemocytometer chamber and the cells in the four corner squares (each measuring 1 sq. mm.) were counted, the sum being multiplied by 50 to get the number of white cells per cubic millimeter of blood.

In making the differential counts, a small drop of blood was placed on a clean slide and a thin smear was prepared. The slide was placed on a staining bridge and flooded with 1 c.c. Wright's stain for one minute. Next, 2 c.c. of buffer solution were added to the Wright's stain and both fluids mixed thoroughly, by blowing gently

through a fine pipette - and finally left to react for a four minute period.

Before settling on Wright's stain as the most practical method, Geimsa's stain was used. This method proved disadvantageous since the staining time was at least quadrupled and the results were not superior.

Buffer solutions of pH values varying from 6.5 - 7.5 were prepared in mixing 2% citric acid with 3% di-sodium phosphate according to the following chart. (Figure 7) (13).



The results in staining varied with the pH of the buffer used.

The optimal results were obtained in using a

buffer solution of pH 6.7. This buffer was prepared by mixing 5 c.c. of 2% citric acid, 15 c.c. 3% disodium phosphate and 30 c.c. distilled water.

The use of this specific buffer yielded dark purple nuclei in the white blood cells in contrast to the pink erythrocytes.

After blood was taken, the wounds were swabbed with cotton soaked in ferric chloride to induce immediate clotting.

All blood for analysis was taken during the afternoon to eliminate as far as possible the influence of diurnal fluctuations in the leucocyte numbers. Concerning this matter, Wintrobe (29) states:

"Fluctuations occur during a single day as well from day to day. The suggestion that these follow a characteristic hourly rhythm has not been confirmed, nor has the occurence of a "digestive leucocytosis" been established conclusively. The random activity of ordinary routine may be associated with a moderate increase and a somewhat higher level is common in the afternoon."

CYST CALCIUM ANALYSIS

It has long been believed that the determination of calcium in tissues should be carried out after destroy-

ing the organic matter in the tissues analyzed by means of ashing. Corey and Denis in 1925 (2) claimed that autoclaving was a much more practical method for calcium determination. They used autoclave digestion with NaOH to determine calcium in tissue, feces and milk. Both these procedures are extremely tedious, time consuming, and are unsuitable for routine work.

Recently, since Schwarzenbach et al., 1956 (23) developed the chelation of divalent and trivalent cations by ethylendiamine tetra-acetic acid (E.D.T.A.), numerous methods of calcium determination have been reported. Originally murexide and Eriochrome Black T were used as indicators. The Eriochrome Black T formed a coloured complex more easily with magnesium than calcium in a mixture of these cations, while the murexide yielded poorly defined endpoints.

Diehl and Ellingboe (1956) (5) reported a new indicator, Fluoresceinbismethyleneiminodiacetic acid (calcein) which proved suitable for the complexometric analysis of calcium in the cyst material. No tedious ashing process was necessary and with some practice the endpoints became readily discernable. Thus calcium could be titrated directly in trichloroacetic acid treated juice of these cysts above a pH of 12 with C.D.T.A.

(disodium salt of 1,2-diamino cyclohexane N, N, N^1, N^1,-tetracetic acid) using calcein as the indicator.

The method employed in the cyst analysis was a modification of Mori's method (19).

Materials Required for Complexometric Analysis of Calcium

Reagents:

1. Potassium hydroxide 3 N

2. Indicator solution 4 mg. of calcein (Fluoresceinbis-methyleneiminodiacetic acid)-(commercially obtained from the J.R. McJannet Co., Regd. Montreal) was dissolved in 100 ml. of 0.25 NaOH. Since the solution is very unstable, it was kept refrigerated and prepared fresh every week.

3. C.D.T.A. solution (titrant)

(disodium salt of 1,-2-diaminocyclohexane-N, N, N^1, N^1-tetracetic acid) 45 g. of C.D.T.A. were dissolved in one liter of distilled water to prepare a stock solution. This in turn was diluted to 100 times to prepare the working solution.

4. Stock standard calcium solution

Calcium carbonate was dried in an oven overnight at 110°C. Then, .1001 g. was transferred to the 100 ml. volumetric flask with about 25 ml. of distilled water. Five ml. of 1N HCl were added to the flask, which was

heated on a hotplate to approx. 60°C. to insure complete solution and the evolution of CO_2. When the solution cooled to room temperature, it was brought up to the mark with distilled water. One ml. of this stock standard solution corresponds to 20 μ equ. calcium.

5. Working standard calcium solution

The stock solution was exactly diluted by 1:20 with distilled water. One ml. of this working solution contains 1 μ equ. calcium.

6. Trichloroacetic acid (T.C.A.) 10% aqueous solution.

Method for Analysis

Standardization of C.D.T.A. solution.

One ml. of working standard calcium solution was pipetted into a small beaker; 3 ml. of 3N KOH and several drops of calcein indicator were added and then the mixture was shaken well. This mixture was titrated with the working C.D.T.A. solution until the color changed from yellowish green (with fluorescence) to pink (without fluorescence). It was easier to detect the endpoint when the titration was carried out against a black background. Thus a factor for the C.D.T.A. solution could be calculated.

Example of factor calculation:

Working Std. Ca. Soln.

1 ml. x 1 m.eq.Ca./liter = x ml. C.D.T.A. x F (factor).

For example, assume 1 ml. C.D.T.A. was required to bring about desired endpoint.

Then 1 x 1 = 1 x F. Therefore, factor (F) = 1.

Mori's Method of Calcium Determination in Tissues

The tissues were cut into 2-4 pieces according to their size, blotted on filter paper to remove blood, and then placed in a pre-weighed small glass vessel containing 5 ml. 10% T.C.A. (The wet weight of the tissue pieces used for analysis varied from 0.5 - 1.2 g.). The vessel was then weighed and its contents were transferred into a micro-stainless steel Waring Blender (having a total capacity of 25 ml.) with an additional 5 ml. of 10% of T.C.A., then homogenized for 2-6 minutes. The final volume of the homogenate was then centrifuged at 2500 r.p.m. for 15 minutes. The resultant, clear supernatant was used for the analysis of calcium.

One (or two) milliliters of the supernatant was pipetted into a small beaker containing 5 (or 8) ml. of 3N KOH. Several drops of indicator were added and the mixture titrated against the standardized C.D.T.A. solution. A blank titration was carried out, replacing the

sample with 1 (or 2) ml. of 10% T.C.A.

Modification of Mori's Method for Cyst Calcium Analysis

The cysts were thawed completely and ground in a mortar containing a small amount of sea sand (washed and ignited (Fisher)) and 5 ml. T.C.A. The fluid was emptied into a centrifuge tube. This procedure was repeated with 5 additional ml. T.C.A. The resulting solution was centrifuged for 15 minutes and the relatively clear supernatant filtered through fine (#42) filter paper. (This filtration was essential, since a flocculation occured when the 3N KOH was added to the original cloudy supernatant, making the endpoint extremely difficult to determine.)

0.1 ml. aliquots of this filtrate were pipetted into small cleaned beakers and 3 ml. 3N KOH added to each sample to assure the proper pH; then several drops of calcein indicator were added and the beakers stirred well. The solution was then titrated against the standardized C.D.T.A. until the pink non-fluorescent endpoint was reached.

Sample calculation:

$$\frac{\text{Factor.}}{1.01^{*}} \text{ using 0.1 ml. aliquots cyst soln.} \quad \frac{\text{Cyst weight}}{1.82 \text{ g.}}$$

Five readings	Mean amount CDTA required 1.60 ml.
1.65 ml.	0.1 ml. cyst soln. contains
1.63	1.60 x 1.01* = 1.616 μ eq. Ca.
1.59	
1.57	
1.60	10 ml. cyst soln. contains 1.61 x
8.04	
Mean = 1.60 ml.	$\frac{100}{1000}$ = 0.162 M.eq.Ca.

This = 0.162 x 20 = 3.24 mg. Ca.

or $\frac{3.24}{1.82} = \frac{1.77 \text{ mg.Ca.}}{\text{g. cyst}}$

BLOOD CALCIUM ANALYSIS

Sufficient blood for this purpose was obtained through cardiac puncture. The blood sample was taken from control animals and also at the 3 and 6 month infective period. (Three mice were sampled at each period).

Into a small beaker containing 3 ml. 3N KOH, 0.1 ml. of blood serum was introduced. Several drops of calcein indicator were added and the beaker shaken well. Then the mixture was titrated against the standardized C.D.T.A. solution to the pink non-fluorescent endpoint. (Blank titrations were conducted with beakers containing 3 ml. KOH).

Sample calculation:	Factor
Using 0.1 ml. aliquots serum	1.0*

Am't. C.D.T.A. required = 0.53 ml.

Thus 0.53 x 1.0* x 10

$$= \frac{5.3 \text{ m. eq. Ca.}}{\text{liter}}$$

Five readings

0.56 ml.
0.50
0.53
0.52
0.54
2.15

Mean = 0.53 ml.

RESULTS

FIGURE 8. Size of Calcareous Corpuscles from Four Month (Rat Strain) Cysts in Black Hybrid Mice.

