450 Lf/mg protein. Both intact and nicked toxin[7] showed similar behavior.

In order to investigate the mechanism of the interaction between toxin and dye, we also performed experiments using difference spectroscopy, measuring the spectral shift produced by the protein when it binds the free dye in solution. Figure 2 shows that the addition of diphtheria toxin to Cibacron Blue produces a very weak difference spectrum, that cannot be ascribed to a hydrophobic interaction. For comparison, the figure also shows the spectrum produced in the same conditions by a mixture of Cibacron Blue and human serum albumin[8] which presents the typical shape resulting from a hydrophobic interaction[5].

From the results of our experiments we may conclude that the interaction of diphtheria toxin with Cibacron Blue takes place on a region of the protein molecule which presents structural characteristics different from those found in albumin or in nucleotide-requiring enzymes.

The binding of the toxin to the immobilized dye is therefore likely to be ionic rather than hydrophobic; the high degree of specificity of this interaction could eventually be explained by a favorable spatial arrangement of positive charges in the protein molecule, which would facilitate an ionic interaction with the negatively charged sulfonate groups of the dye. Moreover, it is important to bear in mind that the chromatography of the crude broth culture of *C. diphtheriae* on Cibacron Blue-Sepharose produced a toxin of high specific immunologic activity, and therefore it could be applied to purify this protein.

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0014-4754/83/080885-02$1.50 + 0.20/0

A histofluorescent procedure for identifying marijuana cannabinoids[1]

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Summary. A rapid and reliable fluorescence procedure is described as a test for the microscopical identification of the glandular hairs of *Cannabis sativa*. The proposed method, designated as the IFIM test (induced fluorescence identification for marijuana test), is based on the induction of a red fluorescence in cannabinoids by a hot clearing solution. The results, compared to those obtained by the classical RIM test, offer the possibility of more satisfactory identification of cannabis, hashish or marijuana in suspected samples.

Several histochemical tests for marijuana have previously appeared[4–7], but only 2 microscopic procedures are routinely used in many countries for the legal identification of marijuana: the RIM and the vanillin tests[8,9]. The specificity of these methods has been recently confirmed[10], but some uncertainty in the identification of marijuana has been noted due to the weak intensity of the color response and the lack of clarity in the microscopic preparations. In spite of the high potential of fluorescence microscopy, the application of fluorescence methods in pharmacognosy represents a much-neglected field of research[11–13]. The present study was carried out to explore the feasibility of using induced-fluorescence microscopy for localizing the cannabinoids in marijuana plants. Samples of fresh or dried plant material of *Cannabis sativa* 'fiber cultigen', collected locally, and authentic marijuana plants and resin samples, obtained from police seizures, were processed directly on microscope slides with the following procedures: a) direct mounting in water to observe the autofluorescence that naturally occurs under different excitation wavelengths. Thus, it is possible to detect many cellular components such as chlorophyll, proteins, lipids, lignin, suberin, etc., which emit primary fluorescence that can mislead during the microscopic observation of fresh material; b) observation of fluorescence emission when the specimen is mounted in cold clearing solution composed of chloral hydrate (7.5 g), propylene glycol (1.0 ml) and distilled water sufficient to make 10 ml. This clearing procedure dissolves proteins, plant pigments and other autofluorescent substances which tend to confuse the diagnostic criteria of identification. The autofluorescence that resists cold chloral hydrate treatment is normally due to lipids, phenols[14] and, in the case of *Cannabis*, cannabinoids[15]; c) IFIM test (induced fluorescence identification for marijuana test). This new procedure is based on a 1-min heating of samples on a microburner with 4–5 drops of the above-cited clearing solution; d)

Color responses of cannabinoid standard and capitate gland heads of fiber and drug strains of *Cannabis sativa* to IFIM test and other cannabinoid indicators

Cannabinoid indicators	Cannabinoid standard	Capitate glandular trichomes		Color of reactions
		Fiber	Drug	
IFIM	+ +	–	+ + +	red (fluorescence)
RIM	+ +	–	+ +	red
vanillin/ethanolic H_2SO_4	+	–	+	pink

+ + + Intense reaction; + + moderate reaction; + weak reaction; – no reaction.

RIM[8], vanillin/ethanolic H_2SO_4[9] tests were employed as classical cannabinoid indicators for purposes of comparison with to the IFIM test.

