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PROTON-INDUCED AND X-RAY INDUCED FLUORESCENCE ANALYSIS OF SCOLIOTIC TISSUE*

Adolescent idiopathic scoliosis is characterized by a curvature or assymetry of the spine which may become progressively more severe, with clinical symptoms appearing just prior to, or during, puberty. The incidence for scoliosis in the age group from 12 to 14 years of age has been reported as high as 8-10%, with more than 80% of the cases occurring in females.[1] Although pathologic changes exist in muscles from both sides of the spinal curvature, and "no statistically significant side differences have been reported", morphologic changes suggest that the concave side is the most affected.[2] This paper reports our preliminary data on the elemental composition of individual muscle fibers derived from convex, concave and gluteal scoliotic muscle, and erythrocytes from scoliotic and normal patients, analyzed by proton induced x-ray emission (PIXE) and x-ray fluorescence spectroscopy (XRF). A new type of specimen holder was designed for this study which offers low x-ray background, minimal absorption and maintenance of a moist environment around the specimen.

Methods

The muscle fibers used in this study were obtained from residual muscle tissue removed during surgery to correct curvature of the spine. Small samples (8 mm x 4 mm) of paraspinal muscle tissue (from an 18 year old female with idiopathic scoliosis), was removed on the convex and

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concave sides of the spine at the apex of the curvature during corrective surgery. A specimen from the gluteus maximus was also removed for analysis. After excision, the tissue was immediately fixed in 3% glutaraldehyde in 0.1 M cacodylic buffer (7.4 pH) at room temperature for 3 hours. The fixative was then removed and replaced with fresh fixative and the specimens were stored in fresh fixative for 3 days at $4^{\circ}C$.

Individual muscle fibers were separated by dissecting away the outer layers of muscle tissue which had come in contact with the scalpel during surgery. Wooden and glass dissection instruments were used to prevent metal contamination of the muscle fibers. The isolated muscle fibers were washed in buffer and placed on a formvar film stretched across a 25 mm diameter hole of a lucite frame (Fig. 1).

Lucite frames measuring 30 x 30 x 1 mm were washed several times in glass distilled, deionized water. A formvar film (40-70 nm thick) was placed across the lucite frame, and stretched across the central circular opening in a uniform sheet. The muscle fibers, which had been placed in the center of this formvar diaphragm with some fresh buffer, were covered with an equally thin sheet of formvar, forming a formvar sandwich which sealed the fibers within a marginal bubble of buffer (Figs. 1B and 1C). Two muscle fibers from the gluteus maximus and two sets of muscle fibers from convex and concave paraspinal muscles were analyzed in this way.

The muscle fibers were scanned with a proton microprobe[3,4] in air using 2.5 MeV protons to stimulate characteristic x-rays from

elements within the sample. The x-rays were detected with a high resolution Si(Li) energy dispersive spectrometer placed ~ 1 cm from the sample which was itself placed ≤ 2 mm from the emergent port of the Van de Graaff accelerator (Fig. 1C). The beam spot available in this mode is ~ 25 μm and scans of the fibers were made at ~ 0.1 mm intervals along the fibers. Energy and concentration calibrations were carried out using commercially available trace element standards in a representative matrix. Data analysis was carried out by standard computer integration of spectral areas. Unfortunately an anomolously high x-ray background was present in addition to copious arsenic characteristic x-rays from the buffer, and little information above 8 keV ($Z > 29$) could be obtained by the scans. However, the samples remained intact and viable, albeit somewhat dehydrated, a problem which will be discussed.

The samples were then irradiated by a broad, uniform beam of protons in vacuum in a standard PIXE system having somewhat greater elemental sensitivity (down to ppm for many elements), particularly for lighter elements, $Z < 26$. The samples were also studied by XRF using a hardened, filtered x-ray beam from an x-ray tube-Si(Li) detector system. This XRF system is complementary to the PIXE system in that its sensitivity emphasizes elements with $Z > 26$.

