CUPROPROTEINS OF HEPATOCYTE LYSOSOMES IN NORMAL AND FATTY LIVER

NAOKI HISHIDA, AKIRA YAGI, TOMOYUKI HIGUCHI, HISAO HAYASHI,* and NOBUO SAKAMOTO

Third Department of Medicine, Nagoya University School of Medicine, Nagoya, Japan

ABSTRACT

The pathophysiological significance of cuproproteins in hepatocyte lysosomes was studied in 7 patients with structurally normal livers and 13 patients with fatty livers by energy-dispersion X-ray microanalysis. In most patients, in both groups, copper and sulfur coexisted in hepatocyte lysosomes. The correlation between copper and sulfur indicated that copper transfer between the cytosol and lysosomes was mediated by a cuproprotein. The copper indices of the lysosomal proteins and transfer proteins were high in some fatty livers. These observations suggest that cuproproteins in hepatocytes are not necessarily pathological, but are present in greater amounts in some fatty livers, probably because of impaired bile drainage.

Key Words: Copper-Sulfur-Cuproproteins-Lysosomes-Fatty liver

INTRODUCTION

After being transported to the liver, copper stimulates the synthesis of metallothionein, a thiol-rich protein with a low molecular weight. Binding to this protein detoxifies the liver from this hepatotoxic metal. The copperthionein thus formed and its degraded products are eliminated in the bile.[1,2] When the biliary tract is obstructed, cuproproteins are retained within the hepatocytes. With prolonged obstruction, copper-sulfur complexes appear in the hepatocyte lysosomes, but their relationship to cytosolic copperthionein or biliary cuproproteins is obscure. A high binding capacity of the copper-sulfur complexes for copper has been suggested by X-ray microanalysis. An advantage of energy-dispersion X-ray microanalysis (EDX)[3] over wavelength dispersion X-ray microanalysis[4,5] is the simultaneous measurement of all elements heavier than Na; in this way one can measure trace elements using standard elements and identify the interaction of all coexisting elements.[6] Using EDX on glutaradehyde-fixed, frozen sectioned specimens, Hanaichi *et al.*[2] found a sulfur-rich protein bound to copper atoms. In a study involving livers of hamsters fed copper and standard samples of codfish cakes with known copper contents, we reported that Cu Kα X-ray intensity can be used to measure the copper content of hepatocyte lysosomes.[8] Using standard samples made of codfish for comparison, we have employed EDX to investigate the behavior of the lysosomal cuproproteins in almost normal livers.

MATERIALS AND METHODS

Preparation of standard samples:

Standard codfish samples were prepared by the method of Yagi *et al.*[8] Briefly, codfish cakes containing 5.0 % sodium chloride and graded amounts of cupric sulfate were baked on a hot plate. The chlorine was later used as the internal standard. A portion of the cakes was dried and incinerated, and its copper content was measured by atomic absorption spectrometry. For X-ray microanalysis, 1 mm^3 pieces of freshly prepared cakes were placed on specimen holders, and were frozen in liquid nitrogen (−180°C) until being sectioned. A glass knife and the specimen holder holding a frozen specimen were cooled at −100°C using an LKB cryokit, and the frozen specimen was cut into 80-nm ultrathin sections with an LKB ultratome. These ultrathin sections were mounted on Maxtaform gold grids coated with carbon (Gluticulus, London, England) without transfer solution and dried at room temperature. Washing and electron staining were omitted. EDX was done under a Hitachi H-800 electron microscope with a Kevex 7000-Q energy-dispersion X-ray analyser. The specimen chamber was cooled with liquid nitrogen to reduce contamination with silicon from the detector window. The accelerating voltage was 100 kV and the current beam was 10^{-10} A. Au Mα appears between P Kα and S Kα, so we examined central areas as far from the gold wires as possible to minimize overlapping. First, an area 0.45 μm square was scanned at random for 200 seconds by selected area analysis. Then an area 250 μm square was analyzed with a scattered electron beam under the same conditions. The specific Kα X-rays for sodium, phosphorus, sulfur, chlorine, potassium, and copper were recorded as X-ray counts and as a molar percentage of all elements detected. Each sample was examined in two areas, 10 times each. Chlorine, a major constant component of the crude fish cake irrespective of the amount of copper, was found to be the element best suited to serve as the internal standard. The thickness of the ultrathin sections, mechanically pre-set, was measured directly by tilting the sections from −10 to 10 degrees at the magnification of x10,000. The thickness thus measured was correlated with the X-ray counts of chlorine. The mean value of chlorine in sections 80-nm thick was 10950 counts/200 seconds. Kα X-ray counts of the elements were adjusted to the 80 nm sample thickness. The mean value of the adjusted Kα X-ray counts of sodium, phosphorus, chlorine, and potassium was statistically the same regardless of the size of the area scanned, but the standard deviation varied; the deviation in the scanning of small areas was large, but was reduced to a satisfactory level in the scanning of large areas, mainly due to the scattered crystals of phosphate in the standard sample. The Cu Kα X-ray counts obtained from the scanning of large areas were adjusted to 80-nm thickness by the chlorine index and the mean value of the adjusted Cu Kα X-ray counts was defined as the Cu X-ray intensity. We tested the correlation between the Cu X-ray intensity and the copper content of the codfish cakes, measured by atomic absorption spectrometry.

