In addition to morphine and trimethobenzamide, codeine, propoxyphene, meperidine, phenazocine, and levorphanol were identified in the urines of patients receiving one or more of these drugs. A two-dimensional chromatogram is shown in Figure 2 in which development was carried out first with solvent S4 and then with solvent S3, which allowed the separation and identification of each of the components of a ten-component mixture.

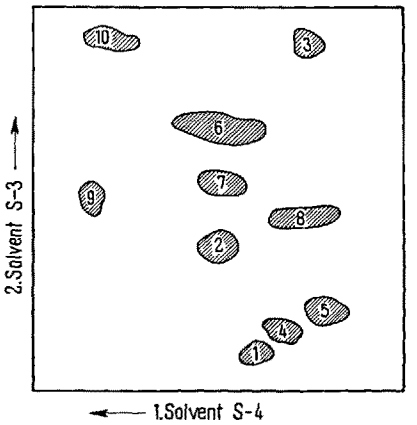

Fig. 2. Two-dimensional chromatogram (silica gel G) of (1) morphine, (2) meperidine, (3) cocaine, (4) codeine, (5) levorphanol, (6)l methadone, (7) chlorpromazine, (8) tripelennamine, (9) nalorphine, and (10) propoxyphene.

Discussion. Thin-layer chromatography was found to be a useful technique for the identification of a variety of drugs in urine extracts. The method is very fast and can be completed conveniently in 5–6 h. It is quite sensitive, detecting 5–10 μg of substance. No interfering substances have been found in control urines which were known to contain caffeine and nicotine[10]. Although the Rf values may be altered by salts and other contaminants of the urine extracts, this can be circumvented by co-chromatographing the reference substances with extracts of control urines. The Rf values were also found to vary somewhat from plate to plate, so that for positive identification it is necessary to chromatograph the reference compounds on the same plate with the unknown mixture.

The patient whose urine was analyzed for morphine had received 15 mg of morphine sulfate parenterally, and the urine was then collected for the subsequent 4 h. Despite the relatively small amount of drug involved and the presence of many other drugs that had been given at the same time, no difficulty was encountered in identifying the morphine excreted, even without hydrolysis of the urine. In the case of addicts taking heroin, identification would be somewhat more difficult because of the small amounts of drug involved, but concentration of large amounts of urine can be effected by flash evaporation.

Thin-layer chromatography is also very useful for *in vitro* and *in vivo* studies of drug metabolism. Such closely related compounds as morphine and normorphine, levorphanol and methorphan, and morphine and dihydromorphinone are easily separated. McMAHON[11] has separated propoxyphene and the propoxyphene N-oxide, which were not separable on paper. This technique, with slight modifications in the usual extraction procedure[12], is also useful for the identification of the alkaloids in the opium poppy itself. The analysis of barbiturates and phenothiazene derivatives using the technique of thin-layer chromatography is now under investigation in this laboratory[13].

Zusammenfassung. Es wird eine einfache, schnelle und empfindliche Methode zur Isolierung und Identifizierung von Analgetica mittels der Dünnschichtchromatographie beschrieben. Die Methode ist zur Charakterisierung dieser Stoffe im Harn und für toxikologische, forensische und Stoffwechseluntersuchungen brauchbar.

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Laboratory of Chemistry, NIAMD, National Institute of Health, Bethesda (Maryland USA), August 18, 1961.

[10] Nicotine has been identified in very concentrated urine extracts of patients but does not interfere at normal levels.

[11] R. E. McMAHON, personal communication.

[12] K. GENEST and C. G. FARMILO, Bulletin on Narcotics *8*, 15 (1960).

[13] *Note added in proof:* Direct extraction of tissues (homogenized 1 in 4 with isotonic KCl) and of whole blood has been carried out at pH 9.0 as described for urine. Prior precipitation of proteins is not necessary. Using this procedure, isolation and identification of analgesics added to tissues and blood and of parenterally administered drugs has been effected.

