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DIFFERENTIAL PULSE-POLAROGRAPHIC ANALYSIS OF TOBACCO MOSAIC VIRUS

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Summary. — The tobacco mosaic virus (TMV) strain vulgare and its mutant TMV 483 (with glutamine-9 replaced by histidine) and the denatured protein of TMV vulgare were analysed by direct current (d. c.) and differential (derivative) pulse polarography (DPP) in the basic electrolyte composed of 0.001 M $Co(NH_3)_6Cl_3$, 0.1 M NH_4Cl and 0.1 M NH_3 at 0 °C. The DPP method gave a substantially better resolution of the polarographic catalytic maxima A and B, but a much lower resolution of the maxima B and C, as compared with the d. c. polarographic method. The clear differentiation of the maximum A from maximum B by DPP permitted to study the variation of maximum A in the course of alkaline degradation of TMV. But for the study of TMV protein denaturation the d. c. polarography is preferable, because the denaturation is accompanied by the appearance and rise of maximum C which can be clearly differentiated from maximum B by d. c. polarography rather than by DPP. The DPP method was more sensitive than d. c. polarography. The denatured TMV protein can be determined by DPP at concentrations around 0.1 μg/ml.

Key words: tobacco mosaic virus; protein denaturation; pulse polarography

Introduction

Proteins containing cysteine (or cystine) in the presence of cobalt and ammonium ions give a polarographic reaction commonly known as the "protein double wave" (Brdička, 1933). Direct current (d. c.) polarographic studies of tobacco mosaic virus (TMV) and cytochrome c revealed that the "double wave" of these proteins is formed by three partially superimposed polarographic catalytic maxima, designated A, B and C (Ruttkay-Nedecký and Anderleová, 1967). Further studies (Ruttkay-Nedecký and Bezúch, 1971*a*) showed that the depolymerization and reversible denaturation of TMV protein is accompanied by marked changes on the d. c. polarographic curves under standard conditions (for specification of "standard conditions"

see Materials and Methods). Untreated TMV vulgare as well as its isolated (native) protein manifest themselves under these conditions predominantly by maximum B. Maximum A forms merely a slight inflection on the positive part of maximum B and maximum C is absent. Depolymerization of TMV protein (by mild alkali treatment) results in an increase of maximum B, but this increase can be detected only when the virus is polarographed up to a concentration of about 5 mg/ml. At higher concentrations the maximum B approaches its limiting height. Denaturation of the protein results in the appearance and rise of maximum C at more negative potentials with respect to that of maximum B (Ruttkay-Nedecký and Bezúch, 1971*a*,*b*). For a detailed discussion of the origin of maxima A, B and C and their relationship to the TMV structure see Ruttkay-Nedecký and Veselá (1977) and Ruttkay-Nedecký *et al.* (1977).

Maximum C can be well resolved from maximum B, but a good resolution of maximum A from maximum B has not been achieved so far at standard conditions. Therefore, the variation of maximum A in the course of TMV degradation cannot be studied by the d. c. polarographic technique.

Since a few years there has been an increasing tendency to replace the classical d. c. polarographic analysis by differential (derivative) pulse polarography (DPP) because of a higher sensitivity and resolution of the latter (Paleček, 1971; Kůta and Paleček, in press). The DPP method has been used for the analysis of proteins (Paleček and Pechan, 1971; Vorlíčková and Paleček, 1973; Paleček *et al.*, 1977). In the present work we attempted to apply DPP for the analysis of TMV with the aim at improving the resolution of the polarographic catalytic maxima and increasing the sensitivity of the polarographic analysis.

In our experiments we used TMV vulgare and its artificial mutant TMV 483 (Singer and Fraenkel-Conrat, 1966). These two virus strains have the same amino acid sequences except at position 9, where TMV vulgare and the mutant 483 have glutamine and histidine, respectively (Rombauts and Fraenkel-Conrat, 1968). It was shown (Ruttkay-Nedecký and Veselá, 1977) by the d. c. polarographic technique that the mutant TMV 483 has a substantially lowered structural stability as compared with TMV vulgare.

Materials and Methods

Viruses and their purification. TMV vulgare was kindly supplied by Prof. G. Melchers, Tübingen, F.R.G., and TMV strain 483 by Prof. H. Fraenkel-Conrat, Berkeley, U.S.A. Both strains were propagated in tobacco plants and purified by 4 cycles of differential centrifugation as described (Ruttkay-Nedecký and Veselá, 1977). The final virus pellet was dissolved in distilled water. The desired virus concentration was adjusted by the interferometric device of an Antweiler s microelectrophoretic apparatus (BOSKAMP Geräte-Bau K. G., Hersel, FRG).

Denaturation of the virus protein. Five ml of an aqueous solution of TMV vulgare (3 mg/ml) was dialysed in a cellophane bag for 12 hr at 4 °C against 100 ml of 8 M urea dissolved in 0.01 M NaOH. As TMV-RNA is polarographically inactive under the conditions used and does not interfere with the polarographic reaction of the denatured TMV protein (Ruttkay-Nedecký and Špánik, 1962), it was not removed from the analysed solution.