Preparation of liver samples from humans:

Portions of histologically normal liver were obtained with the informed consent from 7 patients who underwent partial hepatectomy because of space-occupying lesions in the liver. Their blood biochemistry was near normal. Fatty liver was diagnosed by diffuse high echogenicity in ultrasonography and confirmed by liver biopsy in 13 patients. The histological diagnostic criteria were fat infiltrates in 50 % or more of the hepatocytes, and the absence of morphological features indicating viral hepatitis, either acute or chronic. Five patients had mildly elevated serum levels of transaminases. Two patients had slightly elevated serum levels of alkaline phosphatase, and one patient had a hyperbilirubinemia of 1.9 mg/dl. Serum levels of copper were normal in all patients. All of the patients with normal livers and those with fatty livers were negative for

histochemical copper stained with p-dimethylaminobenzylidine rhodanine.

A portion of each liver specimen was fixed for 2 hours in a chilled solution of 0.5 % glutaral-dehyde and 0.2 M cacodylate buffer (pH 7.4) as reported previously.[7] After being rinsed in distilled water, 1-mm cubes of the fixerd liver specimen were treated with 1.5 M sucrose, mounted on aluminium holders, and frozen in liquid nitrogen (−180°C). Ultrathin frozen sections were cut with an LKB ultratome equipped with a cryokit as described for the standard samples, and were transferred to carbon-coated gold grids using a 1.5 M sucrose solution as the carrier. Electron staining was not done. EDX was done under the same conditions as those employed for the standard samples. First, line-scanning of hepatocytes was done with Cu Kα X-rays to identify where copper was in the cells. This element was usually found in peri-bile canalicular lysosomes of high density. Second, ten or more lysosomes with varying density were selected per sample from each patient. An area 0.45 μm square was scanned for 200 seconds by selected area analysis. Lysosomal copper deposits were considered negative when five copper-positive dense bodies were not found even after an extensive examination. The specific Kα X-rays for sodium, phosphorus, sulfur, iron, and copper were recorded as the X-ray counts and as a molar percentage of all elements detected in the dense body. The mean of the three largest counts of Cu Kα X-rays was defined as the Cu X-ray intensity of the patient, and it was used to estimate the lysosomal copper content from the standard samples. The molar percentages of copper and sulfur were divided by the molar percentage of phosphorus; moreover, we calculated the correlation between the copper and phosphorus ratio (Cu/P) and the sulfur and phosphorus ratio (S/P), and also between the iron and phosphorus ratio (Fe/P) and the S/P for each patient. When we found a correlation between Cu/P and S/P, we concluded that copper transfer between the cytosol and lysosomes was mediated by a cuproprotein. Δ (Cu/P)/Δ (S/P) was used to express the copper binding ratio of the copper transfer protein.

RESULTS

The ultrathin sections of codfish cake without cupric sulfate did not generate any Cu Kα signals in the X-ray spectrum, their copper content being 0.005 mg/g dry weight. Ultrathin sections with a copper content of 0.496 mg/g dry weight gave the minimum reading for identification by the autoprocessor. Their Cu X-ray intensity was 172 counts/200 seconds. Ultrathin sections with a copper content of 16.240 mg/g dry weight yielded an X-ray intensity of 3722. The X-ray intensity of copper (X: counts/200 seconds) and the biochemical copper content in the codfish cake (Y: mg/g dry weight) were correlated in the following formula for copper estimation in ultrathin sections 80 nm thick: $Y = 4.5 \times 10^{-3}X + 0.16$; $r = 0.99$, $P < 0.01$ (Fig. 1). The formula was used to estimate the copper contents of hepatocyte lysosomes in patients with normal liver and in those with fatty liver.

