Sodium fluorescein and fluorescein-labelled dextrans with a mol. wt smaller than 70,000, fluoresced more weakly 30 min following the injection than 2 min after it (figure 4). Dextrans 70,000 and 150,000 also fluoresced intensively 30 min after the injection (figures 5 and 6). Specific fluorescence was never observed in the parenchyma of the cerebral cortex.

If both the artery and vein of both kidneys were ligated before injection of the tracer, even the smallest label, sodium fluorescein, fluoresced brightly in the cortical capillaries 30 min after the infusion but did not, however, leak into the brain tissues (figure 2). In all experiments, the parendryma of cerebral cortex showed only a weak background fluorescence.

Discussion. Fluorescein-labelled dextrans are very stable molecules[7,8] and suitable for microcirculation studies[7,9]. Several successful methods for localizing sodium fluorescein in freeze-dried specimens habe been reported[9-12]. In the present study, the freeze-drying technique of Rodriquez-Peralta[11] and Baurman[12] yielded a good localization of the label fluorescence and well reproducible results with both Na-fluorescein and FITC-Dextrans.

Neither Na-fluorescein nor any of the FITC-Dextrans used penetrated the BBB. Similar results, concerning the permeability of retinal vessels to Na-fluorescein[13] or to FITC-Dextrans[9], have been reported previously.

The fluorescence in the capillaries of the cerebral cortex was weaker 30 min rather than 2 min after an injection with a tracer smaller than FITC-Dextran 70,000. This seems to be explained both by re-distribution of the marker into other tissues of the body and by its elimination through renal excretion. In man, dextrans with a mol. wt smaller than 50,000 are rapidly excreted into urine[14]. In the present study the fluorescence due to the smaller tracers (mol. wt < 70,000) persisted for over 30 min in the cortical capillaries if both the renal artery and vein were closed bilaterally. This operation is very likely to be followed by disruption of the BBB. This, however, did not take place during the first 30 min.

Both the eminentia mediana and the area postrema were brightly fluorescent as early as 2 min after an injection with any of the tracers, which indicates that the fenestrated capillaries in these brain regions are permeable to all the labels used and that 2 min was not too short a time for demonstrating transcapillary leakage.

Both Na-fluorescein and fluorescein-labelled dextrans proved to be reliable and suitable for blood-tissue barrier research. It seems that the transcapillary vesicular transport[15] rather than the possible 7-Å pores proposed before[6], are involved in the exchange of water-soluble particles through continuous brain capillaries[16]. However, non-fenestrated capillaries and barrier systems with pore size inside the range of dextran molecules will undoubtedly be an even more suitable subject for studies with FITC-Dextrans of graded mol. wts.

1 Part of the experimental work was carried out when F.J. worked at the Department of Anatomy, University of Helsinki on a fellowship from the Finnish-Hungarian Exchange Programme. The authors are grateful to Miss Irma Hiltunen for excellent technical assistance.

2 T.S. Reese and M.J. Karnovsky, J. Cell Biol. *34*, 207 (1967).
3 W. Bloom and D.W. Fawcett, in: Textbook of Histology, chapter 12. Nervous Tissue. W.B. Saunders Comp. Philadelphia.
4 F. Joó, Br. J. exp. Path. *52*, 646 (1971).
5 J.R. Pappenheimer, Physiol. Rev. *33*, 387 (1953).
6 J.D. Fenstermacher and J.A. Johnson, Am. J. Physiol. *211*, 341 (1966).
7 K.-E. Arfors and H. Hint, Microvasc. Res. *3*, 440 (1971).
8 U. Schröder, K.-R. Arfors and O. Tangen, Microvasc. Res. *11*, 33 (1976).
9 G. Chioralia, L. Salminen, H. Baurman and F. Kremer, Acta ophthal. *54*, 665 (1976).
10 M. Ota and I. Tsukahara, Acta Soc. ophthal. jap. *75*, 1856 (1971).
11 L.A. Rodriquez-Peralta, J. comp. Neurol. *126*, 109 (1966).
12 H. Baurman, Acta ophthal. *24*, 204 (1971).
13 K. Sasaki, K. Otsuki and K. Mizuno, Jap. J. Ophthal. *17*, 323 (1973).
14 G. Arthuson and G. Wallenius, Scand. J. clin. Lab. Invest. *16*, 81 (1964).
15 M. Simionescu, N. Simionescu and G. Palade, J. Cell Biol. *64*, 586 (1975).
16 J.R. Wolff, in: Microculation, vol.I, p.95. Ed. G. Kaley and B.M. Altura. Univ. Park Press, Baltimore 1977.