Amethyst Violet as a Stain for Distinguishing Cells with a Damaged Membrane from Normal Cells

In routine serial passage in mice of Ehrlich's ascites tumour cells, it is indispensable to recognize with fairly close approximation the number of live cells inoculated. This need has given rise to various staining techniques for differentiating between live and dead cells. Eosyn Y[1,2] has been used especially and, more recently, nigrosine[3]. The latter, which stains dead or non-vital cells black because of the greater ease in passing through their damaged membranes, is the most widely used at present, also because of its low toxic action.

For the last two years I have successfully used an amethyst violet solution in the routine technique for serial transplantation of Ehrlichs ascites tumour cells. I observed that this stain gives an ever clearer and more constant differentiation between the two kinds of cells; moreover, it is possible to obtain a supra-vital staining of leukocytes. Independently of their vitality, they are more permeable to the stain and it is therefore easier to exclude them, together with the dead cancer cells, from cellular counts of ascites fluid.

Amethyst violet is a basic stain (contrary to eosin Y and nigrosine, which are acid stains), belonging to the azynes series. Its molecular weight is 434.997. It was used by BRENNER[4] for supravital staining of mitochondria.

[1] H. M. PATT, M. E. BLACKFORD, and R. L. STRAUBE, Proc. Soc. exp. Biol. Med. *80*, 92 (1953).

[2] R. SCHRECK, Amer. J. Cancer *28*, 389 (1936).

[3] J. P. KALTENBACH, MERLE H. KALTENBACH, and W. B. LYONS, Exp. Cell Res. *15*, 112 (1958).

[4] S. BRENNER, Stain techn. *163* (1950).

A very dilute solution (1:10000) stains mitochondria of lymphocytes dark purple. I have used amethyst violet for isolated mitochondria *in vitro* myself[5], with satisfactory results. But I have not been able to stain mitochondria of whole cells, except leukocytes which, as already stated, are more permeable to the stain. By suitably modifying the permeability of the cell membrane, a diffuse staining of the whole protoplasm may be obtained.

The method is carried out as follows. An aqueous 1% stock solution of amethyst violet is made up. Immediately before use, to 1 ml stock solution are added 9 ml NaCl isotonic solution or Ringer's fluid, buffered with phosphate buffer pH 7.

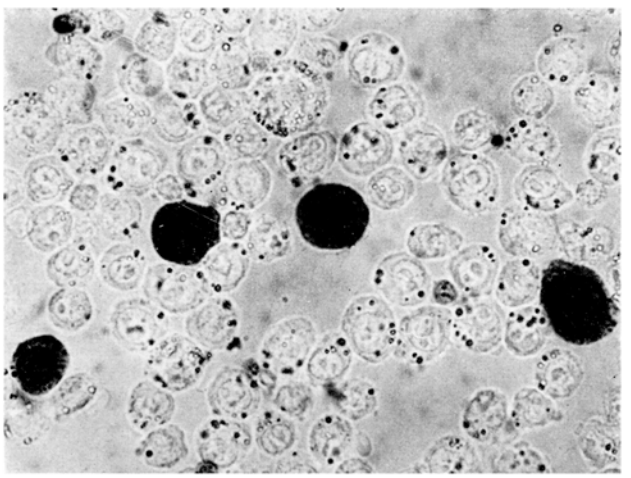

Fig. 1. Ascites cells suspended in isotonic medium with amethyst violet: only dead cells are stained (· 300).

