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Properties and Distribution of Liver Fluorescence in Porphyria Cutanea Tarda (PCT)

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Summary. The livers of patients with porphyria cutanea tarda (PCT) contain preformed porphyrins, demonstrable by fluorescence microscopy due to their red fluorescence. The properties and distribution of the porphyrin fluorescence were studied in fine needle aspiration biopsies and sections from conventional thick needle biopsies using various histological techniques. Contrary to previous reports, the fluorescent porphyrins were found to occur in the cytoplasm of liver cells but in a freely diffusible form necessitating the use of water-free techniques for the demonstration of the correct localization. Freeze-drying and ethanol fixation proved satisfactory but only if the sections were flattened in the dry state. In sections floated on water a redistribution of fluorescent material to nuclei of liver cells, red blood cells and portal connective tissue was observed.

A simple screening test based on aspiration biopsy and semiquantitative grading of the fluorescent material is described which should be a useful diagnostic tool in cases of PCT.

The basic biochemical derangement in the various forms of hepatic porphyrias is considered to be a defective porphyrin synthesis in the liver, which usually contains increased amounts of porphyrins or porphyrin precursors (Schmid *et al.*, 1954).

Under normal conditions only trace amounts of porphyrins can be demonstrated in the liver but in hepatic cutaneous prophyria (PCT) the liver has been shown to contain large amounts of preformed porphyrins, mainly uroporphyrin, and, to a lesser extent, coproporphyrin (Schmid *et al.*, 1954). The porphyrins are strongly fluorescent compounds with emission bands in the red region of the spectrum after activation with visible or ultraviolet light (Vanotti, 1954). This fluorescence can be detected by visual examination of gross biopsy specimens under an ultraviolet lamp or by fluorescence microscopy of liver sections (Schmid *et al.*, 1954; Uys *et al.*, 1963; Niebauer, 1964; Huang *et al.*, 1965; Sparks *et al.*, 1965). The distribution and properties of this fluorescence are, however, not fully understood.

The purpose of the present investigation was to study the liver cell fluorescence in PCT with various microtechniques in order to find optimal techniques for the preservation of the fluorescent material and to apply such techniques to study the gross and cellular distribution of fluorescent porphyrins in the liver.

Materials and Methods

Biopsy specimens were obtained from 19 patients with symptomatic PCT. Five had clinically manifest disease. Nine patients had no skin symptoms at the time of the study

but had had typical symptoms earlier. The diagnosis was ascertained by biochemical analysis of urine and faeces. Excretion of precursors was normal in all. Uroporphyrin excretion in urine was greatly increased in all patients with active disease. In patients with latent disease the urinary uroporphyrin excretion was in most cases slightly increased, but was quantitatively normal in two. Porphyrin excretion data are given in another report (Lundvall and Enerbäck, 1969).

The control material comprised 22 patients on whom liver biopsy was performed, in most instances because of alcohol abuse. In none was there any history or clinical signs of PCT.

Fine needle aspiration biopsy was performed according to a technique previously described (cf Söderström, 1967). The aspirated specimens were as a rule immediately smeared onto clean slides and allowed to dry in air. In a few instances wet slides were fixed in ethanol and air-dried. The slides were then examined in the dry state without further treatment. In some instances smears were covered with a cover slip and a few drops of glycerol containing HCl, NaOH or Mc Ilvaine buffer solutions (one part to 5 parts glycerol) to obtain pH : s of 1, 4, 7, 8 and 11.

The solubility of the fluorescent material was further examined by incubating dry smears in 4% neutral buffered formaldehyde, xylene or ethanol. The slides were allowed to stand for 2 hours and then dried at 37° C before examination in the fluorescence microscope. The effect of pH on the fluorescence was further tested by treating dry smears for a few minutes with fumes from HCl, glacial acetic acid or ammonia.

The effect of heating was studied in a few instances by placing smears in an oven at 100° C for 2 hours before examination in the fluorescence microscope.

Conventional thick needle biopsy was performed in six of the patients and the tissue samples treated according to the following techniques:

1) Fixation in formaldehyde or ethanol, followed by dehydration in ethanol, and paraffin embedding, according to conventional histological techniques. The sections were either floated on warm water or applied in the dry state to albuminized slides preheated to about 50° C, and flattened by further heating the slide to the melting point of the paraffin. The sections were deparaffinized with xylene and mounted in a xylene-entellan® mixture.