Hepatocellular iron-containing deposits in relatives of patients with latent idiopathic hemochromatosis: a qualitative and quantitative approach[1]

J.A. Grimaud, A. Ferzli, P. Paliard and A. Vachon[2]

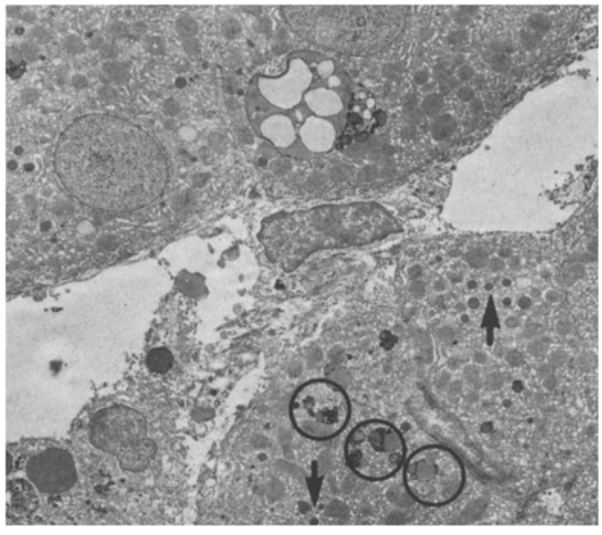
Centre de Microscopie Electronique et de Pathologie Ultrastructurale, Institut Pasteur, 77, rue Pasteur, F-69007 Lyon; Clinique Médicale C, Hôtel-Dieu, F-69002 Lyon, and Pavillon H Bis, Hôpital Edouard Herriot, place d'Arsonval, F-69000 Lyon (France), 31 May 1978

Summary. The quantitative and qualitative study in electron microscopy of hepatic fragments taken in apparently healthy collaterals of idiopathic hemochromatosis, shows the presence of siderosis, undetectable by Perls reaction comparatively with normal liver samples.

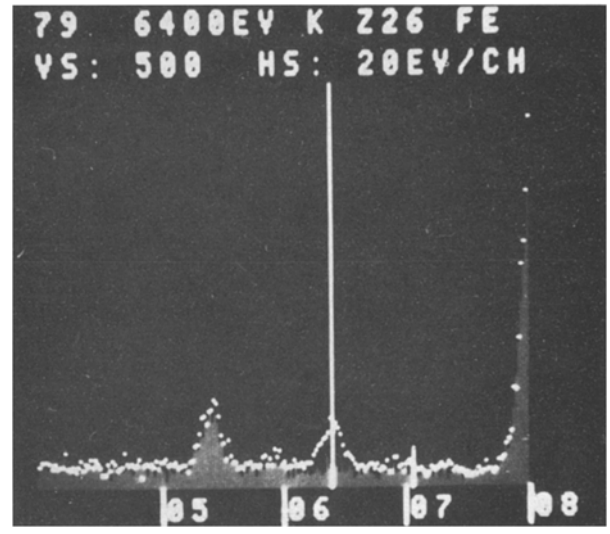
Previous clinical[3,4] and histological[5,6] studies of hemochromatosis relatives have already been made. Hepatic biopsy, associated with ferritine determination[7], is one the most important criteria to appreciate the iron overload[8] in the liver and to detect the nonsymptomatic stades of the disease. This morphological investigation attempts to clarify the cellular mechanisms of tissular overload which remain obscure.

The purpose of the present study is to correlate an electron microscopy quantitative approach of the ferric inclusion in the hepatocytes[9], with a light microscopy evaluation of heterogenous lipofuscin[10] aggregates in hemochromatosis. This correlation is made between the liver of the relatives of patients with latent idiopathic hemochromatosis and patients with normal liver.

Material and method. The propositus (table 1, II A 1), 32 years old, was admitted to the hospital for hepatomegaly and diabetes. His seric iron level was 275 µg/ml and the needle hepatic biopsy showed, after Perls reaction, a massive ferric overload, associated with fibrosis.



1



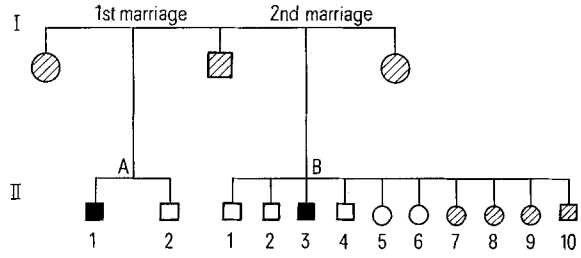
2

Fig. 1. Hepatocellular deposits in relatives of patients with latent idiopatic hemochromatosis. ○: Lipofuscin aggregates. →: Siderosomes.

