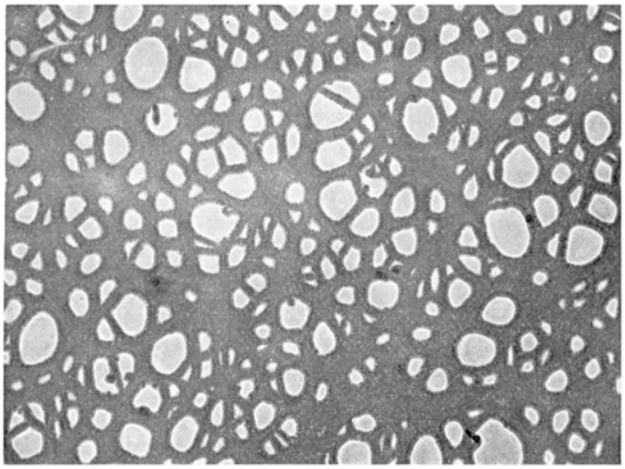


Fig. 1. Holey film prepared with water emulsion (×40000).

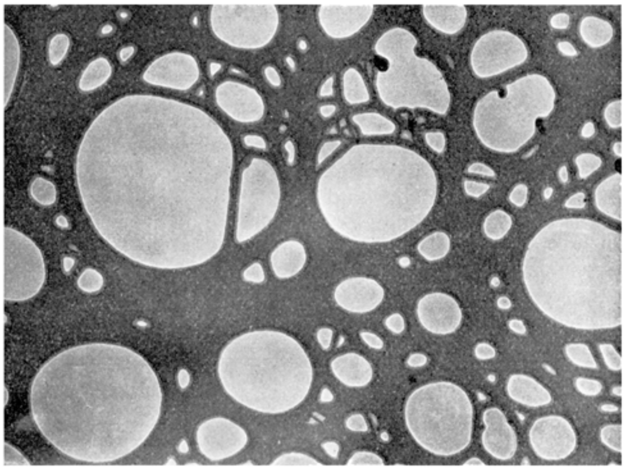


Fig. 2. Holey film. For preparation see text (×40000).

Fifth Avenue, New York 19). We have found it convenient to hold the grid 1 cm from the mouth of the nebulizer and spray three or four times at 10 sec intervals. Too much spray causes the film to break down[6].

Zusammenfassung. Vier verschiedene Methoden zur Gewinnung von Lochfolien für elektronenmikroskopische Belange werden beschrieben. Damit lassen sich unter anderem leicht Anzahl der Löcher pro Flächeneinheit und Lochdurchmesser variieren, so dass eine weitgehende Anpassung der Filmqualität an die sonstigen Versuchsbedingungen ermöglicht wird.

M. E. BAYER and T. F. ANDERSON

The Institute for Cancer Research, Philadelphia (Pennsylvania, U.S.A.), February 25, 1963.

---

[6] This work was supported in part by grant NSF-G12491 from the National Science Foundation.

Preparation of Tritiated Vincaleukoblastine[1] by the Wilzbach Technique

The alkaloid vincaleukoblastine (VLB) has created considerable interest as a chemotherapeutic agent in experimental neoplasias[2]. It was felt that the studies on the mechanism of action of these new agents would be greatly aided if radiolabeled alkaloid were available. Since direct synthesis of specifically labeled VLB is not possible at this time, the use of the Wilzbach gas exposure method[3–5] for tritium labeling was explored.

Experimental and Results. Crystalline $VLB:SO_4$ (950 mg) and amorphous VLB base (650 mg) were each exposed to 15 c of tritium gas (660 mm Hg) for a period of 14 days at 27° by New England Nuclear Inc. Exchangeable tritium was removed by equilibration with methanol. The sulfate salt was purified by recrystallizing six times from methanol-ethanol mixtures. Insoluble materials were removed by filtration of the hot solutions. The tritiated VLB base was converted to the sulfate salt and it too was purified by crystallizing six times from methanol-ethanol. The progress of the purification was followed by determining the specific activity after each crystallization (Table).

The progress of the purification was also followed by thin layer chromatography on silica gel. A small amount of the hot VLB sulfate was added to a mixture of equal parts of cold VLB sulfate and cold dihydro-VLB sulfate and about 25 μg of the material was applied to the plate. A drop of ether saturated with ammonia was then applied to the spot to liberate the free bases. After drying, the chromatogram was developed with methanol. The VLB and dihydro-VLB spots were then located by placing the plate in a chamber containing iodine vapor. The distribution of radioactivity on the plate was determined by scraping 1 cm lengths of silica gel into counting vials. Care was taken to make a clean separation between the VLB spot and the dihydro-VLB spot. After adding 5 ml of scintillator solution to each vial, the radioactivity was

Specific activities of VLB-t following recrystallization

Recrystallization Number	VLB tritiated as sulfate salt	VLB tritiated as free base
0	193 (950 mg)	1230 μc/mg (650 mg)
1	29.6	143
2	23.3	92.3
3	20.0	78.7
4	18.0	62.8
5	17.5	62.8
6	15.5 (320 mg)	61.4 (40 mg)

---

[1] Supplied as VELBAN (vinblastine SO_4-Lilly).

[2] G. H. SVOBODA, I. S. JOHNSON, M. GORMAN, and N. NEUSS, J. Amer. pharm. Assoc. *51*, 707 (1962).

[3] K. E. WILZBACH, J. Amer. chem. Soc. *79*, 1013 (1962).

[4] M. L. WHISMAN and B. H. ECCLESTON, Nucleonics *20*, 98 (1962).

