A CHEMICAL METHOD FOR THE PALYNOLOGICAL PROCESSING OF CHALK

by

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Summary

A general chemical method is described for the palynological processing of chalk. It includes descriptions of the acetylation, flotation, oxidation and filtration techniques. In addition, methods are given for the preparation of single-specimen mounts.

Introduction

Chalk deposits constitute a major percentage of the Upper Cretaceous and Danian rocks of western Europe and dominate in the type sections for the international stages and substages of this interval. It is important, therefore, that a general method should be available for the extraction of dinoflagellates, acritarchs, spores, pollen and other palynomorphs from these sediments, particularly since these microfossils have now attained considerable importance in Cretaceous and Tertiary stratigraphy.

Until recently most of the palynological work on Upper Cretaceous and Danian limestone groups dealt with the associated minor flint horizons; thin flint chips were simply mounted in canada balsam and examined under a microscope. However it is now generally considered preferable to process the limestone and separate the fossils completely from the matrix. In his current research the writer has found that the palynomorphs of European late Cretaceous chalks, although occurring mostly in low concentrations, are well preserved.

Important recent contributions to the palynology of Upper Cretaceous chalk deposits have been made by Clarke and Verdier (1967) and Davey (1966, 1969, 1970). Danian limestones have been examined palynologically by Morgenroth (1968). The writer is currently examining dinoflagellate assemblages from European Campanian, Maastrichtian and Danian deposits, principally chalks, and this paper describes the processing schedule which he has found most generally suitable.

The chemical method described in this paper cannot be used if it is necessary to preserve any of the calcareous or siliceous fossils contained in the rock, since all are destroyed. If a complete faunal and floral extract is required then mechanical separation techniques must be employed. The silicate-depressant method described by Arms (1960), which involves the use of flotation agents, could probably be adapted for use with chalk samples.

Maceration Technique

With the exception of associated thin dark marl bands, for instance the well known Fish Clay (or "Fiskeler") horizon at the base of the Danish Danian, the chalks consist mainly of calcium carbonate, which must be removed completely with hydrochloric acid before other reagents are used. Since the concentration of palynomorphs in chalk is less than in most shales and siltstones it is necessary to process somewhat larger samples in order to obtain an adequate yield. Fine grained chalks should be used whenever possible since these usually have a much greater concentration of palynomorphs than coarse chalks. The writer has found that 250-gram samples are satisfactory for the purest chalks; for impure chalks 150-gram samples normally suffice; for marls the sample is usually 10-50 grams.

Removal of Carbonates

The cleaned chalk sample, in small pieces of about 1 cc, (larger lumps for pure chalks, much smaller pieces for very impure chalks) is placed in a 2-litre beaker and moistened with 50% acetone. Cold 20% HCl is slowly added and, if necessary, concentrated acetone from a wash bottle is sprayed into the beaker to reduce the surface tension of the acid solution and thus help to suppress the resulting violent effervescence. Ideally the beaker should be stirred by placing on an oscillating hotplate, with or without the heat switched on. From time to time fresh HCl and acetone are added until the reaction ceases, which can take up to 12 hours, depending on the purity of the chalk sample. Care should be taken to prevent the froth deposit from drying on the side of the beaker. The froth produced by the $HCl/CaCO_3$ reaction is usually very fossiliferous and a processing technique utilizing this property has been described by Brown (1960, p. 42) for treatment of marls; however not all of the palynomorphs are contained in the froth. Excess acid is decanted and the residue is sieved through a No. 8 mesh sieve to remove solids, such as flint pieces, of more than 2 mm. The residue is transferred either to 100 ml or 50 ml centrifuge tubes, depending on the amount of residue, and is then centrifuged and washed thoroughly. Calcium ions must be removed completely to prevent subsequent formation of a flocculate of the sparingly soluble solid, calcium fluoride. For centrifuging, the writer uses capped polypropylene centrifuge tubes (which can be used for all chemical reactions used throughout palynological macerations) and a centrifuge of the "swing-out" head type.

