SIZE MODIFICATION OF RECENT POLLEN GRAINS UNDER DIFFERENT TREATMENTS

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(Received January 7, 1969)

SUMMARY

The effect of various chemicals on the size of recent pollen grains of *Corylus avellana* L. and *Quercus robur* L. was studied. The size of acetolysed grains was affected by the treatment prior to acetolysis and moreover by the duration of acetolysis. Preparation methods, which produce comparable sizes and shapes, are given for both fresh and dried polliniferous material. It is concluded that size and shape are valuable for the determination of types. A new method is described for isolating anthers from herbarium sheets without causing great damage to the flower. Glycerin jelly proved to be a good mounting medium, provided that the cover glass is supported by granules of modelling clay.

INTRODUCTION

It is common knowledge among palynologists that the size of pollen grains is affected by both chemical treatment (CHRISTENSEN, 1946) and mounting media (ANDERSEN, 1960). As pointed out by Christensen the change in size of pollen grains under various conditions is of considerable interest because the dimensions of such grains can be used as a means of characterization and identification. Unfortunately such an identification based on the dimensions of a pollen grain may be erroneous if no attention is given to the method applied.

While examining pollen grains of *Cornus sanguinea* L. during a pollen morphological study of the Cornaceae s.l., it was found that there were slides prepared from dried material and slides prepared from fresh flowers, preserved in glacial acetic acid. The materials in both kinds of slides originated from exactly the same plant specimen. It was a surprise to see that the pollen grains in slides from herbarium material were larger than the grains taken from fresh-flower material. The pollen obtained from herbarium material was measured immediately after preparation of the slide and compared with that made from fresh material two years after mounting. Such an increase in size became more apparent when

both were mounted in glycerin jelly, according to the method described by PUNT (1962).

Consequently, a study of the changes in size of pollen grains under various treatments is deemed necessary.

CURRENT PREPARATION METHODS

Currently there are the three following basic methods of treatment:

(*1*) The glycerin-jelly method described by WODEHOUSE (1935) based on a non-chemical treatment. Pollen grains are picked out of the anthers and placed on a microscope slide, stained and mounted in glycerin jelly. The disadvantage of this method is that the contents of the pollen grain does not dissolve and consequently it remains not fully transparant after treatment.

This method is still in use by pollen-allergy specialists, students of honey plants and a few pollen morphologists.

(*2*) The potassium hydroxide method described by VON POST (1933) and modified by FIRBAS (1937). Polliniferous material is boiled in a 10% potassium-hydroxide solution for 10 min and then mounted in glycerin jelly. This treatment removes the intine and cell contents without damaging the exine. Since pollen grains treated in this way have little colour, some specialists prefer staining the exine.

This method proved to be most suitable for delicate pollen grains such as those occurring in Cannaceae, Juncaceae, Lauraceae, Marantaceae, Musaceae and Zingiberaceae p.p. (ERDTMAN, 1952; TRAVERSE, 1966; and others).

(*3*) The acetolysis method introduced by ERDTMAN and ERDTMAN (1933), and ERDTMAN (1934) and revised by him in 1960. This method is based on a chemical destruction of cellulosic and cytoplasmic material by means of a mixture of acetic anhydride and concentrated sulphuric acid. During this process the exine is coloured.

This method is commonly used by pollen analists and a large number of pollen morphologists.

TRAVERSE (1965) pointed out that the correct term for this chemical destruction is acetylation. However, I still prefer to use the term acetolysis here, not in a chemical sense but as a pollen preparation method after ERDTMAN (1952). The more so because this term has found its place in the palynological literature.

The effect of preparation methods on pollen size

Several palynologists found that the size of recent pollen grains depends on the treatment used.

CAIN (1944) studied the size-frequencies of pollen grains of *Abies fraseri.*

He concluded that treatment with acetolysis mixture after boiling with potassium hydroxide causes abnormal swelling of the pollen grains.

CHRISTENSEN (1946) examined size changes of recent material of *Corylus avellana*. His main results were: (*1*) Acetolysis expands pollen grains spontaneously at the moment that these grains are brought into contact with the hot acetolysis mixture. (*2*) Size decreases during the process of acetolysis. (*3*) Acetolysis after treatment with potassium hydroxide causes swelling of the pollen grains to sizes much larger than treatment without potassium hydroxide.

WENNER (1947) compared the difference in size of pollen grains caused by boiling in potassium hydroxide and by boiling in an acetolysis mixture. He found that in most cases acetolysis caused a significant size increase if compared with the potassium-hydroxide treatment.

FAEGRI and DEUSE (1960) found the effect of prolonged boiling in acetolysis mixture and potassium hydroxide (up to 21 h) negligible.

WHITEHEAD (1965) compared the sizes of pollen grains of Juglandaceae as reported by WODEHOUSE (1935), ERDTMAN (1943, 1952), HEIMSCH (1944), STACHURSKA (1961), and STONE (1963) with his own results. He concluded that the size is affected by the treatment used.

TING (1966) examined changes in size of pollen grains of *Pinus* under various conditions. According to his results acetolysed pollen grains produce the same mean size as untreated pollen grains mounted in glycerin jelly. He stated: "... the cause of expansion is vaporization of latent moisture owing to intense heating, either directly applied or generated by chemical reaction".

BJÖRK (1967) studied the influence of acetolysis mixture on the size of *Phragmites australis*[1] pollen. Prolonged boiling causes an increase in size.

CURRENT MOUNTING METHODS

The oldest mounting media reported in the literature are glycerin and glycerin jelly. More recently many other mounting media have been recommended by various specialists, but most of these media have not been accepted by palynologists. This does not apply to silicone oil, suggested by ANDERSEN (1960). Other mounting media can be found in BROWN (1960), who offers a comprehensive list.

The effect of mounting media on pollen size

Various specialists report that pollen grains mounted in glycerin jelly often swell markedly. The same applies to glycerin as mounting medium, but to a lesser extent.

CHRISTENSEN (1954) called attention to the tendency of exines to swell in

[1] According to CLAYTON (1968), *Phragmites australis* (CAV.) TRIN. ex STEUDEL is the correct name for *Phragmites communis* TRIN.

glycerin and glycerin jelly. He stated that this effect was probably due to absorption of water.

ERDTMAN and PRAGLOWSKI (1959), ANDERSEN (1960), AYTUG (1960), DONNER and VUORELA (1966), LOBREAU (1966) and BJÖRK (1967) reported the same effect and shared more or less the opinion of CHRISTENSEN (1954) about the cause of this swelling. FAEGRI and DEUSE (1960), however, stated "... we still do not know anything about what causes the sometimes abnormal behaviour of pollen grains in glycerol jelly preparations".

CUSHING (1961) reported a relationship between the distance of the cover glass and the slide, which he called the thickness of the slide, and the swelling of pollen grains. The size was found to be directly proportional to the thickness of the slide, in other words to the amount of pressure on the grains by the cover glass. Pollen grains mounted in slides thicker than the grains did not show any swelling, even after long storage.

TING (1966) pointed out that "... the expansion in glycerin jelly with time is caused by the acid reaction of the glycerin jelly".

PURPOSE OF THE PRESENT STUDY

Study of the effects of preservation on pollen size

There are two methods of preservation of polliniferous material available, namely: (*1*) Preservation by drying fresh material, which here is called physical preservation; (*2*) Preservation by storing fresh material in solutions like glacial acetic acid etc., which in this study is called chemical preservation.

The effect of preservation on the size of pollen grains will be discussed.

Study of the effect of "wetting agent" on pollen size

It is well known that detergent solutions like "Teepol", etc. soften dried flowers (DAVIS and HEYWOOD, 1963). This softening is based on a decrease of the surface tension by the detergent. Plant taxonomists in the Division of Palaeobotany and Pollen Morphology in Utrecht use "wetting agent"[1] to soften flowers. The effect of "wetting agent" on the size of pollen grains will be discussed later.