Mean = 22.13 ± .62 μ

Standard Deviation = 6.24 μ

Coefficient of Variation = 28.2%

Observed Limits of Variation = 16.6 μ - 35.3 μ

FIG. 8

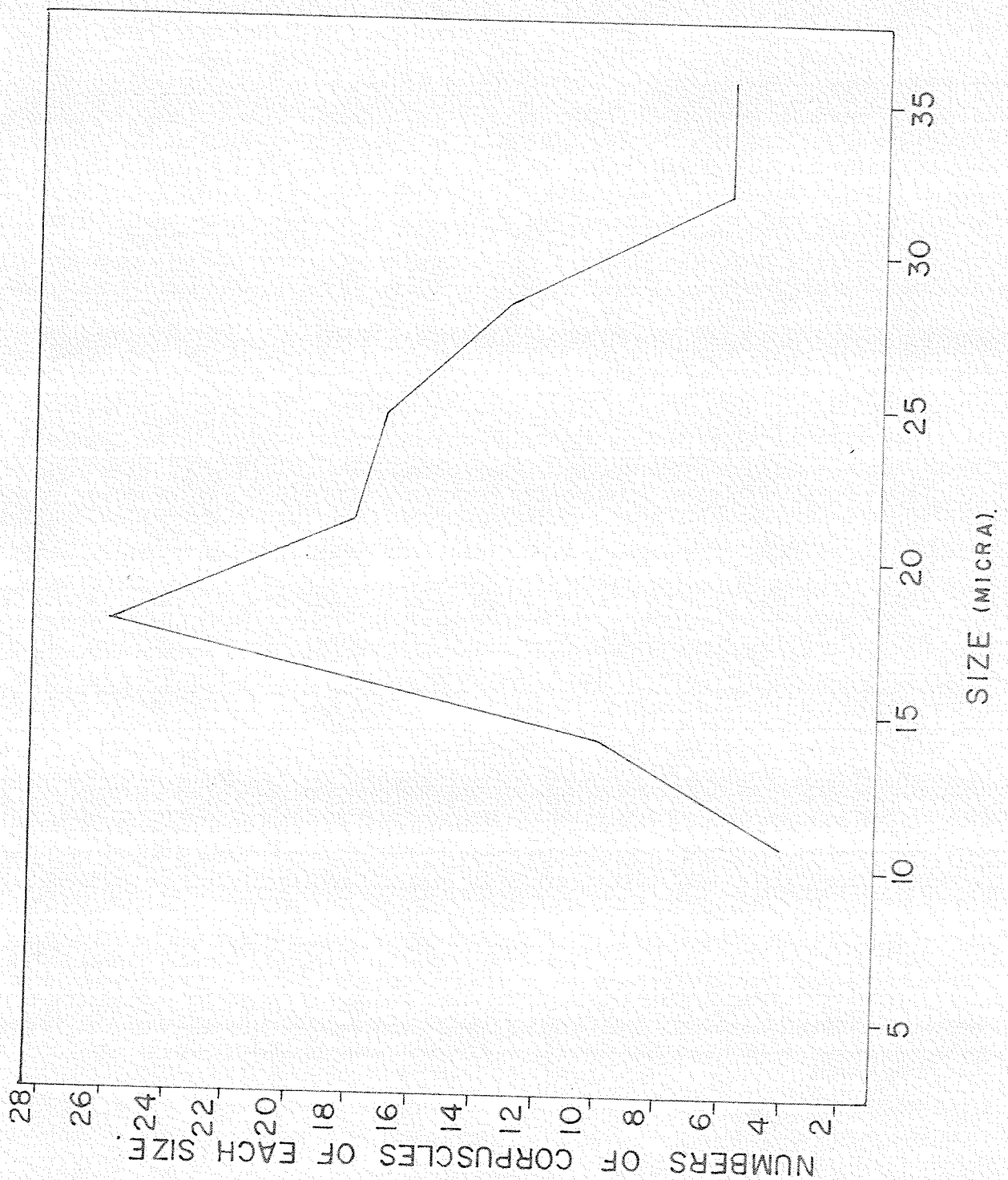

NUMBERS OF CORPUSCLES OF EACH SIZE.
2
4
6
8
10
12
14
16
18
20
22
24
26
28
5
10
15
20
25
30
35
SIZE (MICRA).

FIGURE 9. Size of Calcareous Corpuscles from Eight Month (Rat Strain) Cysts in Black Hybrid Mice.

Mean = 22.52μ ± .52μ

Standard Deviation = 5.15 μ

Coefficient of Variation = 22.9%

Observed Limits of Variation
= 10.59 μ - 35.3 μ

FIG. 9

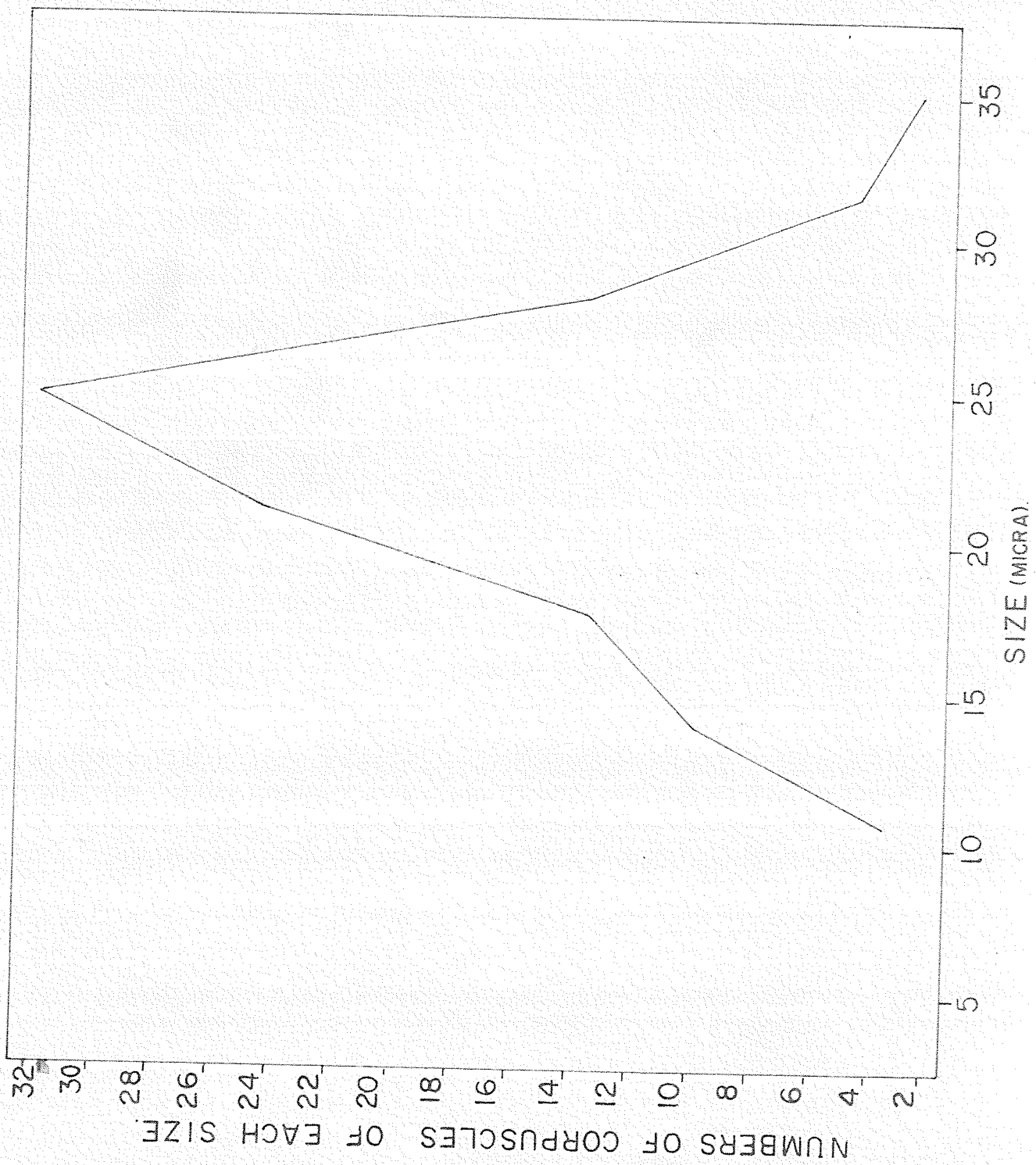

NUMBERS OF CORPUSCLES OF EACH SIZE
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
5
10
15
20
25
30
35
SIZE (MICRA).

FIGURE 10. Size of Calcareous Corpuscles from Ten Month (Rat Strain) Cysts in Black Hybrid Mice.

Mean = 22.98 μ ± .64 μ

Standard Deviation = 6.44 μ

Coefficient of Variation = 28.0%

Observed Limits of Variation
= 10.59 μ - 41.77 μ

FIG. 10

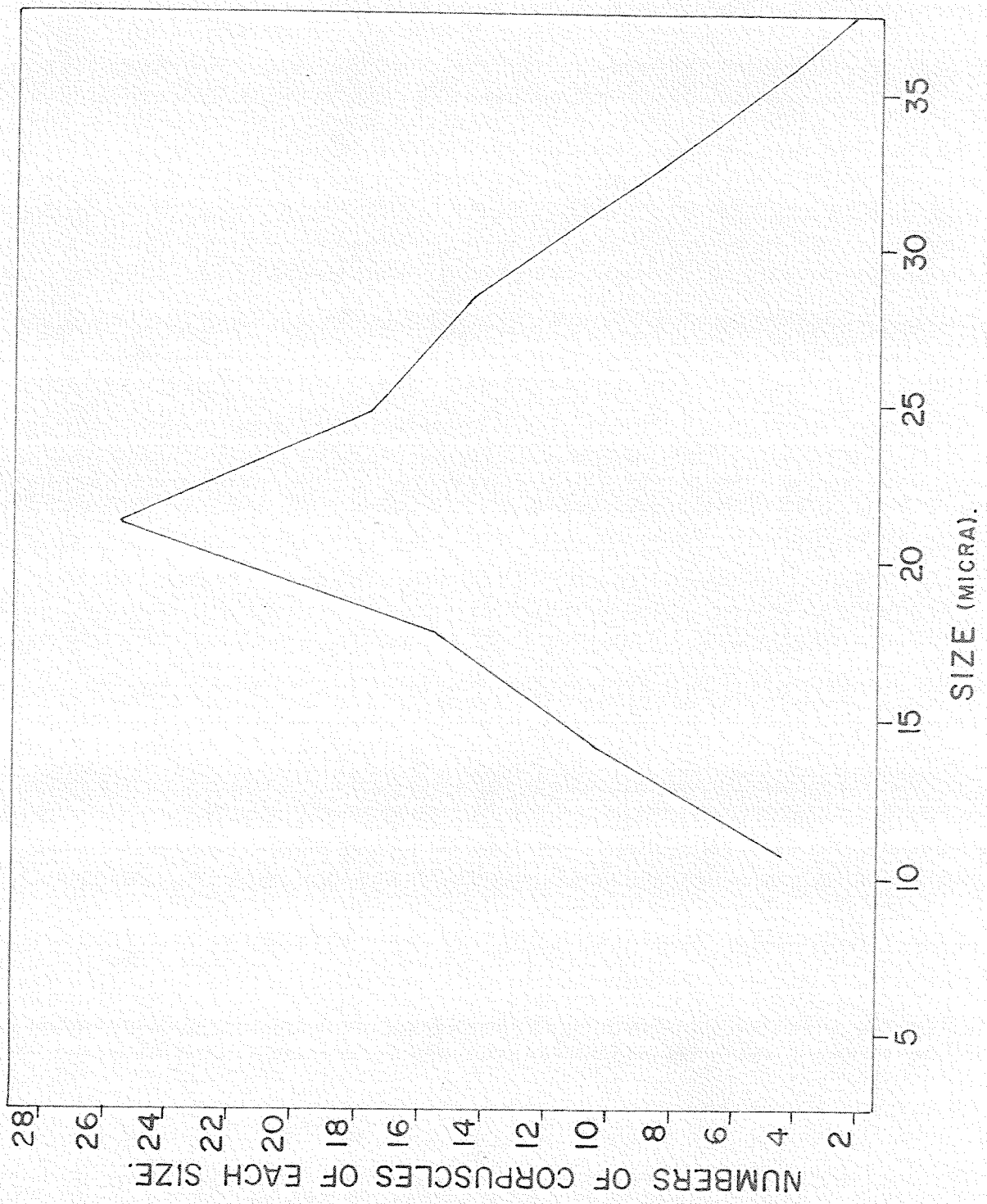

NUMBERS OF CORPUSCLES OF EACH SIZE.
2
4
6
8
10
12
14
16
18
20
22
24
26
28
5
10
15
20
25
30
35
SIZE (MICRA).