2) Quenching of the tissue sample in propane, cooled by liquid nitrogen, followed by freeze-drying in a Speedivac-Pearse Tissue Dryer for 24 hours at −40° C, fixation in formaldehyde gas for 1 hour, and paraffin-embedding in vacuo. The sections were flattened onto dry slides as above and mounted in xylene-entellan®.

Sections and slides were examined in a Zeiss fluorescence microscope equipped with a high pressure mercury lamp (Osram HBO 200). The exciting light was filtered through a Schott BG 12 filter and the emitted light through a barrier filter with 50% transmission above 500 nm. Dark field condensers were used. Fluorescent structures were identified by subsequent staining with toluidine blue or according to May-Grünwald Giemsa. Fluorescence photomicrographs were obtained using Kodak High Speed Ectachrome (daylight type) or Tri X films, exposure times $^1/_2$ to 2 minutes.

Results

Properties of the Fluorescence and its Cellular Distribution

Aspiration biopsy smears contained a varying number of liver cells dispersed separately or occurring in small groups. Most smears also contained small fragments of liver tissue, containing several layers of liver cells. In the controls the liver cells exhibited a dull green autofluorescence but the lumps of liver tissue fluoresced more intensely green. In addition most smears also contained blood cells, fluorescing weakly green or not at all. In some instances, however, a dull red color was observed in small areas mostly in the periphery of the liver fragments. Examination of these areas in transmitted light revealed that they contained a layer of red blood cells. This color could easily be distinguished from the porphyrin fluorescence (see below). Apart from the green autofluorescence the only fluores-

cence that was observed in control specimens consisted of yellow or orange fluorescent lipofuscin granules, easily identified by their characteristic shape and size.

Unfixed and air-dried smears from porphyrics regularly contained liver cells exhibiting red fluorescence. In some specimens this occurred only in a limited number of the liver cells, but in others, virtually all liver cells fluoresced red. The fluorescence occurred both in cytoplasm and nuclei but was always of a higher intensity in the latter. In cases with a low fluorescence intensity only the nuclei exhibited red fluorescence. Smears with a high fluorescence intensity often contained a dull red background of diffusely dispersed fluorescent material. In most instances the fragments of liver tissue fluoresced more intensely than the separate liver cells. The cellular distribution of this fluorescende could not be studied due to the thickness of the fragments.

The red fluorescence of the liver cells faded rather rapidly during illumination with ultraviolet light and was in most instances very substantially reduced after 2 minutes illumination. The decrease in fluorescence was most obvious in cells with a low initial fluorescence intensity where the fluorescence often disappeared completely after prolonged illumination.

Heating of the slides to 100° C for 2 hours did not result in a noticeable increase in fluorescence intensity in specimens with a low initial fluorescence intensity.

Mounting of the dry smears under cover slips with a few drops of glycerol had a deleterious effect on the fluorescence, regardless of the pH of the glycerol. When the smears were studied immediately after application of the glycerol the fluorescent material was found to diffuse gradually from the fluorescent cells, and finally, after 1—2 hours, form a diffuse red background on the slide.

The effect of pH on the porphyrin fluorescence was tested by treating dry smears with fumes from hydrochloric acid, glacial acetic acid or ammonia. Treatment with hydrochloric acid fumes resulted in small increases in fluorescence intensity, but the other treatments were without noticeable effect. The solubility of the fluorescent material was further tested by immersion of dry slides in 4% formaldehyde solution, ethanol and xylene for two hours. Formalin treatment resulted in a complete loss of fluorescence but ethanol and xylene had no noticable effect on the fluorescence intensity.

A comparison was made of the intensity and distribution of the fluorescence in liver sections prepared by the various histological techniques. On each occassion about 10 mm long specimens of liver tissue were obtained, halved, and each half subjected to the two techniques according to Table 1. Comparison of the methods for flattening of sections was made on adjacent sections from the same tissue block. The results which appear in Table 1 show that the fluorescent porphyrins were best preserved in freeze-dried and ethanol-fixed specimens provided that the sections were flattened in the dry state. Flattening of the sections on water reduced the fluorescence intensity and changed the distribution of the fluorescent material.

The localization of the fluorescent material in ethanol-fixed specimens flattened without contact with water was confined to the cytoplasm of the liver cells. In most cells the nuclei appeared as nonfluorescent spots.