Study of the effect of mounting media on pollen size

It seems clear that mounting media like glycerin and glycerin jelly may affect the size of pollen grains. In this study attention will be given to the factors causing such changes in size.

[1] "Wetting agent" is made by Kodak Ltd., London

CHOICE OF MATERIAL

In this study pollen grains of *Corylus avellana* L. and *Quercus robur* L. were used. Reason for the selection of *Corylus* is that this species has already been studied before. It may be valuable to compare the results with those of other specialists. On the other hand *Quercus*, although less used for experiments, has a rather different kind of pollen type, namely 3-colpate versus 3-porate in *Corylus*.

Fresh polliniferous material was collected from a single specimen of *Corylus avellana* L. and from one specimen of *Quercus robur* L., both from the vicinity of Utrecht.

METHODS

Chemicals used

In this study the following chemicals were used:
acetic anhydride (acidum aceticum anhydridicum)
glacial acetic acid (acidum aceticum concentratum): 97–98%
glycerin (glycerinum): 50%
glycerin jelly prepared according to Sass; formula: gelatin 5 g, water 20 cc, glycerin 30 cc, phenol crystals 5 g
lactic acid (acidum lacticum): ca. 85%
potassium hydroxide (kalium hydricum): 10%
sulphuric acid (acidum sulphuricum): 94–96%
water; in all cases tap-water was used instead of distilled water (it appeared that tap-water, at least that of Utrecht, gave the same results as distilled water)
"Wetting agent": 1%

Preparation methods

The polliniferous material of both species was homologized alternately and afterwards divided into two parts. One part was preserved physically by drying in a stove at 75°C for 10 days and the other part chemically by storing in the following solutions: 10% potassium hydroxide, water, glacial acetic acid and lactic acid.

Physically preserved material

Pollen samples were boiled in a water bath with the same solutions as used for chemical preservation, namely 10% potassium hydroxide, water, glacial acetic acid and lactic acid. The time selected for boiling was in the case of 10% potassium hydroxide, water and glacial acetic acid 2, 4, 8, 16 and 32 min and in lactic acid 2 min only. After treatment, each sample was boiled in a mixture of 9 volumes

acetic anhydride and 1 volume concentrated sulphuric acid (acetolysis mixture) for 1, 2, 4, 8 and 16 min, respectively, in a water bath.

Chemically preserved material

Each sample was boiled in acetolysis mixture for 1, 2, 4, 8 and 16 min, respectively, in a water bath after storage for 3 and 12 months.

Mounting method

Pollen grains were mounted in glycerin jelly. According to the method described by PUNT (1962) granules of modelling clay were used as cover glass supports. The glycerin jelly was prepared according to Sass.

Measurements, statistics and graphs

All measurements have been carried out by this writer personally, for reasons mentioned by CHRISTENSEN (1946). The microscope used was a Leitz Ortholux, objective Leitz plan apo öl 100/1.32, eyepiece Leitz periplan GF × 10M and a Leitz eypiece micrometer. Each scale degree of the micrometer represents 1,03 μ (size unit).

Determination of size

To determine the size of the pollen grains of *Corylus* the equatorial diameter was measured in polar view. The size of pollen grains of *Quercus* was determined by measuring the polar axis in equatorial view.

Significance of the size differences

To test the significance of the size differences the "Student test" was used (see SNEDECOR, 1959); 100 pollen grains of each collection were measured. The class interval was 0,515 μ, i.e., half the size unit.

Composition of graphs

The sizes obtained by the measurements were plotted on a graph and connected with each other by a smooth line.

Physically preserved material. The relationships between size and the various treatments are shown in two ways: (*1*) A graph showing the relationship between size and boiling time with acetolysis mixture. The time of boiling with the solutions prior to acetolysis is constant. (*2*) A graph showing the relationship between size and boiling time with solutions prior to acetolysis. The time of acetolysis is constant.

Chemically preserved material. The relationships between size and boiling time with acetolysis mixture after preservation in one of the above mentioned solutions are shown in graphs.

THE EFFECTS OF PREPARATION METHODS ON PHYSICALLY PRESERVED MATERIAL

Effect of 10% potassium hydroxide on non-acetolysed grains

Prolonged boiling caused a slight increase in size of the pollen grains, although not significant. This applies to both *Corylus* (see Fig.13, curve *1*) and *Quercus* (see Fig.13, curve *2*).

Effect of 10% potassium hydroxide on acetolysed grains

Prolonged boiling causes a slight increase in size, although not significant. This applies to both *Corylus* (Fig.1) and *Quercus* (Fig.2). The size of acetolysed pollen grains is not influenced by the time of boiling in potassium hydroxide.

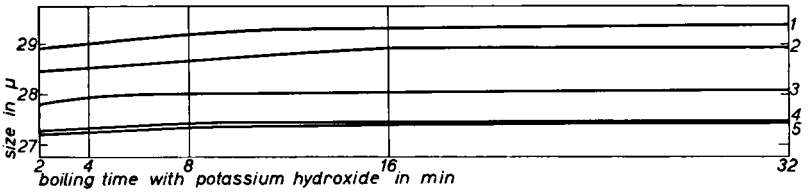

Fig.1. Influence of potassium hydroxide on the size of acetolysed pollen grains of *Corylus*. Curve *1*, acetolysis for 1 min; curve *2*, acetolysis for 2 min; curve *3*, acetolysis for 4 min; curve *4*, acetolysis for 8 min; curve *5*, acetolysis for 16 min.

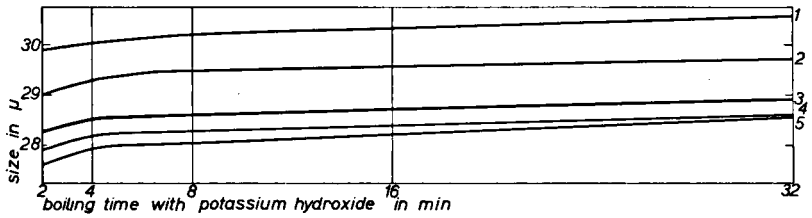

Fig.2. Influence of potassium hydroxide on the size of acetolysed pollen grains of *Quercus*. Curve *1*, acetolysis for 1 min; curve *2*, acetolysis for 2 min; curve *3*, acetolysis for 4 min; curve *4*, acetolysis for 8 min; curve *5*, acetolysis for 16 min.

Effect of acetolysis mixture on grains boiled in 10% potassium hydroxide

Continuous acetolysis for 4 min causes a decrease in size of the pollen grains for both *Corylus* (Fig.3, curves *1–5*) and *Quercus* (Fig.4, curves *1–5*). This decrease is no longer significant after 4 min acetolysis.

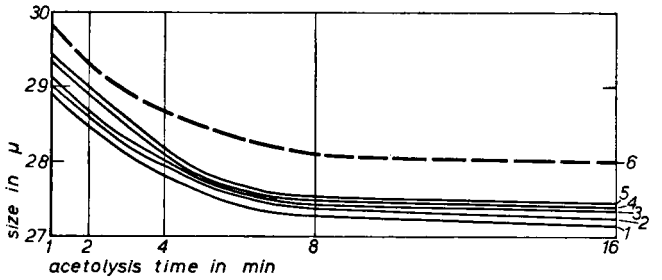

Fig.3. Influence of acetolysis mixture on the size of pollen grains of *Corylus* after treatment with potassium hydroxide. Curves *1–5*, dried material boiled in potassium hydroxide: curve *1*, boiled for 2 min; curve *2*, boiled for 4 min; curve *3*, boiled for 8 min; curve *4*, boiled for 16 min; curve *5*, boiled for 32 min. Curve *6:* fresh material preserved in potassium hydroxide, preservation for 3 months.

