sind jedoch keine Ergebnisse veröffentlicht worden, die mit dieser Methode am Tabakrauch erhalten wurden. Ein anderes, aussichtsreiches Verfahren arbeitet gaschromatographisch[32].

Die bisherigen grob quantitativen Ergebnisse mit diesen Identifizierungsmethoden erscheinen jedoch zuverlässig genug, um auszusagen, dass der Tabakrauch auch im Falle basen- und nitratreicher Tabake höchstens Mengen um 1 µg N-Nitroso-Verbindungen pro Zigarette enthält. Die höheren, von SERFONTEIN und HURTER im Zigarettenrauch gefundenen Werte müssen nach den vorhergehenden Ausführungen aus methodischen Gründen auf Artefakte zurückgeführt werden.

Über die biologische Wirkung dieser von uns als wahrscheinlich angenommenen Mengen von Nitrosaminen im Tabakrauch kann bisher noch keine Aussage gemacht werden. Alle bisher beschriebenen Tierversuche sind mit wesentlich grösseren, chronischen Gaben von Nitrosaminen durchgeführt worden, so dass Vergleiche kaum gezogen werden können. Da die einzelnen Nitrosamine eine ausserordentlich starke organotrope Wirkung entfalten, kann bisher nicht gesagt werden, ob eine solche Organotropie auch beim Menschen denkbar ist.

Bei der heute meist angewandten epikutanen Applikation von Tabakrauchkondensaten an der Maus und bei subkutaner Prüfung können Nitrosamine keine lokale Wirkung entfalten[33]. Es sind noch eine Reihe von analytischen und tierexperimentellen Problemen zu lösen, die sich durch die ausserordentlich geringen Mengen N-Nitroso-Verbindungen, wie sie im Tabakrauch vorkommen, stellen, bevor eine eindeutige Aussage über die Möglichkeit einer biologischen Wirkung der im Rauch enthaltenen Nitrosamine getroffen werden kann.

Summary. Important facts in connection with the occurrence of N-Nitroso compounds in tobacco smoke have been summarized. This paper reports on analytical methods for the identification of N-Nitroso compounds. The figures known about nitrate content and volatile bases of tobacco, as well as the nitrogen oxides and volatile bases of tobacco smoke as precursors of N-Nitroso compounds, are summarized. The third order reaction of secondary amines with an equimolar mixture of nitric oxide and nitrogen dioxide is a precondition for the formation of N-Nitroso compounds in tobacco smoke. While examining tobacco smoke for N-Nitroso compounds, temperature and time conditions have to be adapted to the natural smoking process. The use of solvent as well as cold traps has to be avoided to exclude the formation of artifacts. Analyses completed under these conditions furnished results of 0.004 µg of a mixture consisting of N-Nitroso-dimethylamine and N-Nitroso-pyrrolidine, calculated as N-NO per cigarette. A number of animal-experimental preconditions are still lacking, to judge the biological effects of these extraordinarily small amounts of N-Nitroso compounds.

G. NEURATH

Zentrallaboratorium H. F. & Ph. F. Reemtsma, Hamburg (Deutschland), 3. Januar 1967.

[32] E. NESEMANN, Privatmitteilung.

[33] J. H. WEISBURGER und E. K. WEISBURGER, Chem. Engng News *1966*, 124.

PRO EXPERIMENTIS

A Simple Method of Purifying Human Sera for the Assay of Long-Acting Thyroid Stimulator

Most investigators screening sera from patients suspected of Graves' disease for the presence of the long-acting thyroid stimulator (LATS)[1] employ the mouse bioassay introduced by MCKENZIE[2]. The use of this assay is complicated by the appreciable toxicity of many human sera to the assay animals, as well as by the occurrence of 'non-specific responses' associated with the albumin fraction of some sera[3]. In our experience more than $^1/_3$ of sera tested have killed half of the assay animals or more when 0.5 ml of untreated serum was administered by tail vein injection. This toxicity has made necessary the employment of laborious detoxification procedures, including chromatography on Sephadex columns[4] and alcohol fractionation of sera at low temperatures[3]. Only the last method would be expected to remove the 'non-specific' stimulatory albumin fraction.

We have used a modification of the procedure of BAUMSTARK, LAFFIN and BARDAWIL[5] to effect a rapid and convenient detoxification of sera for the MCKENZIE assay. Since the method was devised for the purification of 7 S γ-globulins from serum in high yield it would be expected to be applicable to the purification of LATS which has been shown to be an Immunoglobin G[6].

DEAE-Sephadex A-50 in the chloride cycle was equilibrated with M/100 phosphate buffer of pH 6.5. 2 ml of a 50% (v/v) suspension of the gel was delivered into 16 · 125 mm test tubes. After centrifugation the supernatant buffer was discarded. 5 ml of serum was added to the gel and the mixture shaken for 10 min and then centrifuged. The supernatant was transferred to a second tube containing 1 ml of packed DEAE-Sephadex and the shaking and centrifugation repeated. The final supernate was used for injection into the test mice. It was well tolerated, the mortality of test animals in a 21 h assay being about 3%, which is not significantly different from the mortality of saline-injected controls.

To assess possible losses of LATS activity due to adsorption on DEAE-Sephadex we assayed 16 sera before

[1] D. D. ADAMS, J. clin. Endocr. Metab. *18*, 699 (1958).

[2] J. M. MCKENZIE, Endocrinology *62*, 865 (1958).

[3] D. D. ADAMS, T. H. KENNEDY and H. D. PURVES, Aust. J. exp. Biol. med. Sci. *44*, 355 (1966).

[4] J. M. MCENZIE, J. biol. Chem. *237*, *PC* 3571 (1962).