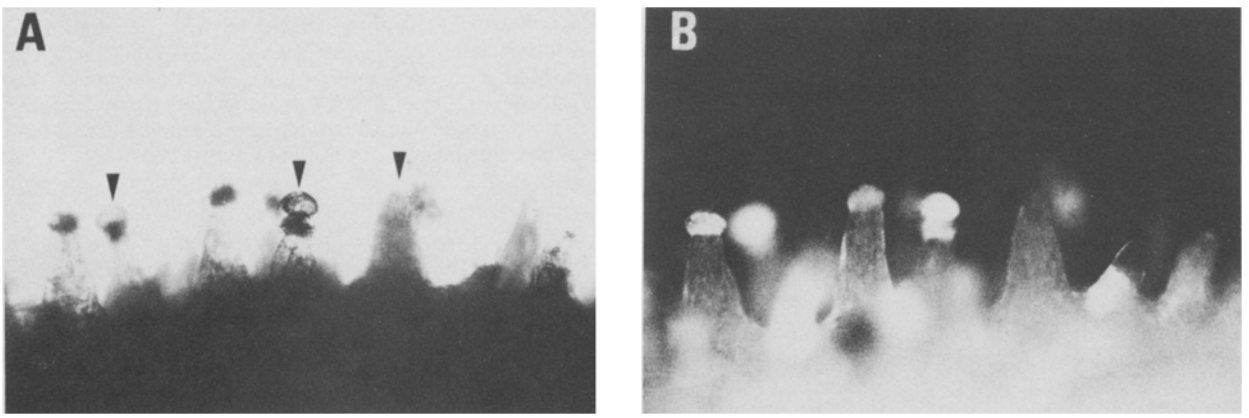
The slides were viewed with a Zeiss Photomicroscope II equipped with an incident fluorescence condenser (F1, II), and an XBO-75W xenon arc source for fluorescence and a tungsten lamp for white light. The following Zeiss filter sets for the epi-fluorescence condensers were used: No. 1 = G365, FT420, LP418; No. 2 = BP390-440, FT460, LP475; No. 3 = BP450-490, FT510, LP520; No. 4 = BP546/10, FT580, LP590. Other microscope companies furnish equivalent filter sets. Standard filters were deliberately chosen when choosing the filter combinations for fluorescence screening so that even smaller laboratories, such as those of the police, could readily analyze suspected material. As a control, marijuana samples were extracted in petroleum ether and the mother liquor was concentrated in vacuo at room temperature. This concentrate was then dissolved in 1 ml of ethanol and drops of the alcoholic fraction were placed singly on microscope slides and evaporated to dryness. These slides were used as a standard for the cannabinoid detecting tests described above. Authentic and non-authentic marijuana plants were also tested to determine the content of cannabinoids by TLC and GLC using classical procedures[7].

The response of fresh and dried plant material to cannabinoid indicators is reported in the table. Samples confiscated from the illicit drug trade, when mounted in water or in a cold clearing solution and examined by direct fluorochromy, show a strong autofluorescence of a wide variety of compounds normally present in fresh and dried plant tissues. The cold clearing solution does not give rise to the induced fluorescence phenomena, and cannabinoids 'per se' are not fluorescent.

The 'drug cultivar' specimens show a very peculiar fluorescence reactivity when processed by the IFIM procedure. The best results are obtained with the No.2 and No.3 filter sets. The 1st combination is recommended as standard violet excitation, transmitting only the range between 390 and 440 nm. The emission is selected by a long-wave pass filter from 475 nm. With this set a small amount of cellular component can emit a weak blue-green autofluorescence that is resistant to chloral hydrate treatment and reduces the contrast. On the contrary, the No.3 set shows a higher contrast producing a very dark background. The transmittance range, 450-490 nm with band pass shape, furnishes a more specific exciting radiation which gives rise to a limited amount of unspecific fluorescence. The barrier filter at 520 nm stops the blue-green fluorescence, enhancing the contrast. If the available filter set permits it, better results are obtained by changing the LP520 filter to an LP590 filter. In fact, cannabinoids show a range of excitation between 435 and 490 nm, and a fluorescence emission spectrum between 680 and 700 nm.

With the No.3 standard filter set, the glandular head of stalked hairs emits a strong red fluorescence, while the cystolith hairs appear pale green. Chlorophyll and other autofluorescent compounds are destroyed by heating with chloral hydrate so that the red fluorescence of the glandular head can be observed more easily. The red fluorescence is particularly evident in the case of the illicit marijuana glandular hairs while, in 'fiber cultivar' plants the fluorescence emission is weak and pinkish. Very young leaves of 'drug cultivar' marijuana show different stages in glandular differentiation. Immature hairs fluoresce green, while mature glandular heads appear strongly red fluorescent. The specimens processed by the RIM and vanillin tests show a lower capacity for localization of the glandular hairs in comparison to that exhibited by the IFIM test. In addition, observation in transmitted light introduces artifacts due to the typical diffraction of thick specimens, and sometimes the chromatic reaction is difficult to detect. When the samples processed with the RIM test are observed by fluorescence microscopy, the glandular heads emit a red fluorescence having an intensity comparable to that resulting from the IFIM test. Since the treatment by hot clearing solution is also needed in the RIM test, and since Fast Blue B, the chromogenic reagent of the RIM test, is not fluorescent, it is possible to use the same specimens for the 2 different types of histochemical analyses, by conventional transmitted light and by fluorescence incident emission.