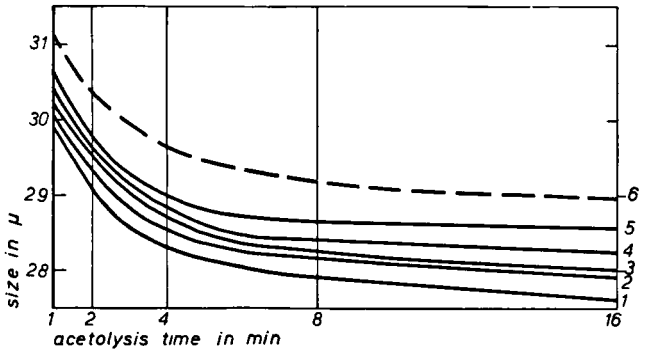

Fig.4. Influence of acetolysis mixture on the size of pollen grains of *Quercus* after treatment with potassium hydroxide. Curves *1–5*, dried material boiled in potassium hydroxide: curve *1*, boiled for 2 min; curve *2*, boiled for 4 min; curve *3*, boiled for 8 min; curve *4*, boiled for 16 min; curve *5*, boiled for 32 min. Curve *6*, fresh material preserved in potassium hydroxide, preservation for 3 months.

Effect of water on non-acetolysed grains

Maximum size increase of *Corylus* pollen grains is attained after 6 min (see Fig.13, curve *3*) and of *Quercus* after 5 min (see Fig.13, curve *4*) of continuous boiling.

Effect of water on acetolysed grains

Maximum size increase of *Corylus* pollen grains is reached after 20 min (Fig.5) and of *Quercus* after 12 min of boiling (Fig.6). The size of acetolysed pollen grains thus depends on the length of time of boiling in water.

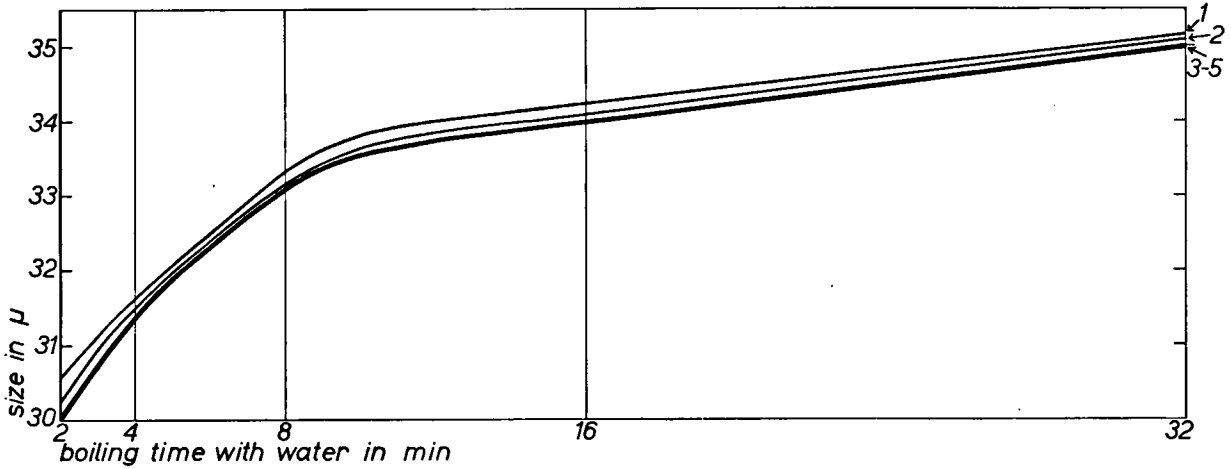

Fig.5. Influence of water on the size of acetolysed pollen grains of *Corylus*. Curve *1*, acetolysis for 1 min; curve *2*, acetolysis for 2 min; curve *3*, acetolysis for 4 min; curve *4*, acetolysis for 8 min; curve *5*, acetolysis for 16 min.

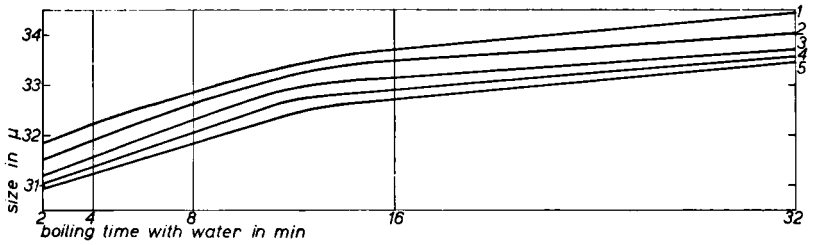

Fig.6. Influence of water on the size of acetolysed pollen grains of *Quercus*. Curve *1*, acetolysis for 1 min; curve *2*, acetolysis for 2 min; curve *3*, acetolysis for 4 min; curve *4*, acetolysis for 8 min; curve *5*, acetolysis for 16 min.

Effect of acetolysis mixture on grains boiled in water

Both *Corylus* (Fig.7, curves *1–5*) and *Quercus* pollen grains (Fig.8, curves *1–5*) show a minor though unimportant decrease in size after prolonged acetolysis.

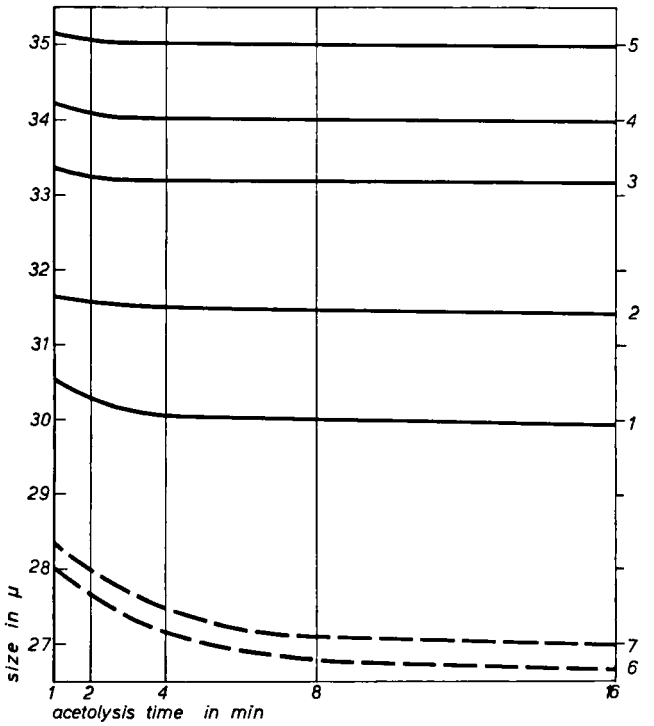

Fig.7. Influence of acetolysis mixture on the size of pollen grains of *Corylus* after treatment with water. Curves *1–5*, dried material boiled in water: curve *1*, boiled for 2 min; curve *2*, boiled for 4 min; curve *3*, boiled for 8 min; curve *4*, boiled for 16 min; curve *5*, boiled for 32 min. Curves *6–7*, fresh material preserved in water: curve *6*, preservation for 3 months; curve *7*, preservation for 12 months.

