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.

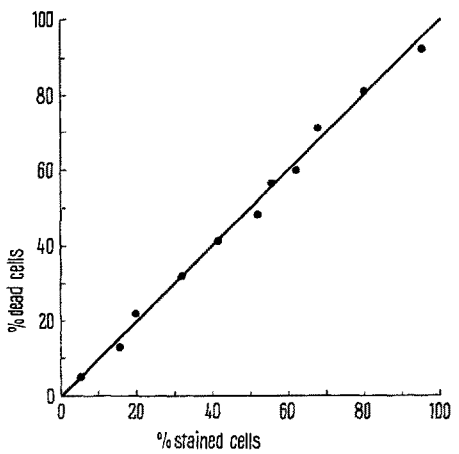
(2) Nile Blue can penetrate into the intact yeast cells as a vital dye under certain experimental conditions[7]. This can result in too high counts of damaged cells. In our own experience with Nile Blue, uncoloured, dark-coloured, and many more or less faintly coloured cells were seen on microscopic 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).

(3) The results with these dyes are always more or less variable, with high experimental errors, especially at high % of cytolysis[5]. The present authors found the same poor correlation between the number of dead and coloured cells.

Trying to find a better staining method for dead yeast cells, good results were obtained with a combination of the negative dye Ponceau Red and uranyl nitrate, commonly used for the tricomplex staining of phospholipids[8]. It is known that the phospholipids of the intact yeast cell membrane bind Ponceau Red and uranyl ions in a similar way[9]. This 'tricomplex staining' of the membrane phospholipids is not visible microscopically. Over a wide concentration range Ponceau Red and uranyl nitrate appear to have no cytolytic properties. Moreover, neither Ponceau Red[9] nor uranyl ions[10] permeate into the intact yeast cells. It appeared, however, that they do permeate into damaged cells, giving a bright staining of the cell contents. After preliminary experiments to find optimal staining conditions, the following procedure was adopted.

Reagents. (1) 0.04% Ponceau Red solution, (2) 5% $UO_2(NO_3)_2 \cdot 6H_2O$ solution, (3) 0.075 molar maleic acid, dissolved in $18 \cdot 10^{-3} N$ NaOH.



Relationship between % dead cells and % stained cells, after exposure to varying concentrations of alkyl dimethyl benzylammonium chloride. The number of dead cells was determined with the millipore-plate method; at each detergent concentration 12 plates, each containing about 100 cells, were prepared.

A suspension of yeast cells, exposed to a cytolytic agent, is centrifuged and the supernatant is discarded. The yeast cells are resuspended in ice cold water, to give a 0.2–0.5% suspension. This suspension is kept in melting ice for 10 min. 1 ml of this suspension is added to a mixture of 10 ml Ponceau Red, 2 ml uranyl nitrate and 2 ml maleic acid solution and kept at room temperature for 5 min, with continuous stirring. The final pH of this suspension should be 2.48–2.52. Subsequently proportional counts of stained and unstained cells are made by direct microscopic examination, utilizing a cell counting chamber. With this procedure accurate and reproducible results were obtained with various cytolytic agents (Figure).

Staining is positive, if the whole cytoplasm is coloured more or less uniformly. A local staining at a single spot should be considered negative; such local spots are presumably coloured cellular debris, attached to the surface of the cells. When 500 cells are counted, the experimental error is about 2%. In contrast to the methylene blue and Nile Blue method, the results are accurate over the whole range from 0–100% cytolysis. It seems probable that the same procedure can be used with respect to other microorganisms[11].

Zusammenfassung. Es wird eine neue Methode zur selektiven Färbung beschädigter (toter) Hefezellen mit Hilfe eines Gemischs von Uranylnitrat und Ponceau-Rot beschrieben, die grosse praktische Vorteile gegenüber der klassischen Methylenblau- und Nilblaufärbung besitzt.

MAGDALENA MAAS and J. VAN STEVENINCK

Laboratory for Medical Chemistry, Leiden (The Netherlands), 12th December 1966.

[8] G. J. M. HOOGHWINKEL and H. P. G. A. VAN NIEKERK, Proc. K. ned. Akad. Wet. Series B, 258 (1960).
[9] J. VAN STEVENINCK and H. L. BOOIJ, J. gen. Physiol. *48*, 43 (1964).
[10] A. ROTHSTEIN, Protoplasmatologia B2, E4 (1954).
[11] Acknowledgment. The authors thank Unilever N.V. for financial support of this work.

Synthesis of a Pentapeptide by the MERRIFIELD Method

We wish to report the synthesis of the pentapeptide His-Phe-Arg-Trp-Gly by the solid phase method of MERRIFIELD[1], as an attempt to introduce arginine in a sequence without a protecting group on the guanidinium end of the molecule. ter-Butyloxycarbonyl (t-BOC) Gly was linked to the polymer according to the classical process and, after acidolysis of the t-BOC protecting group, was reacted with t-BOC Trp in methylene chloride using dicyclohexylcarbodiimide (DCCI) as coupling agent. A few drops of dimethylformamide (DMF) were added to ensure a better solubility of the t-BOC Trp in methylene chloride. No destruction of the indole nucleus seemed to occur during the acidolysis of the t-BOC group with HCl/HAc, as we were indeed able to isolate and identify (Ehrlich positive) the dipeptide Trp-Gly after refluxing an aliquot of the resin with sodium ethylate in absolute ethanol.

The next step of the synthesis was the addition of the Arg residue; α-t-BOC G.H.$^+$ Arg was used. This protected amino acid was prepared as follows: t-BOC Arg was synthesized according to the general method of

[1] R. B. MERRIFIELD, J. Am. chem. Soc. *85*, 2149 (1963); Biochemistry N.Y. *3*, 1385 (1964); J. org. Chem. *29*, 3100 (1964).