Removal of Silicates

Most chalks contain a small percentage of silicate minerals which must be removed with hydrofluoric acid. For this purpose it is best to use small quantities of 60 - 70% hydrofluoric acid where adequate fume cupboard facilities are available. This acid is very toxic and corrosive and it is essential that all work involving its use should be done in an efficient fume cupboard. Centrifuging should also be done in a fume cupboard if a bench model centrifuge is available or, if a floor model centrifuge is used, then it should be equipped with a blower and exhaust system, connected to the fume cupboard, such as the type described by Lennie (1968). Centrifuge tubes should have tight fitting plastic caps and the technician should wear appropriate breathing apparatus (a small respirator equipped with a filter for acid gases is satisfactory), face mask and rubber gloves. The centrifuge bowl and interior fittings should be coated either with a plastic spray or silicone grease. If traces of the acid are detected, despite the above precautions, then the less dangerous 40% HF, as supplied for analysis, should be used.

Ten to twenty millilitres of cold hydrofluoric acid are carefully added to each tube containing not more than 20 cc of sample. The capped tube is then shaken with a vortex mixer and a further 20-40 ml of acid are added. The samples are left to digest in the hydrofluoric acid for 24 hours, during which time they should preferably be gently shaken at least twice. The digestion is accelerated if the tubes are heated, by suspension in a thermostatically controlled electric heating bath operating at 120°C. The use of a bath filled with Lissapol detergent, (an I.C.I. product), suitable for this purpose, has been mentioned by Lennie (1968). Water-filled heating baths have many faults, caused mainly by the comparatively low boiling point of water, and should not be used during this or any other stage of the processing schedule. After centrifugation, which should be carried out at 2,500 r.p.m. for three minutes, the samples are washed thoroughly in water. The residual hydrofluoric acid should be poured into a large fume cupboard sink containing at least 20 litres of water and the greatly diluted acid may then be flushed down the sink with excess water.

The next step involves the removal of fluorides and silicofluorides with hot hydrochloric acid. Hydrochloric acid, of about 20% strength, is heated to boiling point on a hotplate and about 10 ml added to each sample. The mixture is quickly stirred with a vortex mixer and the tubes are filled with more boiling HCl. The tubes are centrifuged at a standard speed of 2,500 r.p.m. for three minutes and, after decantation of the yellowish orange supernatant liquid, the residue is washed twice with water. The process of washing with boiling HCl is repeated several times until the supernatant liquid is colourless (or pale green if certain commercial grade acids are used). Finally, the tubes are allowed to stand in a heating bath, kept at 120°C,

for 20 minutes. A substantial reduction in residue bulk occurs during the HF/HCl process and the residue can usually be transferred to 15 ml conical glass centrifuge tubes at this stage. The residue is then washed thoroughly, centrifuged, and inspection mounts are made.

Oxidation and Filtration

Residues which contain finely divided organic debris should be filtered through a sintered glass filter using fuming (95%) nitric acid. The use of this reagent was first suggested to the writer by Dr. J.P. Verdier. It removes extraneous organic matter and facilitates the passage of fluids through the sintered glass filter plate. However it should be noted that fuming nitric acid is a highly toxic and corrosive chemical and must be treated with same care that is vital when using hydrofluoric acid; since it rapidly attacks rubber gloves these should be washed thoroughly after handling the acid. The choice of filter pore size depends on the size of the palynomorphs being studied; for anything but the smallest pollens and acritarchs a sintered glass filter of porosity grade 3 (20-30 μ) is most satisfactory.

Five millilitres of fuming nitric acid are added to each 15 ml tube and the mixture is stirred with the vortex mixer. A further 5 ml is added and the mixture filtered. A method of filtration using a sintered glass plate and an air compressor, providing an intermittent upward flow of air through the filter, has been described by Neves and Dale (1963). The system used by the writer employs the same principle but, instead of a mechanical pump, he has found that an occasional squeeze on an empty plastic wash bottle attached by a rubber tube to the arm of the Buchner flask produces a sufficiently strong upward current of air to free the sintered glass plate of debris. The residue is washed through the sintered glass plate with excess fuming nitric acid and then thoroughly washed with water. The filtrate is discarded. The filter funnel is afterwards placed in a cleaning solution in an ultrasonic tank; the sintered glass filter should not be allowed to dry before cleaning.

