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Final **REPORT ON**

CONTRACT NO DA-92-557-FEC-34631

INCLUSIVE DATES 15 February 1962 TO 14 February 1963

CA. 100  
AS AD IN. 400306  
FOOTIA

### SUBJECT OF INVESTIGATION

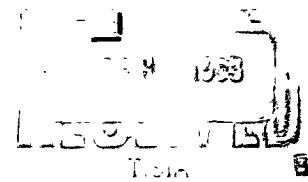
ANALYSIS OF THE DEVELOPMENT OF JAPANESE  
B ENCEPHALITIS VIRUS WITH THE  
ELECTRON MICROSCOPE

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Office of the Chief of Research and Development

United States Army

APO 343

AD Div 16/2  
Nara Medical College, Nara, Japan  
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### Abstract

The cerebral tissue of mice, especially medulla oblongata, infected with Japanese B encephalitis virus has been studied in serial thin sections in the electron microscope. The fine structure of neurons, oligodendroglia, microglia and astrocyte, which were infected with Japanese B encephalitis virus, has been observed under consideration of that in the normal ones. It has been revealed that most of the Japanese B encephalitis virus particles 36 to 40 m $\mu$  in diameter have developed within the neurons and microglia cells. The multiplication process of the virus particles has been traced in the ganglion cell. Concurrently with the multiplication of the virus particles, protein synthesis occurs in the cytoplasm of infected cells. An x-ray scanning microanalysis has shown that the elemental phosphate increases in its amount in the cerebral tissue infected with the Japanese B encephalitis virus.

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1. Purpose of the Study
2. Materials and Methods
  - a. Virus
  - b. Animals
  - c. Inoculation
  - d. Preparation of Electron Microscope Specimens
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  - e. Electron Microscopy
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## 1. Purpose of the Study

Numerous reports have been made of the Japanese B encephalitis concerning its clinical and pathological findings as well as etiological purpose since 1924 when Japan saw more than 6,000 victims of the disease. However, it is surprising that little work has been done so far on the structure and development of the Japanese B encephalitis virus in cerebral tissue (1, 2). It has been attempted to illustrate and describe the manner in which the virus particles differentiate within the mouse cerebral tissue infected as revealed in thin sections of materials fixed with osmium tetroxide by the electron microscope. In the present study, the thalamus, hypothalamus and medulla oblongata have been cut off the mouse in which the paralysis in the hind legs has developed. Furthermore, the potassium permanganate fixation has been made in the non-infected, normal cerebral tissue in order to examine virus particles' attitude toward its fixation.

## 2. Materials and Methods

a. Virus. The Nakayama strain (G 19) of Japanese B encephalitis virus, supplied by Institute for Microbiology, Osaka University, were passed intracerebrally in mice through several generations. The infectivity titer of the the passage material was  $10^{-68}$  at  $LD_{50}$ .

b. Animals. Mice, ddY strain, were observed for 4-6 weeks before inoculation to confirm their apparent healthy. Their average weight was 15 gm.

c. Inoculation. Intracerebral inoculation was carried out. Paralysis occurred in the hind legs 4 to 5 days after inoculation.

### d. Preparation of electron microscope specimens.

- (1) Normal cerebral tissue. Cerebral cortex and thalamus were selected.
- (2) Cerebral tissue infected. Thalamus, hypothalamus and medulla oblongata, 4 to 5 days after inoculation, were examined.
- (3) Fixation, embedding, cutting, and staining. A fresh solution of 2% osmium tetroxide (pH 7.4) buffered with veronal-acetate was dripped from a pipette onto the exposed mouse brain. The cerebral tissue was then excised and transferred

to 2% osmium tetroxide (containing 0.5% CaCl) buffered to pH 7.4 with veronal-acetate. Blocks ca. 1 mm in thickness were fixed for 60 to 90 minutes at 4°C.

For permanganate (KMnO<sub>4</sub>) fixation of normal cerebral tissue, the following procedure was devised. The fixative consisted of 2% KMnO<sub>4</sub> in veronal acetate buffer, pH 7.6, containing 0.56% NaCl and 0.028% CaCl<sub>2</sub>. The fixation was continued for 2 hours at 4°C.