These results support the conclusion that the histofluorescent method presented here can be usefully employed in parallel with, or as an alternative to the classical RIM test. The specificity of the IFIM and RIM tests is comparable, but the examination by induced fluorescence permits a clearer detection of glandular heads, being independent of the efficiency of a very unstable reagent such as Fast Blue B. In addition to previous methods for characterizing cannabis resin, the IFIM test may also be used to follow cannabinoids in 'ripening' glandular hairs. The mechanism by which red fluorescence is induced in glandular hairs of authentic marijuana is not described here, but it may be assumed that, as in the RIM test, the heating process causes important changes in cannabinoids. It is well known[16-18], in



Microscopical appearance of stalked glandular trichomes of marijuana plant material. *A* After the RIM test. Note that the cannabinoid-containing glandular heads (arrows) can be easily confused with the other glandular appendages. *B* The same specimen as in A, observed after the IFIM test. The cannabinoid-rich glandular heads emit a strong red fluorescence that permits a clearer identification (× 100).

fact, that the heating step leads to decarboxylation of any cannabinoid acids present in marijuana, forming the corresponding neutral phenolic cannabinoids. With the RIM test, the localization of cannabinoids is possible using Fast Blue B, a chromogenic reagent for phenols. Using the IFIM test, cannabinoid identification is achieved by induced fluorescence probably caused by the condensation of the neutral phenolic cannabinoids. The formation of highly fluorescent derivatives from the cannabinoid condensation is already documented in literature[19]. To shed some light on cannabinoid fluorescence induced by heating, studies are in progress in our laboratories.

1 Paper supported by grants from Consiglio Nazionale delle Ricerche (Contract No. 82.02016.04) and Ministero della Pubblica Istruzione of Italy awarded to Prof. G. Dall'Olio.
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0014-4754/83/080886-03$1.50 + 0.20/0

Heme oxygenase activity is decreased by D-penicillamine in neonates

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Summary. A 3-day D-penicillamine treatment of neonatal rats caused a significant decrease in heme oxygenase activity. This change was not observed in adult rats. The data indicate age-related differences in the effects of D-penicillamine.

Since 1973, D-penicillamine (D-PA) has been used in our department for the treatment of neonatal hyperbilirubinemia[1], and on the basis of more recent experimental and clinical observations for the prevention of retrolental fibroplasia[2]. In our previous animal experiments we found that D-PA enhances the activities of heme-containing enzymes, playing an important role in the defence against oxygen toxicity[3]. Since heme metabolism is a crucial stage in bilirubin production, it was of interest to examine the activity of heme oxygenase (E.C. 1.14.99.3.), the initial and rate-limiting enzyme of heme degradation.

Materials and methods. Experiments were performed on neonatal and on 6-week-old adult male CFY rats. The animals received 1000 mg/kg D-PA i.p. between 08.00 h and 10.00 h in a single dose daily for 3 days. The treatment of the neonatal rats was introduced on the 1st day of their lives. The control group was treated with the same volume of physiological NaCl solution. On the day following the final injection the animals were exsanguinated after decapitation, and the livers were washed with ice-cold phosphate buffer, blotted dry, and weighed. Livers not used immediately were stored at −20 °C.

Heme oxygenase was determined as described by Eaton et al.[4]. Livers were homogenized in 4 vol. of phosphate buffer with a motor-driven Potter-Elvehjem tissue homogenizer. Homogenates were kept on ice until used. The liver homogenate was centrifuged at 15,000 × g for 15 min at 4 °C. A 2-ml aliquot of the supernatant was used for the determination of heme oxygenase activity.

Protein content was measured by the method of Lowry et al.[5]. D-PA (Metalcaptase®) was a gift from Knoll AG, Ludwigshafen/Berlin. The chemicals employed were commercial products of Reanal (Budapest) and Sigma (USA), and were used without further purification.

Results and discussion. The results are given in the table. The 3-day D-PA treatment of the adult animals did not lead to any significant change in the heme oxygenase activity. In contrast, in the neonates a marked reduction in heme oxygenase activity was observed following D-PA treatment.

The rapid degradation of fetal hemoglobin and the oxidation of its heme moiety are contributing factors in the development of postparturition hyperbilirubinemia. It has been shown that in those species in which postparturition hemolysis takes place, the activity of heme oxygenase is enhanced in the newborn period[7]. Heme oxygenase, which constitutes the rate-limiting enzyme in the degradation of heme, utilizes as the substrate not only hemoglobin heme, but the heme moiety of cellular hemoproteins.

Changes in enzyme activities following 3-day D-penicillamine treatment

		Heme oxygenase nmoles bilirubin/mg protein/h
Adult	Control (n = 7)	29.0 ± 9.0
	D-PA (n = 7)	29.9 ± 10.9
Neonate (4-day old)	Control (n = 7)	24.8 ± 9.7
	D-PA (n = 8)	9.5 ± 2.20[a]

[a] $p < 0.01$ (Student's t-test).