bers would be expected. Such a behavior has been observed by Rosenberg [3] for different metal chelates of amino acids, where, for example, the carboxyl frequency at 1 590 cm^{-1} of the Na^+ salt showed a shift of 29 cm^{-1} in Cu^{++} chelates and of 55 cm^{-1} in Pt^{++} compounds. The carboxyl frequency in the Zn^{++} and Ni^{++} complexes of PGA shows no such trend. On the contrary, a small shift towards lower wave numbers can be observed. If this shift has any significance of its own is difficult to decide as the position of the band for the Na^+ salt is very approximative owing to its broadness; but it is clear, anyhow, that the carboxyl group shows no evidence for covalent bond formation with Zn^{++} and Ni^{++}.

The absorption due to the other dominant group, the ionized phosphate, exhibits remarkable changes when Na^+ is substituted by Ni^{++} or Zn^{++}. Unfortunately, there is very little information available on the infrared absorption of ionized phosphate groups [7], which makes it difficult to interpret the changes in the spectra. The intense absorption band between 1 150 and 1 050 cm^{-1} (8.7—9.5 μ) (probably due to the ionized group and P—O—C stretchings) loses much of its intensity in the Zn^{++} and Ni^{++} complexes. In the Zn^{++} complex, the absorption peak shows a tendency to split into two bands and at the same time small changes in the position of the maximum absorption can be detected. The absorption bands in the 1 050—950 cm^{-1} (9.5—10.5 μ) region, also characteristic of the phosphate group, show a more complex picture in the Zn^{++} and Ni^{++} compounds compared to the Na^+ salt.

Zn^{++} activates enolase while the Ni^{++} enzyme is inactive [8]. As an explanation for this, it has been suggested, on the basis of ultraviolet absorption measurements, that the Ni^{++} complex with the substrate is of a different type than the complexes with the activating ions [8]. The infrared absorption data reported here show that such a difference, if it exists, cannot be the amount of covalent character of the interaction with the carboxyl group, since this is completely ionic in both complexes. On the other hand, distinct differences exist between the phosphate absorption bands of the Zn^{++} and Ni^{++} complexes, but unfortunately these cannot be properly evaluated at present.

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1. Malmström, B. G. *Arch. Biochem. and Biophys.* **49** (1954) 335.
2. Smith, E. L. *Advances in Enzymol.* **12** (1951) 191.
3. Rosenberg, A. *In preparation.*
4. Kiessling, W. *Ber.* **68** (1935) 243.
5. Warburg, O. and Christian, W. *Biochem. Z.* **310** (1942) 385.
6. Schiedt, V. and Reinwein, H. *Z. Naturforsch.* **7b** (1952) 270.
7. Bellamy, L. J. *The Infra-Red Spectra of Complex Molecules*, Methuen, London 1954.
8. Malmström, B. G. *Arch. Biochem. and Biophys.* **58** (1955) 381.

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Free Amino Acids in Pollen

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In the summer 1954 we studied the composition of the free amino acid fraction in the pollen especially of wind-pollinated plants. The method used was that generally employed in this laboratory for the identification of free amino acids in green plants, *i. e.* homogenisation of the plant material, extraction with 70 % alcohol, separation of amino acids with Amberlite IR-120, elution with 1 N ammonia, and subjection of the evaporated extract to two-dimensional paper chromatography with butanol-acetic acid and phenol-ammonia.

The wind-pollinated plants investigated are: *Alnus incana, Betula alba, Corylus avellana, Quercus robur, Pinus silvestris, Populus balsamifera, Populus tremula, Secale cereale, Salix caprea.* For the identification of the spots of hydroxyproline and citrulline a special colour reaction was used: the spot of hydroxyproline turns red[1], and that of citrulline yellow [2], when treated with isatin + *p*-dimethylaminobenzaldehyde (PDB). The amounts of the amino acids were roughly estimated on the basis of the intensity of the spots.