The specimens fixed were dehydrated without washing directly in a series of increasing concentrations of ethanol. The specimens fixed in osmium tetroxide were embedded in a mixture of methyl- and *n*-butyl methacrylates or Epon epoxy resin. The specimens fixed in permanganate were embedded in Epon epoxy resin. Sections were cut on a Porter-Blum microtome, with glass knives, and mounted on copper grids coated with formvar.

e. Electron microscopy. The sections were stained according to Watson's lead acetate procedure, with slight modification, or with uranyl acetate, and a thin coat of carbon evaporated onto them. They were examined in an Akashi TRS-50E<sub>1</sub>, TRS-60B or TRS-80 electron microscope or an electron microscope of the Japan Electron Company, model JEM-6C.

f. X-ray scanning microanalysis. The apparatus itself consists of three main parts: an electron optical system, an x-ray spectrograph, and an optical microscope. The specimen is prepared in the usual way as for optical microscopy. The x-radiation, emitted at the focal spot where the electrons strike the specimens, is analyzed with a curved crystal, Geiger-Müller counter, and vacuum spectrograph.

Procedures for x-ray scanning microanalysis were as follows: sections 1 μ or more thick were mounted on nickel plates with a supporting membrane; they were stained (without removing the plastic) with 0.5% basic fuchsin solution to identify individual cells, and covered with a carbon film; they were then examined with Akashi x-ray scanning microanalyzer, model Tronalyzer-TRA type, with an accelerating voltage of 22 kV and a probe current of 0.35 μA.

### 3. Results

Electron micrographs of thin sections of the normal thalamus fixed with potassium permanganate permit the study of morphological relationships among the various cellular elements. In Fig. 1 an oligodendroglia is identified by its scant rim of the cytoplasm and relatively large nucleus. The cytoplasm contains the endoplasmic reticulum in a small amount. The double layer of the nuclear envelope and its porous structure are clearly visible in the permanganate fixation. No mitochondrion can be seen in the cytoplasm (at least in the field), but it is visible in the process. The processes of the oligodendroglia are characteristically scanty in cell organelles.

The double limiting membrane of the mitochondria and their cristae appear clearly in cross sections through unmyelinated nerve fibers.

Fig. 2 shows a perikaryon containing part of the nucleus and cytoplasm which consists of the Golgi complex, mitochondria and endoplasmic reticulum. The conspicuous Golgi complex is comprized of parallel arrays of membranes and associated vesicles of varying size and shape. The karyoplasm appears almost to be homogeneous. The nuclear envelope demonstrates numerous porous structure. A large number of unmyelinated nerve fibers are visible closely attached to one another. A process of the astrocyte is identified by its scanty cytoplasmic matrix. Moreover, it is noticeable that tubular elements are visible within the cross sections of unmyelinated nerve fibers.

Fig. 3 demonstrates an electron micrograph of a thin section of the normal cerebral cortex. At the upper left corner of the figure, a part of the cytoplasm of a small ganglion cell is visible, which contains an oval-shaped dense body surrounded by an apparently single membrane, a small mitochondrion, and circular or elongated endoplasmic reticulum. A synapsis appears closely attached to the ganglion cell, in which numerous synatpsis vesicles can be seen. Many of cross sections of unmyelinated nerve fibers contain mitochondria of varying size and shape, and small vesicles characteristic for nerve fibers. The mitochondria are sometimes devoid of their limiting membrane. Some mitochondria are confused to a single body, being devoid of cristae.

Fig. 4 shows clearly a P K<sub>α</sub> spectrum obtained from the normal cerebrum cortex of a mouse.

Fig. 5 depicts also a P K<sub>α</sub> spectrum obtained from the cerebrum cortex infected with Japanese B encephalitis virus, in which the phosphate element increases in its amount as compared with that of the normal one. Another spectrum showing a high peak at wavelength 6.200 Å

belongs to an unknown pattern.