TABLE I

CALCIUM ANALYSIS OF THE RAT STRAIN CYSTS IN MICE

Analysis	mg. Ca. / g. cyst	Mean cyst weight (g.)
2 Month cysts		
1	0.52	
2	0.40	0.65
3	0.57	
Mean -	0.49	
4 Month cysts		
1	1.14	
2	1.51	1.18
3	1.20	
Mean -	1.28	
6 Month cysts		
1	1.84	
2	1.92	1.65
3	2.16	
Mean -	1.97	
8 Month cysts		
1	2.28	
2	1.92	1.60
3	2.84	
Mean -	2.34	

FIGURE 11. Growth of Subcutaneous Cysts (Rat Strain) in Black Hybrid Mice and the Calcium Content of These Cysts Expressed as

$$\frac{\text{mg. } Ca.^{++}}{\text{g. cyst}}$$

FIG. 11

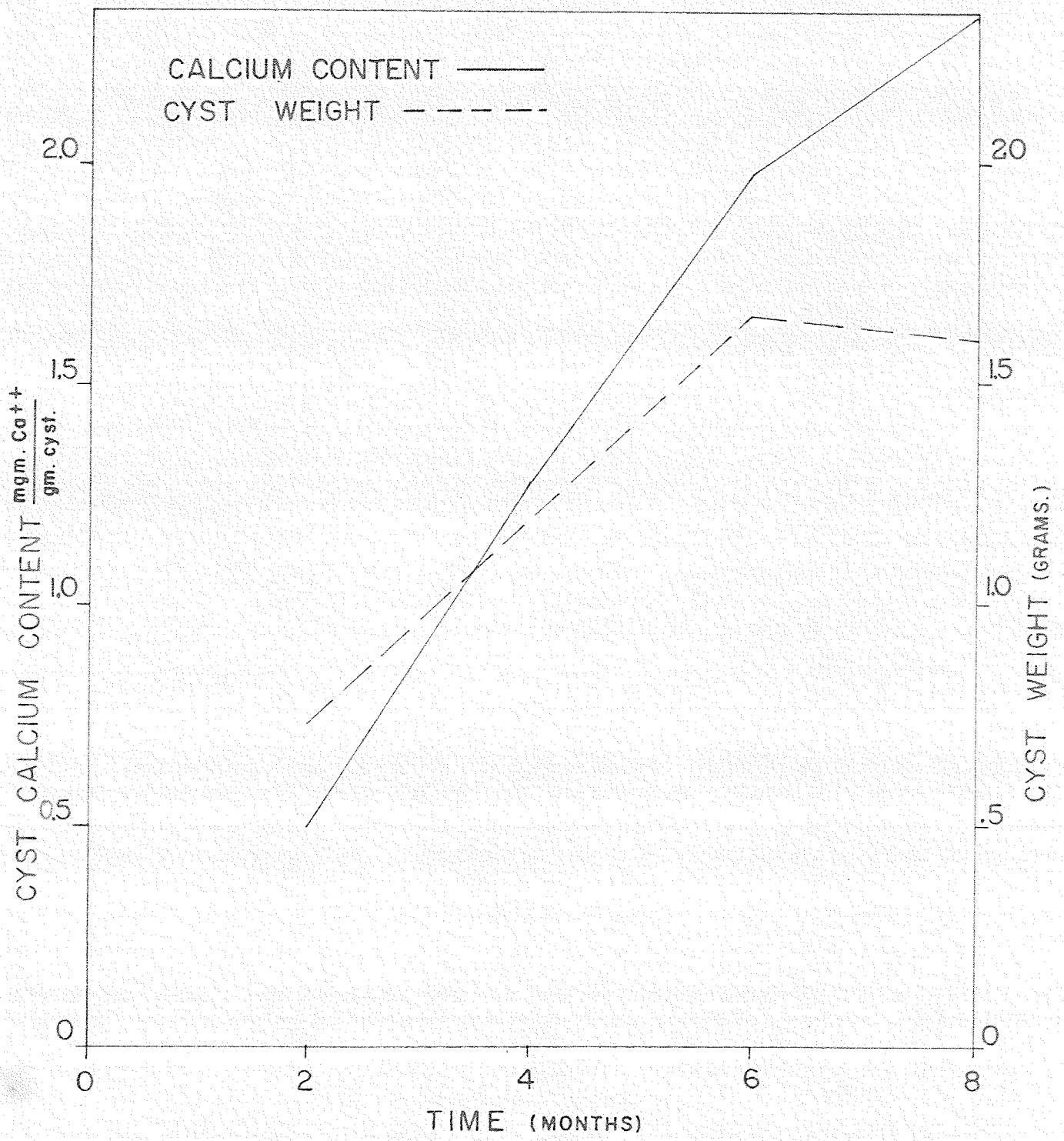

CALCIUM CONTENT
CYST WEIGHT
2.0
1.5
1.0
.5
0
CYST CALCIUM CONTENT mgm. Ca++ / gm. cyst.
2.0
1.5
1.0
.5
0
CYST WEIGHT (GRAMS.)
0
2
4
6
8
TIME (MONTHS)

TABLE II

CALCIUM ANALYSIS OF MOUSE STRAIN CYSTS IN MICE

Analysis	mg. Ca. / g. cyst	Mean cyst weight (g.)
2 Month cysts		
1	1.00	
2	1.15	0.85
3	0.86	
Mean -	1.01	
4 Month Cysts		
1	1.47	
2	1.38	0.96
3	1.72	
Mean -	1.52	
6 Month cysts		
1	2.31	
2	1.76	1.33
3	2.05	
Mean -	2.03	
8 Month cysts		
1	1.98	
2	2.43	1.89
3	4.38	
Mean -	2.24	

FIGURE 12. Growth of Subcutaneous Cysts (Mouse Strain) in Black Hybrid Mice and the Calcium Content of These Cysts Expressed as

$$\frac{\text{mg. Ca.}^{++}}{\text{g. cyst}}$$

FIG. 12

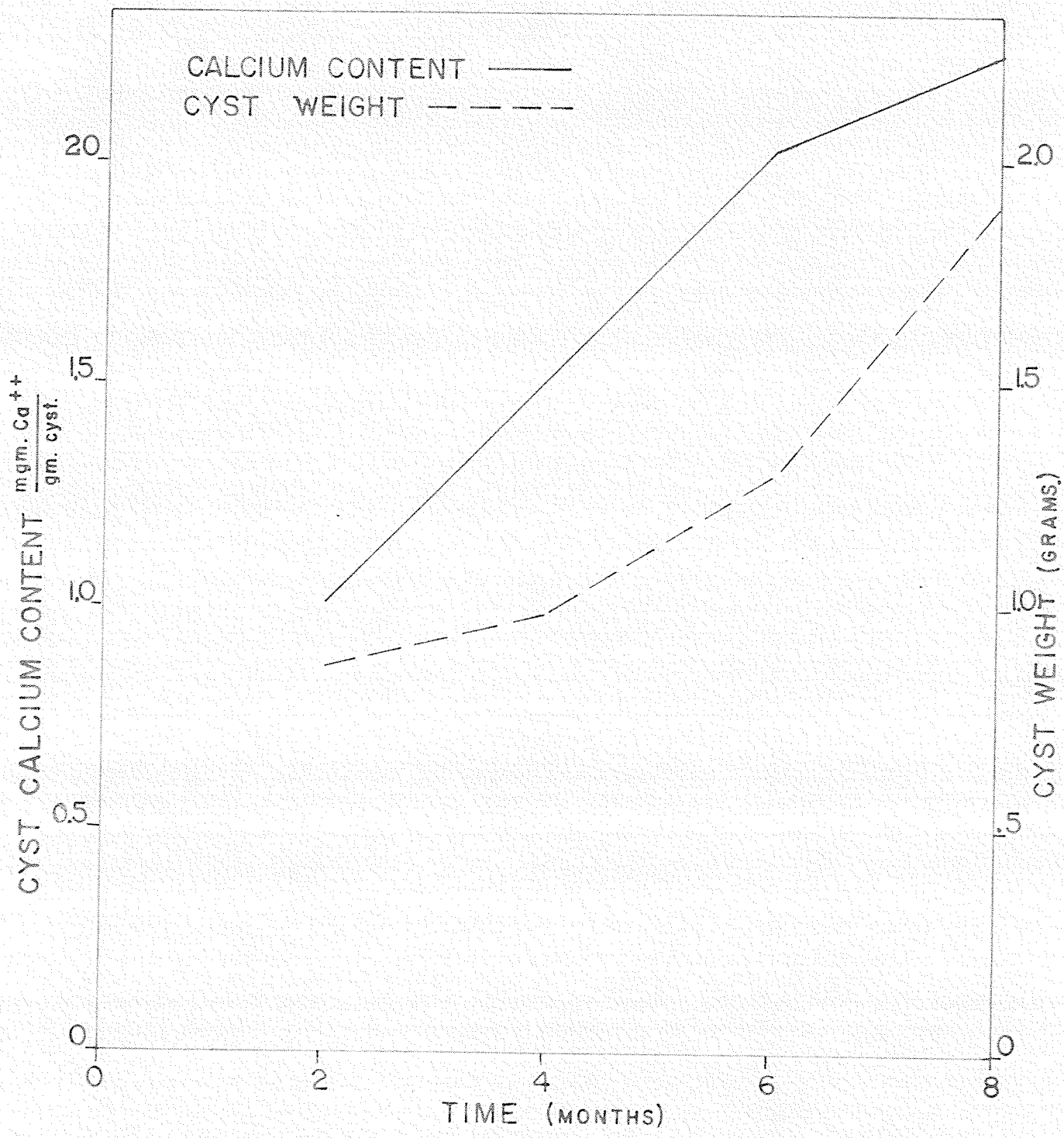

CALCIUM CONTENT
CYST WEIGHT
CYST CALCIUM CONTENT mgm. Ca++ / gm. cyst.
CYST WEIGHT (GRAMS)
TIME (MONTHS)
0
0.5
1.0
1.5
2.0
0
2
4
6
8
0
.5
1.0
1.5
2.0