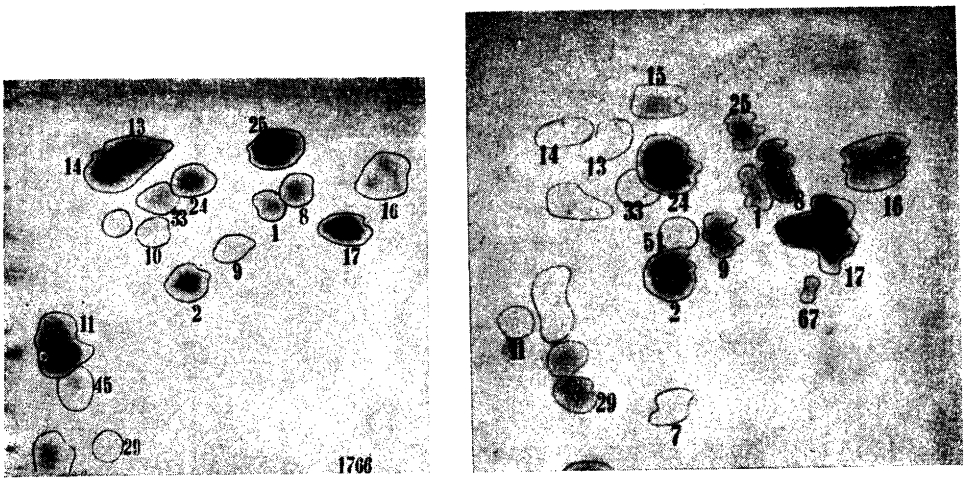


Fig. 1. Free amino acids of Betula alba. *Twodimensional paper chromatogram (phenol-NH_3 and butanol-acetic acid). A: pollen (210 µg N), B: leaves (182 µg N). 1 = gly, 2 = ala, 3 = val, 7 = tyr, 8 = ser, 9 = threo, 10 = OH-pro, 11 = pro, 13 = hist, 14 = arg, 15 = lys, 16 = asp, 17 = glu, 19 = glutathione, 24 = glu-NH_2, 25 = asp-NH_2, 29 = γ-aminobut, 33 = citr, 45 = ethanolamine, 51 = homoser, 60 = pipecolic acid, 67 = α-aminoadipic acid.*

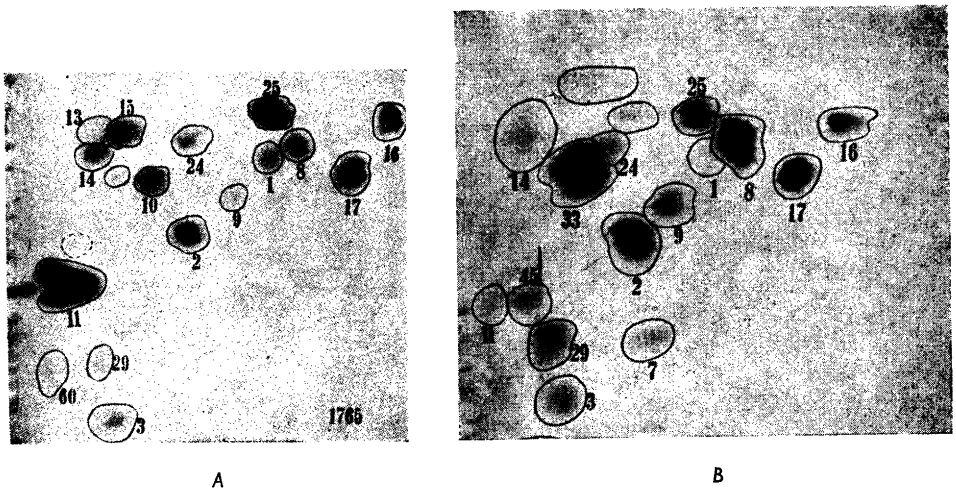
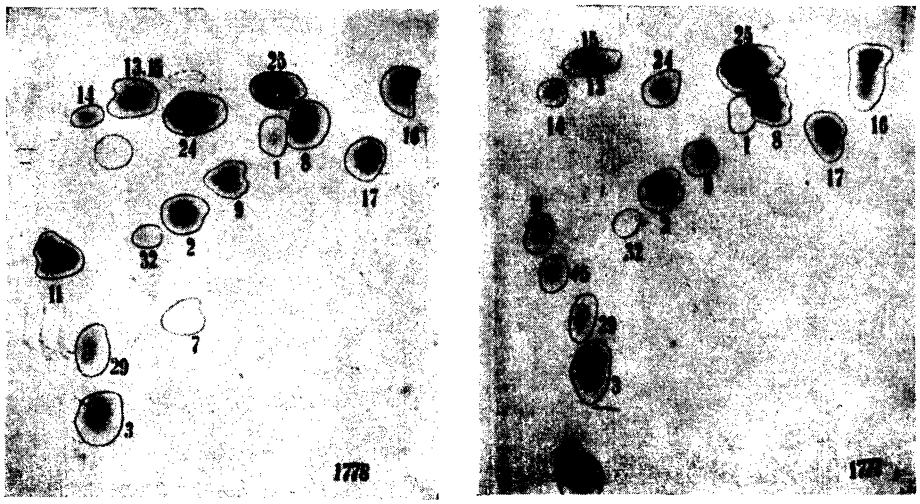