Subcellular organelles could be seen in the unstained ultrathin sections (Fig. 2). Peri-bile canalicular lysosomes were dense when the contents of copper and iron were large. Mitochondria were easily recognized because of their scattered distribution and low density. The results of microanalysis of normal and fatty livers are summarized in Tables 1 and 2, respectively. Lysosomal copper deposits were detected in 5 of the 7 patients with normal livers and in 12 of the 13 patients with fatty livers. The Cu X-ray intensity of the patients was between 512 and 1286 in the normal livers, and between 942 and 1796 in the fatty livers. Figure 3 shows the distribution of all Cu Kα X-ray counts obtained from a patient with fatty liver. Figure 4 shows the correlation of lysosomal S/P and Cu/P for this same patient. In 4 of the 7 patients with normal liver and 10 of the 13 patients with fatty liver, there was a correlation between S/P and Cu/P. The
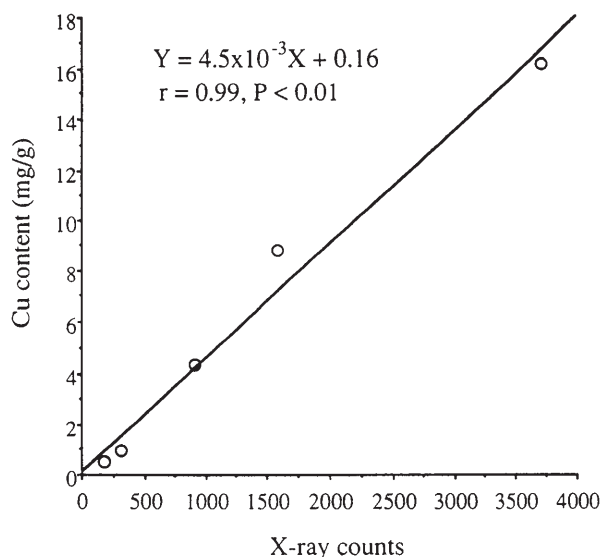
Naoki Hishida *et al.*

Fig. 1   Correlation between the copper content in fish cakes and the X-ray intensity of Cu. The X-ray intensity is expressed by the Cu Kα counts adjusted by the 10950 count of Cl Kα X-rays that correspond to an ultra-thin section 80-nm thick.
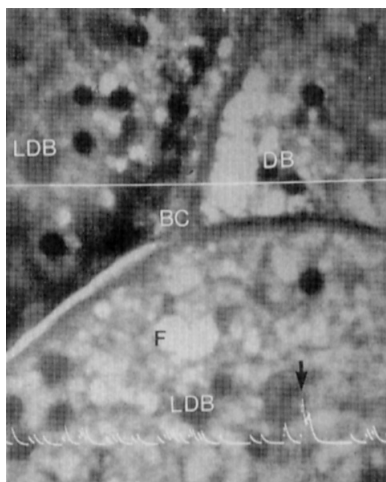

Fig. 2.   Scanning transmission electron micrograph from a patient with fatty liver (patient No. 10) showing subcellular structures of hepatocytes such as fat droplets (F), electron-dense bodies (DB), less dense bodies (LDB), and a bile canaliculus (BC). Scanning of the hepatocyte by Cu Kα X-rays was done on the mid-horizontal straight line. The spectrum of the lower irregular line shows the yield of Cu Kα X-ray (arrow) in a DB. Most electron-dense lysosomes are near the canaliculi and contain much copper, but the less dense, copper-negative mitochondria are scattered within the cytoplasm. ×10,000

binding ratio, Δ (Cu/P)/Δ (S/P), was between 0.07 and 0.11 in the normal livers, and 0.10 and 0.48 in the fatty livers. In a patient with fatty liver, lysosomal copper deposits were negative. This patient's lysosomes contained much iron. Two patients with normal liver were negative for Cu X-rays of hepatocyte lysosomes. No abnormalities were found in their lysosomes. In 3

patients (1 with normal liver and 2 with fatty livers), lysosomal copper accumulation was detected by the X-ray analysis, but no correlation between S/P and Cu/P was found because of the narrow copper content distribution and low copper concentration. There was no correlation between indices of cuproteins and biochemical data except for bilirubin. The patient with a transient hyperbilirubinemia showed the maximum Cu X-ray intensity.