#### 4. Discussion

During the past one year, the mouse cerebral tissue infected with JBE virus has been studied with the electron microscope in serial thin sections. The fine structure of the ganglion cells, oligodendroglia, astrocyte and microglia, which were infected with JBE virus, has been described in the first report.

In the second report the dense particles 36 to 40  $\mu$  in diameter isolated or in clusters have been found within the cytoplasm of perikarya and microglia, but never found in their nuclei, oligodendroglia, and astrocytes. The particles have sometimes been observed in unmyelinated nerve fibers and spaces between the nerve fibers. In the mice in which paralysis occurred by inoculation of JBE virus, any other virus particles have never been observed in the medulla oblongata. Thus the dense particles mentioned above must be **decided to be JBE virus**.

However, measurements of Yaci et al. (3) on ultra-filtration preparations of virus gave an average diameter of 20  $\mu$ . The value is similar to that of immature one which has been described the Final Report No. 1 (4).

In the third report the JBE virus particles have been found to be phagocytized by ganglion cells in the medulla oblongata of the mice. The particles are ingested by an invagination process of the plasma membrane resulting in the formation of intracellular vesicles. Ultimately large amounts of the virus particles accumulate in the cell, occupying substantial portions of the cytoplasm. At the same time, numerous particles of ribosomes, and a large amount of rough surfaced endoplasmic reticulum make their appearance in the cytoplasm. These structure changes have been interpreted as indicative of protein synthesis.

As Fukai (5) has described that the JBE virus particles are composed of ribonucleoprotein, potassium permanganate fixation technique has been tried in the normal cerebral tissue of mice. Such technique will be carried out in the cerebral tissue infected with JBE virus in near future.

With the advancement of descriptive cytology brought about by electron microscopy, there has arisen a need for cytochemical methods at high resolution. The method of x-ray scanning microanalysis has already found numerous applications in the field of metallography, and may also be extended to the study of biological specimens. Yasuzumi et al. (6), and Yasuzumi (7-8) have succeeded in showing an  $\text{Fe K}_\alpha$  spectrum in biological specimens, and Boyde et al. (9) in taking images formed by  $\text{Ca K}_\alpha$  and  $\text{Fe K}_\alpha$  radiations in dental tissue. In the present study, identification of

the elemental phosphate has been pursued by the application of the x-ray scanning microanalyser which has revealed it in the infected cerebral tissue much more than in the normal tissue. The increase of the elemental phosphate in its amount in the cerebral tissue infected with JBE virus seems to be an interesting finding, but its functional significance remains to discuss in detail in future.

#### 5. Summary

The cerebral tissue of mice, especially medulla oblongata, infected with Japanese B encephalitis virus, has been studied with an electron microscope and an x-ray scanning microanalyser. It has been revealed that most of the virus particles 36 to 40 mu in diameter have developed in the neurons and microglia examined. An x-ray scanning microanalysis has shown that the elemental phosphate increases in its amount in the cerebral tissue infected with the virus.

#### 6. References

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#### 7. Explanation of Figures

Fig. 1. Electron micrograph of a section of the thalamus of a normal mouse. A part of the oligodendroglia cell is visible at the upper side of the figure, showing the nucleus (N), the nuclear envelope with a porous structure, circular or elongated profiles of the endoplasmic reticulum (ER) and tangential section through the cytoplasmic membrane system (CM). Processes (P) of the oligodendroglia appear attached to the cell. Mitochondria (M) of varying size and shape, and small vesicular (V) or tubular (T) elements can be seen in cross-sections of the nerve fibers (NF). X 43,000.