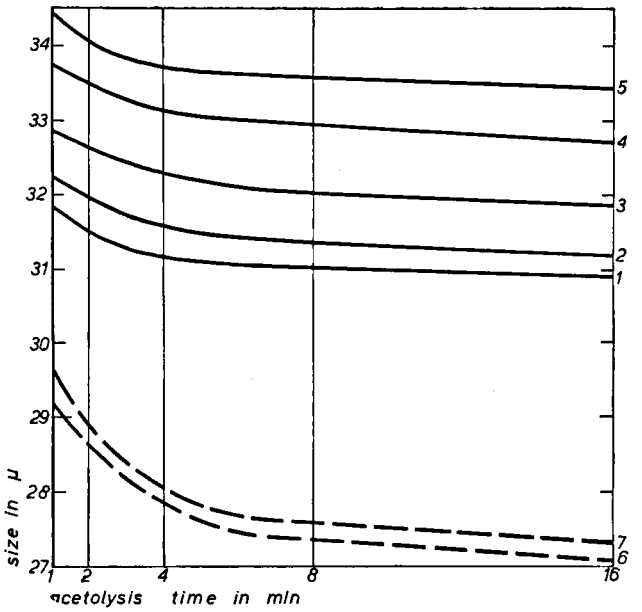

Fig.8. Influence of acetolysis mixture on the size of pollen grains of *Quercus* after treatment with water. Curves *1–5*, dried material boiled in water: curve *1*, boiled for 2 min; curve *2*, boiled for 4 min; curve *3*, boiled for 8 min; curve *4*, boiled for 16 min; curve *5*, boiled for 32 min. Curves *6–7*, fresh material preserved in water: curve *6*, preservation for 3 months; curve *7*, preservation for 12 months.

Effect of glacial acetic acid on non-acetolysed grains

Both *Corylus* (see Fig.13, curve *5*) and *Quercus* (see Fig.13, curve *6*) show a minor, but not important decrease in pollen-grain size after continuous boiling.

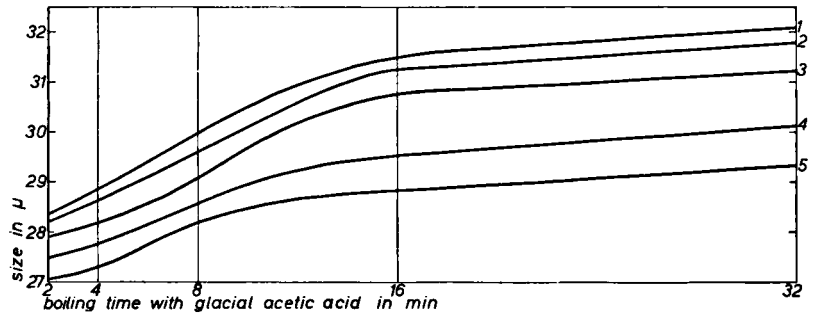

Fig.9. Influence of glacial acetic acid on the size of acetolysed pollen grains of *Corylus*. Curve *1*, acetolysis for 1 min; curve *2*, acetolysis for 2 min; curve *3*, acetolysis for 4 min; curve *4*, acetolysis for 8 min; curve *5*, acetolysis for 16 min.

Effect of glacial acetic acid on acetolysed grains

Prolonged boiling causes an increase in size of the pollen grains. This increase is no longer significant after 14 min or more for *Corylus* (Fig.9). The same holds for *Quercus* after 8 min or more (Fig.10). Moreover, it is obvious that boiling for 8 min or longer is detrimental to the cytoplasm, which can withstand up to 4 min of acetolysing. However, this does not apply to all pollen grains.

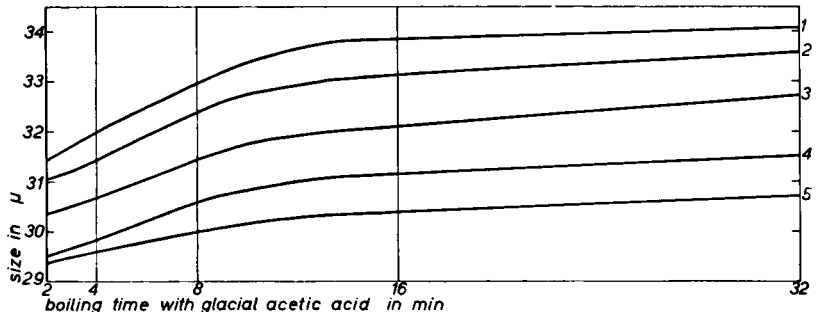

Fig.10. Influence of glacial acetic acid on the size of acetolysed pollen grains of *Quercus*. Curve *1*, acetolysis for 1 min; curve *2*, acetolysis for 2 min; curve *3*, acetolysis for 4 min; curve *4*, acetolysis for 8 min; curve *5*, acetolysis for 16 min.

Effect of acetolysis mixture on grains boiled in glacial acetic acid

Prolonged acetolysis causes a decrease in size. This decrease is no longer significant after 4–9 min or more, dependent on boiling time in glacial acetic acid,

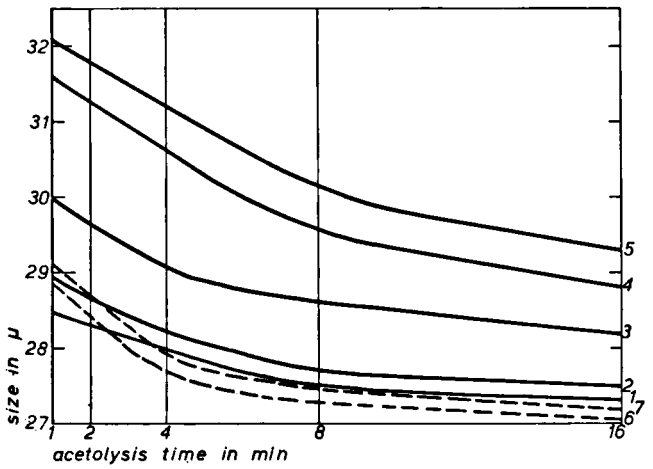

Fig.11. Influence of acetolysis mixture on the size of pollen grains of *Corylus* after treatment with glacial acetic acid. Curves *1–5*, dried material boiled in glacial acetic acid: curve *1*, boiled for 2 min; curve *2*, boiled for 4 min; curve *3*, boiled for 8 min; curve *4*, boiled for 16 min; curve *5*, boiled for 32 min. Curves *6–7*, fresh material preserved in glacial acetic acid: curve *6*, preservation for 3 months; curve *7*, preservation for 12 months.

for *Corylus* (Fig.11, curves *1–5*). The same holds for *Quercus* pollen grains after 4–7 min or more (Fig.12, curves *1–5*).

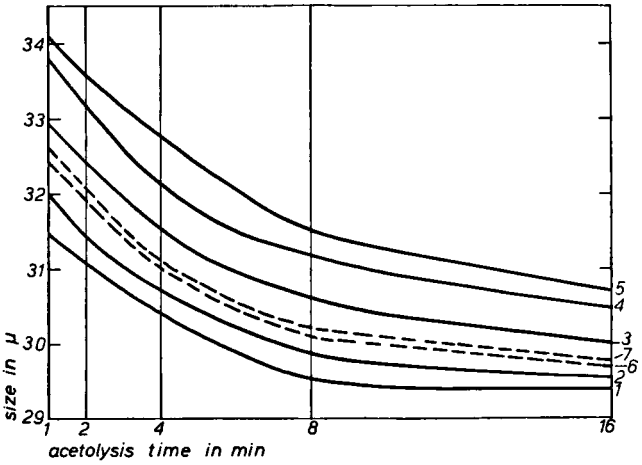

Fig.12. Influence of acetolysis mixture on the size of pollen grains of *Quercus* after treatment with glacial acetic acid. Curves *1–5*, dried material boiled in glacial acetic acid: curve *1*, boiled for 2 min; curve *2*, boiled for 4 min; curve *3*, boiled for 8 min; curve *4*, boiled for 16 min; curve *5*, boiled for 32 min. Curves *6–7*, fresh material preserved in glacial acetic acid: curve *6*, preservation for 3 months; curve *7*, preservation for 12 months.