Fig. 2. Free amino acids of Corylus avellana. *A: pollen (210 µg N), B: leaves (200 µg N).*



A B

Fig. 3. Free amino acids of Populus balsamifera. *A: pollen (210 μg N), B: leaves (196 μg N). 32 = β – al.*

As examples of our results chromatograms of the free amino acids in *Betula*, *Corylus*, and *Populus balsamifera* are presented in Figs. 1–3. From these chromatograms some of the typical differences between the free amino acids in the pollen and in the leaves of the plants appear.

The most important results of our investigations are:

1. There is much more of free proline in the pollen than in the green parts of plants. Also the absolute amount of free proline is mostly high in the pollen. Because the dry matter content of pollen is very high, about 90 %, it is probable that the high proline content in pollen depends on this. Since the water content of the green parts of plants is usually high, 75–90 %, the formation of pollen is accompanied by a sharp decrease in the water content. Kemble and Macpherson [3] recently discovered that during the wilting of perennial rye grass the proline content increases so sharply that a considerable amount of proline must be synthesized during wilting. Glutamic acid is probably the precursor of proline because the enzymatic reaction: L-glutamic acid ⇌ L-proline is established[4].

2. Free hydroxyproline, the occurrence of which is comparatively rare in plants, is to be found in the pollen of four of the investigated plants *(Alnus, Corylus, Betula, Salix)*, but not in the green parts of any of these plants. It is possible that hydroxyproline is formed *via* oxidation of proline as has been found to be the case in the animal organism [5].

3. In addition to the high content of proline an imino acid containing a 6-membered ring, pipecolic acid (piperidine-2-carboxylic acid) is found in the pollen of most of the investigated plants *(Alnus, Corylus, Quercus, Betula, Salix, Secale)*, but not in the green parts. The biosynthesis of this acid from lysine is established [6]. The reaction is reversible. It is not known if drying promotes this synthesis.

The occurrence of proline in much higher amounts in the pollen than in the leaves of wind-pollinated plants is an interesting phenomenon. The formation of proline from glutamic acid leads to neutralization of this acidic amino acid and also to enrichment of carbon and nitrogen in the molecule. In the pollen of some plants pipecolic acid is formed from lysine (*cf.* above) which causes neutralization of this basic amino acid. Because the formation of proline is, however, many times greater than that of pipecolic acid, ringformation in its entirety lowers the acidity of the free amino acid fraction. From these imino acids the corresponding acidic and basic amino acids, respectively, may be formed

again during fructification. These circumstances can be of physiological significance.

