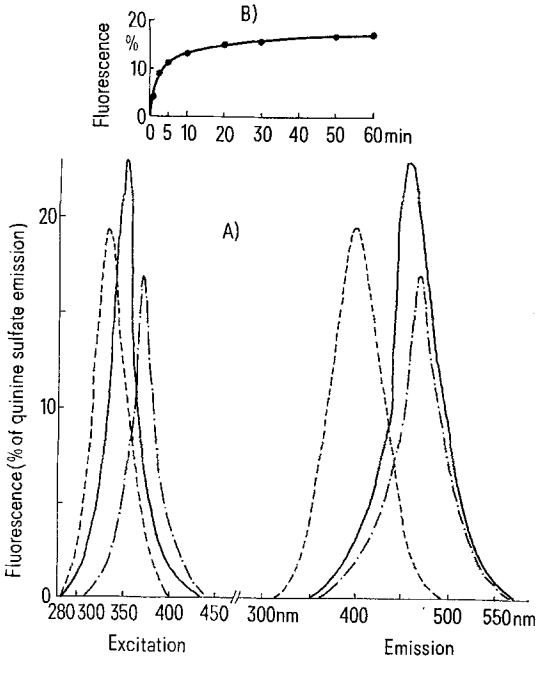
Preliminary Note on the Occurrence of a Biological Compound Showing Fluorescence Characteristics Similar to that of NADH

In the course of experiments on the relationship between amino acids and energy metabolism in nerve tissues, we have undertaken a study of the effect of various amino acids on the redox state of the cellular pyridine nucleotides, using a microfluorimeter similar to that constructed by JÖBSIS et al.[1]. Preliminary results showed a striking increase in signal emission upon addition of pyridoxal phosphate (P-5-P) to an incubating saline already supplemented with an amino acid. Further studies of that phenomenon indicate that the fluorescence signal results from a reaction occurring between P-5-P and the amino acid in the absence of any biological material. This preliminary report deals with some properties of the fluorescent material formed from P-5-P and glutamic acid.

The Figure shows both excitation and fluorescence spectra of P-5-P, NADH and of a mixture of P-5-P and glutamate in *Tris* buffer pH 7.4. P-5-P has fluorescence characteristics similar to those of the other compounds of the vitamin B-6 group[2]. As pyridoxine, pyridoxamine or pyridoxal, it indeed presents a fluorescence maximum in the 400 nm region for an excitation maximum around 330 nm. Addition of glutamate to P-5-P induces a shift in both excitation and fluorescence maxima which become respectively 370 and 468 nm. These values are close to those reported for NADH[3,4] (see Figure). This fluorescence emission is probably due to the Schiff-base which is known to form from pyridoxal or pyridoxal phosphate and an amino acid[5,6]. In the course of spectrophotometric studies, FASELLA et al.[7] indicated the fluorescence of such Schiff-bases. These authors, however, did not study the fluorescence characteristics of these compounds. On the other hand, it is known that the formation of the Schiff-base is enhanced in the presence of various metal ions such as Ni^{2+}, Cu^{2+} or Al^{3+}, with which it can form co-ordinated complexes[6,8]. The important increase in fluorescence signal we observe when adding $AlCl_3$ to a mixture of P-5-P and an amino acid can thus be interpreted as resulting from the formation of a Schiff-base chelate. In acetate buffer 20 m*M* at pH 5.0, addition of $AlCl_3$ 10^{-4} *M* to a reaction medium containing P-5-P (3 µg/ml) and an excess glutamate (3.75 m*M*) indeed increases the fluorescence emission from 40% to 67.5% of the emission of a standard quinine sulfate solution at 0.5 µg/ml H_2SO_4 0.1 *N*.