Since adolescent idiopathic scoliosis is believed to have a genetic as well as environmental itiology,[1] it appears feasible to assume that the genetic defect may express itself in tissues of the body other than the musculoskeletal system. To see if any unusual electrolytes could

be found in the blood of scoliotic patients, clotted, heparinized and glutaraldehyde fixed (1.5% in 0.1 M cacodylic buffer) preparations of whole blood were placed on formvar-lucite frames and analyzed by PIXE and XRF. Initial trials demonstrated that fixation increased the elemental background and did not preserve sufficient sample integrity to warrant its use. Subsequent blood samples were collected with and without heparin and centrifuged in hematocrit capillary tubes to separate the erythrocytes from leukocytes and serum. A 2-5 μg aliquot of blood was smeared onto the formvar (90 nm thick) window, and all samples were analyzed for ~ 20 minutes by PIXE and ~ 60 minutes by XRF. A total of 21 blood samples were surveyed with donors ranging from 12-34 years of age.

Results and Discussion

By XRF analysis, Yarom et al.[5] reported somewhat high copper and zinc concentrations in freeze-dried thick sections of scoliotic muscle when compared to normal specimens. He notes that convex muscle reveals somewhat higher calcium levels than the concave paraspinal tissue, and significantly less calcium than observed in glutei.

The spectra we observed from hydrated muscle fibers analyzed by PIXE and XRF show no significant variations in calcium, phosphorus, zinc or copper x-ray events emitted from convex, concave or glutei muscle fibers. However, the muscle fibers from the concave sample reveal spectra with small selenium (Se) K_α (11.21 keV) and K_β (12.49 keV) x-ray events. Initially it was not possible to distinguish the Se signals from the arsenic (As) K_α (10.54 keV) and K_β (11.72 keV) x-ray events produced

by the cacodylate buffer. By resetting the gain to maximize Se signal above background using PIXE, the Se was observed. XRF spectroscopy supported the PIXE data and reveals no Se signal from gluteal muscle fibers and significant Se signal from concave, and to a lesser extent convex muscle fibers (Figs. 2 and 3). After 2 days of observation, the muscle fibers became desiccated due to the presence of minute pores in the formvar. We later learned that this can be prevented by using only anhydrous ethylene dichloride to dissolve the formvar; thereby eliminating water vapor contamination. Subsequent experiments using rabbit muscle fibers demonstrated that the wet chambers can be maintained for 3 to 5 days. We were also able to introduce polyethylene catheter tubing (1.5 mm bore) into the chambers to provide a constant flow of oxygenated ringers to the tissue.

X-ray spectra from most of the scoliotic erythrocyte samples show significant Se signal; whereas the spectra from normal samples show none. Non-heparinized samples show significantly greater Se content than heparinized identical specimens. It is unfortunate that some of the pathological blood samples could not be included in the tabulated results due to the possibility of undiagnosed blood dyscrasias and possible endocrinopathies. Further studies will have to be done with a known sample population to eliminate these variables.

Conclusions

Additional work must be done to conclusively determine if the presence of Se in concave muscle (and to some extent convex) from

patients with idiopathic scoliosis is universally observed. The fact that no Se is found in gluteal muscle fibers, and is found in not only the paraspinal muscle fibers adjacent to the curvature in the spine but also associated with the red blood cells of affected patients, strongly suggests that Se may be a marker element for idiopathic scoliosis. It is too soon to speculate whether Se is a by-product of abnormal neuromuscular function or if its presence may be the genetic expression of altered enzyme or transport systems.