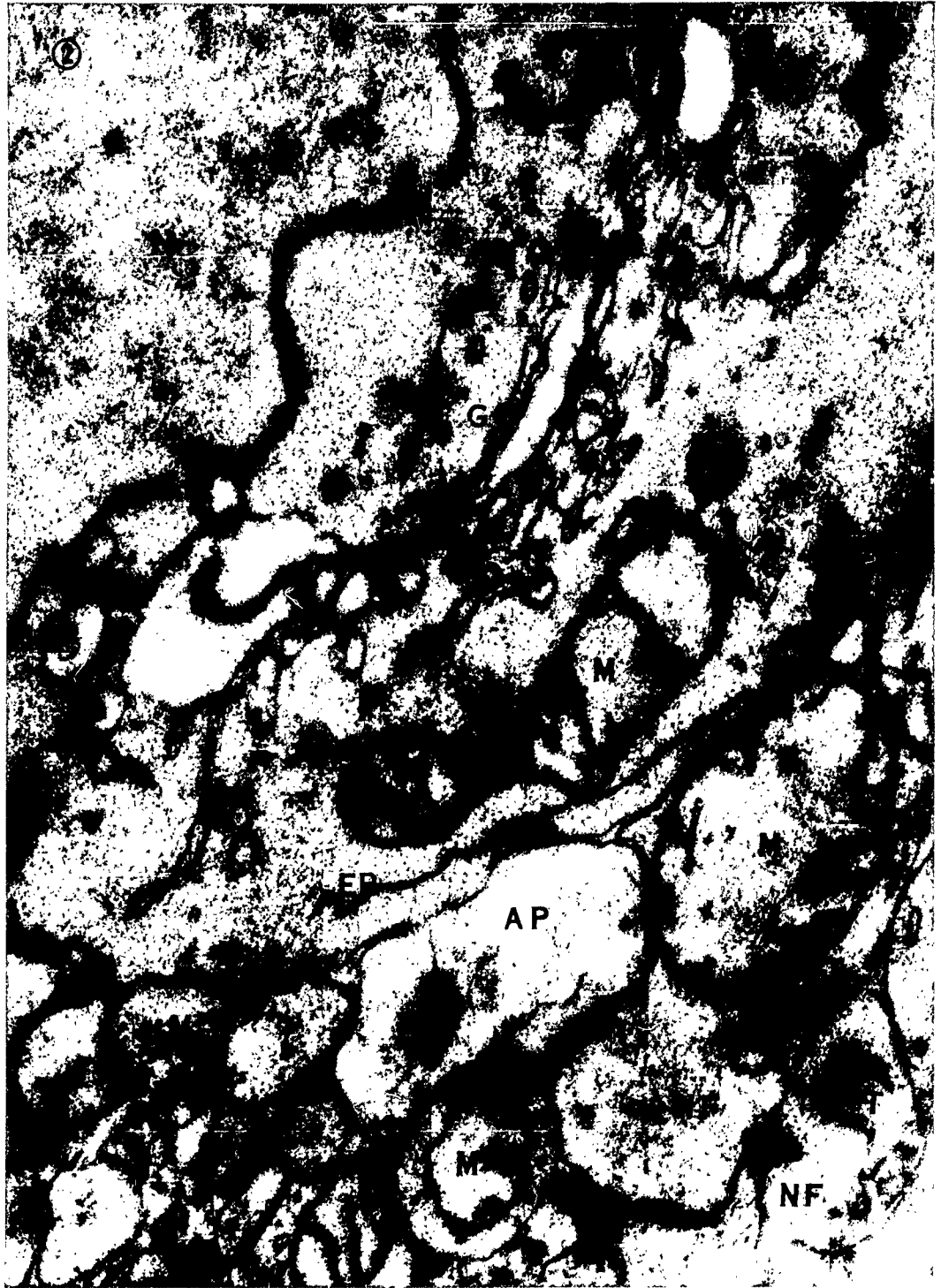
Fig. 2. Thin section of the thalamus of a normal mouse. The field is almost occupied by the ganglion cell in which the nucleus (N) and the nuclear envelope with a porous structure can be seen at the upper left corner. The cytoplasm contains the Golgi complex (GC) consisting of an array of membranes and associated vesicles of different sizes and shapes. Mitochondria (M) and elongated endoplasmic reticulum (ER) are also visible. Mitochondria (M), and small vesicular (V) or tubular (T) elements are clearly visualized in cross-sections of the nerve fibers (NF). An astrocyte process (AP) is identified by its watery character. X 45,000.