Lysosomal iron was detected in 10 of the 13 patients with fatty livers and in all patients with normal livers. There was no correlation between Fe/P and S/P. The third lysosomal trace

Table 1.   Results of X-ray Microanalysis of Normal Livers

Patient	Sex	Age	X-ray intensity (/200 sec)	Transfer protein	
				$\Delta Cu/\Delta S$	p
1	M	59	no Cu[a]	nc[b]	nc
2	M	45	no Cu	nc	nc
3	F	51	754	0.02	<0.54[c]
4	M	54	512	0.11	<0.04
5	M	32	886	0.10	<0.01
6	F	19	1267	0.08	<0.01
7	F	39	1286	0.07	<0.01

a) The copper content of hepatocyte lysosomes is below the detection limit of the EDX used.
b) nc; not calculated.
c) Copper element is detectable but a copper transfer protein between the cytosol and lysosomes is not identified.

Table 2.   Results of X-ray Microanalysis of Fatty Livers

Patient	Sex	Age	X-ray intensity (/200 sec)	Transfer protein	
				$\Delta Cu/\Delta S$	p
1	M	68	no Cu[a]	nc[b]	nc
2	M	46	1079	0.04	<0.08[c]
3	M	42	1310	0.01	<0.76[c]
4	M	22	942	0.10	<0.01
5	M	71	1092	0.27	<0.01
6	M	41	1107	0.38	<0.01
7	F	59	1234	0.01	<0.01
8	M	30	1238	0.34	<0.04
9	M	38	1313	0.19	<0.01
10	F	72	1516	0.48	<0.01
11	M	30	1675	0.12	<0.02
12	F	28	1747	0.16	<0.01
13[d]	M	35	1796	0.21	<0.01

a) The copper content of hepatocyte lysosomes is below the detection limit of the EDX used.
b) nc; not calculated.
c) Copper element is detectable but a copper transfer protein between the cytosol and lysosomes is not identified.
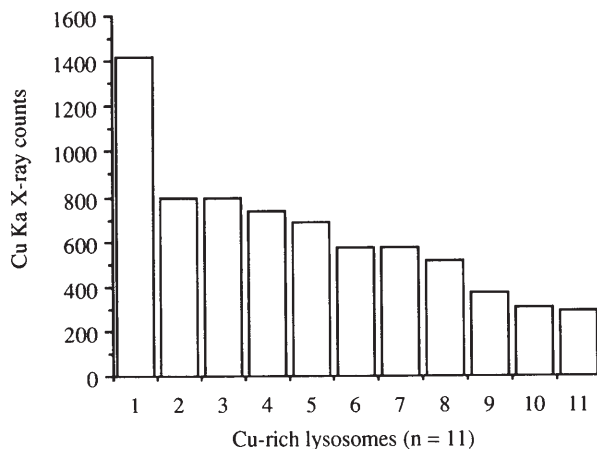d) The patient showed a transient hyperbilirubinemia.

Naoki Hishida *et al.*

Fig. 3. Cu KαX-ray counts of hepatocyte lysosomes from a patient with fatty liver (patient No. 6). The counts of eleven lysosomes ranged between 292 and 1419 counts/200 seconds. The mean value of the three largest counts (here, 1107) was considered as the Cu X-ray intensity of the patient.
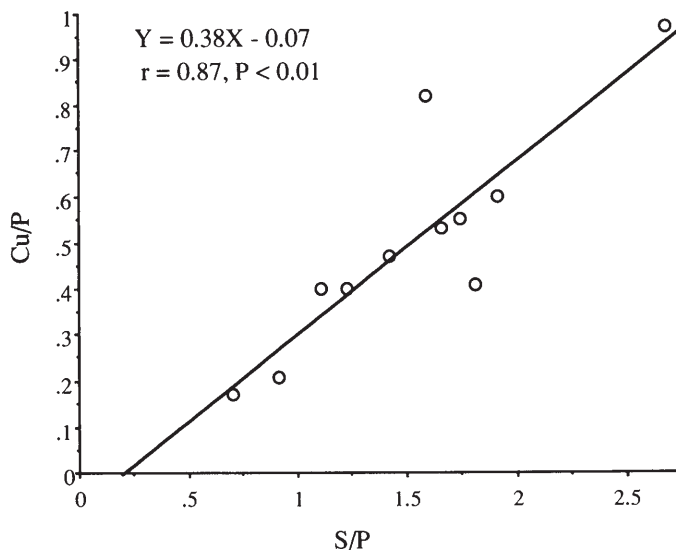