These preliminary results indicate that compounds of biological importance present fluorescence characteristics close to that of NADH. Moreover, the Schiff-base signal is observed not only in *Tris*, acetate or phosphate buffer at various pH, but also in deproteinized tissular extracts at pH 7.4. This makes it likely that the Schiff-base fluorescence emission also occurs in intact tissues when excitated around 360 nm. When considering the microfluorimetric technique, it seems impossible to discriminate between the Schiff-base and the NADH fluorescence emission from intact tissues by use of very selective filters. The emission maxima of these compounds are indeed very close, and such a procedure would considerably decrease the intensity of the signal on the 'fluorescence' photomultiplier. The rather slow development of the fluorescence emission from the Schiff-base (Figure B) cannot be used either to discriminate between a change in signal due to modification of the pyridine nucleotides redox state or to a variation in Schiff-base concentration. Indeed, maximum emission from the complex is immediately reached when aspartate aminotransferase, which catalyzes in biological material the transamination between glutamate and oxaloacetate, is added to the mixture of P-5-P and glutamate. Moreover this result brings further evidence for the formation of a Schiff-base complex as the first step not only of chemical transamination but also of enzyme catalyzed transamination.



A) Uncorrected excitation and emission spectra of pyridoxal phosphate, 3 µg/ml (– – –); NADH, 1.6×10^{-6} *M* (—) and of a mixture of pyridoxal phosphate, 3 µg/ml and glutamate, 3.75 m*M* (— · —). All the solutions are made up in *Tris* buffer 20 m*M* pH 7.4. The mixture pyridoxal-phosphate-glutamate is kept for 45 min in darkness before running the spectra. B) Time evolution of the fluorescence emission of the mixture pyridoxal-phosphate-glutamate. The results are expressed as % of the emission of a quinine sulfate solution at 0.5 µg/ml in H_2SO_4 0.1 *N*. The fluorimeter used is a Zeiss spectrofluorimeter equipped with 2 monochromators and a xenon arc lamp Osram XBO 450 W/P.

[1] F. JÖBSIS, V. LEGALLAIS and M. O'CONNOR, IEEE Trans. biomed. Engng. *13*, 93 (1966).
[2] D. B. COURSIN and V. C. BROWN, Proc. Soc. exp. Biol. Med. *98*, 315 (1958).
[3] O. H. LOWRY, N. R. ROBERTS and J. I. KAPPHAHN, J. biol. Chem. *224*, 1047 (1957).
[4] B. CHANCE, P. COHEN, F. JÖBSIS and B. SCHOENER, Science *137*, 499 (1962).
[5] D. E. METZLER, M. IKAWA and E. E. SNELL, J. Am. chem. Soc. *76*, 648 (1954).
[6] D. E. METZLER, J. B. LONGENECKER and E. E. SNELL, J. Am. chem. Soc. *76*, 639 (1954).
[7] P. FASELLA, H. LIS, N. SILIPRANDI and C. BAGLIONI, Biochim. biophys. Acta *23*, 417 (1957).
[8] G. L. EICHHORN and J. W. DAWES, J. Am. chem. Soc. *76*, 5663 (1954).

It should be pointed out that in so-called physiological conditions of pH and concentration, the NADH signal is much larger than that produced by the Schiff-base. Indeed, NADH 10^{-5} *M* in *Tris* buffer 20 m*M* pH 7.4 gives a fluorescence signal of 137% of that of the standard quinine sulfate solution, while the emission of a mixture of P-5-P 3 μg/ml and glutamate 3.75 m*M* is only 19.5% of the emission of the reference solution. Nevertheless, not only glutamate but also the other amino acids can participate in the formation of Schiff-bases, and care should be taken in the interpretation of the results of experiments which are not inducing changes in tissue fluorescence emission large enough to be safely attributed to a modification in the redox state of the pyridine nucleotides.

In the course of our experiments, a good correlation between the emission intensity at 468 nm and the concentration of P-5-P in the concentration range between 0.01 μg to 1 μg/ml has been observed in a reaction medium heated at 50 °C for 5 min and containing glutamate 5 m*M* and $AlCl_3$ 2 m*M* in acetate buffer 20 m*M* at pH 5. This indicates the possibility of the use of the fluorescence characteristics of the Schiff-base for the development of a specific technique of determination of free pyridoxal phosphate concentration in biological material.