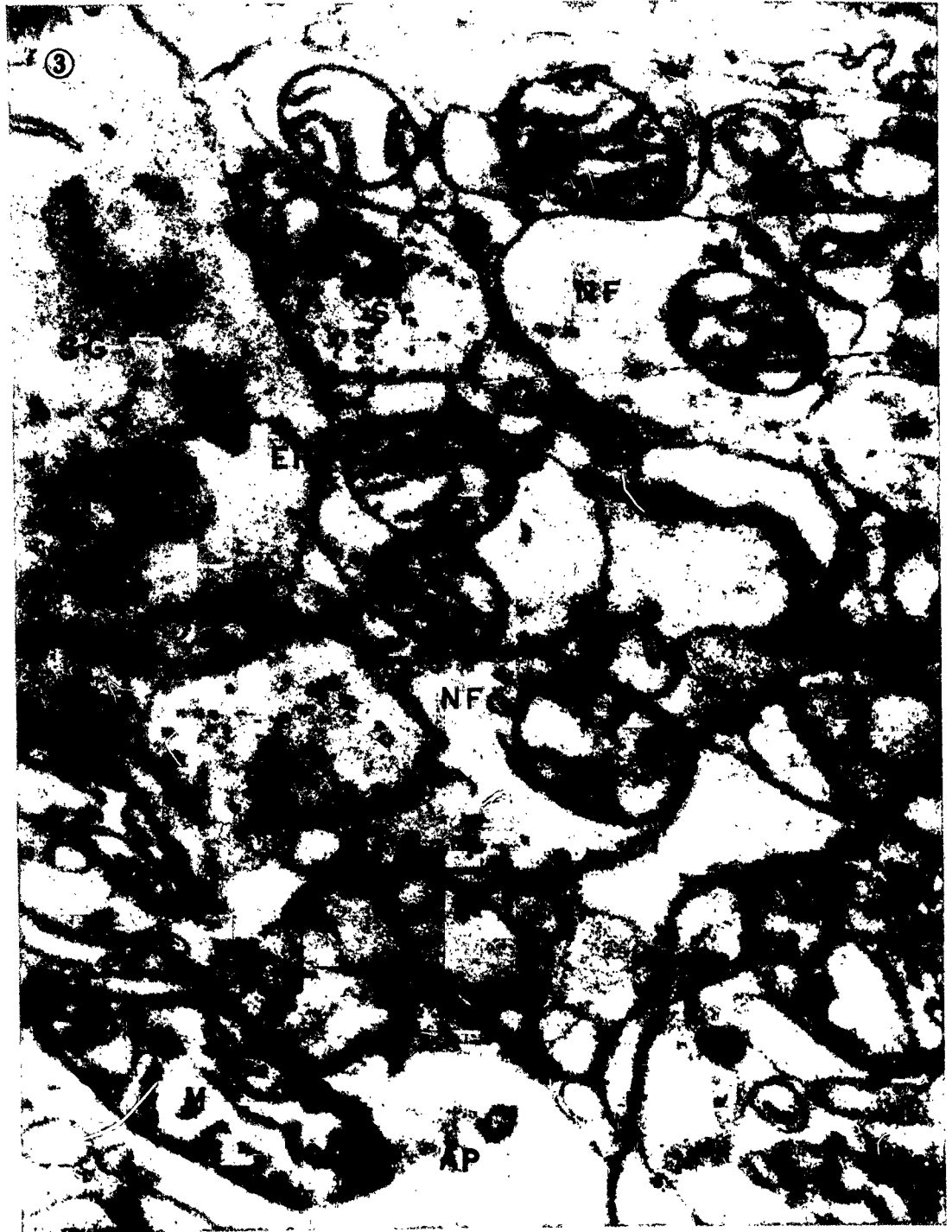
Fig. 3. An electron micrograph of the cortex of the cerebrum of a normal mouse, showing a small ganglion cell (SG) with mitochondria (M), endoplasmic reticulum (ER) and oval-shaped homogeneous body (OB), synapsis (SY), cross-sections of nerve fibers (NF), and an astrocyte process (AP) with a large mitochondrion (M). X 41,000.