In conclusion, it appears that PIXE and XRF analysis offer a very direct and sensitive means of detecting trace elements in intact muscle fibers. Furthermore, experience with these techniques indicates possible accuracies of 10-20% at $\sim$ 10 ppm trace levels. Ideally the tissue should not be fixed, but quench frozen in liquid nitrogen and examined frozen by PIXE or freeze dried by XRF, to avoid exogenous elemental contamination and reduce the background. The proton μ-beam has the efficiency and sensitivity to detect small amounts of endogenous elements in biological samples with comparable accuracy to XRF. Because samples can be elementally analyzed as intact structures at atmospheric pressure and in a wet cell, the proton microprobe has a marked advantage as a biological research tool.

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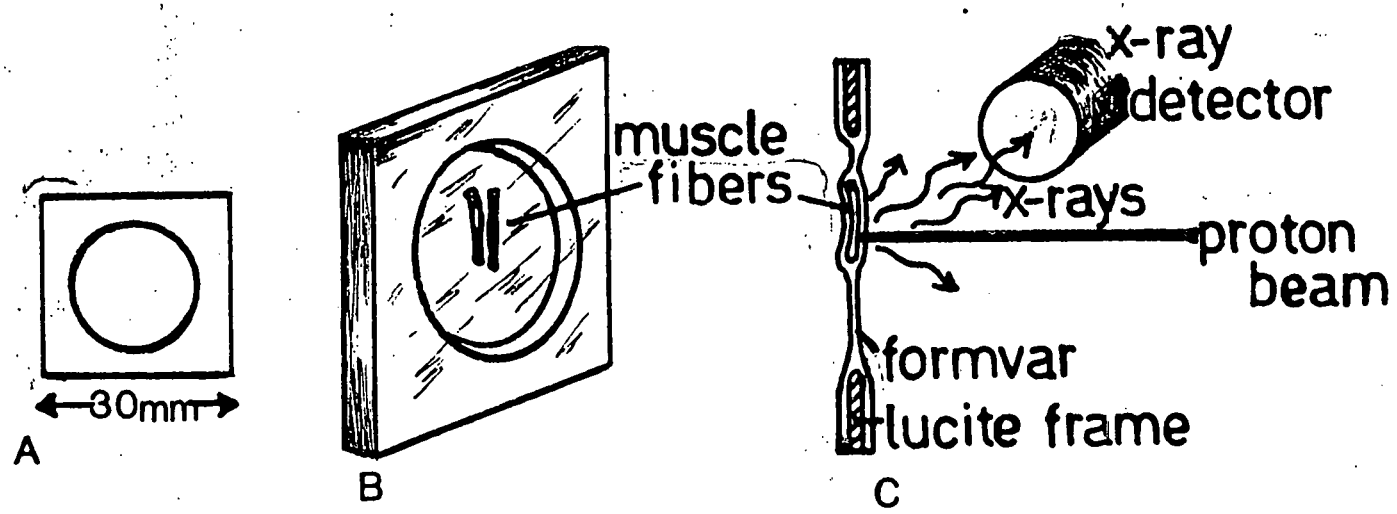
Figure Captions

Figures 1A, 1B, 1C — A lucite frame with a circular opening (35 mm dia.) was covered with a thin formvar film. The muscle fibers were placed in the center of the formvar window (B) and coated with another layer of formvar. Trapped buffer accumulates around the muscle fibers and keeps it moist throughout proton beam excitation (C).

Figures 2A, 2B — Proton induced x-ray emission (PIXE) spectra of isolated muscle fibers routinely showed the presence of Se in the fibers taken from the concave paraspinal musculature (A), and a total lack of Se in fibers from the glutei (B).