Fig. 2. Nondispersive X-ray analysis of iron deposits: 2 different analyses are superposed and obtained from ferric and nonferric inclusion.

In the course of the familial investigation, 7 members of the family have been examined: one case of latent hemochromatosis has been detected (table 1, II B 3) and 4 relatives (table 1, II B 2, 4, 4, 5), 20–26 years old, without any clinical or biological manifestation, but among which 3 showed a slight hepatomegaly, have accepted a needle liver biopsy (Menghini needle technique).

Table 1. Pedigree of the studied family



Studied subjects: ■ Hemochromatosis;
○, □ Infra-clinical manifestations.
Nonstudied subjects: ⊘ ▨.

These liver biopsies have been compared to those of 5 human normal liver (table 2, t), free from any clinical or biological liver diseases and the iron seric level of which was normal. These human normal liver were obtained by surgical biopsy, realized during a laparotomy made for various digestive diseases (namely 4 duodenal ulcus and 1 gastric carcinoma), without previous bleeding.

Fixation. The fragments were divided in 2 groups: The first one was immersed in Bouin aqueous fixative and processed through conventional histological methods. The Perls reaction was systematically done. The second one was fixed in buffered cacodylate osmic acid (2%), pH 7.4, 0.3 M, 400 mOsM. Dehydration was carried through ethanol and embedded in EPON 812.

Semi-thin sections. They were contrasted according to Richardson's technique (azur II-methylen blue) and the count of the cytoplasmic inclusions found in the hepatocytes was carried out with a Zeiss integrator (obj. 40, occul. 10).

A sufficient number of blocks was cut to allow 3 different readings, corresponding, respectively to periportal, intermediary and pericentrolobular zones. A total number of

Table 2. Count of intrahepatocytary inclusions

Studied subjects	Perls reaction	Steatosis	Light microscopy (semi-thin sections) Average number of inclusions per 150 hepatocytes	Electron microscopy (ultra-thin sections) Average number of inclusions per 150 hepatocytes Lipofuscin (L)	Siderosome (S)	Total (T) (L+S)
C_1	±	+	307	262	371	633
C_2	0	+	294	170	432	602
C_3	0	+	283	208	304	512
C_4	0	0	310	355	484	839
C: m±SE			298±54	248.75±34.75	397.75±33.65	646.5
t_1	0	0	90	169	63	232
t_2	0	0	52	258	55	313
t_3	0	0	333	285	35	320
t_4	0	0	202	158	104	262
t_5	0	0	171	260	264	524
C: m±SE			169.6±43.80	226±23.27	104.2±25.95	330.2

C: Collaterals of hemochromatosis, t: normal livers, SE: standard error.

Table 3. Histological data and iron seric level in collaterals

	Age (years)	Liver biopsy Perls reaction	Steatosis	Iron seric level (μg/100 ml)	IBC (μg/100 ml)	CS (%)
II B 2	26	±	+	100	360	27
II B 4	23	0	+	155	310	50
II B 5	22	0	+	70	380	18
II B 6	20	0	0	130	300	43

150 (±10) cells have been chosen as representative of each patient.

Electron microscopy. For each patient, 3 blocks of hepatic tissue corresponding to 3 lobular zones were selected. On each block, a series of ultra-thin sections (600–800 Å) was made and laid on 200-mesh grids (of 85 μm^2 of length). Each block allowed the reading of about 50 hepatic cells on 5 grids-square. The count was realized on a positive print (4 prints for each square, totalizing thus 60 prints per patient), i.e. 150 cells (±10) located in 3 different zones of the hepatic lobule.

The noncontrasted sections which were examined show 2 different kinds of inclusions which will be counted separately: on the one hand, the lipofuscin inclusions containing some ferritin or hemosiderosis clusters, and, on the other hand, the smaller siderosomes, the nature of which has been controlled by using high magnification and nondispersive X-ray analysis (EDAX system) (figure 1).

The timing of the count was chosen between 100 and 200 sec; the finding of the iron peak (Kα at 6.4 KeV and Kβ at KeV) allowed the identification of siderosomes. Each analysis was memorized and superposed on a nonferric inclusion isolated from the same studied square (figure 2).