FIGURE 13. Calcium Content of Subcutaneous Cysts of Black Hybrid Mice (Rat and Mouse Strain) Expressed as

$$\frac{\text{mg. } Ca.^{++}}{\text{g. cyst}}$$

FIG. 13

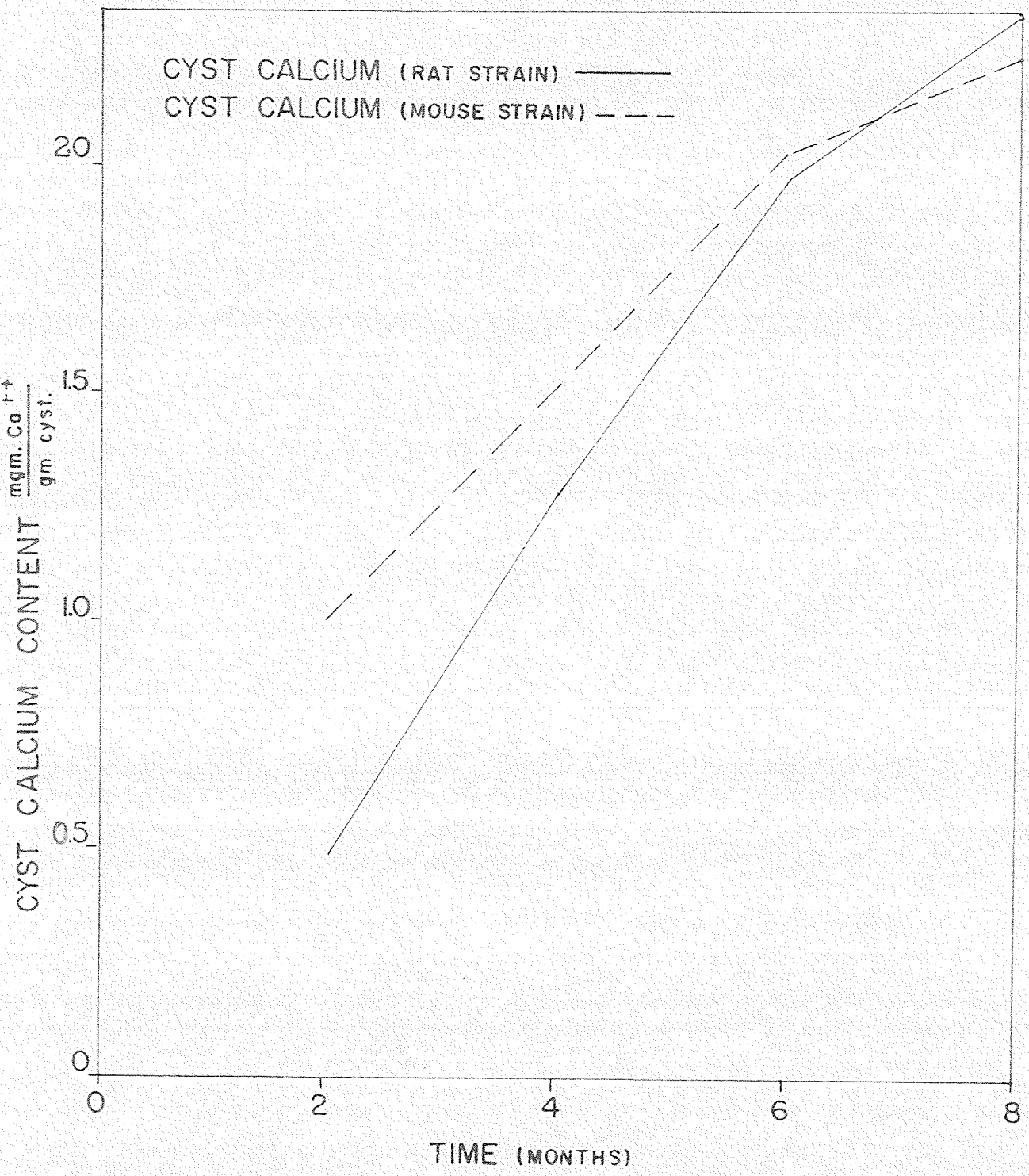

CYST CALCIUM (RAT STRAIN)
CYST CALCIUM (MOUSE STRAIN)
CYST CALCIUM CONTENT mgm. Ca++ / gm cyst.
0
0.5
1.0
1.5
2.0
0
2
4
6
8
TIME (MONTHS)

TABLE III

CALCIUM ANALYSIS OF RAT STRAIN CYSTS IN MICE
(EXTENDED STUDY)

Analysis	mg. Ca. / g. cyst	Mean cyst weight (g.)
10 Month cysts		
1	2.41	
2	1.46	1.30
3	1.70	
	Mean - 1.86	
12 Month cysts		
1	1.77	
2	2.10	1.31
3	1.57	
	Mean - 1.81	
15 Month cysts		
1	1.52	
2	1.88	1.37
3	1.64	
	Mean - 1.64	

FIGURE 14. Growth of Subcutaneous Cysts (Rat Strain) in Black Hybrid Mice and the Calcium Content of Cysts Expressed as

$$\frac{\text{mg. } Ca.^{++}}{\text{g. cyst}}$$

(Extended study).

FIG. 14

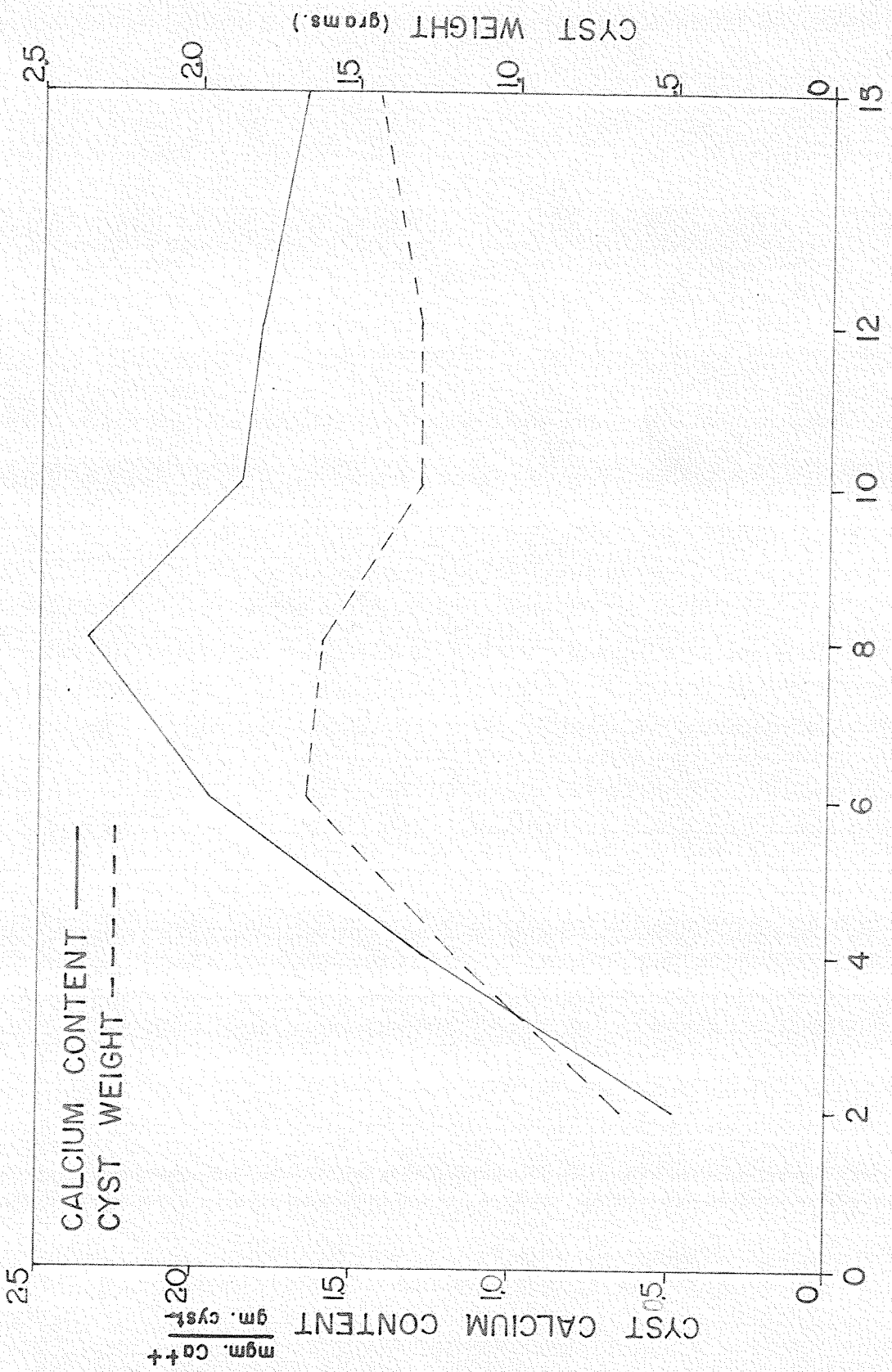

CYST WEIGHT (grams.)
CYST CALCIUM CONTENT mgm. Ca++ / gm. cyst
TIME (MONTHS)
CALCIUM CONTENT ——
CYST WEIGHT - - - - - -
0
5
10
15
20
25
2
4
6
8
10
12
13

TABLE IV

BLOOD CALCIUM ANALYSIS IN MICE INFECTED WITH RAT STRAIN

	Mouse	m.equ.Ca. / liter		mgm.Ca. / 100 c.c. plasma
Controls	1	5.38		
	2	5.15		
	3	5.21		
	Mean	5.26	-	10.52
3 Months infected	1	5.05		
	2	4.85		
	3	5.65		
	Mean	5.19	-	10.38
6 Months infected	1	5.30		
	2	5.51		
	3	4.82		
	Mean	5.21	-	10.42

TABLE V

TOTAL AND DIFFERENTIAL LEUCOCYTE COUNTS IN MICE INFECTED WITH MOUSE STRAIN

Total numbers of leucocytes cu. mm. blood	Percentage of					
	Lymph.	Mono.	Stab.	Seg.	Eos.	Bas.
Controls - 4,650	69.0	7.0	12.0	10.0	2.0	0.25
1 Month - 12,345	56.1	7.0	22.5	16.5	1.3	0.25
2 Months - 9,657	59.7	4.1	18.6	15.3	2.7	0.25
3 Months - 10,029	59.0	5.4	15.4	16.5	6.3	0.37
4 Months - 10,099	49.8	6.3	20.5	20.1	5.0	0.50
5 Months - 9,350	52.7	4.4	20.5	18.4	3.2	0.25
6 Months - 9,458	62.5	6.4	13.5	13.0	4.5	0.12
7 Months - 8,883	61.4	4.9	16.1	14.6	2.7	0.25
8 Months - 8,633	63.1	6.2	15.0	13.7	3.1	0.25

* Lymph. = Lymphocytes

Mono. = Monocytes

Stab. = Stab Neutrophils

Seg. = Segmented Neutrophils

Eos. = Eosinophils

Bas. = Basophils

TABLE VI

TOTAL AND DIFFERENTIAL LEUCOCYTE COUNTS IN MICE INFECTED WITH MOUSE STRAIN

Total numbers of leucocytes cu. mm. blood	Absolute Number of					
	Lymph.	Mono.	Stab.	Seg.	Eos.	Bas.
Controls - 4,650	3,208	325	558	465	93	12
1 Month -12,345	6,913	866	2,777	2,036	160	31
2 Months - 9,657	4,756	396	1,835	1,478	263	24
3 Months - 10,029	5,917	542	1,544	1,655	632	37
4 Months - 10,099	5,029	637	2,170	2,030	504	50
5 Months - 9,350	4,927	411	1,916	1,729	299	47
6 Months - 9,458	5,911	605	1,277	1,220	425	11
7 Months - 8,883	5,454	435	1,420	1,297	240	23
8 Months - 8,633	5,439	537	1,294	1,183	268	22

FIGURE 15. Total and Absolute Leucocyte Counts in Mice Infected with the Mouse Strain.