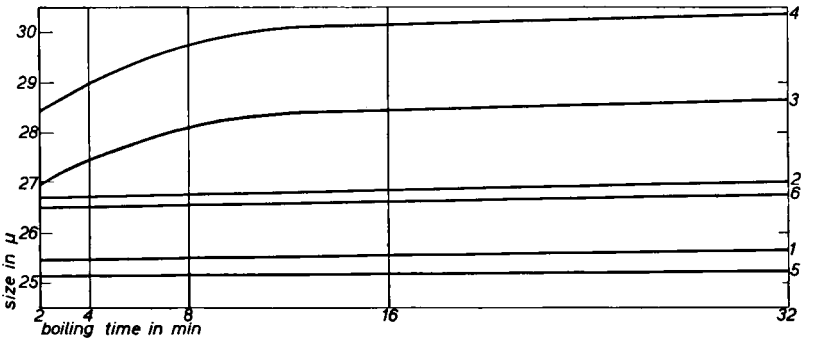

Fig.13. Influence of prolonged boiling in various liquids on the size of non-acetolysed pollen grains. Curve *1*, influence of potassium hydroxide on the size of pollen grains of *Corylus*; curve *2*, influence of potassium hydroxide on the size of pollen grains of *Quercus*; curve *3*, influence of water on the size of pollen grains of *Corylus*; curve *4*, influence of water on the size of pollen grains of *Quercus*; curve *5*, influence of glacial acetic acid on the size of pollen grains of *Corylus*; curve *6*, influence of glacial acetic acid on the size of pollen grains of *Quercus*.

Effect of acetolysis mixture on grains boiled in lactic acid

Prolonged acetolysis of both *Corylus* (Fig.14, curve *1*) and *Quercus* pollen grains (Fig.15, curve *1*) show a decrease in size after 2 min of boiling in lactic acid. This decrease is no longer important after 3 min.

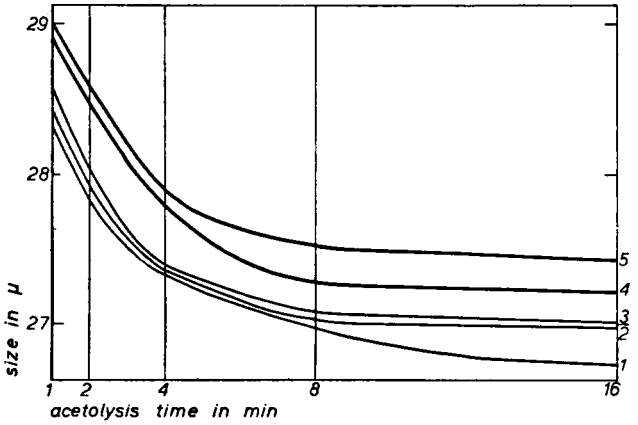

Fig.14. Influence of acetolysis mixture on the size of pollen grains of *Corylus* after various treatments. Curve *1*, dried material boiled in lactic acid for 2 min; curve *2*, dried material treated with "wetting agent"; curve *3*, fresh material preserved in lactic acid for 3 months; curve *4*, dried material boiled in potassium hydroxide for 2 min; curve *5*, fresh material preserved in glacial acetic acid for 3 months.

Effect of acetolysis mixture on grains treated with "wetting agent"

Continued acetolysis, after treatment with "wetting agent", of both *Corylus* (Fig.14, curve *2*) and *Quercus* pollen grains (Fig.15, curve *2*) shows a decrease in size. This decrease becomes less important after 3 min.

Treatment after ERDTMAN (1960)

The pollen grains were treated exactly as prescribed by ERDTMAN (1960). It was difficult to measure the size, because about 80% of the pollen grains were folded and wrinkled. The mean size of *Corylus* grains was 27.4 μ and of those of *Quercus* 28.2 μ.

Treatment after TEPPNER (1966)

TEPPNER (1966) recommended the use of hot glycerin after acetolysis but before mounting in glycerin jelly. After boiling in 10% potassium hydroxide for 2 min and acetolysis for 4 min, the mean size of *Corylus* grains was 30.6 μ and of *Quercus* grains 31.3 μ.

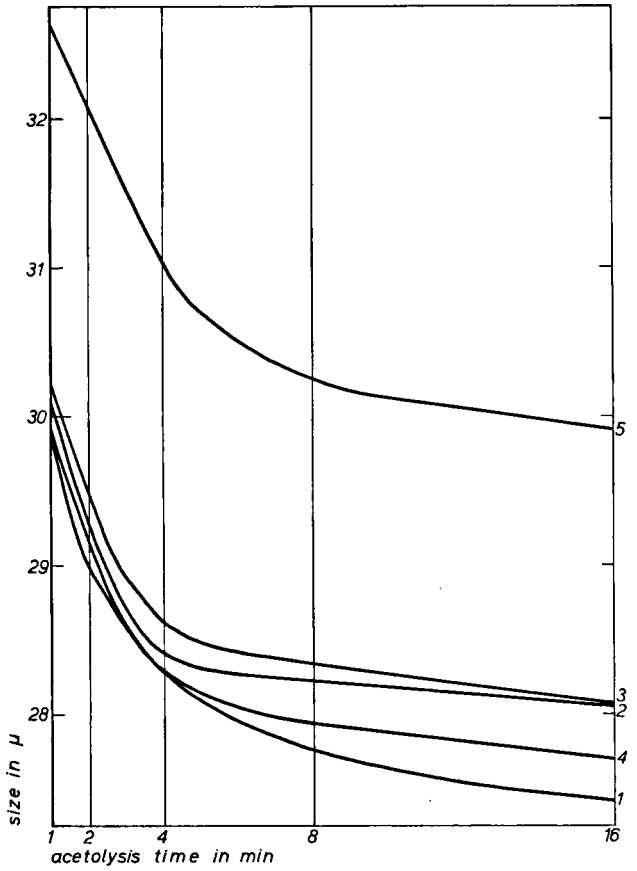

Fig.15. Influence of acetolysis mixture on the size of pollen grains of *Quercus* after various treatments. Curve *1*, dried material boiled in lactic acid for 2 min; curve *2*, dried material treated with "wetting agent"; curve *3*, fresh material preserved in lactic acid for 3 months; curve *4*, dried material boiled in potassium hydroxide for 2 min; curve *5*, fresh material preserved in glacial acetic acid for 3 months.

THE EFFECTS OF PREPARATION METHODS ON CHEMICALLY PRESERVED MATERIAL

Effect of acetolysis mixture on grains preserved in 10% potassium hydroxide

Storage for 3 months

Decrease in size, due to prolonged acetolysis, becomes less important after 4 min for *Corylus* (Fig.3, curve *6*) and after 3 min for *Quercus* grains (Fig.4, curve *6*).

Storage for 12 months

After 12 months storage time the exine was corroded to such an extent that measuring of the grains was no longer possible.

Effect of acetolysis mixture on grains preserved in water

Storage for 3 months

Decrease in size of pollen grains due to continuous acetolysis is no longer significant after 4 min for *Corylus* (Fig.7, curve *6*) and after 3 min for *Quercus* (Fig.8, curve *6*).

Storage for 12 months

Decrease in size due to continued acetolysis is no longer significant after 4 min or more for *Corylus* pollen grains (Fig.7, curve *7*) and after 3 min for *Quercus* (Fig.8, curve *7*).

Effect of acetolysis mixture on grains preserved in water and afterwards boiled in 10% potassium hydroxide

Decrease in size due to prolonged acetolysis is no longer significant after 4 min for *Corylus* grains (Fig.16, curve *2*) and after 3 min for *Quercus* (Fig.17, curve *2*).