Acetylation

This process removes unwanted cellulosic material and improves the fossils for microscopy by darkening their external shells and increasing the contrast of their external features. Firstly it is necessary to wash the residues in glacial acetic acid. Then 5 ml of a freshly prepared mixture of acetic anhydride and concentrated sulphurric acid (9:1) is carefully added, preferably while it is still hot (the addition of sulphuric acid to acetic anhydride produces a highly exothermic reaction and the hot colourless solution turns a rich reddish brown). The mixture is shaken carefully with a vortex mixer and placed in a heating bath at 90°C for 10 minutes. Care is taken not to overtreat the samples as expansion of palynomorphs may occur (Harris, 1956). The final residue is washed several times in water. A detailed description of the acetylation principle and method is given by Gray (1965b).

Removal of Residual Mineral Matter

If the residue still contains mineral matter, it should be treated with a heavy liquid in order to float off the organic material. The writer uses a solution of zinc bromide in 10% HCl, prepared to give a specific gravity of 2.0. The residues are firstly washed in 10% HCl and centrifuged. Five millilitres of zinc bromide solution are added to each residue and mixed thoroughly. After centrifugation the tubes are shaken quickly and the float fraction is decanted into another 15 ml tube. The tube is filled with 10% HCl and the mixture shaken thoroughly and centrifuged. The mineral fraction is tested for possible fossil content and, providing the result is negative, is then discarded.

Further Treatment

At this stage the residue should consist mainly of palynomorphs. If fusain is present, it may be necessary to break down the larger fragments by careful use of ultrasonic irradiation and then remove the finely divided fragments by means of a second filtration or repeated short centrifuging (Funkhouser & Evitt, 1959). Ultrasonic irradiation may also be used to deflocculate

residues (Funkhouser & Evitt, 1959; Stevens *et al.*, 1960), although, in his studies of European chalks, the writer has not yet encountered any residues requiring deflocculation. Clarke and Verdier (1967) are of the opinion that ultrasonic treatment can cause damage to dinoflagellate cysts, especially large forms with apical archeopyles. In any case excessive ultrasonic treatment should be avoided.

Before preparing final slide mounts, the residue must be washed several times with water.

Mounting of Residues

The residue should be mixed with at least three times its volume of water and should not be allowed to dry in the centrifuge tubes. Before pipetting, the pipette is used to blow air through the residue in order to give a homogeneous, non-layered suspension. Semi-micro pipettes are used for transferring the aqueous residue from tube to slide and, after use with one sample, are discarded - it is difficult to completely clean the small capillary and there is always a risk of contamination if any attempt is made to use the same pipette for more than one sample.

Residues are mounted in glycerine jelly and spread over a 3" x 1" slide by means of a wide flat toothpick (Lennie, 1968). The writer usually aims for a concentration of between 3000 and 20,000 specimens per slide. The strew-mounts are covered with clean 22 x 22 mm "O" gauge coverslips, and when dry are sealed with colourless nail lacquer. The writer prepares two slides using unstained glycerine jelly and at least five slides using safranin glycerine jelly. Although most of the microscopic work, including photomicrography, involves the use of stained specimens, there invariably exist in each assemblage some very opaque thickwalled species which are best examined and photographed unstained. Slides prepared with glycerine jelly can be remelted at a later date if required and the specimens removed. With most other common mountants, specimens cannot be removed or reorientated once slides have been prepared.

Aqueous residues are kept in labelled 10 ml glass vials to which 1 ml of silicone fluid and a few drops of 5% phenol solution have been added in order to prevent both desiccation and possible fungal growth (Lennie, 1968).