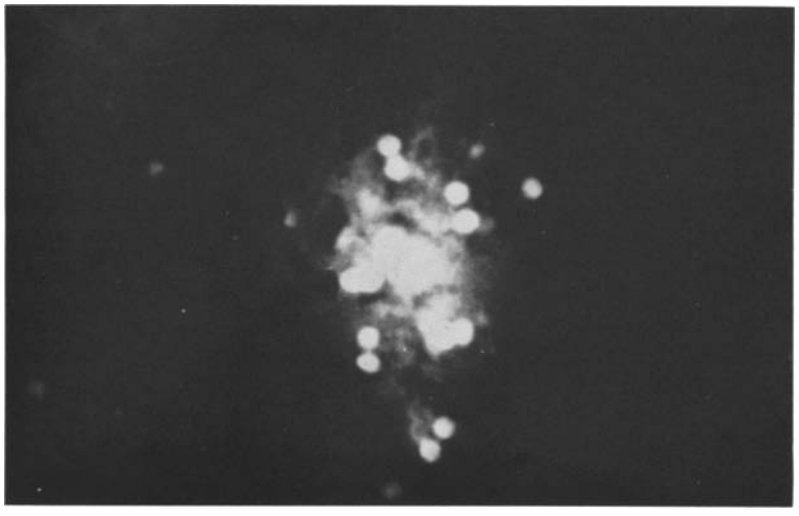

Fig. 1. Fine needle aspiration biopsy smear containing a group of liver cells. Note fluorescence in both nuclei and cytoplasm. Magnification 250×

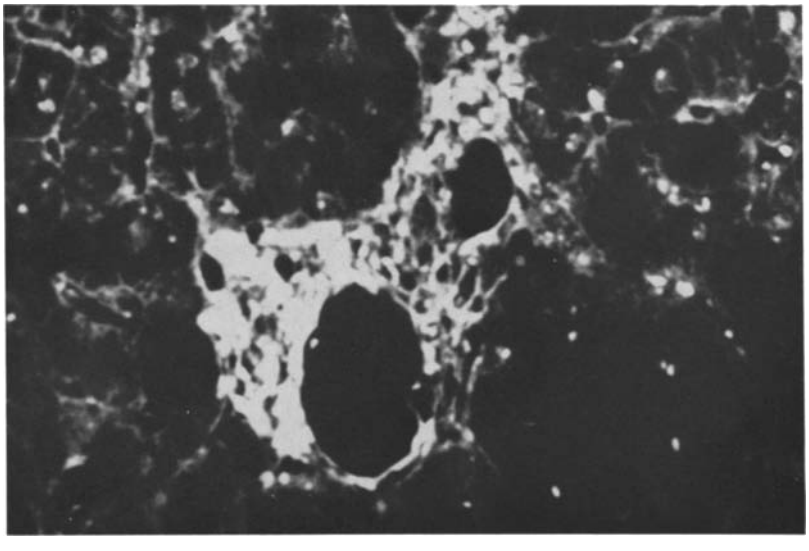

Fig. 2. Section from formalin-fixed biopsy specimen, floated on water. In the center a periportal area exhibiting a strong fluorescence. Also fluorescence in red blood cells of sinusoids and liver cell nuclei. Magnification 250×

Semiquantitation of Liver Cell Fluorescence in Aspiration Biopsy Smears

Smears from patients with latent and manifest PCT were studied with the aim of finding suitable parameters for grading of the amount of fluorescent material. The smears were found to vary both with respect to the fluorescence