Effect of acetolysis mixture on grains preserved in glacial acetic acid

Storage for 3 months

Decrease in size due to prolonged acetolysis is no longer significant after

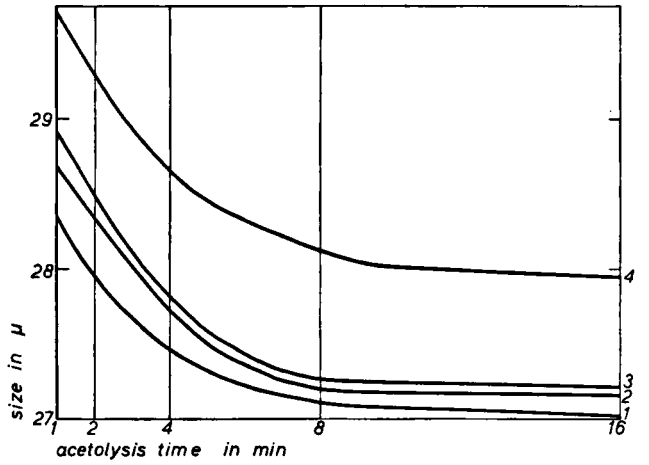

Fig.16. Influence of acetolysis mixture on the size of pollen grains of *Corylus* after various treatments. Curve *1*, fresh material preserved in water for 12 months; curve *2*, fresh material preserved in water for 12 months and afterwards boiled in potassium hydroxide for 2 min; curve *3*, dried material boiled in potassium hydroxide for 2 min; curve *4*, fresh material preserved in potassium hydroxide for 3 months.

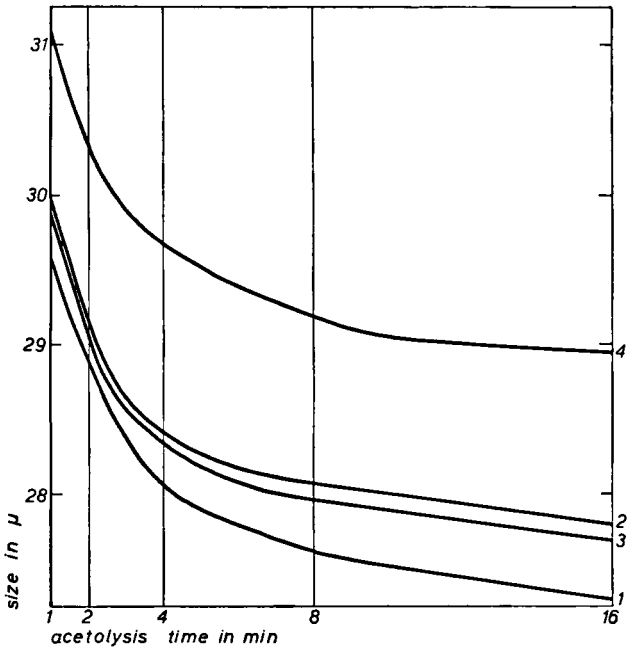

Fig.17. Influence of acetolysis mixture on the size of pollen grains of *Quercus* after various treatments. Curve *1*, fresh material preserved in water for 12 months; curve *2*, fresh material preserved in water for 12 months and afterwards boiled in potassium hydroxide for 2 min; curve *3*, dried material boiled in potassium hydroxide for 2 min; curve *4*, fresh material preserved in potassium hydroxide for 3 months.

4 min for *Corylus* (Fig.11, curve *6*) and after 5 min for *Quercus* (Fig.12, curve *6*).

Storage for 12 months

Decrease in size due to prolonged acetolysis is no longer significant after 4 min for *Corylus* grains (Fig.11, curve *7*) and after 5 min for *Quercus* (Fig.12, curve *7*).

Effect of acetolysis mixture on grains preserved in lactic acid

Storage for 3 months

Decrease in size due to prolonged acetolysis is no longer significant after 3 min for both *Corylus* (Fig.14, curve *3*) and *Quercus* grains (Fig.15, curve *3*).

Storage for 12 months

Decrease in size due to prolonged acetolysis is no longer significant after 3 min for both *Corylus* (Fig.14, curve *3*) and *Quercus* grains (Fig.15, curve *3*).

For these experiments physically preserved material of *Corylus* was acetolysed for 4 min after boiling in 10% potassium hydroxide for 2 min.

The effect of time

After mounting in glycerin jelly:

With cover glass supports. Results are shown in Table I. Time has no effect on the size.

TABLE I

EXPANSION OF POLLEN SIZE[1] IN GLYCERIN JELLY PREPARATIONS

Method	*Time*		
	1 h	*1 day*	*1 year*
With clay supports	27.8	27.8	27.9
Without clay supports	27.8	28.4	28.9[2]

[1] In μ; $N = 100$ pollen grains.
[2] Difference statistically significant.

Without cover glass supports. Results are shown in Table I. Time causes a significant increase in size.

After mounting in pure glycerine:

With cover glass supports. Results are shown in Table II. Time has no effect on the size.

TABLE II

EXPANSION OF POLLEN SIZE[1] IN GLYCERIN PREPARATIONS

Method	*Time*		
	1 h	*1 day*	*1 year*
With clay supports	27.8	27.9	28.0
Without clay supports	27.9	28.1	28.9[2]

[1] In μ; $N = 100$ pollen grains.
[2] Difference statistically significant.

Without cover glass supports. Results are shown in Table II. Time causes a significant increase in size.

STATISTICAL RESULTS

As mentioned on p.180 the size class was 0.515 μ and 100 pollen grains were measured in each slide. The calculated standard deviations of the mean values of *Corylus* are all 0.5 μ and those of *Quercus* are all 0,7 μ.

For *Corylus* all differences of mean values larger than 0.7 μ are significant; for *Quercus* those are 1.1 μ, both for $P = 0{,}05$.

DISCUSSION

The effect of preparation methods on shape

These experiments show that in general the shape of pollen grains does not change as the result of various treatments. This applies to both acetolysed and non-acetolysed pollen grains, but it does not apply to grains treated according to the method prescribed by ERDTMAN (1960) for herbarium material, i.e., physically preserved material. Physically preserved pollen grains treated by this method are often folded and wrinkled. Such grains are never observed in slides, if prepared by other methods. It is obvious that generally these former pollen grains are anomalous in shape. However, a small percentage of pollen grains is neither folded nor wrinkled and from these the shape can be determined. It appears that this shape does not differ from shapes of grains observed in slides, prepared in a different manner.

From these data it can be concluded that the acetolysis mixture cannot be held responsible for the change in shape. Since ERDTMAN (1960) does not prescribe any chemical treatment before acetolysis, the cause of the folding has to be sought in the pollen grain itself, i.e., in the means of preservation. SCHOCH-BODMER (1936) pointed out that living, untreated pollen grains shrink under dry and expand under wet conditions and that this process is reversible. Consequently, this process is a physical one. It seems evident that the amount of shrinkage depends on the solidity of the exine. It follows from the above that physically preserved pollen grains can be expected to shrink.

Since treatment of physically preserved pollen grains with acetolysis mixture does not cause an expansion, it will be necessary to expand dried pollen grains before acetolysis. This can be done either by boiling with 10% potassium hydroxide, water, glacial acetic acid and lactic acid or by treatment of the grains with "wetting agent". Shape can be of great value in the identification of pollen types.

From our experiments it is concluded that the shape remains reliable if the pollen grain is submitted to some sort of expansion prior to acetolysis.

The effect of preparation methods on size

Effect of the acetolysis mixture

From a comparison of sizes of grains prepared with and without acetolysis

and in the presence of an expansion solution it appears that there is a sudden increase in size at the moment the pollen grain is brought into contact with the acetolysis mixture. This is in agreement with the results of CHRISTENSEN (1946). The pollen grain seems to expand with a jump. This jump in size is greater with longer boiling time with an expansion solution. This effect of boiling time on the jump in size does not apply to 10% potassium hydroxide.

After the sudden increase in size the pollen grains decrease gradually during prolonged acetolysis (see CHRISTENSEN, 1946 and BJÖRK, 1967). After a certain time the size decrease becomes insignificant. The time interval, during which the decrease in size takes place, is controlled by the expansion method used.

It will be necessary to decide upon a time limit for acetolysis. This time limit must fulfill the following conditions:

(*1*) The cell contents must be dissolved. Dissolution of the cell contents has already taken place after 1 min of acetolysis. Thus, every acetolysis time fulfills this condition.