Results. Light microscopy. No hepatic histological alteration has been observed on the normal surgical biopsies, whereas a slight steatosis has been noted in 3 of the relatives. The Perls reaction was slightly positive in one of the relatives, under the form of granulations, scattered in some rare hepatocytes. It was negative in all the other cases (table 3).

As regards the 150 hepatocytes counted on semi-thin sections, the number of inclusions was more important in the liver of the relatives than in the normal liver samples, except for the particular case of sample t_3, for which the number of inclusions is high and for which no satisfactory explanation was found, in the absence of clinical or biological abnormalities.

Electron microscopy. Compared to normal liver samples, no significant increase of the number of lipofuscins (L) was noted in the relatives of hemochromatosis, but a strong increase of siderosomes was observed. The total amount of inclusions T (lipofuscins + siderosomes) is significantly more important in the relatives than in the normal samples. Apart from sample t_3 which presents an overload in aggregates of lipofuscins - the reason of which remains obscure - there may be a correlation, according to the small number of studied cases, between the number of inclusions per hepatocytes on the one hand, observed in light microscopy, and the number of siderosomes, and on the other hand, the total number of inclusions in electron microscopy. However, there is no correlation between the number of inclusions per hepatocyte observed in light microscopy and the number of lipofuscins inclusions observed in electron microscopy.

Discussion. The hepatic increase of ferric deposits (ferritin, siderosomes and lipofuscin aggregates) more or less overloaded in iron in relatives of idiopathic hemochromatosis is well-known. This fact suggested the liver needle biopsy as a way to detect infraclinical forms and to measure the ferric concentration in the liver, compared with the uncertain results of other investigations[12,13].

Our study confirms the value of electron microscopic count to appreciate such a ferric overload. The electron microscopic count has shown a strong increase of siderosomes number in 4 apparently normal relatives compared to 5 normal subjects, whereas the Perls reaction was slightly positive in 1 case only.

However, these facts do not permit us to determine which, among these relatives, are those who are liable to present afterwards a patent form of hemochromatosis. No morphological or ultrastructural characteristic permits us to differentiate the observed deposits from those of normal liver parenchyma or other human or experimental hepatosiderosis[14].

In the absence of any positivity to Perls reaction of the liver biopsies, the hepatocellular count of inclusions identified on semi-thin sections (light microscope) may help to appreciate the number of siderosomes, since this inclusion depends more on siderosomes than on lipofuscins. The validity of this count remains more or less unreliable, since various etiologies are responsible for a lipofuscin increase.

In the absence of a patent ferric overload, the histological study, as well qualitative as quantitative, of hepatic tissular iron deposit in apparently healthy relatives, without any elevation of seric iron, shows a ferric overload a minima. This overload takes the form of an excess of siderosomes, identifiable in electron microscopy, but the evolution of which remains unforeseeable.

1 This work was supported by a grant from UER Sud-Ouest Lyon University Research Funds.
2 Acknowledgment. The authors are thankful to Dr Partensky (Service of Prof. Saubier, Pavillon 0) who made the liver surgical biopsies, and to Mrs Crozet for the preparation of the manuscript.
3 R. Debre, J.C. Dreyfus and J. Frezal, Ann. hum. Genet. *23*, 16 (1958).
4 R. Williams, P.C. Pichter, A. Parsonson and H.S. Williams, Lancet *1*, 1243 (1965).
5 R. Williams, P.J. Sheuer and S. Sherlock, J. Med. *3*, 249 (1962).
6 F.B. Brick, Gastroenterology *40*, 210 (1961).
7 J.W. Halliday, A.N. Russo, J.L. Cowlishaw and L.W. Powell, Lancet *2*, 621 (1977).
8 M. Barry, Gut *15*, 411 (1974).
9 G.W. Richter, J. biophys. biochem. Cytol. *4*, 55 (1958).
10 P. Ganter and G. Jolles, in: Histiochimie normale et pathologique, vol. 1, p. 846. Ed. Villard. Gauthier, Paris 1969.
11 F.N. Ghadially, J.M.A. Lalonde and A.F. Oryschak, Virchows Arch. B. Cell Path. *22*, 135 (1976).
12 E. Feller, A. Pont, J. Wands, E. Carter, G. Forster, I. Kourides and K. Isselbacher, New Engl. J. Med. *296*, 1422 (1977).
13 C. Edwards, M. Carroll, P. Bray and G. Cartwright, New Engl. J. Med. *296*, 7 (1977).
14 A. Vachon, J. Barthe, J.A. Grimaud, S. Peyrol and M.F. Barthe, Lyon méd. *230*, 385 (1973).