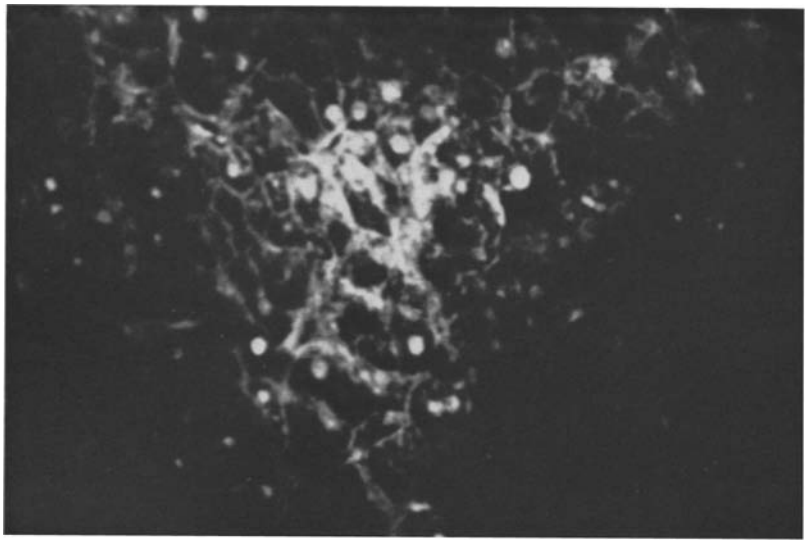

Fig. 3. Section from ethanol fixed biopsy specimen, floated on water. Fluorescence of weak intensity in nuclei and, to a lesser extent, in cytoplasm of liver cells. Magnification 250×

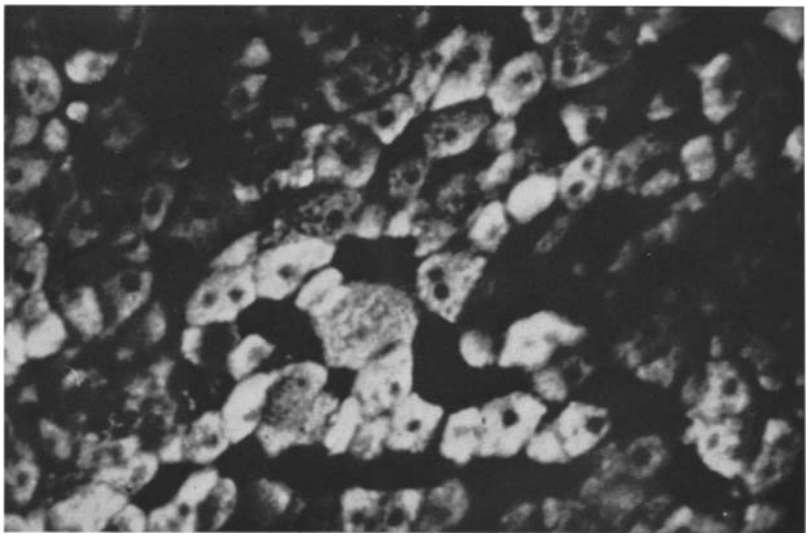

Fig. 4. Section from the same specimen as in Fig. 3 flattened on a dry slide. Strong fluorescence exclusively in the cytoplasm of liver cells. Note absence of fluorescence in nuclei, appearing as dark, non-fluorescent spots. Magnification 250×

intensity of individual liver cells and the relative number of fluorescent cells. The proportion of fluorescent cells in each smear seemed to be correlated to the fluorescence intensity of the fluorescing cells. In specimens with a low fluorescence intensity only a limited number of the liver cells contained fluorescent material

Table 1. *The effect of variations in the histological procedure on the intensity and distribution of fluorescence in sections*

Fixation	Flattening of sections	Fluorescence	
		Intensity	Distribution
Formalin	Water	Moderate	Cytoplasm and nuclei of liver cells. Collagen of portal areas. Red blood cells in sinusoids. Fluorescent rims outside sections
	Dry	Moderate	Only cytoplasm and nuclei of liver cells
Ethanol	Water	Weak	Cytoplasm and nuclei of liver cells. Large fluorescent rims outside sections
	Dry	Strong	Cytoplasm of liver cells. No fluorescence in nuclei
Freeze-drying + Formaldehyde gas	Dry	Strong	Cytoplasm of liver cells

Table 2. *Quantitation of fluorescence in aspiration biopsy smears*

	Fluorescence grade			
	1	2	3	4
Fluorescence intensity	Very weak	Weak	Moderate	Strong
Distribution of cellular fluorescence	Nuclei	Nuclei	Nuclei and cyltoplasm	—
Number of fluorescent cells	Few	Most	Amost all	All
Distribution of fluorescence in tissue fragments	Patchy or non-fluorescent	Patchy	Almost homogeneous	Homogeneous

while the remaining liver cells showed a usually weak, green autofluorescence. In specimens with higher fluorescence intensities virtually all liver cells fluoresced red. The fluorescence of liver cell nuclei was usually more intense than that of cytoplasm. In specimens with low fluorescence intensity the red fluorescence was confined to the nuclei while the cytoplasm fluoresced weakly green. The fragments of liver tissue also varied both with respect to the fluorescence intensity and the distribution of fluorescent material. In specimens with a high fluorescence intensity of individual liver cells the fragments usually exhibited a homogeneously distributed red fluorescence. In specimens with lower fluorescence intensities only parts of the fragments fluoresced in the red, the remaining parts exhibiting green autofluorescence.