Fig. 4. Correlation between the sulfur and phosphorus ratio (S/P) and the copper and phosphorus ratio (Cu/P) in the same patient as in Fig. 3. X-rays of sulfur and copper were expressed as molar ratios and were divided by those of phosphorus which was an internal standard. In this patient, a correlation between the two ratios was found, and the copper transfer protein between the cytosol and lysosomes had a copper-to-sulfur binding ratio of 0.38.

element detected was calcium, which was found in about 30 % of the lysosomes examined in the study. Zinc was detected in only two of about 300 dense bodies. No relationship to other elements was observed for calcium and zinc.

DISCUSSION

Ultrastructural evidence of copper accumulation in nearly normal livers is reported here for the first time. All the liver specimens, whether normal or fatty, lacked copper when tested histochemically. However, copper deposits in hepatocyte lysosomes were found in 5 of the 7 patients with normal livers and in 12 of the 13 patients with fatty livers when investigated by X-ray microanalysis. Copper transfer protein, which was demonstrated by a correlation between the amount of copper and that of sulfur, was identified in 4 of the 7 patients with normal livers and in 10 of the 13 patients with fatty livers. This information on hepatic copper accumulation in relation to the physiological state has been well documented by an EDX study published in 1984 by Hanaichi et al.[7] Some modifications were required in this study because the amount of cuproproteins was not so much in almost normal livers. Cooling the specimen chamber with liquid nitrogen reduced contamination by silicon from the detector window. Consequently, we could identify a low peak of P Kα X-rays, enhancing the detection of cuproproteins.

We needed a new formula to estimate *in situ* copper, because normal hepatocyte lysosomes have little copper. Yagi et al.,[8] in an animal experiment, used samples with a copper content of 10 to 40 mg/g dry weight, but in this study we employed standard samples that contained less than 20 mg of copper per gram (dry weight). Copper contents within this range were proportional to the X-ray intensity of Cu Kα and the correlation obtained showed that the X-ray intensity was a reliable parameter to measure the copper content in less than pathological conditions. The maximum Cu X-ray intensity was 1300 in normal liver and 1800 in fatty liver. These figures correspond to a copper content of about 6 mg/g dry weight and 8 mg/g dry weight, respectively. In 3 patients, the copper content of hepatocyte lysosomes was below the detection limit of our method, estimated to be 0.5 mg/g dry weight. The remaining 17 patients had hepatocyte lysosomes with copper contents of more than 0.5 mg/g dry weight. Another group of 3 patients had lysosomal copper, but Cu/P and S/P did not correlate in either of them. The copper transfer protein between the cytosol and lysosomes was not identified because cuproproteins were not major component of their lysosomal proteins. In all, Cu/P and S/P were correlated in 14 of the 20 patients studied. Correlation of Cu/P and S/P meant that copper transfer between the cytosol and lysosomes was mediated in a patient by a cuproprotein with a binding ratio of Δ (Cu/P)/Δ (S/P). The estimated binding ratio was about 0.1 in normal liver and it ranges between 0.1 and 0.5 in fatty liver. The wide range of the binding ratio supports the concept that this copper transfer protein has multiple binding sites for copper.[4]

The Cu X-ray intensity of hepatocyte lysosomes was less than 3000 counts/200 seconds and the binding ratio of the copper transfer protein was less than 0.5 in our patients. According to the criteria of Yagi et al,[8] which were based on results in hamsters fed excess copper, all of our patients were in stage 1, the initial step in lysosomal copper accumulation. Because the X-ray intensity represents the element content, some fatty livers contain more copper in their hepatocellular lysosomes than normal livers. If the lysosomal copper reflects hepatic copper, it is likely that some fatty livers would contain large amounts of copper. A possible mechanism of copper retention and cuproprotein formation is bile canalicular compression caused by swollen hepatocytes, because bile is the main pathway for copper elimination. Fatty liver is not infrequently associated with cholestasis.[9,10] On the other hand, excess intake of copper is unlikely, because there has been no report of copper toxicosis secondary to copper-rich foods in Japan. Regardless of the mechanism of copper accumulation in hepatocyte lysosomes, cuproprotein formation and its lysosomal storage might be a cytoprotective reaction rather than a cause of fatty liver. When much cuproproteins are formed, the probability of cell damage in fatty liver may be negligible, even though copper *per se* is toxic.

