tainer at rest was 1.7:1 for the yeasts and 1.6:1 for the bacteria. The rate of the yeast disintegration was determined by direct counting of cells and cell walls stained with methylene blue after heating and then transferred onto the graduated screen of a microscope. With bacteria, the disintegration speed was determined by measuring the extinction of their supernatant layer at 280 nm. An orienting control of the disintegration course was carried out by measuring the viscosity of the disintegrated suspensions.

Results and discussion. Continuous disintegration of micro-organisms in the apparatus presented is based on the principle of intensively mixing the suspension passing through the cylindrical container of the disintegrator filled with glass beads. The suspension is conveyed (the direction of flow being indicated by a bold arrow in Figure 1) by a pump (H) to the container of the disintegrator (A). The grooved disks of the horizontally disposed agitator (D) impart a preferential rotatory movement to the individual layers of the mixture; the beads move along the streamlines in the suspension, involving, in addition, colliding and rolling of the beads. The suspension is separated from the beads prior to leaving the disintegrator. The container surface and the bearing of the stirrer are cooled by flowing water or salt water (the direction of flow being indicated by an ordinary arrow in Figure 1). The disintegration temperature of the suspension can be influenced when different cooling liquids are used. Salt water (−4 °C) with a flow rate of 190 l/h for example allows to disintegrate with a temperature of until less than 10 °C (peripheral speed of stirrer 10 m/sec).

The disintegrating effect of the 0.6 l unit is evident from Figure 2 for yeast suspensions with a dry-matter content of 14% and at flow rates of 2.5 to 10 l/h. Figure 3 shows the appearance of the yeast material after different degrees of disintegration and separation of protoplasm released by a single washing-out process with a physiological solution and by centrifuging. The different degrees of disintegration can also be judged by the number of residual unopened cells (Figure 3B), but chiefly by the damage of cell walls (Figure 3D) and the quantity of protoplasm adherring to them.

Protoplasm released from the cells into the solution causes an increase of the whole suspension's viscosity. This circumstance was utilized to perform an orienting determination of the degree of disintegration for yeasts, as is shown by the example in the Table.

Continuous disintegration of bacterial suspensions in a 0.6 l container of the equipment is documented by the results obtained by milling a suspension of *Bacillus subtilis* cells with glass beads at a peripheral speed of the stirrer of 15 m/sec. In this case, 80–85% of the cells is disintegrated at a flow rate of the suspension equal to 1 l/h, 75–80% at 3 l/h, 70–75% at 5 l/h and 60–65% at 7 l/h.

The unit equipped with a 0.3 l container for continuous disintegration was sufficiently effective to achieve the destruction of the fibrillary micro-organisms tested (*Aspergillus niger*) resulting in a 100% disintegration. The 0.3 l container for batch use with stirrer disks bearing tangential grooves of different depths was successfully employed in the disintegration of low-volume samples up to 150 ml[12].