Figure 3 — X-ray induced fluorescence spectra (XRF) also showed the presence of Se in concave paraspinal muscle fibers and a lack of Se in gluteal fibers.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muscle
fibers
B
x-ray
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x-rays
proton
beam
formvar
lucite frame
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1

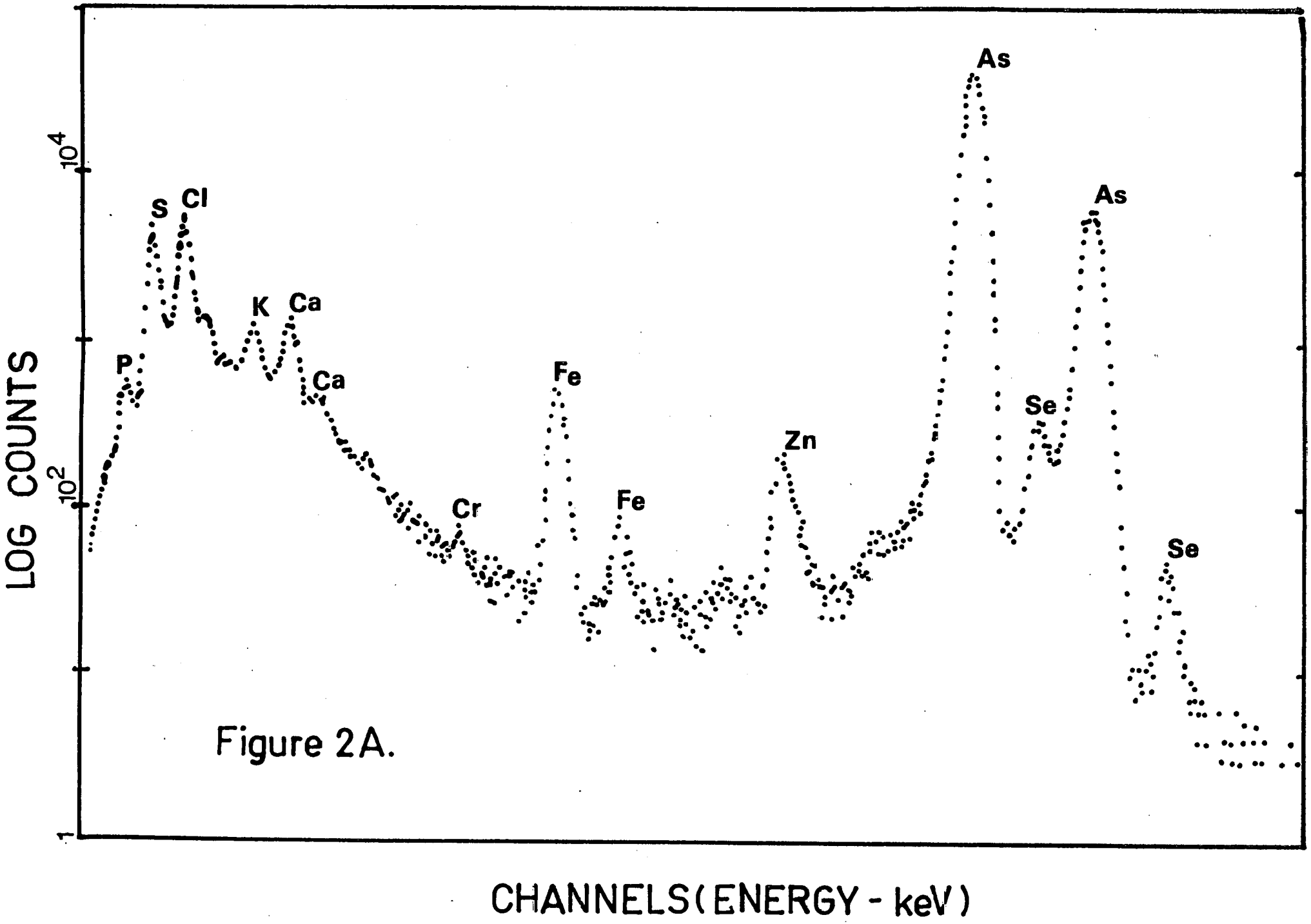
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Figure 2A.