(*2*) Slight variation in acetolysis time may not influence the size significantly. The curves of Fig.3, 4, 7, 8, 11, 12, 14, 15, 16 and 17 show that a variation of 1 min does not cause any significant change in size irrespective of acetolysis time. However, the experiments show that the greatest size decrease takes place during the initial phase of acetolysis. Because in most instances acetolysing for 3 min or more does not affect the size significantly, it is recommended to acetolyse pollen grains for at least 3 min to prevent the size being altered significantly.

(*3*) For morphological reasons it is desirable that the colour of the exine, caused by acetolysis, stands out. The intensity of this colour increases with the duration of acetolysis (BJÖRK, 1967, p.18). To study exine structures well it is desirable that the exine is neither too light nor too dark. It was proven that an acetolysis for 4–8 min gives excellent results.

From the three above-mentioned reasons it is evident that boiling in acetolysis mixture for 4–8 min will give the best results. Acetolysis for 4 min was consequently chosen as standard acetolysis time.

Effect of preservative solutions on expansion

Prolonged boiling in 10% potassium hydroxide has no significant effect on the size (see Fig.13, curves 1 and 2). The same applies to the size of acetolysed pollen grains (see Fig.3 and 4). This agrees with the results of FAEGRI and DEUSE (1960) for *Betula* pollen. From Fig.16, curves *1* and *2 (Corylus)* and Fig.17, curves *1* and *2 (Quercus)* it can be deduced that the sizes of pollen grains, acetolysed after preservation in water, will not differ from those preserved the same way but boiled in potassium hydroxide prior to acetolysis. These two sizes can be compared with those, obtained by acetolysis of physically preserved material after expansion by means of 10% potassium hydroxide.

Comparison of the sizes obtained by using the method of ERDTMAN (1960)

for physically preserved material shows that these sizes do not differ significantly from one another. The acetolysis time after treatment with potassium hydroxide, etc. must be 4 min or more. The fact that pollen grains have to be acetolysed for at least 4 min, is due to the long acetolysis time prescribed by ERDTMAN (1960). His instruction for acetolysis runs as follows: "Move a tube (filled with acetolysis mixture) to a water bath and warm up from room temperature or maximum 70°C to boiling point. Stop heating when the water boils and leave the tube in the water bath for about 15 min." Since the reaction by the acetolysis mixture depends on the temperature, it is not possible to find out either the start or the exact time of acetolysis, but it is clear that the active time of the process is at least 4 min.

The conclusion can be drawn that boiling with 10% potassium hydroxide has no chemical effect on pollen walls. This chemical inertness has also been demonstrated by VAN GIJZEL (1967) who found that alkaline solvents like potassium hydroxide did not affect the fluorescence of palynomorphs. It follows that the curves of Fig.3 *(Corylus)* and of Fig.4 *(Quercus)* merely show the effect of the acetolysis mixture on the size without any chemical effects.

In this study these curves are called the type curves of the acetolysis mixture. Each point of the type curve represents the type size of the pollen grains corresponding to a given acetolysis time.

A type curve can also be constructed when dried pollen grains which have been expanded in lactic acid or "wetting agent" and when fresh pollen grains which have been preserved in lactic acid are treated with acetolysis mixture (see Fig.14, curves *1–3*, *Corylus*; and Fig.15, curves *1–3*, *Quercus*). The size of the pollen grains obtained after these treatments agrees with the type size.

The results show that any other treatment produces sizes and curves, which deviate from the type size and from the type curve.

Boiling in water does affect the exine. This is concluded from the following results:

(*1*) The effect of boiling in water on the size without acetolysis differs considerably from that of boiling in 10% potassium hydroxide without acetolysis (see Fig.13, curves *1* and *3*, *Corylus*; and Fig.13, curves *2* and *4*, *Quercus*).

(*2*) The acetolysis curve is quite different from the type curve (compare Fig.3 and Fig.7, *Corylus*; and Fig.4 and Fig.8, *Quercus*).

(*3*) The size obtained by acetolysis of pollen grains expanded in water differs significantly from the type size.

The same applies to material expanded in glacial acetic acid, although less markedly.

Preservation in 10% potassium hydroxide and in glacial acetic acid also affects the exine, but in a different way from preservation in water. Indeed the sizes obtained by acetolysis after preservation in the above-mentioned solutions differ significantly from the type sizes but the acetolysis process proceeds along a line similar to the type curve. However, there is one exception. The sizes of pollen

grains of *Corylus* obtained by acetolysis after preservation in glacial acetic acid do not differ significantly from the type sizes.

From all this it is obvious that independent of the treatment method used, the type size can be acquired. Consequently it is possible to speak of the actual size of acetolysed pollen grains.

It should be kept in mind that fresh pollen grains show variable sizes (SCHOCH-BODMER, 1936; FAEGRI and IVERSEN, 1964). Schoch-Bodmer pointed out that the process of swelling and shrinking is a reversible reaction, depending on the quantity of available moisture in the air. The degree of moisture fluctuates in nature and so will the size of fresh pollen grains. Thus a definite, constant size for fresh pollen grains does not exist.

One would assume that pollen grains mounted in a liquid are enclosed in a constant environment and consequently would show a constant size. However, according to SCHOCH-BODMER (1936), in this case the process of swelling or shrinking becomes irreversible as soon as the grains come into contact with the mounting liquid. Thus, grains mounted in a liquid cannot be considered untreated. Measurements on pollen grains treated this way do not produce the size of fresh untreated grains, but the size of non-acetolysed grains.

It is reasonable that acetolysis time should be fixed at 4 min (see p.194) and that expansion with 10% potassium hydroxide does not have any effect on the actual size.

The grain size obtained by acetolysis for 4 min of pollen material after expansion by 10% potassium hydroxide is called the acetolysis size in this study.

The next step was to check whether the acetolysis size of pollen grains of other plants agree with the sizes obtained by acetolysis for 4 min of the same material preserved in lactic acid or expanded by means of "wetting agent".

Polliniferous material of the following species was collected in the botanical garden of the State University at Utrecht: *Cerinthe minor* L., *Colchicum autumnale* L., *Delphinium ajacis* L., *Erodium manescari* COSS., *Linaria striata* D.C., *Phyteuma campanuloides* BIEB., *Potentilla nitida* L., *Ruta montanum* L., and *Weigelia florida* SIEB. et ZUCC.

Material of each species was divided into two parts. One part was preserved in lactic acid and the remainder was dried in a stove for 10 days at 75°C. The dried material was expanded with 10% potassium hydroxide or with "wetting agent". All parts were acetolysed for 4 min.

It is evident that the sizes obtained by using the three different kinds of treatment do not differ significantly from each other (Table III). The conclusion seems to be justified that treatment with these three methods always leads to comparable sizes.

Especially the use of "wetting agent" as an expansion method is of great value, because it is possible to collect polliniferous material from herbarium sheets without great damage to the flower.

TABLE III

SIZES[1] OF POLLEN GRAINS ACETOLYSED FOR 4 MIN AFTER VARIOUS TREATMENTS

Species	*Expanded in potassium hydroxide*	*Expanded in "wetting agent"*	*Preserved in lactic acid*
Cerinthe minor	13.6	13.7	13.5
Colchicum autumnale	71.6	71.8	72.0
Delphinium ajacis	26.2	26.1	26.1
Erodium manescari	74.2	74.0	73.8
Linaria striata	17.2	17.2	17.1
Phyteuma campanuloides	33.1	33.2	33.1
Potentilla nitida	22.1	22.0	22.1
Ruta montanum	20.7	20.7	20.8
Weigelia florida	51.3	51.5	51.4

[1] In μ; $N = 100$ pollen grains.