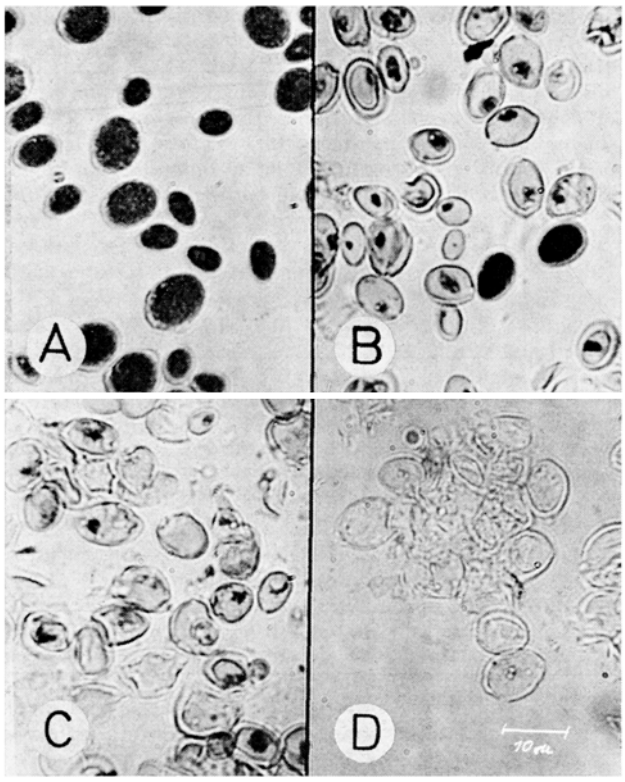

Fig. 3. A) Yeasts prior to disintegration. Cells and cell walls after removal of protoplasm released following continuous disintegration. B) Flow rate 7.5 l/h, and peripheral speed of the stirrer 10 m/sec. C) Flow rate 5.0 l/h, and peripheral speed of the stirrer 15 m/sec. D) Flow rate 2.5 l/h, and peripheral speed of the stirrer 20 m/sec.

Zusammenfassung. Die kontinuierliche Desintegration von Hefen und Bakterien mit Hilfe einer horizontalen, schnellaufenden Labor-Kugelmühle wird beschrieben, deren Arbeitsprinzip auf industrielle Desintegrationsverfahren anwendbar ist.

J. ŘEHÁČEK

Valurba C, CH-1350 Orbe (Switzerland), 8 March 1971.

[12] Thanks are due to Mrs. I. TRAUTMANN for skilled technical assistance.

Carbodiimide Fixation for Immunohistochemistry: Observations on the Fixation of Polypeptide Hormones

Of the fixatives normally used in immunofluorescence[1a], those which act chemically rely heavily on attack of primary amino groups to form cross-links between neighbouring structures. The stimulus to seek alternative fixatives arose recently in connection with our immunohistochemical studies of polypeptide hormones. The use of water-soluble carbodiimides (CDI) suggested itself because these reagents effect cross-linking by initial attack of carboxyl groups, and because native antigenic determinants survive in immunogen conjugates prepared with CDI.

This communication presents our preliminary observations on the potential value of carbodiimides as fixatives.

Materials and methods. The fixative was a 2% solution, prepared just before use, of either 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAP-CDI) (Sigma) or 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulphonate (CME-CDI) (Aldrich) in water or 0.01 *M* phosphate-buffered saline (PBS)[2], pH 7.1. The study was confined to 6 polypeptide hormone antigens: intestinal glucagon (enteroglucagon), pancreatic glucagon, calcitonin, gastrin, ACTH and insulin. Small pieces of the appropriate tissue (human, canine or rat) were either: a) fixed directly in CDI (18–24 h at 4 °C), followed either by paraffin embedding, or by quenching in Arcton (Freon) 22 at −158 °C and cryostat sectioning; or b) quenched in Arcton 22, sectioned in the cryostat, and post-fixed in CDI as above. After fixation, specimens were washed for 24 h in PBS containing 30% sucrose.

Sections were examined by the standard indirect immunofluorescent method, using commercial samples of appropriate fluorescein-labelled antibodies. The respective antihormone antibodies were applied as the sulphate-precipitated globulin fraction of rabbit or guinea-pig antisera[3]. Previous experiments had established that the sera used were active towards the hormone in question. The routine control of specificity was by replacement of the specific antibody by normal rabbit or guinea-pig globulin, but in some cases the specific antibody was pre-treated with the homologous pure antigen.

Results. No difference was noted in the fixative properties of EDAP-CDI and CME-CDI. The former was generally preferred because of its readier dissolution at 4 °C. The Table summarizes the observations on localization by immunofluorescence of the 6 polypeptide antigens using CDI fixation; also noted, for tentative comparison, are the best results obtained with each hormone using established tissue preparation procedures.

The most striking case is that of intestinal glucagon (enteroglucagon), which is excellently preserved by fixation in CDI, while previous attempts to localize this hormone have almost consistently failed. This work is fully described elsewhere[4]. An important general feature seen in the Table is that, for all the antigens thus far studied, specific immunofluorescent localization was good to excellent after CDI fixation and appropriate processing, even though in 2 instances slightly better results could be obtained by a conventional technique. When, as a control, tissue was placed in PBS at 4 °C for 24 h, instead of CDI, results were completely negative. Hence the data in the Table are based on some genuine fixative effect of CDI, and not, for example, on allowing the antigens to remain unfixed in a congenial medium.