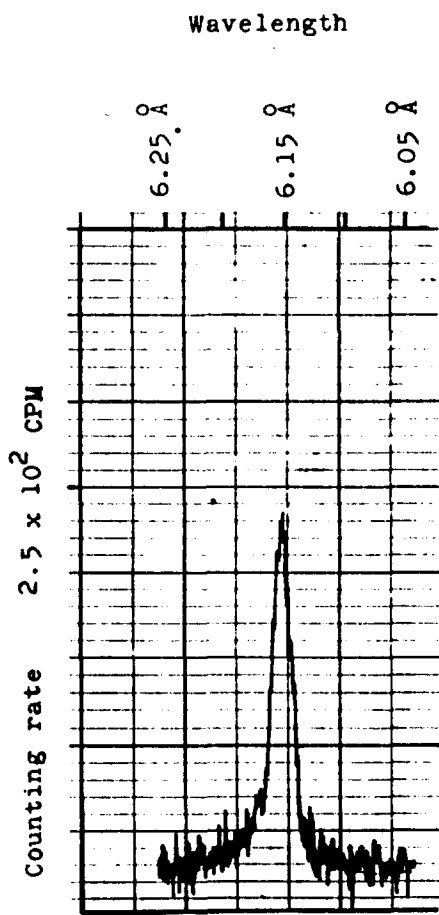
Fig. 4. P K<sub>α</sub> spectrum of the cerebral tissue of a normal mouse.

Fig. 5. P K<sub>α</sub> spectrum of the mouse cerebral tissue infected with Japanese B encephalitis virus.

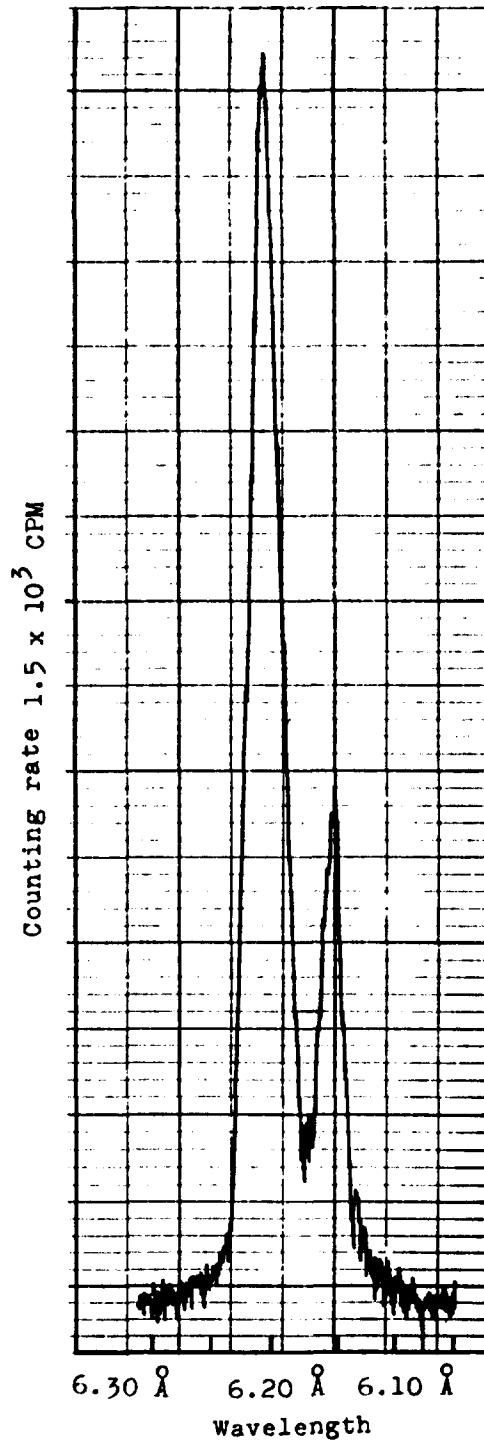








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