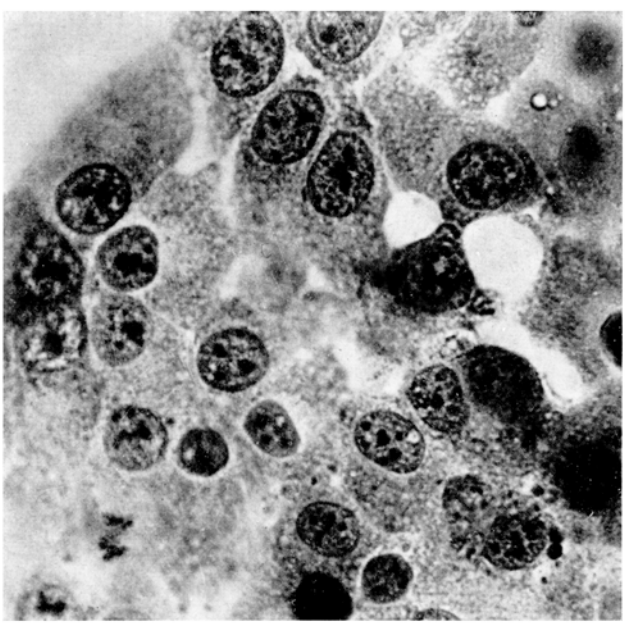

Fig. 2. Epithelial cells of a culture of human kidney stained with amethyst violet after immersion in liquid nitrogen, without any fixing reagents (×380).

The ascites tumour cells are washed in physiological saline and then diluted 10 or 20 times with the amethyst violet solution. The count must be made immediately and on no condition more than 10 min after contact with the stain. After a longer period, the dye may pass into other cells, whose permeability has meanwhile altered. Even so it is possible to differentiate cells which were dead from the start, as they are more heavily stained.

Various kinds of cells were used for testing permeability: cells from mice with advanced ascites (higher percentage of dead cells and leukocytes), cells after washing in water instead of saline, cells suspended for 1 or more hours at room temperature (18–22°C), irradiated cells (2600 r to 4000 r, according to the radiosensitivity test carried out on the same tumour strain by DI VITA and PRANDINI[6]) cells after immersion in liquid nitrogen for a few seconds. I was thus able to observe a constant proportion between duration of the damaging action and staining properties of cells. The most complete staining of cells was found to occur after treatment with liquid nitrogen. In this case, though, there was no diffuse and massive staining, as there is in naturally dead cells, or in cells killed by ageing *in vitro*, by irradiation or by immersion in hypotonic solution. A far more delicate staining appeared, which showed up selectively the various cell structures distinguished by cytological examination.

Treatment with liquid nitrogen, together with quick fixation in ethyl alcohol 95°, was used in previous studies with DI VITA[7] so as to obtain with ease a good fixation of especially delicate material (smears of ascites tumour cells, protozoa, etc.). The action of liquid nitrogen prevents shrinkage of the cells and therefore preserves, as much as possible after fixation, the shape and structure they had during life. By using amethyst violet I was able to obtain a good cytological staining, even without fixing the cells. Staining may be carried out directly on cell suspension after treatment with liquid nitrogen for about 30 sec. The stain is dropped straight on to the coverslip, after it has unfrozen.

This method has also been successfully applied to the morphological study of cells cultured *in vitro*. I have especially satisfactory results with human kidney cells (Figure 2) and with chick and mouse fibroblasts.

Riassunto. Per distinguere le cellule a membrana integra da quelle con membrana alterata può essere vantaggiosamente usata una soluzione isotonica di violetto ametista: il colorante trova utile applicazione nel conteggio delle cellule vive e morte per il trapianto del cancro ascite di Ehrlich. Modificando la permeabilità delle cellule mediante azoto liquido si possono inoltre ottenere delle buone colorazioni delle strutture cellulari indipendentemente dall'uso dei comuni fissatori.

A. NOVELLI

Istituto di Patologia Generale dell'Università di Genova (Italy), March 13, 1962.

[5] L. MICHELAZZI and A. NOVELLI, Atti 2° Congr. naz. Soc. it. Istoch. in Riv. Istoch. norm. pat. *5*, 429 (1959).

[6] G. DI VITA and B. D. PRANDINI, Boll. Soc. Ital. Biol. sper. *35*, 948 (1959).

[7] G. DI VITA and A. NOVELLI, unpublished results.