[5] J. S. BAUMSTARK, R. J. LAFFIN and W. A. BARDAWIL, Archs Biochem. Biophys. *108*, 514 (1964).

[6] J. P. KRISS, V. PLESHAKOV and J. R. CHIEN, J. clin. Endocr. Metab. *24*, 1005 (1964).

and after DEAE-Sephadex treatment. The post-treatment response was 96 ± 5% of the response obtained with untreated sera. Among the 7 sera of this series which gave highly significant LATS responses the recovery was 96 ± 8 (S.E.)%. One serum whose 21:0 h response ratio was 206% declined to 126% after gel adsorption, indicating that its response may bave been non-specific.

We have also explored to what extent the method can be used to free sera of radioactivity associated with iodide and thyroxine. In the assay of sera from patients treated with therapeutic doses of radioiodine serum radioactivity sometimes must be removed by decay or fractionation before assay for LATS becomes possible. However, the procedure as described removes only 60% of labelled thyroxine and 65% of iodide. This may be because in our experience the removal of non-γ-globulins has not been as complete as BAUMSTARK and collaborators[5] found it to be. Starch gel electrophoresis showed the presence of many serum components other than γ-globulin in the treated sera, although their concentration was only $^1/_3$ of the initial levels.

Recently we have used batches of DEAE-Sephadex, purchased from the same source which removed 95% of serum iodide and 98% of thyroxine when sera were shaken 3 times with the resin. Only 10.3% of total serum proteins remained after such treatment. The differences in the performance of various lots of DEAE-Sephadex have not yet been explained. However, even the least efficient lots of the resin eliminated serum toxicity to mice completely.

This simple technique appears to be useful for routine purification of sera for the McKENZIE LATS assay. It permits a significant reduction in the number of mice used to assay each serum without loss of precision. Theoretical considerations as well as our limited experience indicate that the method eliminates the 'non-specific' stimulators associated with serum albumin[3].

Résumé. Pour purifier les sérums pour l'étalonnage du LATS, on les agite deux fois avec l'échangeur DEAE-Sephadex. Le surnageant contient la quantité entière du LATS (96 ± 8%) et un taux fort réduit des autres protéines. Le traitement élimine la toxicité des sérums et la plupart de l'iodure et de la thyroxine sérique.

W. H. FLORSHEIM[7]

Radioisotope Service, U.S. Veterans Hospital and U.C.L.A. Medical School, Long Beach (California 90804, USA), 22nd November 1966.

[7] Aided by grant No. A-4455 from the U.S. Public Health Service.

A Method for Selective Staining of Damaged Yeast Cells

Yeast cells exposed to various agents (like phenothiazine derivatives, mercuric chloride and detergents) suffer loss of K^+. Theoretically it is important to know whether this loss can be characterized as an all-or-none response of individual cells, or as a gradual K^+ loss of all cells simultaneously[1]. With an all-or-none response, the % K^+ recovered from the medium will equal the % of cells that suffered major damage of the cellular membrane and lost their viability. If, however, each cell responds in a graded fashion, the relationship between membrane damage and viability on the one hand, and K^+ loss on the other hand, should be expected to be more complicated. Methods to evaluate membrane damage and loss of viability are either based on selective staining of damaged cells or on the counting plate method. As the methods described in the literature all have certain drawbacks, a new staining technique was developed, giving much better results.

Methods and results. The classical plating method[2] has the disadvantage that a certain concentration of the cytolytic agent has to be incorporated in the growing medium. Better results are obtained with a modification of the plate method, utilizing millipore filters. A suitable amount of yeast suspension is filtered through a millipore filter (HA) and washed with distilled water to remove any surplus of the cytolytic agent. Subsequently the filter is placed on a suitable agar growing medium in a Petri disc. After incubation for 48 h at 28 °C, the number of colonies can be counted and compared with the number of yeast cells originally present in the filtered suspension. The experimental error with this method is relatively high (about 5%) and especially with low fractions of non-viable cells (less than 10%) numerous, time-consuming counts have to be made.

Under these circumstances proportional counts of viable and non-viable cells by direct microscopic examination, after selective staining of non-viable cells, are much more practicable. The dyes most widely used for this purpose are methylene blue[3–5] and Nile Blue[5,6]. Both dyes have a number of disadvantages:

(1) Both dyes have cytolytic properties[4,6]. In the concentration range used in staining experiments, no cytolytic effects are observed. When, however, the cells have been previously exposed to other cytolytic agents, a potentiation of cytolysis by the subcytolytic dye concentration, is not unlikely.

(2) Nile Blue can penetrate into the intact yeast cells as a vital dye under certain experimental conditions[7]. This can result in too high counts of damaged cells. In our own experience with Nile Blue, uncoloured, dark-coloured, and many more or less faintly coloured cells were seen on microscopic examination. These faintly coloured cells were definitely not damaged, so that differentiation of viable and non-viable cells was difficult and subjective.

[1] H. PASSOW, A. ROTHSTEIN and T. W. CLARKSON, Pharmac. Rev. *13*, 185 (1961).
[2] C. H. COLLINS, *Microbiological Methods* (Butterworths Ltd., London 1964) p. 130.
[3] M. G. McFARLANE, Biochem. J. *30*, 1369 (1936).
[4] H. PASSOW, A. ROTHSTEIN and B. LOEWENSTEIN, J. gen. Physiol. *43*, 97 (1959).
[5] H. PASSOW and A. ROTHSTEIN, J. gen. Physiol. *43*, 621 (1960).
[6] T. G. SCHARFF and W. C. MAUPIN, Biochem. Pharmac. *5*, 79 (1960).
[7] A. LEMAN, Protoplasma *59*, 231 (1964).