With CDI fixation, fluorescent background was always very low when the anti-hormone antibody was from guinea-pig antiserum. In contrast, with antihormone antibody made in rabbits, the nature of the CDI became decisive. These small polypeptides had been made immunogenic by coupling them to a protein carrier using a CDI, and it was found that, where rabbits had been immunized with a conjugate made with EDAP-CDI, then there would be moderate background fluorescence with EDAP-CDI-fixed tissue. This generalized uptake could be largely eliminated by fixing the tissue instead with CME-CDI. Non-specific binding occurred also with certain samples of unlabelled normal rabbit globulin (but not with any samples of the fluorescent antibodies). CDI did not induce fluorescence in any tissue.

Discussion. The observations reported here suggest that water-soluble carbodiimides are likely to be of some value as fixatives for immunohistochemistry. One of the polypeptide antigens investigated could be localized immunohistochemically only after CDI fixation; all the other antigens were also well fixed by CDI. These findings hold promise that a single fixation method (followed by either paraffin embedding or cryostat sectioning) may be applicable to a tissue specimen such as an endocrine tumour in which more than one antigen is being sought; at present, any such specimen must be divided up on excision, for divers treatments. The absence of induced fluorescence offers advantages over, e.g., formaldehyde.

[1] M. GOLDMAN, *Fluorescent Antibody Methods* (Academic Press, New York and London 1968), a) chapter 9; b) p. 177.

[2] It is not clear whether there is any reaction between CDI and phosphate under these conditions. The CDI was in any case present in large excess, and the pH remained neutral.

[3] These antisera were, variously, kind gifts of Dr. B. A. L. HURN (Wellcome Research Laboratories), Prof. K. W. REES (University College Hospital), Prof. I. MACINTYRE (Royal Postgraduate Medical School) and Dr. S. BLOOM (Middlesex Hospital).

[4] JULIA M. POLAK, S. BLOOM, I. COULLING and A. G. E. PEARSE, Gut, *12* 311 (1971).

Localization of polypeptide hormone antigens by immunofluorescence after carbodiimide fixation and other tissue preparation procedures

	Intestinal glucagon[a]	Pancreatic glucagon[a, b]	Calcitonin[a]	Gastrin[a, c]	ACTH[c]	Insulin[c]
CDI fixation, paraffin sections	—		—	++		
CDI fixation, cryostat sections	++	+	++	—		++
Cryostat sections, post-fixed in CDI	+	+	++	—	+	++
Best other method	None[d]	Freeze-dried, unfixed[e] ++	Freeze-dried, unfixed[e] ++	Methanol-free formaldehyde, paraffin sections[e] ++	Formol-saline or formol-calcium, paraffin sections[e] ++	Freeze-dried, unfixed[e] ++

Specific immunofluorescence: ++, excellent; +, good; —, negative; (blank denotes not attempted). Species: [a] Dog; [b] rat; [c] man; [d] see POLAK et al.[4]; [e] JULIA M. POLAK, unpublished data.

Fixation by CDI must depend on an attack of carboxyl groups, to give an *O*-acylisourea, which can then react with a neighbouring amino group to cross-link through an amide bond[5]. Thus, gelatin is rapidly gelled at neutral pH by addition of CDI[6]. In effect, the cross-link is formed by elimination of a water molecule; no part of the CDI becomes incorporated into the protein in this reaction. Any explanation of the apparent virtues of fixation by CDI for immunohistochemistry remains partially speculative. The adoption of new fixatives must still rest heavily on empirical evaluation, although it is rational to attempt, as in the present work, to diversify the sites of chemical reaction in the tissue.