4. Citrulline which is present in noticeable amounts in the leaves of *Alnus* and in large amounts in the leaves of *Corylus* could not be found in pollen. In other investigated plants citrulline does not occur, except in *Betula* where a small amount of it was found both in leaves and in pollen.

5. The amount of free basic amino acids is much higher in the pollen than in the leaves of many plants, as in *Betula*, *Populus tremula*, *Quercus*, and *Pinus*. A noticeable increase in amides (asparagine or glutamine or both) is also often found in pollen. When comparing the free amino acid fraction in pollen and in the green parts of plants it seems that the decrease in acidity of this fraction (*via* ringformation, formation of amides, increase in basic amino acids) is characteristic for the pollen of wind-pollinated plants.

Of the pollen of insect-pollinated plants only the free amino acids in the pollen of *Amaryllis* and *Lilium tigrinum* have been investigated. In these plants no typical differences between the amino acids in the pollen and in the green parts of the plants could be found. Because of the scantiness of the material no general conclusions can, however, be drawn about the free amino acids in the pollen of insect-pollinated plants.

1. Jepson, J. B. and Smith, I. *Nature* **172** (1953) 1100.
2. Dent, C. E. *Biochem. J. London* **43** (1948) 173.
3. Kemble, A. R. and Macpherson, H. T. *Biochem. J. London* **58** (1954) 46.
4. Vogel, H. J. and Bonner, D. M. *Proc. Natl. Acad. Sci. U. S.* **40** (1954) 688; Strecker, H. and Mela, P. *Biochim. et Biophys. Acta* **17** (1955) 580; Yura, T. and Vogel, H. J. *Ibid.* **17** (1955) 582.
5. Stetten, M. R. and Schoenheimer, R. *J. Biol. Chem.* **153** (1944) 113; Stetten, M. R. *Ibid.* **189** (1951) 504.
6. Lowy, P. H. *Arch. Biochem. and Biophys.* **47** (1953) 228; Rothstein, M. and Miller, L. L. *J. Biol. Chem.* **211** (1954) 851.

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Dissociation Constants in the Yeast Alcohol Dehydrogenase System, calculated from overall Reaction Velocities

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As shown recently [1], the overall data obtained for yeast alcohol dehydrogenase ** (ADH) are consistent with the formation of a ternary complex where all equilibria are adjusted rapidly except for the transformation of the ternary complex (step 5).

For this scheme, Alberty [2] has shown that K_3, the dissociation constant of the third step, is identical with the Michaelis constant of DPN, K_4 the Michaelis constant of EtOH etc. Furthermore, the new kinetic constant introduced by Alberty [2],

$$K_{\text{EtOH·DPN}} = K_1 \cdot K_3 = K_2 \cdot K_4 \text{ and}$$
$$K_{\text{AldD·PNH}} = K_6 \cdot K_8 = K_7 \cdot K_9.$$

From these relationships the dissociation constants of ADH · EtOH ($= K_1$), of ADH · DPN ($= K_2$) etc. can be calculated. In Table 1 are shown the data obtained using the kinetic constants of the previous work [1]. Although the conditions of

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** The following abbreviations are used: ADH, yeast alcohol dehydrogenase; DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; EtOH, ethyl alcohol; Ald, acetaldehyde.

1. ADH + EtOH ⇌ ADH · EtOH
2. ADH + DPN ⇌ ADH · DPN
3. ADH · EtOH + DPN ⇌ ADH · EtOH·DPN
4. ADH · DPN + EtOH ⇌ ADH·EtOH·DPN
5. ADH · EtOH · DPN ⇌ ADH · Ald · DPNH
6. ADH · Ald · DPNH ⇌ ADH · Ald + DPNH
7. ADH · Ald · DPNH ⇌ ADH · DPNH + Ald
8. ADH · Ald ⇌ ADH + Ald
9. ADH · DPNH ⇌ ADH + DPNH