Based on these observations a four-grade system was adapted. The principles of this grading are summarized in Table 2. The grading was performed on 3 to 5 smears from each patient and the variations between the smears from one patient were found to be less than one grade.

The distribution of the grades of fluorescence in 26 aspiration biopsies from 19 porphyrics is shown in Table 3.

Table 3. *Distribution of fluorescence grades in 19 patients with PCT*

Grade of fluorescence	Number of biopsies
0	0
1	5
2	6
3	10
4	5

Gross Distribution of Porphyrin Fluorescence

The variable proportion of fluorescent liver cells in the aspiration biopsy smears from patients with PCT suggests that the gross distribution of the fluorescent material might also be uneven. To study this, cytological material was aspirated from limited areas at three different depths in the livers of four patients. The fluorescence was graded on coded smears. Three to five smears from each level of aspiration were evaluated. The coded smears from one depth were always found to have the same grade of fluorescence. As shown in Table 4 the fluorescence was equal in the smears from the different parts of the liver in two patients. In the two others the fluorescence was equal in smears from two depths but differed at the third depth. The difference did not amount to a whole grade, however.

Table 4. *Fluorescence in specimens from various depths of the liver of four patients with PCT*

Level of aspiration	Patient no.			
	I	II	III	IV
I: 2—4 cm below liver surface	2	3	1	[a]
II: 6—8 cm below liver surface	2	3	1	2
III: 10—12 cm below liver surface	2+	2+	1	2

[a] Unsufficient material.

In sections from conventional thick needle biopsies processed by the optimal histological technique indicated above, both the distribution and the intensity of the porphyrin fluorescence exhibited wide variations. In 2 of the 6 examined biopsies the cytoplasm of virtually all liver cells were packed with strongly fluorescent material but in the other examined biopsies the red fluorescence was unevenly distributed. In these specimens the liver parenchyma contained weakly fluorescing or non-fluorescent areas, irregularly distributed within the lobuli without preference for central or portal areas. None of the liver sections showed signs of cirrhosis, but all except one of the biopsies contained cytoplasmic fat droplets. One of the livers exhibited a pronounced fatty change, most of the liver cells containing large fat vacuoles. In these specimens the fluorescent porphyrins were seen as an intensely fluorescent rim at the periphery of the fat containing

cells. No correlation between the amount of fat vacuoles and porphyrin fluorescence could be demonstrated. All of the examined livers contained histochemically demonstrable iron located both in parenchymal cells and in Kupffer cells. No correlation between the amount or distribution of iron and porphyrin fluorescence was found.

Discussion

The results show that livers of patients with PCT contain red fluorescence of the porphyrin type varying in intensity and distribution. This fluorescence can be demonstrated both in single liver cells obtained by fine needle aspiration biopsy and in sections from conventional thick needle biopsies. The great advantage with fine needle biopsy specimens from a technical point of view is that smears can be used directly for fluorescence microscopy without further treatment. A possible disadvantage of the method is the considerable negative pressure necessary for aspiration, which might cause damage to cell membranes resulting in a redistribution of diffusible compounds.

The results further show that porphyrins are freely diffusible in liver cells and are easily extracted by water or water-containing media. The material has a low solubility in ethanol and xylene but is very soluble in formalin solutions as indicated by the fluorescence intensity of smears before and after immersion in these media. Aspiration biopsy specimens must therefore be examined in the dry state or mounted in a water-free medium such as the xylene-entellan mixture used for sections in the present study. The smears should be air-dried or fixed in ethanol.

Freeze-drying is an ideal method for the study of highly diffusible material in tissue sections and was accordingly found to give satisfactory results. The results were, however, equally good with ethanol-fixed specimens, provided that a strictly water-free technique was maintained during the entire histotechnical procedure. A critical point in the procedure which is easily overlooked is the flattening of sections which must be performed on dry slides to prevent diffusion of fluorescent porphyrins.

Where the diffusion of porphyrins was prevented by water-free techniques, the porphyrins were found to be confined to the cytoplasm of liver cells, and the nuclei appeared as non-fluorescent spots. In sections floated on water a redistribution of the fluorescent material occurred, which was now found in the nuclei of liver cells, red blood cells and, to a lesser extent, in the collagen stroma of the portal areas.