FIG. 15

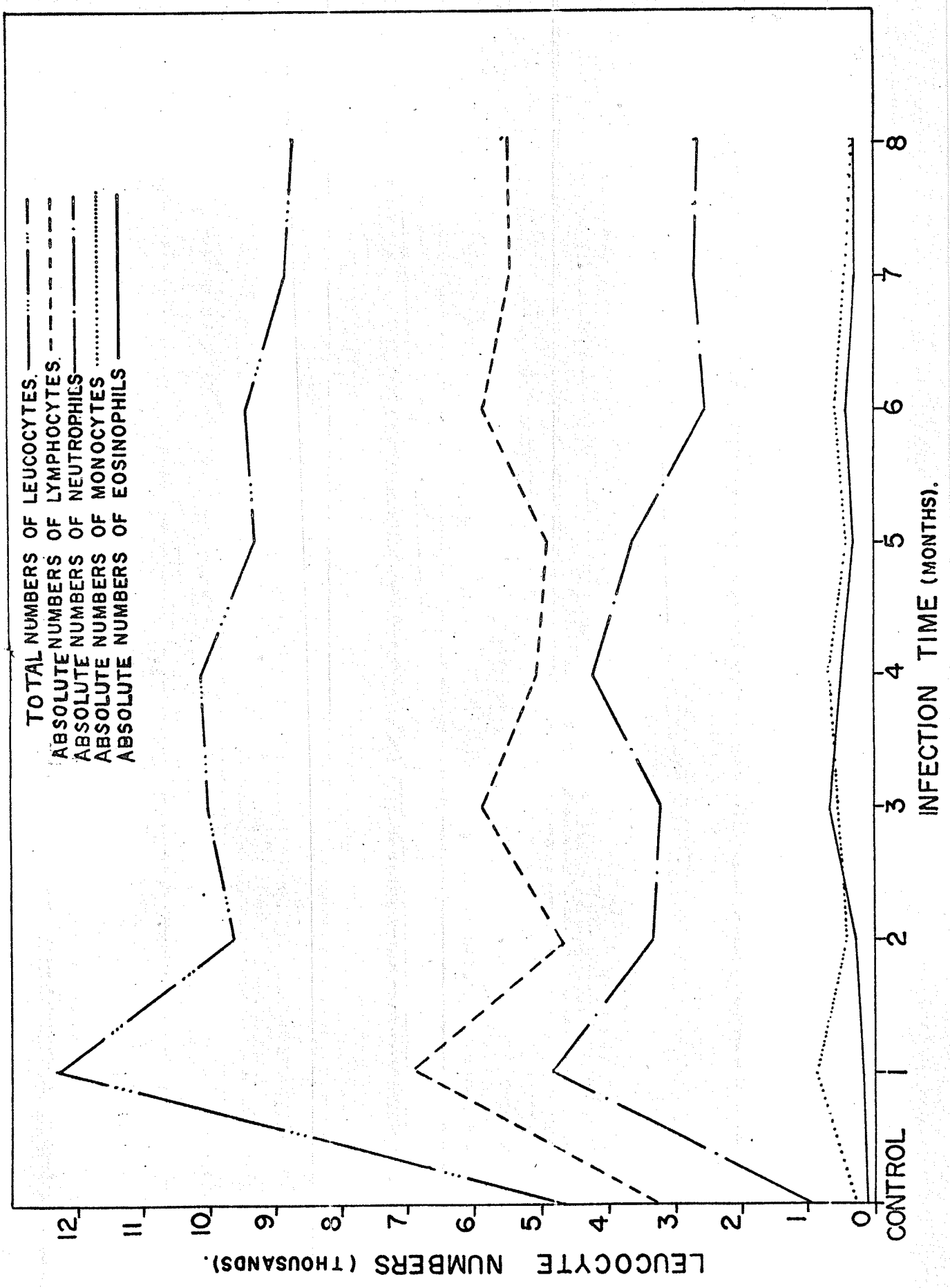

TOTAL NUMBERS OF LEUCOCYTES.
ABSOLUTE NUMBERS OF LYMPHOCYTES.
ABSOLUTE NUMBERS OF NEUTROPHILS
ABSOLUTE NUMBERS OF MONOCYTES
ABSOLUTE NUMBERS OF EOSINOPHILS
LEUCOCYTE NUMBERS (THOUSANDS).
0
1
2
3
4
5
6
7
8
9
10
11
12
CONTROL
1
2
3
4
5
6
7
8
INFECTION TIME (MONTHS).

TABLE VII

TOTAL AND DIFFERENTIAL LEUCOCYTE COUNTS IN MICE INFECTED WITH RAT STRAIN

Total numbers of leucocytes cu. mm. blood	Percentage of					
	Lymph.	Mono.	Stab.	Seg.	Eos.	Bas.
Controls - 4,650	69.0	7.0	12.0	10.0	2.0	0.25
1 Month - 9,377	43.0	8.2	23.2	22.1	3.3	0.50
2 Months - 10,037	54.5	7.0	17.6	14.0	4.8	0.50
3 Months - 11,191	54.0	7.2	17.4	18.0	2.9	0.75
4 Months - 6,700	61.6	3.7	13.9	16.1	7.0	0.75
5 Months - 12,081	62.6	8.0	14.0	12.6	2.5	0.25
6 Months - 8,596	61.6	7.2	15.1	14.2	0.9	0.25
7 Months - 7,895	57.5	5.1	17.2	17.6	2.4	0.40
8 Months - 8,044	63.9	5.7	14.0	12.6	3.7	0.13

* Lymph. = Lymphocytes

Mono. = Monocytes

Stab. = Stab Neutrophils

Seg. = Segmented Neutrophils

Eos. = Eosinophils

Bas. = Basophils

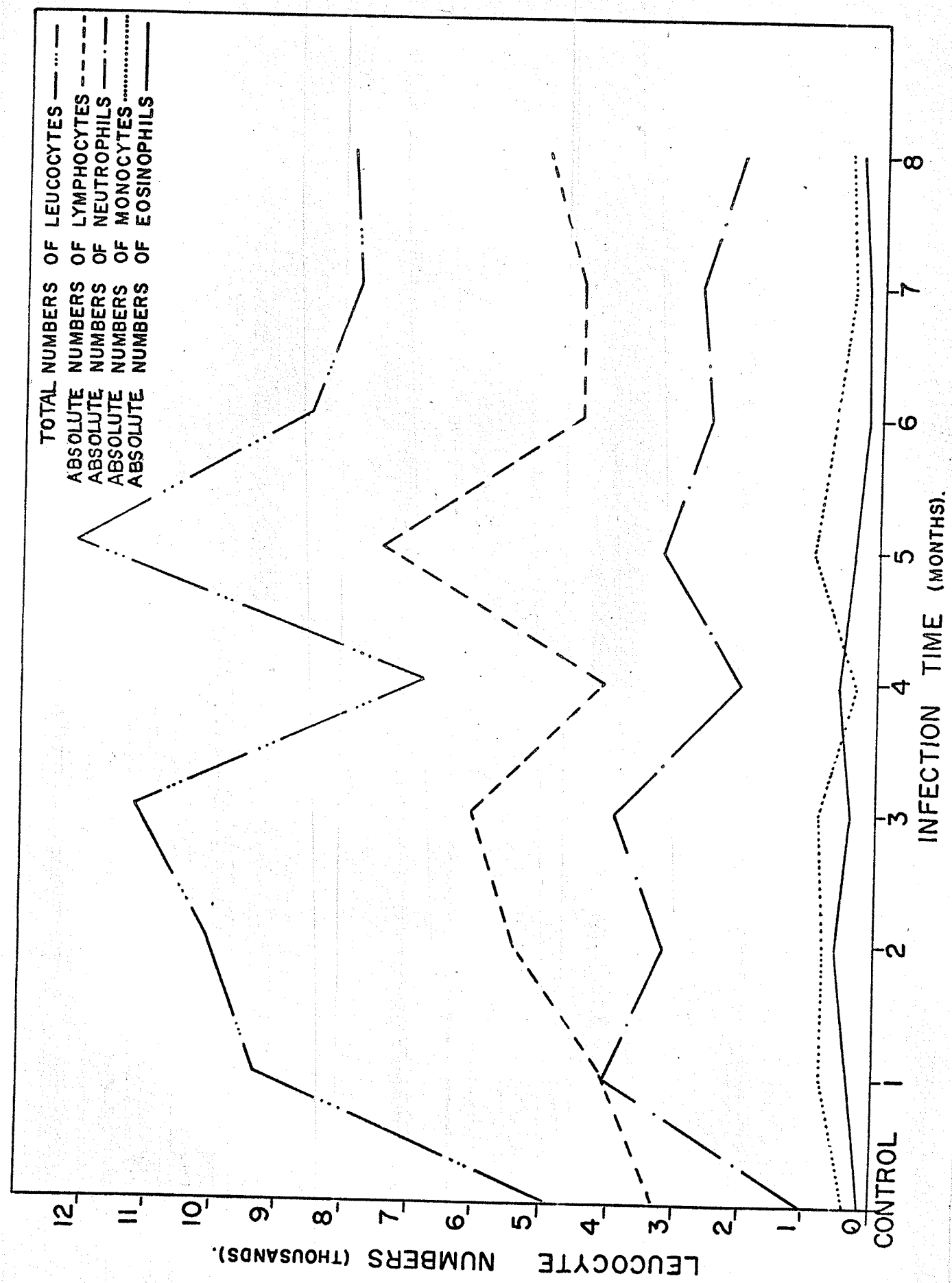
TABLE VIII

TOTAL AND DIFFERENTIAL LEUCOCYTE COUNTS IN MICE INFECTED WITH RAT STRAIN

Total numbers of leucocytes cu. mm. blood	Absolute Number of					
	Lymph.	Mono.	Stab.	Seg.	Eos.	Bas.
Controls - 4,650	3,208	325	558	465	93	13
1 Month - 9,377	4,032	768	2,175	2,072	309	47
2 Months - 10,037	5,420	702	1,767	1,405	482	50
3 Months - 11,191	6,042	805	1,947	2,014	334	84
4 Months - 6,700	4,127	248	931	1,078	469	50
5 Months - 12,081	7,562	966	1,691	1,522	302	30
6 Months - 8,596	4,487	620	1,298	1,221	75	22
7 Months - 7,895	4,540	403	1,358	1,390	189	31
8 Months - 8,044	5,140	458	1,126	1,014	297	11

FIGURE 16. Total and Absolute Leucocyte Counts in Mice Infected with the Rat Strain.