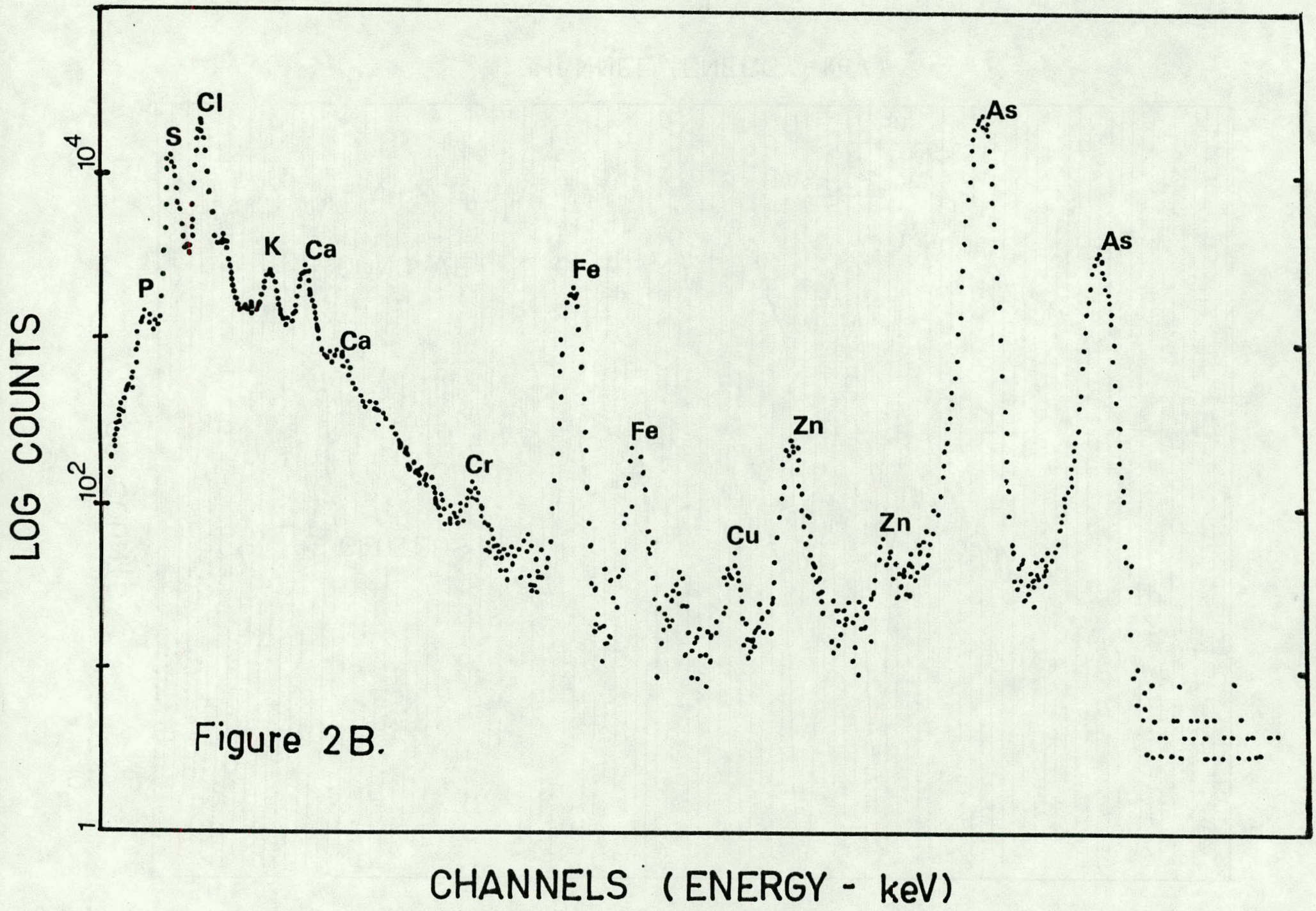
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