Similar results as those reported here were obtained by Holzner and Niebauer (1963). These authors used cryostat sections as well as ethanol and paraffin embedded liver specimens and found that the fluorescent material could be extracted by water containing media. Most reports dealing with the distribution of fluorescent porphyrins in hepatic porphyrias have stressed the occurrence of fluorescence in liver cell nuclei (Schmid *et al.*, 1954; Huang *et al.*, 1965; Sparks *et al.*, 1965). In all of these reports this finding can be ascribed to an unsuitable histological technique such as the use of frozen sections or mounting of sections in buffered glycerol. Smears from fine needle biopsies, however, also contain this nuclear fluorescence even if the smears have been air-dried and examined in the dry state. This apparent diffusion of porphyrins might occur either during the

aspiration or during the drying of the slides. But nuclear fluorescence is also present if the smears have been fixed in ethanol in the wet state immediately after aspiration. Attempts to prevent the nuclear fluorescence from occurring by employing various measures, such as lessening the negative pressure during aspiration, or ejecting the aspirated material directly into ethanol instead of smearing, have so far proved unsuccessful.

The very simple method of direct fluorescence microscopic examination of aspiration biopsy smears might be useful as a diagnostic procedure in cases of PCT. Of special interest ist the question whether such examinations can yield quantitative information on the amounts of porphyrins stored in the liver. In general, the fluorescence intensity is proportional to the amount of fluorophor provided that the concentration is not high enough to cause a significant absorption of the activating or emitted light (Udenfriend, 1962). Visual estimation is a very crude measure of the fluorescence intensity. The human eye has a high capacity for distinguishing between adjacent fields of almost equal brightness provided that they are of the same color, but, since for practical reasons, the comparisons must be made between fluorescent cells in different specimens and on different occasions, the errors in visual estimations of fluorescence intensity must increase considerably. To compensate for such errors in estimation, additional criteria which could be used in a semiquantitative grading were sought. It was found that in cases with a high fluorescence intensity of the liver cells, practically all cells fluoresced red. A decrease in the fluorescence intensity was accompanied by a decreasing number of fluorescent cells.

Although the porphyrin content of individual liver cells thus varies within wide limits, the gross variation of the fluorescence seems to be small (Table 4) and should not influence the grading, at least not if the aspiration is performed with long needles.

Thus the present grading based on fluorescence intensity and relative number of fluorescent cells should give reproducible results and can be expected to give a reasonably accurate measure of the porphyrin content of the liver. The sensitivity of this visual grading method can however only be determined by comparing with liver porphyrin concentration determined chemically. The small amounts of porphyrins present in non-porphyric livers are below the limit for detection by fluorescence microscopy.

We are not aware of any compounds occurring in the liver and showing fluorescence properties which might be misinterpreted as porphyrin fluorescence. Lipofuscin granules have a yellow or orange fluorescence quite different from the deep red porphyrin fluorescence. Many drugs accumulate in the liver. So far, we do not know of any drug giving red fluorescence. But fluoresecence in liver biopsies obtained from patients treated with drugs of unknown fluorescence properties must be evaluated, keeping the possibility of drug-induced fluorescence in mind. The dull red colour, which in some instances was observed in the periphery of liver tissue fragments, is most probably due to an absorption phenomenon. These areas contained red blood cells, partly covering the autofluorescent tissue lumps. The tissue autofluorescence, mainly caused by proteins with a high content of aromatic amino acids, appears blue-green with the filter combination used in the present study. The emission curve is however broad and flat, and

extends into the red region of the spectrum (Enerbäck, unpublished observation). A partial absorption of the autofluorescence by haemoglobin-containing red blood cells might therefore modify the colour of the autofluorescence towards a dull red. However, this phenomenon is easily recognized and can be distinguished from porphyrin fluorescence.

The potential value of the aspiration biopsy method as a diagnostic procedure in cases of PCT has been tested in a study of the correlation between the grade of liver fluorescence and the porphyrin excretion data, the stage of clinical activity of the disease, and the effect of treatment (Lundvall and Enerbäck, 1969). The findings so far indicate that the method is a valuable diagnostic tool in cases of PCT and well suited as a screening test for the disease. The finding of liver fluorescence in patients with clinically latent forms of the disease, and with porphyrin excretion data within the normal range, indicate that this simple method might in fact be a more sensitive diagnostic procedure than the currently used biochemical methods for the detection of porphyrins in the excreta.

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