FIG. 16


TOTAL NUMBERS OF LEUCOCYTES
ABSOLUTE NUMBERS OF LYMPHOCYTES
ABSOLUTE NUMBERS OF NEUTROPHILS
ABSOLUTE NUMBERS OF MONOCYTES
ABSOLUTE NUMBERS OF EOSINOPHILS
LEUCOCYTE NUMBERS (THOUSANDS).
0
1
2
3
4
5
6
7
8
9
10
11
12
CONTROL
1
2
3
4
5
6
7
8
INFECTION TIME (MONTHS).

DISCUSSION

DISCUSSION

MORPHOLOGICAL STUDIES

Squashes of larval echinococcus cysts were prepared and examined microscopically with the aid of polarized light.

Initially the shape of the calcareous corpuscles was studied. The great majority were sub-spherical while a few were elipsoid in shape. They varied in size ranging from 10.59 micra to 41.77 micra in diameter. Two hundred such corpuscles were measured at random. This procedure was followed when examining cysts of 4, 8, and 10 months of age. The range of variability in corpuscle size was independent of the age of the cyst. The differences in size of the corpuscles from cysts of different ages were not statistically significant.

This perhaps may best be expressed by indicating the values obtained. In order for the differences between the sizes of corpuscles from cysts of different ages to be statistically significant

$$\frac{M_1 - M_2}{\sqrt{m_1^2 + m_2^2}} \text{ must equal or exceed } 3$$

where M_1 and M_2 are the mean values for the size of the corpuscles and m_1 and m_2 are the errors of these means. Now considering the corpuscles from 4 and 6 month cysts:

$$\frac{22.52 - 22.13}{\sqrt{.52^2 + .62^2}} = .48$$

the 10 and 6 month old cysts:

$$\frac{22.98 - 22.52}{\sqrt{.64^2 + .52^2}} = .55$$

the 10 and 4 month old cysts:

$$\frac{22.98 - 22.13}{\sqrt{.64^2 + .62^2}} = .95$$

Thus it now becomes obvious that differences in size between calcareous corpuscles in the 4, 6 and 10 month cysts are not statistically significant.

The very young cysts appeared almost transparent and filled with a clear fluid. They contained relatively few calcareous corpuscles. With progressing age the cysts become more and more opaque. Old cysts are literally stuffed with scolices and calcareous corpuscles.

Thus the calcareous corpuscles in the younger cysts are not as a rule smaller than those in the more mature cysts. The large difference in calcium content between the younger and older cysts is at least partially due to the far greater number of corpuscles which have accumulated in the older cysts.

CALCIUM ANALYSIS

The examination of the calcium content of a 2 month old rat strain cyst has shown that the calcium concentration was considerably higher than that in the blood of a normal mouse (49 mg.ca./100 c.c. - 10 mg. Ca./ 100 c.c.).

The concentration of calcium in the two month old cysts of the mouse strain, on the other hand, was higher than that in the rat strain cysts of the same age. The cysts of the mouse strain transplanted into mice developed and matured faster than cysts of the rat strain did. The mouse strain cysts in mice matured earlier and produced within a relatively short time a considerable number of scolices and calcareous corpuscles, while the rat strain cysts implanted into mice developed initially more slowly and the scolices and calcareous corpuscles, at least during the first two months of development were produced at a lower rate. In the 4 and 6 month old cysts there is a marked increase in the calcium concentration and the values now approach those of the rat strain cysts.

Unfortunately, because of unexpected circumstances the mouse strain studies were carried no further than 8 months (see page 94).

An extended study was made of cysts of 10, 12 and

15 months of age from mice infected with rat strain. Calcium analysis of cysts of these ages yielded most interesting results. The maximum calcium concentration was reached in the 8 month old cysts and after this the level began to decline gradually. The older cysts appeared solid, packed with calcareous corpuscles and scolices. The reduction in the calcium level of the 10, 12 and 15 month old cysts suggests a dissolution of the calcareous corpuscles, their released calcium salts being resorbed into the host's tissues.

The function of the calcareous corpuscles has been enigmatic since their first discovery late in the 18th century. Early observers thought that these corpuscles in larval echinococcus were eggs. Others regarded them as lymph corpuscles. V. Siebold (10), thought it possible to regard these as a dermal skeleton.

In 1939, Starcoff (25), (Almost a century later) stated that these corpuscles were not simply conglomerations of calcium salts but possessed a definite structure and probably a complex function. Starcoff progressed no further and did not even attempt to attribute a function to the corpuscles.

Logachov, in 1951 (11) claimed that the calcareous corpuscles arise as a result of the binding of carbon diox-

ide in the course of anaerobic metabolism and, in the adult worm, serve in the neutralization of intestinal acids. He regarded them also as places where the products of metabolism are deposited.

The author feels that the host probably actively secretes calcium into the parasite, attempting to petrify it through calcification. The calcareous cells or macrophage amoebocytes described by Logachov (page 21) accumulate this excess calcium and gradually become transformed into "naked" corpuscles. In this fashion the scolices of the larval cestodes are protected against calcification. In the later stages the cysts become almost entirely calcified and there is a tremendous number of calcareous corpuscles. This theory might also apply to adult cestodes which may become almost completely calcified.

A case of tetany in a heavily intraperitoneally infected gerbil containing vast amounts of cyst material (observed by Dr. Lubinsky, 4) led to several blood calcium analyses in the infected animals. The author felt, however, that the small subcutaneous cysts would induce no change in the blood calcium level.

It is interesting that the normal calcium level in human blood is 10 mg.% (1), while in the Sprague

Dawley strain of rats it was 10.9 mg.% (19). In the normal control "black hybrids" it was 10.4 mg.%.

HAEMATOLOGICAL STUDY

Absolute and Differential Leucocyte Counts

In mice infected with the mouse strain of echinococcus there was a marked leucocytosis during the first month, the total number of leucocytes at that time almost tripling (from 4,650 to 12,345). During the second month the leucocyte numbers dropped rapidly and then remained fairly constant at a level of 9 to 10 thousand per c.mm. until the end of the sixth month. The absolute numbers of all types of leucocytes rose sharply during the first month of infection. The numbers of lymphocytes, monocytes and basophils were doubled, while those of the neutrophils increased almost five times.

The absolute number of lymphocytes generally paralleled the total number of leucocytes, reaching its peak of almost 7 thousand at the end of the first month of infection. The relative number however, decreased from 69% to 56% because of the sharp increase in the number of neutrophils.

The number of neutrophils in the course of infection also paralleled the total leucocyte count, reaching

a peak in the first month. The neutrophilia was the most striking trait of the leucocyte picture. The absolute numbers of neutrophils increased almost five times during the first month (from 1,023 to 4,815).

The monocytes also reached a peak at the end of the first month of infection, having increased from 325 to 866. Their numbers subsided during the second month and further showed only minor fluctuations.

The eosinophilia reached its peak during the third month of infection, when the absolute number of eosinophils increased seven fold over the control values (from 93 to 632). On the other hand the relative number of these cells was only tripled. After the third month the numbers of eosinophils gradually declined.

The number of basophils increased from 12 per c. mm. to a maximum of 50 at the end of the fourth month, after which it slowly declined approaching the control values in the course of the sixth month.

The total number of leucocytes in mice infected with the rat strain also rose sharply during the first month of infection to over 9,000 per c. mm. and continued to rise until the fourth month. During the fourth month there was a large unexpected drop in leucocyte numbers which affected both the total and differential values

of all types of leucocytes with the exception of the eosinophils. This unusual leucopenia can be attributed to the fact that the blood was examined following a furnace failure on a particularly cold night in February during which the experimental animals were severely cooled. During the fifth month the total number of leucocytes rose to a new peak of over 12,000 per c. mm. and later declined sharply, levelling off during the seventh and eighth month.

The differential counts showed that the lymphocyte numbers closely paralleled the total W.B.C. counts, the peaks and low points coinciding almost exactly. The relative number of lymphocytes remained fairly constant throughout the eight month test period.

There was a pronounced neutrophilia during the first month (similar to that seen in the mouse strain), the numbers of neutrophils increasing four times, from 1,023 to 4,247. The number of neutrophils remained relatively high until the third month, when it began to decrease. The neutrophils levelled off during the sixth and seventh month, their relative numbers falling towards the normal values during the eigth month.

The relative number of monocytes showed little variation during the test period though their absolute numbers increased and reached their maximum of almost a

thousand at five months.

The dynamics of the eosinophils was similar to that observed in the mice infected with the mouse strain. There was a marked eosinophilia during the first three months, the number of eosinophils increasing five times (from 93 to 482). After the fifth month the eosinophils declined gradually, levelling off during the seventh and eighth months, then subsiding towards the control values in the last two months.

The total leucocyte numbers in the control animals closely coincided with those reported by Snell in 1941 (24).

COMPARATIVE STUDY

It is interesting to study the relationships between the total leucocyte numbers, cyst growth, and the cyst calcium content, Figure 19, page 90.

Upon injection of the cyst material, the host reacted strongly to the foreign protein by severe leucocytosis during the first month. (Unfortunately at this time the cysts were too tiny for practical calcium analysis and it was not until the second month that the first analysis was made). After this the total number of leucocytes decreased considerably (though still remained

FIGURE 17. Control and Maximum Absolute Leucocyte Numbers in Mice Infected with the Mouse Strain.

