Technical Papers

Dissociation of Chick Embryo Cells by Means of a Chelating Compound

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A number of recent investigations have involved the dissociation of the component cells of an embryo or organ rudiment (*1–5*). The dissociative agents have been high *p*H (*1, 4*), tryptic action (*2, 5*), or mechanical grinding (*3*). In two current problems, we found that the chelating compound di-sodium ethylene diamine tetraacetic acid—di-sodium versenate (*6*)—is very effective for dissociating cells from chick embryos (*7*).

One of these problems involves the dissociation of entire definitive primitive-streak-stage chick embryos into a suspension of cells. This is accomplished on a fairly large scale by the following procedure: The blastoderms are removed from the yolk, the area pellucida is dissected free in Tyrode's solution and then washed in three or four changes of Moscona's (*2*) Ca- and Mg-free solution. The blastoderms (six to eight in number) are then pipetted into 15-ml centrifuge tubes containing 2 ml of the versenate solution (100 mg percent, made up in Moscona's solution) which has been brought to approximately *p*H 8.5 with 0.4 ml of 1-percent KOH. After 20 to 30 min incubation at 37.5°C, the centrifuge tubes are agitated with a rotating motion. This agitation converts the blastoderms into a suspension of cells very rapidly. These cells may then be compacted by mild centrifugation, washed in Moscona's solution, and, again following centrifugation, resuspended in a standard tissue-culture medium (horse serum, Tyrode's solution, and embryo extract, in the proportions 2 : 2 : 1).

Microscopic examination prior to the last step reveals that the majority of cells are completely free and that the largest clumps contain only three or four cells. Very few of the cells show any visible indication of damage. Cells suspended by this procedure reaggregate in the culture medium. When such aggregates are kept in depression slides, they regularly form healthy cultures, which spread over the glass surface and live for at least 2 or 3 days. In a few instances, some of the larger aggregates have been placed on the chorioallantoic membranes of appropriate chick embryo hosts. They have yielded healthy, growing grafts.

In the second problem, we found it desirable to remove the ectoderm from the limb buds of 3-day chick embryos. The object was to leave the mesoderm as unaltered as possible. Trypsin skinning (*2*) was not appropriate for this work. It was found that the following procedure yields limb-bud mesenchyme which is firm and amenable to further manipulation: The limb buds are removed from the embryo surgically in Tyrode's solution, washed in three or four changes of Moscona's solution, and then placed in the versenate solution described in a foregoing paragraph (alkali not added). After 20 to 30 min at 37.5°C, the ectoderm is sufficiently macerated so that it can be scraped off (a longer sojourn in the versenate solution, especially at higher *p*H, results in the dissociation of the mesenchyme). This leaves the limb-bud mesenchyme smooth and firm and, after washing in Tyrode's solution or Tyrode's solution and horse serum (1:1), viable.

These observations suggest that di-sodium ethylene diamine tetraacetic acid may be a very useful adjuvant for other biological procedures that require Ca- and Mg-free solutions.

References and Notes

1. J. Holtfreter, *Symposia Soc. Exptl. Biol.* **2**, 17 (1948).
2. A. and H. Moscona, *J. Anat.* **86**, 287 (1952).
3. P. Weiss and G. Andres, *J. Exptl. Zool.* **121**, 449 (1952).
4. S. Mookerjee, *Nature* **171**, 796 (1953).
5. G. S. St. Amand and S. R. Tipton, *Science* **119**, 93 (1954).
6. Kindly supplied by the Bersworth Chemical Co., Framingham, Mass.
7. This investigation was supported in part by a research grant [C-1790 (R)] from the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

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Crystalline Neoretinene b

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We have prepared, in crystalline form, the isomer of vitamin-A aldehyde, which Hubbard and Wald (*1*) have called neoretinene b and identified as the precursor *in vivo* of the visual pigment rhodopsin. In this preliminary report (*2*) we describe the method of concentration and crystallization of the isomer, its physical properties, and its reduction to the corresponding vitamin-A isomer.

The preparative procedure was as follows: A solution of crystalline, all-trans vitamin-A aldehyde (4.0 g) in ethanol (1000 ml in a 1000-ml flask) was exposed for 4 hr to bright sunlight (outdoors, midsummer), filtered through a combination of Corning Nos. 3966 and 3850 filters. Alternatively, three photoflood lamps (No. 2), at a distance of 8 in. from the solution, were used as the light source, with the same filters, for an exposure time of 8 hr. These procedures proved to be preferable to the method used by Hubbard and Wald (*1*) for preparing larger amounts of the new isomer.

After removing the alcohol by distillation under reduced pressure, the residual mixed vitamin-A aldehyde isomers were dissolved in petroleum ether (bp 30° to 60°C) and chromatographed on a column (2 by 24

in.) of sodium silico-aluminate (400 g) (*3*). The column had been previously washed with 10 percent aqueous acetone (250 ml) to remove any free alkali and then with petroleum ether (500 ml) to remove acetone. The chromatogram was developed with petroleum ether to give first a nearly colorless filtrate (500 ml, 0.11 g), followed by a yellow-colored filtrate (1000 ml). The latter fraction gave, after removal of solvent by distillation under reduced pressure, a concentrate of neoretinene b (1.2 g, E(1 percent, 1 cm) (254, 375 mμ) = 445, 1115.