The position of specimens may be recorded on the coverslip by means of a marking objective which, basically, is an objective with a diamond point instead of lenses. It may be used to etch circles, of diameters ranging from about 400μ to 4000μ, on the coverslip. For very small specimens (say less than 40μ) it is best to inscribe two or more concentric circles. Neither ink nor paint should be used to mark the position of specimens, since both are easily erased from glass and also tend to obscure other specimens.

Slides should be stored horizontally, preferably away from excessive heat.

Preparation of Single-Specimen Mounts

Single-specimen mounts are essential for systematic work and may be prepared either from specimens contained in a glycerine jelly strew mount or from unmounted specimens contained in an aqueous residue.

A fairly simple method of picking specimens from a strew-mount slide was suggested to the writer by Dr. J.P. Verdier; it utilizes the marking objective, which is used to cut through the coverslip around the palynomorph. The circle diameter should be considerably larger than the maximum dimension of the fossil. The slide is then heated very gently and the glass fragment lifted off with a needle. The needle is then dipped into a drop of melted glycerine jelly contained on a heated slide and, while the desired specimen is viewed under the microscope with a low power objective (the writer uses a 6.3x objective in conjunction with 10x eyepieces), the specimen is carefully picked up with the needle and transferred to the drop of glycerine

jelly. The strew mount slide, when cool, is repaired by placing a very small drop of clear nail lacquer over the small circular hole left by the marking objective. The glycerine jelly is left to partially harden before a small circular coverslip (the writer uses 19 mm coverslips) is placed on top of the jelly. If too little glycerine jelly is used and a coverslip applied before the jelly hardens, the specimen is often crushed by the weight of the coverslip; if too much glycerine jelly is used, the specimen usually migrates to the edge of the coverslip. The orientation of the specimen can be controlled by carefully moving the coverslip sideways. Any pieces of debris adhering to the fossil can usually be removed by sliding and twisting the coverslip just before the jelly hardens. When the desired orientation has been obtained, the hardening of the glycerine jelly should be accelerated by applying ice to the slide or by directing a jet of cold air on to it.

It is important to note that, for elongated specimens which are to be studied at a series of different orientations (holotypes should be viewed at three standard orientations if possible), each specimen should firstly be mounted with the longest axis uppermost and finally with the shortest axis uppermost. It is very difficult to reverse the order without breaking the specimen. After about 12 hours, when the mountant has hardened, excess glycerine jelly must be removed with a tissue moistened with ethanol. The coverslip should be sealed with a dark coloured nail laquer, which should be applied with a small brush completely over the surface of the coverslip, except immediately above the fossil, in order to facilitate specimen location. For more precise specimen location the writer inscribes one or more circles around the fossil with a marking objective.

When a large number of specimens are to be picked from one strew mount it is best to remove the large coverslip completely from the slide instead of picking "through the coverslip". The lacquer seal must firstly be removed with acetone before the slide is placed on a hotplate. The coverslip is then carefully pulled off by sliding it sideways and, before the jelly hardens, the coated side of the coverslip is scraped along the edge of a clean glass slide so that all of the fossil-bearing jelly is transferred to the upper surface of the slide. One drop of water is added to the two slides and the residues are spread with a toothpick. In order to pick specimens it is necessary to gently warm the slide, after a specimen has been located, so that the specimen can be removed with a needle. It is then possible to proceed as described above. This is the method used by the writer over the past six years for the preparation of most of his collection of 2000 single-specimen palynological slides. The strew mount slides may be covered with a fresh coverslip after use.

Single-specimen slides may also be prepared from specimens contained in an aqueous residue. The layer of silicone fluid is first removed from the residue with a small pipette and 1 ml of glycerine is added to the aqueous residue to prevent desiccation. A few drops of the thoroughly mixed residue are placed on a slide and the desired specimen is picked out as above. In this case it is not necessary to heat the picking slide.

Further details on the preparation of single grain slides are given by Faegri (1939), Klaus (1953), Takeoka & Stix (1963), Tsukada (1964) and Wilson (1968). Mädler (1956) has described a method for the preparation of multi-specimen palynological slides.