FIG. 17

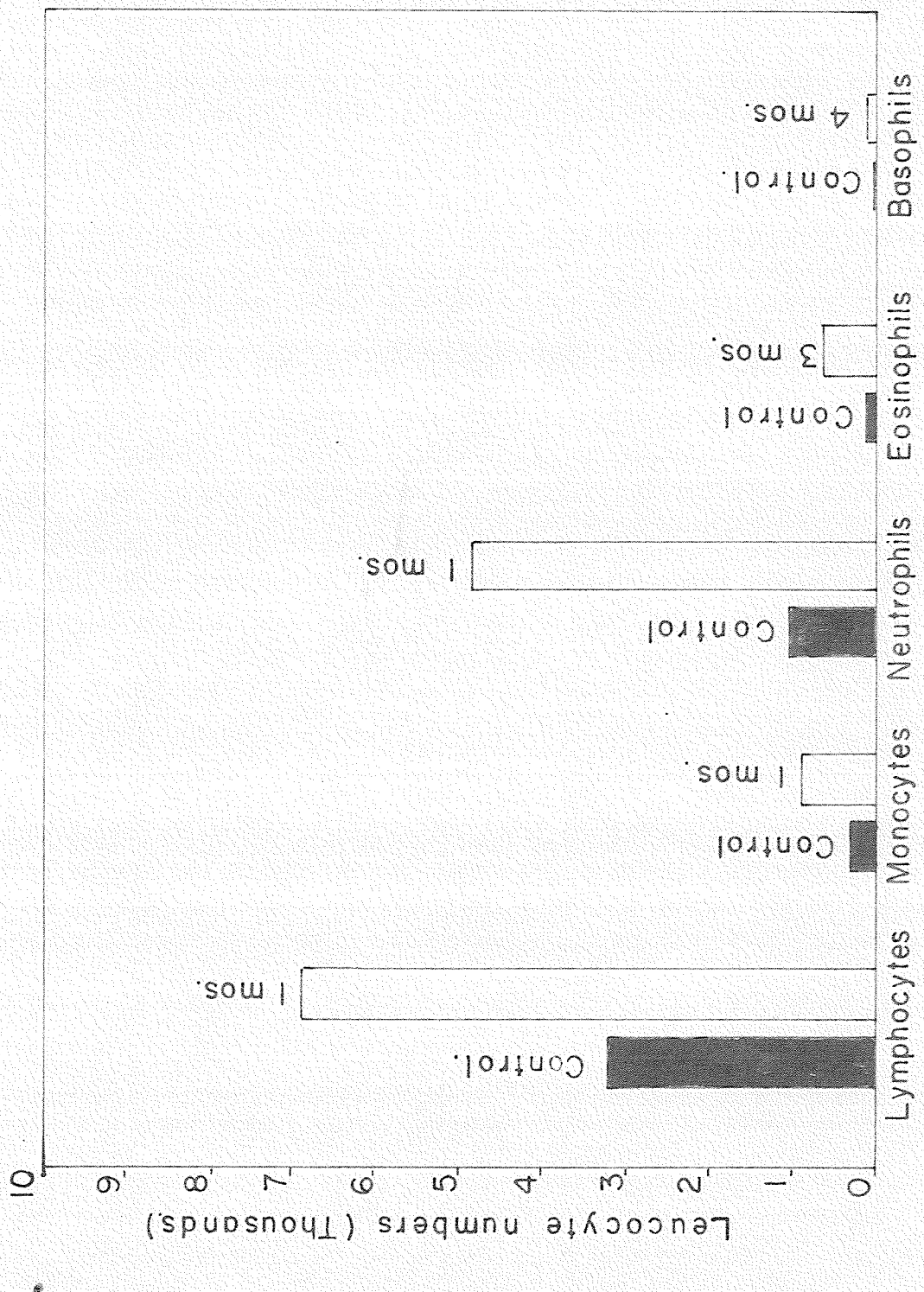
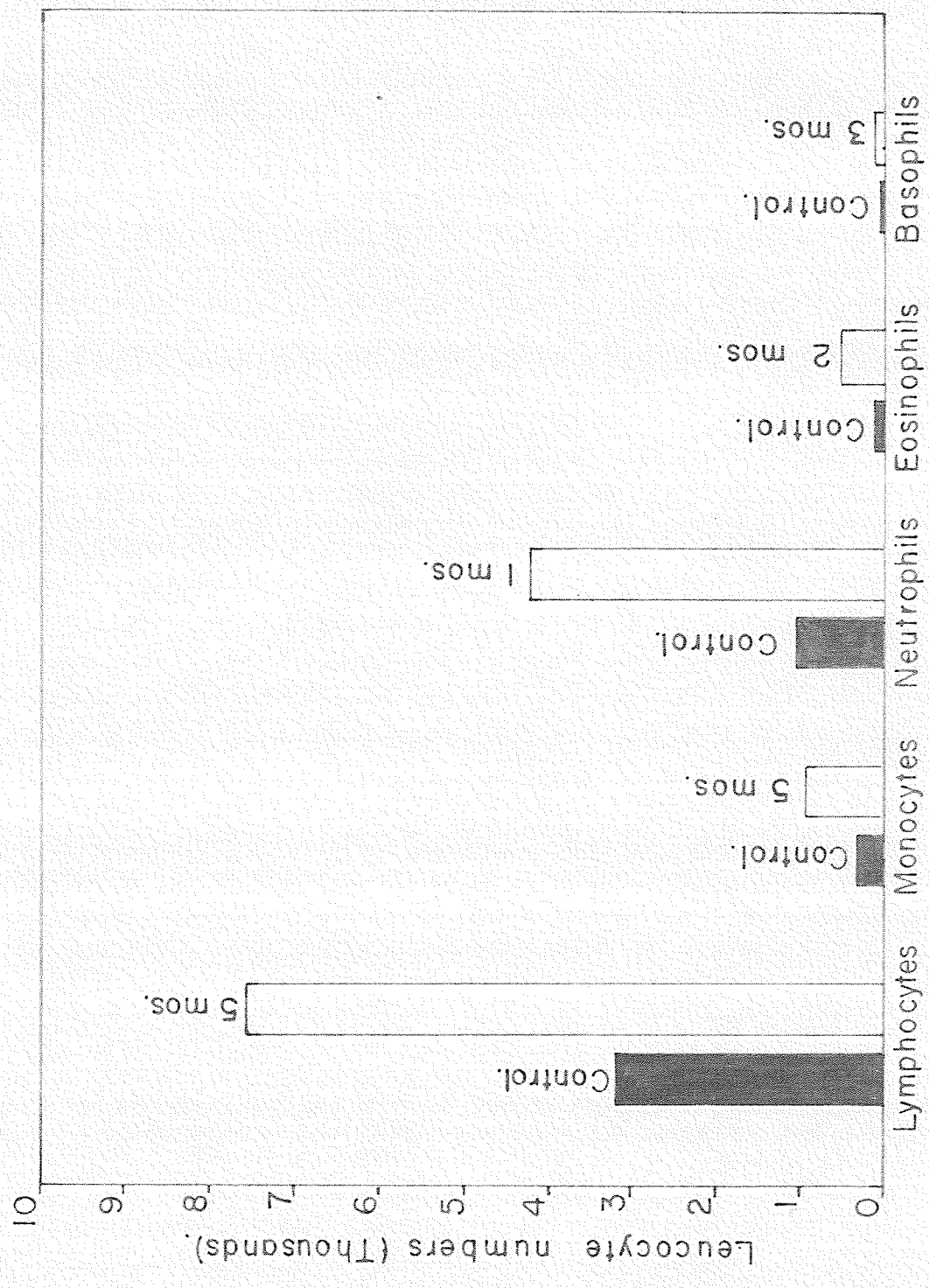

Leucocyte numbers (Thousands.)
0
1
2
3
4
5
6
7
8
9
10
Lymphocytes
Control.
1 mos.
Monocytes
Control
1 mos.
Neutrophils
Control
1 mos.
Eosinophils
Control
3 mos.
Basophils
Control.
4 mos.

FIGURE 18. Control and Maximum Absolute Leucocyte Numbers in Mice Infected with the Rat Strain.

FIG. 18


Leucocyte numbers (Thousands).
0
1
2
3
4
5
6
7
8
9
10
Lymphocytes
Control.
5 mos.
Monocytes
Control.
5 mos.
Neutrophils
Control.
1 mos.
Eosinophils
Control.
2 mos.
Basophils
Control.
3 mos.

far greater than that of the control animals) and became stabilized at a lower level hereafter from two to six months. During this time both the cyst size and calcium content increased progressively. After the six month period the leucocyte numbers began to decline gradually as the cyst size and calcium content attained maximum values.

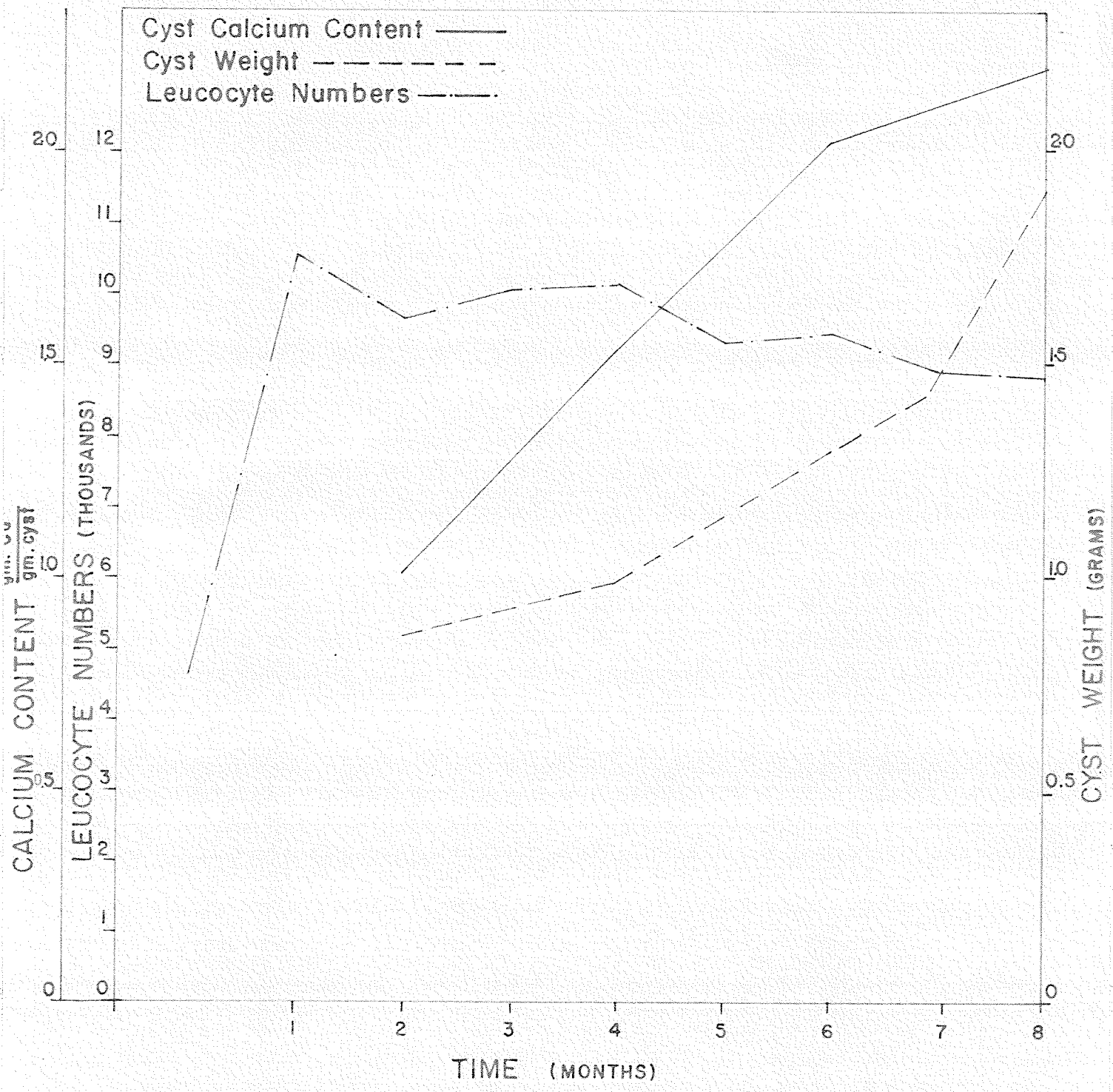
It is interesting to recall at this point the extended rat strain study (see page 62), where both the maximum cyst calcium concentration and cyst size were reached during eighth month. After this time the calcium concentration slowly declined and further cyst growth ceased.