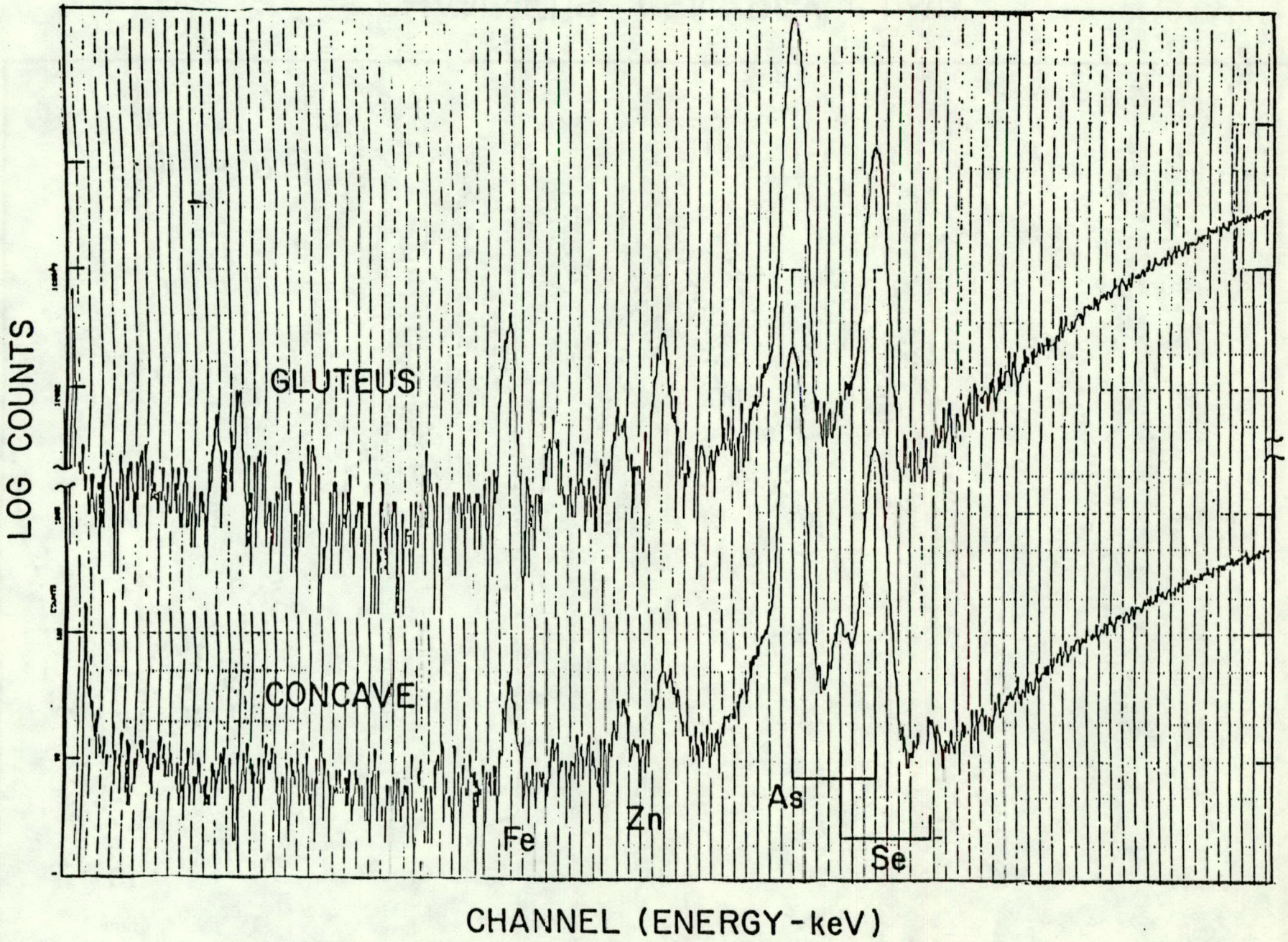
Figure 2B.



Ficg. 3