It was predicted that antibodies to hormone-carrier conjugates prepared with a CDI would bind non-specifically to tissue fixed in the same CDI, since JAFFE et al.[7] observed that such antibodies (from rabbits or goats) had affinity for groups present in the particular CDI. This effect was indeed observed in the case of rabbit antibodies, but was negligible with guinea-pig antibodies. Presumably some haptenic groups arise from CDI molecules which become integrally incorporated into the conjugate, and into the tissue, by rearrangement of unstable *O*-acylisourea to the stable acylurea[5]; this hapten is evidently far less immunogenic in guinea-pigs than in rabbits or goats. Introduction of substituted urea groups by the rearrangement just mentioned would make the tissue more basic, since carboxylate would be replaced by a positive group (one of the substituents in water-soluble CDI is basic). Surprisingly, CDI-fixed material shows little tendency to bind fluorescent globulin non-specifically, a phenomenon frequently attributed to charge interaction[1b], which increases with increasing acidophilia of the tissue.

CDI was inferior to aldehydes for preserving general structure, probably because CDI demands that carboxyl and amino groups be in close proximity for cross-links to be formed. By incorporating some bifunctional nucleophile, e.g. a suitable diamine, in the CDI fixative, cross-linking between tissue carboxyl groups should be greatly favoured. The use of CDI to preserve well both antigenicity and structure at light and electron microscope levels is the subject of current investigation[8].

Experiments performed since this manuscript was submitted suggest that EDAP-CDI may fix antigens more effectively than CME-CDI, which is a much larger molecule. Furthermore, a higher concentration (e.g. 10%) of CDI may be advantageous.

Zusammenfassung. Wasserlösliche Carbodiimide werden als Fixiermittel für immunohistochemische Untersuchungen vorgeschlagen, da sie die Verwendung von Carboxylgruppen bei der Fixierung erlauben.

P. A. KENDALL, JULIA M. POLAK and A. G. E. PEARSE

Department of Histochemistry, Royal Postgraduate Medical School, Hammersmith Hospital, London W12 OHS (England), 16 February 1971.

[5] For a recent summary of reactions of CDI with proteins, see G. R. STARK, Adv. Protein Chem. *24*, 261 (1970).
[6] J. C. SHEEHAN and J. J. HLAVKA, J. Am. chem. Soc. *79*, 4528 (1957).
[7] B. M. JAFFE, W. T. NEWTON and J. E. MCGUIGAN, Immunochemistry *7*, 715 (1970).
[8] This work was supported by grants from the Cancer Research Campaign and the Wellcome Foundation.

CORRIGENDUM

F. PESARO und H. KOBLET: *Reinigung von F-Antigen*, Experientia *27*, p. 235 (1971). Wir verdanken R. UTZINGER und J. LINDENMANN den Hinweis, wonach Lysozym auf Sephadex verzögert eluiert werden kann[1], und zwar vermutlich aufgrund einer Affinität für β-1,4-Bindungen zwischen Hexosen. Somit besteht die Möglichkeit, dass das Molekulargewichts-Aequivalent für F-Antigen höher als 40 000 bis 45 000 ist, was die offensichtliche Diskrepanz zwischen den Bestimmungen mit Sephadex, der Polyacryamidgel-Elektrophorese und der analytischen Ultrazentrifugation zwanglos erklären würde. Molekulargewichts-Aequivalente auf Sephadex sind ohnehin vorsichtig zu interpretieren, solange nichts über die Molekülradien bekannt ist[2].

F. PESARO und H. KOBLET

Institut für Virologie der Universität Zürich, CH-8006 Zürich (Schweiz).

[1] H. M. RAUEN, *Biochemisches Taschenbuch*. 2. Auflage (Springer-Verlag, Berlin 1964), vol. 2, p. 910.
[2] D. RODBARD und A. CHRAMBACH, Proc. natn. Acad. Sci. USA *65*, 970 (1970).

CONGRESSUS

India
8th International Symposium on the Chemistry of Natural Products

in New Delhi, 6–12 February 1972

The Symposium will be devoted mainly to the following topics for which it is proposed to organize separate sections: 1. Alkaloids. 2. Polyphenolics. 3. Terpenoids and steroids. 4. Macromolecules of biological interest (proteins, peptides, nucleic acids, etc.). 5. Carbohydrates, lipids and related substances. 6. Other topics in natural products chemistry including physical methods of structure and determination.

The deadline for sending in abstracts is 1 September 1971. Further information by Prof. S. Rangaswami, Secretary, 8th IUPAC Symposium, Indian National Science Academy, Bahadur Shah Zafar Marg, New Delhi 1 (India).