[5] S. ROTHCHILD (Editor), *Advances in Tracer Methodology*, vol. 1 (Plenum Press, New York 1963), p. 4.

determined in a β-ray spectrometer. The Figure presents the results of thin layer chromatography of each sample of tritiated VLB before purification and again after six recrystallizations.

Discussion. When either VLB or VLB sulfate was tritiated by the Wilzbach method the bulk of the radioactivity in the product is not associated with VLB. Two major tritiated by-products, one fast moving, the other slow moving, are formed (Figure). Both are readily removed by recrystallization of VLB as the sulfate salt.

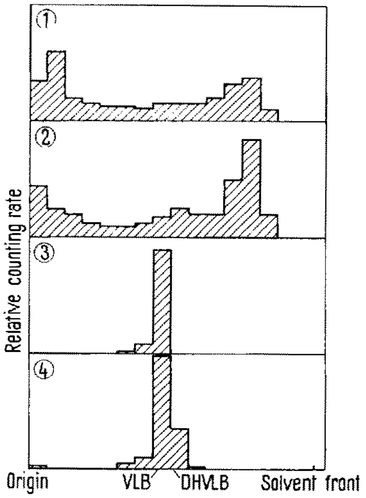
There are several interesting differences between the results obtained by tritiation of the salt compared to the free base. For example, six recrystallizations of VLB sulfate obtained from exposure of the free base produces material of nearly constant specific activity, while in case of the material exposed as the salt constant specific activity is not reached. The specific activity of the product is also considerably higher in the former case. The most notable difference, however, is that the product from exposure of the salt contains substantial amounts of dihydro VLB while the material exposed as free base is essentially free of this reduced by-product (Figure). Since reduction of double bonds is known[5] to be an important side reaction during Wilzbach tritiation, dihydro VLB had been expected to be present as an impurity. The structure of VLB is now well enough known[6] so that it can be said that the double bond involved in dihydro-VLB formation is in the vindoline portion of the molecule. This double bond is in a position allylic to a basic nitrogen:

$$\begin{tikzcd}
\mathrm{N{-}CH_2CH{=}CH{-}} \arrow[r, "\text{Salt}", "\text{Formation}"'] \arrow[d, "T_2"] & \overset{+}{\mathrm{N}}\mathrm{{-}CH_2{-}CH{=}CH{-}} \arrow[d, "T_2"] \\
\mathrm{N{-}CHTCH{=}CH{-}} & \overset{+}{\mathrm{N}}\mathrm{{-}CH_2{-}CHT{-}CHT{-}}
\end{tikzcd}$$

It is not possible at present to explain why the double bond in the salt form is susceptible to reduction while that in the free base is not. It is interesting to note, however, that the catalytic reduction of the similarity placed double bond of vindoline goes readily in acid solution but not in the presence of base[7]. Furthermore, in recent experiments in these laboratories by NEUSS, it has been found that the catalytic reduction of VLB to dihydro-VLB proceeds only when VLB is present in the salt form.

The reason for the higher specific activity obtained during tritiation of the free base is not apparent, but it is possible that in this case the methylene carbons adjacent to nitrogen are more readily exchangeable than is true when the nitrogen is ionized. Another possibility is that the base in the amorphous form offers more surface for exchange than does the crystalline salt form.

Thus it has been possible to prepare tritiated VLB of constant specific activity which contains no dihydro-VLB. It is of course possible that other impurities which co-crystallize with VLB:SO_4 and co-chromatograph with VLB may be present[8].



Thin-layer chromatography of various vinkaleucoblastine samples. (1) VLB:SO_4 after tritiation; (2) VLB base after tritiation; (3) same as (2) but after six recrystallizations as sulfate salt; (4) same as (1) after six recrystallizations.

Zusammenfassung. Das Alkaloid Vincaleukoblastine (VLB) wurde als Base bei Anwendung der Wilzbach-Technik titriert. Die Verbindung, bis zur konstanten Aktivität gereinigt, enthielt kein Dihydro-VLB mehr. Das Alkaloid, als Sulfatsalz dem Tritium unterworfen, inkorporiert die Substanz nur geringfügig, wobei das Tritium enhaltende Dihydro-VLB ensteht.

R. E. McMAHON

Lilly Research Laboratories, Indianapolis (Indiana, U.S.A.), March 18, 1963.

---

[6] N. NEUSS, M. GORMAN, H. E. BOAZ, and N. J. CONE, J. Amer. chem. Soc. *84*, 1509 (1962).

[7] M. GORMAN, N. NEUSS, and K. BIEMANN, J. Amer. chem. Soc. *24*, 1058 (1962).

[8] *Acknowledgment.* We wish to thank Dr. N. NEUSS of these laboratories for samples of dihydro-VLB:SO_4 and for information concerning its preparation.

Determination of Adenine in Hydrolyzates of Ribonucleic Acids with the Aid of Comparative Titration

One of the principal methods of research into nucleic acids is the determination of the molecular proportions of the bases of these acids. At present, several methods are used to determine the proportions of bases. The most widely used method is that of paper chromatography which, upon separation of the individual bases, permits their measurement—spectrophotometry in UV. Another method used in practice consists in fractionation of a mixture of bases with the aid of an ion exchanger and, upon elution, in repeated measurements in UV. These methods yield sufficiently accurate results; however, their performance is rather time consuming.

For this reason, it is advisable to look for new methods which would essentially speed up the process of analysis. Thus, the method of direct spectrophotometry of RNA hydrolyzate has been worked out; however, it is little used, for the treatment of the results is rather intricate, and moreover the determination in the presence of substances absorbing in UV is inaccurate.

Polarographic methods for the determination of the components of nucleic acids were used for the first time by