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Summary of maceration and mounting schedule

1. Removal of Carbonates. Sample thoroughly cleaned and broken into small pieces. 150-250 grams of chalk normally required, depending on purity and grade. Sample placed in a 2-litre beaker and moistened with acetone. Dilute (20%) HCl added slowly and additional acetone sprayed into mixture to suppress resulting effervescence. Mixture stirred frequently during reaction time of 2-12 hours. Excess HCl decanted, residue sieved through No. 8 mesh sieve and coarser fraction discarded. Fine fraction transferred to capped 50 ml or 100 ml polypropylene tubes and washed thoroughly with water.

2. Removal of Silicates: Hydrofluoric acid (60-70%) mixed very carefully with residue in centrifuge tube and left to digest for 24 hours. Heating in 120°C "Lissapol" bath speeds reaction. Residue washed thoroughly with water.

3. Removal of Fluorides and Silicofluorides: Residue washed several times with boiling 20% HCl until supernatant liquid, after centrifuging, is colourless. Finally, tube containing HCl allowed to stand in 120°C heating bath for 20 minutes. Residue washed thoroughly with water and transferred to 15 ml conical glass tube if sufficient reduction in volume has occurred.

4. Oxidation and Filtration: Residue washed through No. 3 porosity sintered glass filter with fuming HNO_3 (care!), then washed thoroughly with water.

5. Acetylation: Residue washed with glacial acetic acid. 5 ml. of hot acetylation mixture (9 parts acetic anhydride/1 part conc. sulphuric acid) carefully added and mixture heated to 90°C for 10 minutes (avoiding overtreatment). Residue washed firstly with acetone then several times with water.

6. Heavy Liquid Flotation: Residue washed with 10% HCl and centrifuged. 5 ml of $ZnBr_2$/HCl solution (s.g. 2.0) added and mixed with residue which is then centrifuged and quickly shaken. Float fraction decanted into another tube and washed firstly with 10% HCl then several times with water.

7. Further Treatment: Fusain, if present, can be broken down by careful ultrasonic treatment and then removed either by filtration or repeated short centrifuging. Ultrasonic irradiation can also be used for deflocculation if necessary.

8. Storage of Residues: Thoroughly washed acqueous residue can be kept for long periods in labelled 10 ml glass vials containing small amounts of silicone oil and 5% phenol solution. It may subsequently be used for preparation of either strew mounts or single-specimen mounts.

9. Preparation of Strew Mounts: Residue washed thoroughly in water, centrifuged, and mixed by blowing air through it with a semi-micro pipette. Pipette used for transferring one or two drops of residue to a glass slide where it is mixed thoroughly with small quantity of melted

glycerine jelly using toothpick. Mounted residue covered with 22 x 22 mm "O" gauge coverslip and sealed with clear nail lacquer. Each slide should preferably contain 3000 - 20,000 palynomorphs.

10. Preparation of Single-Specimen Mounts:

(a) When small number of palynomorphs required from one strew mount it is convenient to pick "through the coverslip". Small hole cut around the desired specimen with marking objective. Specimen transferred by needle from the warmed slide to separate slide containing a drop of melted glycerine jelly and small circular coverslip placed on jelly when partially hardened. Orientation of specimen can be altered and most adhering debris removed by controlled sliding and twisting of coverslip. Coverslip sealed and painted with dark coloured nail lacquer, leaving small area clear for specimen examination; precise specimen position should be etched on coverslip with marking objective. Hole left in coverslip of strew mount must be sealed with clear nail lacquer.

(b) When large number of palynomorphs required from one strew mount, coverslip should be removed from warmed slide and any residue still adhering to it transferred to another slide by scraping hot coverslip along top edge of slide. Specimens may then be removed as in (a).

(c) Specimens may be picked from aqueous residue after it has been washed thoroughly with water and had a small amount of aqueous glycerine added. Procedure then as in (a).

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