When this concentrate, in petroleum ether (2 ml), was cooled to −18°C, crystals were obtained (0.6 g) that consisted of neoretinene b mixed with some neovitamin-A aldehyde [neoretinene a (*1*)]. The orange prisms of neoretinene b were separated manually from the yellow needles of neovitamin-A aldehyde and were recrystallized from petroleum ether.

The neoretinene b thus prepared had mp 63.5° to 64.4°C and E(1 percent, 1 cm) (255, 376 mμ) = 595, 857 (ethanol, Cary instrument, Fig. 1). The ratio of the absorption at the so-called "cis" peak at 254 mμ to that at the main absorption maximum (376 mμ) was 0.69. Wald kindly arranged for a test on a sample of the crystals by incubation with opsin and confirmed its identification as neoretinene b.

Hubbard and Wald (*1*) have described the concentration of neoretinene b and the preparation of some crystalline material, but the amount was reported to be too small to determine its extinction coefficient. It was found to have an absorption maximum at 377.5 mμ (ethanol) and an extinction coefficient estimated to be between 900 and 1000. Its "cis" peak ratio was about 0.5. By private communication, Wald has indicated that other highly purified, although noncrystalline, preparations have subsequently been made in his laboratory.

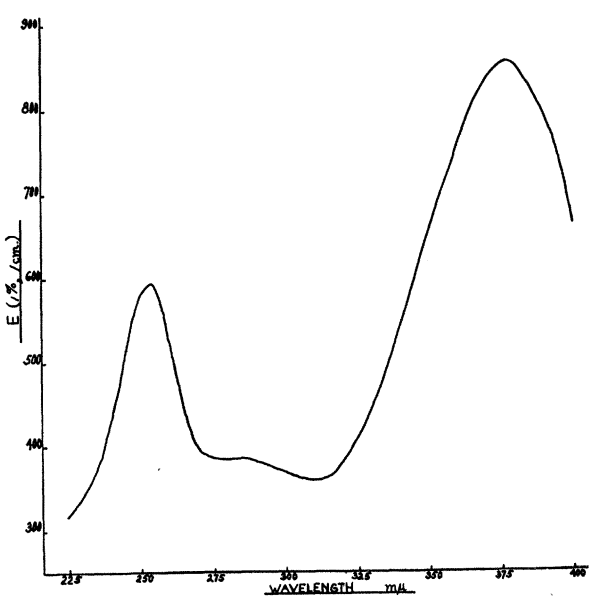

Fig. 1. Spectrophotometric curve of neoretinene b.

Neoretinene b was reduced with both lithium aluminum hydride and sodium borohydride to give the corresponding vitamin A. The absorption curve of a noncrystalline preparation, made by reduction of the aldehyde with lithium aluminum hydride, had a rather broad maximum with E(1 percent, 1 cm) (322 mμ) = 940 (ethanol) and a subsidiary maximum at 233 mμ having E(1 percent, 1 cm) = 270. From the data on the corresponding vitamin-A aldehyde isomers, it was expected that the extinction coefficient of this vitamin-A isomer [temporarily called neovitamin Ab (*1*)] would be substantially lower than that of all-trans vitamin A E(1 percent, 1 cm) (325 mμ) = 1830. Crystallization experiments on the isomer are in progress.

A preliminary bioassay by our biochemistry department, using the rat liver-storage method, has indicated that neoretinene b has about half the biological potency of all-trans vitamin-A aldehyde. Thus, this bioassay method does not reflect the biological importance that neoretinene b is considered to have in the formation of the visual pigment rhodopsin. The bioassay work will be described in a separate publication from these laboratories.

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References and Notes

1. R. Hubbard and G. Wald, *J. Gen. Physiol.* **36**, 269 (1952).
2. Communication No. 202 from the Research Laboratories, Distillation Products Industries, Division of Eastman Kodak Co., Rochester, N.Y.
3. Doucil from Philadelphia Quartz Co.

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The Radical Dissociation of Aryldisulfides

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A considerable time ago (*1–3*) I investigated the problem of the dissociation of simple aryldisulfides, such as phenyldisulfide, into radicals with monovalent sulfur. In these studies I concluded that the investigated aryldisulfides do not undergo a *reversible* dissociation into *long-life* radicals. Later, Schönberg, Rupp, and Gumlich (*4*) reached the contrary conclusion, based partly on a different interpretation of my experiments and partly on new experimental data furnished by Schönberg and his associates. Schönberg's views have been shared by several subsequent authors (*5–7*). Considering the theoretical importance of the problem indicated, a review of existing data has been made and additional experimental evidence has been obtained supporting a position that differs from that of Schönberg and his followers.

1) We do not know all the factors that cause the formation and relative stability of long-life organic