If the maximum lysosomal copper content is 8 mg/g and the hepatic copper content is less than 150 µg/g dry weight in fatty livers,[11] hepatocellular lysosomes must take up copper against the high gradient through a special mechanism. In near-normal hepatocytes, copper is concentrated in hepatocyte lysosomes as cuproproteins. The amount of the lysosomal cuproproteins and the binding ratio of the copper transfer protein are low, but it is difficult to characterize the copper accumulation of almost normal livers from the histochemical copper accumulation seen in pathological conditions.[12] Lysosomal accumulation of curoproteins may be a cytoprotective mechanism and also may be useful as a storing of this essential element, although toxic when in excess.

CONCLUSION

Energy-dispersion X-ray microanalysis was useful to detect copper accumulation of hepatocyte lysosomes of patients with normal liver and fatty liver. In most patients studied, coexistence of copper and sulfur was found in hepatocyte lysosomes. The correlation between the content of copper and that of sulfur indicates that copper transfer between the cytosol and lysosomes is mediated by a cuproprotein. The copper content of hepatocyte lysosomes and the copper binding ratio to the transfer protein were high in some fatty livers. These observations suggest that lysosomal accumulation of the cuproproteins in hepatocytes is not necessarily pathological, but it is greater in some fatty livers, probably because of impaired bile drainage.

REFERENCES

1) Bloomer, L. C. and Lee, G. R.: Normal hepatic copper metabolism. In Metal and the liver, edited by Powell, L. W., pp. 179–239 (1978), Marcel Dekker, New York.
2) Sternlieb, I.: Hepatic lysosomal copper thionein. In Metallothionein 2, edited by Kägi, H. R. and Kojima, Y., pp. 647–653 (1987), Birkhäuser Verlag, Basel.
3) Nakanuma, Y., Karino, T. and Ohta, G.: Orcein positive granules in the hepatocytes in chronic intrahepatic cholestasis: morphological, histochemical and electron X-ray microanalytical examination. *Virch. Arch. A Path. Anat. Histol.* 382, 21–30 (1979).
4) Sipponen, P., Hjelt, L., Törnkvist, T. and Salaspuro, M: X-ray microanalysis of copper accumulation in liver in secondary biliary cirrhosis. *Arch. Pathol. Lab. Med.* 100, 664–666 (1976).
5) Humbert, W., Aprahamian, M., Stock, C. and Grenier, J. F.: Copper accumulation in primary biliary cirrhosis: an electron and X-ray microanalytical study. *Histochemistry,* 74, 85–93 (1982).
6) Janssens, A. R., Van-Noord, M. J., Van-Hoek, C. J., Ruiter, D. J., Mauw, B. J. and Van-den Hamer, C. J: The lysosomal copper concentration in the liver in primary biliary cirrhosis. *Liver,* 4,396–401 (1984).
7) Hanaichi, T., Kidokoro, R., Hayashi, H. and Sakamoto, N.: Electron probe X-ray analysis on human hepatocellular lysosomes with copper deposits: copper binding to a thiol-protein in lysosomes. *Lab. Invest.* 51, 592–597 (1984).
8) Yagi, A., Hayashi, H., Higuchi, T., Hishida, N and Sakamoto, N.: Three stages of copper accumulation in hepatocellular lysosomes: X-ray microanalysis of copper-loaded golden hamsters. *Int. J. Exp. Med.* 73, 85–94 (1992).
9) Yazaki, Y.: Hepatic copper concentrations in various liver diseases. *Acta. Hepatol. Jpn.* 24, 1260–1272 (1983).
10) Sherlock, S.: Acute fatty liver of pregnancy and the microvesicular fat diseases. *Gut,* 24, 265–269 (1983).
11) Capron, J. P., Gineston, J. L., Herve, M. A. and Braillon, A.: Metronidazole in prevention of cholestasis associated with total parenteral nutrition. *Lancet,* 1,446–447 (1983).
12) Sasa, A., Hayashi, H., Yagi, A., Ohguchi, S., Kidokoro, R., Sato, Y. and Sakamoto, N.: A copper-sulfur complex in the liver of a patient with Wilson's disease. *Gastroenterol. Jpn.* 21, 633–637 (1986).