The decline in cyst calcium concentration and stabilization of cyst size after eight months seems to coincide with the decline in the total number of leucocytes.

In studying the literature on the effect of echinococcus on the white blood picture of experimental animals, relatively little pertinent information was found. All the data available were based on human material. It has been known for many years that certain parasites cause a pronounced eosinophilia. The most striking example of this is the eosinophilia in trichiniasis described by Brown in 1897. Wintrobe, (27) states that

FIGURE 19. Comparison of Cyst Calcium Content, Cyst Size and Variation in the Total Number of Leucocytes in Mice Infected with the Mouse Strain.

FIG. 19


Cyst Calcium Content
Cyst Weight
Leucocyte Numbers
CALCIUM CONTENT mgm. / gm. cyst
LEUCOCYTE NUMBERS (THOUSANDS)
CYST WEIGHT (GRAMS)
TIME (MONTHS)
0
1
2
3
4
5
6
7
8
0
0.5
1
2
3
4
5
6
7
8
9
10
11
12
0
5
10
15
20
0
5
10
15
20

in echinococcus disease, eosinophilia of about 50% does occur, but is uncommon. It is more likely to be observed when the parasite is alive. On the other hand Whitby and Britton (28) stated that in cases of echinococcus infection eosinophilia is most marked when the hydatid cyst is ruptured or leaking. If the cyst dies, becomes calcified or walled off with a dense fibrous tissue, the eosinophilia disappears and at the same time the Casoni skin test (used in the detection of the parasite) and the complement fixation test become negative.

There was no pronounced eosinophilia in the experimental animals. (In trichiniasis, values for eosinophils as high as 85% with absolute counts as high as 15,000 per c. mm. have been reported. The highest value observed in our infected animals was 7% with an absolute eosinophil count of 632 per c. mm.). This is probably due to the fact that the small localized subcutaneous cysts did not rupture or release their foreign protein into the tissues of the host thus inducing the eosinophilia.

SUMMARY

1. Morphological examination of the calcareous corpuscles from the cysts of Echinococcus multilocularis has shown that the size of the corpuscles does not depend on the age of the cysts. The number of corpuscles does however increase considerably with progressing age.
2. The calcium content of the cysts increases gradually in both the rat and mouse strains. The two-month rat strain cysts contained 49 mg./% calcium (almost 5 times the concentration in the blood of a normal mouse, i.e. 10 mg./%): this concentration rose to a maximum of 240 mg./% in the eight-month cysts. The two-month old mouse strain cysts contained considerably more calcium than the rat strain cysts of the same age. In the mouse strain cysts the calcium content (101 mg./%) exceeded 10 times the value for normal mouse blood. By the fourth month of infection the values for the mouse strain cysts approached those of the rat strain. A maximum concentration of 224 mg./% calcium was reached in eight-month old mouse strain cysts.
3. The extended study of the rat strain cysts in mice has shown that the calcium concentration in cysts over

eight-months old gradually decreases, falling to a level of 164 mg./% in fifteen-month old cysts. (This value still exceeds that of normal mouse blood by sixteen times).

4. The cyst size generally increases up to the age of eight months when a plateau is reached. The volume of subcutaneous cysts often begins to decrease slightly after this age.

5. Mice infected subcutaneously with either strain of Echinococcus develop a pronounced leucocytosis in the course of the first month of infection. In those infected with the rat strain the total W.B.C. counts increased from 4600 to 9400, while those infected with the mouse strain showed corresponding increases up to 12000 W.B.C.'s/c. mm. blood.

6. There was a corresponding sharp increase in the absolute numbers of almost all types of leucocytes. In mice infected with the mouse strain the absolute numbers of monocytes tripled, the neutrophils increased five times, and the eosinophils six times. The numbers of monocytes and neutrophils reached their peak during the first month of infection, while that for the eosinophils occurred in the third month. In mice infected with the rat strain the absolute

number of monocytes also tripled, (reaching its peak during the fifth month of infection)while the neutrophils more than quadrupled in the course of the first month. Here, as in the mouse strain, there was a marked eosinophilia reaching its peak in the second month of infection.

7. After eight months of infection, when the cyst size and calcium concentration reach a plateau, there is a corresponding stabilization in both the absolute and relative numbers of leucocytes.

VEGETATIVELY PROPAGATED STRAIN OF *ECHINOCOCCUS MULTILOCULARIS* AND MALIGNANT TUMORS

In 1959, when maintaining a strain of larval *E. multilocularis* at the Institute of Parasitology, McGill University, Dr. Lubinsky observed in a subcutaneously inoculated white mouse a sarcoma, which had arisen in the vicinity of the cysts and attained a size of 22 x 18 x 7 mm.

In June 1962, 40 male hybrid mice (A/Jax ♀ x C57Bl ♂) were injected subcutaneously with *E. multilocularis*, 20 with the mouse strain, and 20 with the rat strain. The donor mouse had been intraperitoneally infected and when the peritoneal cavity was opened it was found to

contain the expected typical cysts similar to those represented in Figure 5 (page 36). In removing cyst material for the transfer, portions were taken at random from several locations in the peritoneal cavity. All the animals inoculated with the mouse strain developed sarcomas at the point of inoculation and died within 6 weeks (12). There was no indication in any of these animals of echinococcus growth. It appeared obvious that the tumors simply overgrew the echinococcus cysts.

Tumor material from one of these experimental black hybrid mice was used to initiate a strain of sarcoma. Serial transfers were made by injecting groups of mice of the same strain with a suspension of sarcomatose tissue. These transfers were made using the same technique as that for cyst transfers (see page 32). Five such serial transfers were made. It was observed that the tumors grew more quickly in younger mice. An attempt was made to transfer the tumor strain to 4 A/Jax white mice. However, the implanted tumors of the "black hybrids" did not take in these mice.

These observations suggest that malignant tumors do arise in close proximity to E. multilocularis cysts. How often this happens and to what extent the "normal" frequency of occurrence of tumors in various strains of

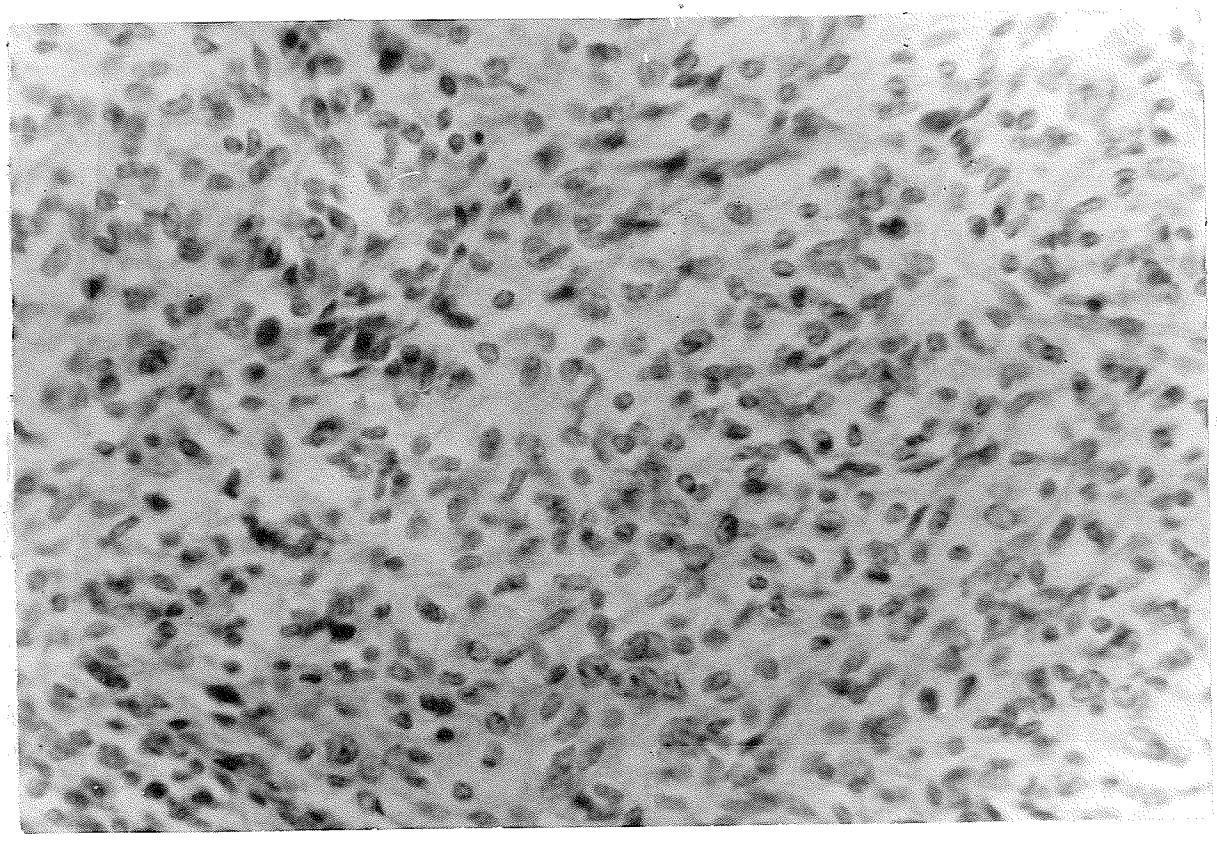
mice can be influenced by the presence of echinococcus cysts can be determined only on the basis of extensive experimentation.

A similarity in both macroscopic and microscopic structure of the tumors and also in the circumstances leading to their development was observed by the author between the previously described sarcomas and those arising in mice inoculated with Ak cell-free leukemic extracts. When injecting experimental animals with echinococcus cyst material containing scolices the author was as surprised to discover the rapidly growing sarcomas as Gross was in 1953 (7), when he observed instead of the expected leukemia the development of solid tumors in the neck region of his mice. In a later paper Gross (8) stated that the leukemic filtrates contained at least two oncogenic agents, i.e. a leukemic agent and an agent causing parotid tumors and possibly other neoplasms.

Both the tumors found in association with the echinococcus cysts and the subcutaneous sarcomas described by Gross were of the spindle cell fibrosarcoma type. It is conceivable that the sarcomas found in association with E. multilocularis were caused by a similar polyoma virus activated by the presence of these cysts. This supposition, however, must also be checked by intensive experimentation.

FIGURE 20. Thirty-five-day Old Sarcoma (5th transfer) in "Black Hybrid" (A/Jax x C57Bl) Mouse.

FIGURE 21. Photomicrograph of Subcutaneous Spindle Cell Fibrosarcoma from Mouse in Figure 20 x 263.



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