Résumé. La base de Schiff formée à partir de pyridoxalphosphate et d'un acide aminé présente une fluorescence dont les caractéristiques spectrales sont similaires à celles du NADH. Les possibilités éventuelles de faire la distinction entre le signal émis par la base de Schiff et le NADH au cours de travaux réalisés avec les microfluorimètres utilisés pour étudier les modifications du niveau d'oxydoréduction des nucléotides de la pyridine sont discutées.

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A Simple Technique for Demonstrating Heterochromatin in *Nigella*

This is to report a new technique which enabled us to document the presence of heterochromatin in *Nigella*, a species in which a lack of demonstratable heterochromatin was reported previously[1]. The technique is a modification of the cytological procedures used for the demonstration of heterochromatin in rye chromosomes[2].

Actively growing primary root tips of *Nigella damascena* var Miss. Jekyl, were pretreated in monobromonaphtalene solution for 3 h at room temperature and fixed in glacial acetic acid overnight (22–24 h). After fixation, the root tips were softened by immersing in an enzyme solution[3] for ½ h at room temperature. The root tips were then squashed. The cover slips were separated from the slides by CO_2 freezing, dehydrated in absolute ethanol for 30 min and dried by blowing air. The chromosome preparations, were then immersed in a freshly prepared saturated barium hydroxide solution for 5 min at room temperature. After through rinsing in several changes of distilled water and air drying, the slides were incubated in a 2X SSC solution[4] for 1 h at 60 °C, washed thoroughly in distilled water, air dried, stained in a Giemsa staining solution[5] for 2 min, rinsed and air dried. The stained slides were left in Xylene overnight and mounted in Canada balsam.

All the chromosomes of *Nigella damascana* var. Miss. Jekyl (5 pairs metacentric and 1 pair acrocentric) showed the presence of heterochromatin (C-bands) in the centromeric area (Figure). Another characteristic feature of the heterochromatin: chromocenter formation in interphase nuclei, were also seen. Their number varied from 3 to 5 per nucleus (Figure).

Zusammenfassung. Feststellung von C-Banden in *Nigella damascena* var. Miss Jekyl mittels eines neuen zytologischen Verfahrens. Es waren 3–5 Chromozentren pro Nukleus zu erkennen.

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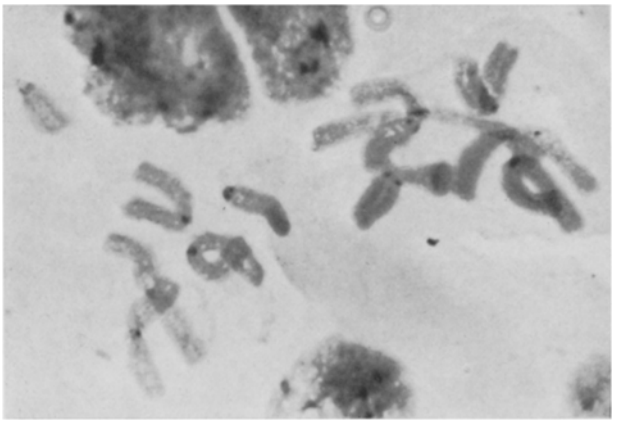

Metaphase spread showing the centromeric localization of heterochromatin. Chromocenters are visible in the interphase nuclei.

[1] A. T. Natarajan and G. Ahnstrom, Chromosoma *28*, 48 (1969).

[2] B. S. Gill, G. Kimber, Proc. natn. Acad. Sci., USA, in press.

[3] The enzyme mixture is prepared by mixing equal parts of 5% pectinase and cellulase solutions to which 2–3 drops of 1*N* HCl were added for each 5 ml of the mixture.

[4] The 2X-SSC solution is made with 0.887 g of NACl and 0.411 g of trisodium citrate in 100 ml of distilled water.

[5] The stock Giemsa solution is made by mixing 1 g Giemsa powder (Fisher) in 66 ml of glycerine and heating it for 2 h in a 60 °C oven with frequent agitation. Then 66 ml of absolute methanol is added and this mixture is cooked for 2 days in 60 °C oven with frequent agitation. The working staining solution is made by mixing 5 ml of stock Giemsa solution, 1.5 ml MacIlvaine buffer (pH 7.0), 1.5 ml absolute methanol and 50 ml of distilled water. This staining mixture is filtered and used.