The effect of the acetolysis mixture on the exine

Acetolysis gives rise to a sudden size increase of the pollen grain. This increase cannot be compared with the expansion caused by the above-mentioned expansion solutions. The shape of the acetolysed pollen grains depends on the shape before treatment with acetolysis mixture. This might be due to a chemical reaction of the acetolysis mixture with the pollen wall, i.e., the sporopollenine.

On the other hand expansion could also be the result of a physical reaction of the following expansion solutions with the cell contents (see p.193): 10% potassium hydroxide, lactic acid and "wetting agent". This belief contradicts the opinion of TING (1966), who stated: ". . . the cause for expansion in both cases is vaporization of latent moisture, owing to intense heating".

Moreover he stated: "Little or no expansion will occur by overheating an acetolysed sample mounted in glycerin jelly, since the response to acetolysed heat (sic) has already been achieved." This statement is also contrary to the results of the present study, which shows that treatment of acetolysed pollen grains with hot glycerin causes a significant increase in size (see p.188).

Acetolysis, by itself, causes an increase in the size of pollen grains without reaching a maximum. Indeed, it seems likely that the exine retains its elasticity after the process of acetolysis. This is confirmed by the work of ANDERSEN (1960) who reported size changes in acetolysed pollen grains when transferred to another chemical medium. It is also substantiated by the size decrease on prolonged acetolysis.

Since the chemical composition of the exine is not exactly known, it is impossible to explain the cause of the decrease in size during acetolysis. The

acetolysis mixture also changes the colour of the exine. The exine of untreated pollen grains shows a faint colour. However, after acetolysis the colour of the exine becomes yellow-brown (see also BJÖRK, 1967). By accident it was found that the same colour can be obtained if some of the acetolysis mixture is dropped on unstained white wood.

Since one of the main components of wood is lignin, it is obvious to consider lignin responsible for this reaction. SHAW and YEADON (1964) found a lignin-like fraction as one of the components of the exine. They stated, however, that this fraction, sandwiched between the cellulose and the lipid fraction, did not show any reaction.

HESLOP-HARRISON (1968), however, found that the exine responds more positively to lignin tests in early stages of development. It is possible that the colour is caused by a chemical reaction between this lignin fraction and the acetolysis mixture after removing one of the layers, i.e., the cellulose (see also HESLOP-HARRISON, 1968). This is supported by the fact that the intensity of the colour increases if acetolysis is continued.

Not all pollen grains show the same colour after acetolysis. This may be explained by the following hypotheses: (*1*) there are different types of lignin-fractions; (*2*) there are varying amounts of the lignin-fraction; (*3*) the permeability of the protecting layers is variable; (*4*) there is a combination of the three possibilities.

The effect of mounting methods

From the results it is evident that glycerin and glycerin jelly can affect the size of pollen grains. However, this effect is nullified when cover glass supports are used. As pointed out by CUSHING (1961), the main reason for this size increase is the pressure of the cover glass.

Pollen slides are usually sealed with paraffin and this paraffin shrinks during hardening. This shrinking process, undergone by the paraffin, increases the pressure of the cover glass on the glycerin jelly. However the use of cover-glass supports nullifies this pressure to a large extent.

As pointed out before, hot glycerin has the effect of increasing the size of the pollen grains. It is possible that temperature also has an effect on the glycerin jelly. Although heating, until the glycerin jelly bubbles, causes no change in size (TING, 1966), it is advisable to avoid high temperature during the mounting procedure as much as possible.

ANDERSEN (1960) stated that the size of pollen grains of *Corylus* from Holocene deposits, does not differ significantly from that of recent *Corylus* pollen after treatment with potassium hydroxide and acetolysis mixture, provided that silicone oil is used as a mounting medium.

WHITEHEAD (1965) calculated the conversion factor for pollen grains mounted

in glycerin jelly and those mounted in silicone oil. The measurements from glycerin jelly preparation must first be divided by 1.06 if used as comparison with the measurements from silicone oil preparations. When using the conversion factor of Whitehead, it is evident that the sizes given by ANDERSEN (1960) do not differ significantly from those given by the present author.

It follows that glycerin jelly can be a good mounting medium for pollen grains provided that cover glass supports are used. Even pollen in 10 years old slides from the reference collection in our Division of Palaeobotany and Pollen Morphology in Utrecht did not show any tendency to increase in size.

RECOMMENDED SCHEME FOR PREPARATION

Treatment of herbarium material

Potassium hydroxide method

(*1*) Boil a number of flowers, buds or isolated stamens for a short time in 10% potassium hydroxide or in lactic acid.

(*2*) Isolate the anthers, if necessary with the help of a binocular microscope.

(*3*) Grind the anthers through a fine phosphor-bronze screen into a dish and wash with water. (N.B. The selection of the meshes of the screen depends on the size of the pollen grains; the smaller the meshes the cleaner the slides).

(*4*) Decant the liquid containing the pollen grains into a centrifuge tube.

(*5*) Centrifuge and decant.

(*6*) Wash with glacial acetic acid.

(*7*) Centrifuge and decant.

(*8*) Add acetolysis mixture to the residue.

(*9*) Transfer the tube to a water bath with boiling water and heat for 4 min.

(*10*) Centrifuge and decant.

(*11*) Wash with water.

(*12*) Centrifuge and decant.

(*13*) Wash with 50% glycerin in which some phenol crystals have been dissolved to prevent bacterial growth.

(*14*) Centrifuge, decant and place the tube upside-down on a filter paper for about 24 h at room temperature to drain and dry. (N.B. After decanting one may not place the tube upright, for it is possible that after placing the tupe up-side-down again the residue will not remain attached to the bottom of the tube.)

"Wetting agent" method

(*1*) Moisten the flower with some drops of a 1% "wetting agent" solution.

(*2*) Isolate some anthers after the flower has been softened. This preparation must be done carefully if damage to the flower is to be prevented.

(*3*) Grind the anthers through a screen into a dish. Wash with some lactic acid and thereafter with water. (N.B. It is possible to dry the flower and to replace it on the herbarium sheet.)

(*4*) Follow steps *4–14* of the potassium-hydroxide method mentioned above.

Treatment of fresh material

(*1*) Fresh polliniferous material should be preserved for a few hours or longer in well-closed bottles filled with lactic acid or water.

(*2*) Isolate the anthers.

(*3*) Follow steps *3–14* of the potassium-hydroxide method mentioned before.

RECOMMENDED SCHEME FOR MOUNTING

(*1*) Take some glycerin jelly about the size of a pinhead on a clean needle.

(*2*) Bring the glycerin jelly into contact with the dry residue of pollen grains.

(*3*) Place the glycerin jelly provided with pollen grains on a slide.

(*4*) Melt the glycerin jelly by gentle heating and stir with a needle to spread the pollen grains.

(*5*) Put two granules of modelling clay on either side of the glycerin jelly. (N.B. Do not use synthetic material (plasticine) because the melting-point of this clay is more or less the same as that of paraffin.)

(*6*) Place a cover glass on the two granules and press with a needle until the cover glass touches the polliniferous blob.

(*7*) Place some paraffin wax near the cover glass.

(*8*) Melt the paraffin wax by careful heating and let the melted paraffin run under the cover glass.

(*9*) Wait until the paraffin has hardened and then clean the slide with xylene.

ACKNOWLEDGEMENTS

The writer wishes to express his deep gratitude to Prof. Dr. F. P. Jonker, under whose supervision this work was carried out. He is also grateful to Dr. W. Punt, to Dr. C. R. Janssen, to Mrs. J. H. A. van Konijnenburg and to Mr. H. Visscher, who offered fruitful discussions and kindly criticized the manuscript. Credit is due to Mr. H. Rijpkema for the drawings, to D. K. Ferguson, B.Sc., M.Sc. for linguistic advice, and to Mrs. H. A. M. Wouters for typing the manuscript.

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