Polarographic analyses. Current-sampled d. c. and DP polarographic measurements were carried out on a Model 174 Polarographic Analyzer (Princeton Applied Research Corp.) using

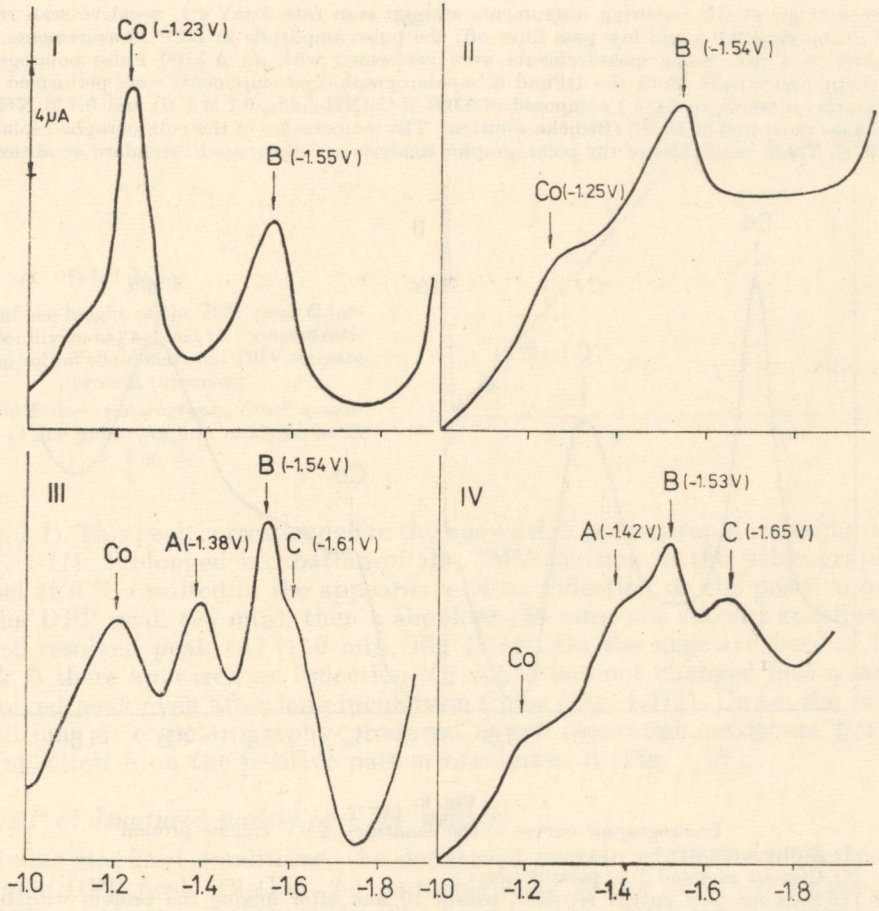

Fig. 1.

Polarographic curves of TMV vulgare

I and III: DP polarograms. The peak of cobalt is marked Co and the protein peaks A, B and C.
II and IV: Current-sampled d. c. polarograms. The diffusion wave of cobalt is marked Co and the protein catalytic maxima A, B and C.
I and II: Polarographic curves recorded within 20 min after mixing of the virus with b. e.
III and IV: Solutions polarographed in I and II, recorded again after 150 min incubation in b. e. in the polarographic vessel.
I — IV: Concentration of TMV in b. e. : 2.5 mg/ml. Temperature during the incubation of the virus in the polarographic vessel (including the registration of the polarographic curve) was 0 °C.
Abscissae: V.

a two-electrode system with dropping mercury electrode (DME) and a mercury pool on the bottom of a Novák's polarographic vessel as a reference electrode. DME had the following constants: at the mercury column height h = 45 cm the flow rate m = 1.85 mg s^{-1} (the value of m was measured in distilled water at open circuit). The measurements were performed in the

presence of air at the following instruments setting: scan rate 5 mV s^{-1}, negative scan range 1.5 V, drop time 2.0 s and low pass filter off; the pulse amplitude in DPP measurements was adjusted to 5 mV. Some measurements were performed with an A 3100 Pulse polarograph (Southern Analytical). Both the DPand d. c.polarographic measurements were performed in a background electrolyte (b. e.) composed of 0.001 M $Co(NH_3)_6Cl_3$, 0.1 M NH_3 and 0.1 M NH_4Cl, pH 9.4 (as measured at 25 °C) (Brdička solution). The temperature of the polarographed solution was 0 °C. These conditions of the polarographic analysis are designated "standard conditions".

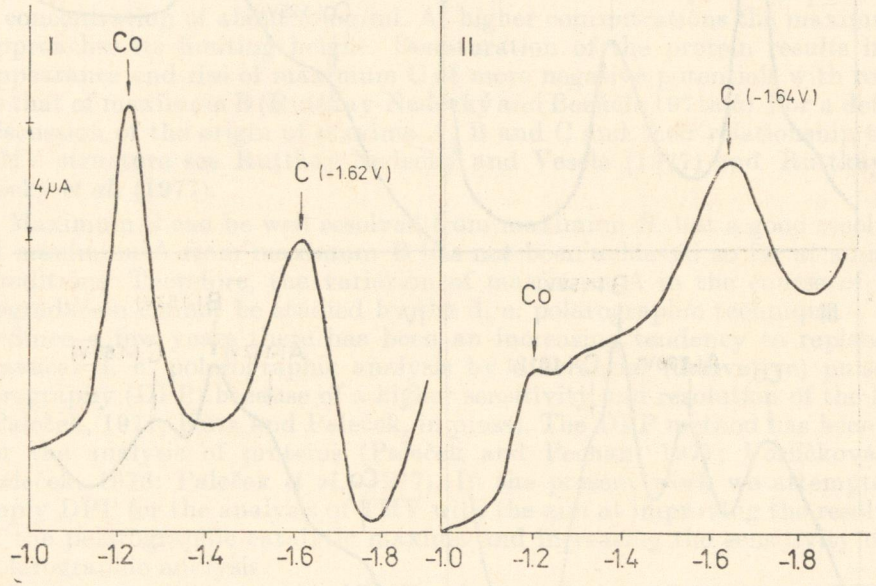

Fig. 2.

Polarographic curves of the denatured TMV vulgare protein

I: DP polarogram.

II: Current-sampled d. c. polarogram.

I and II: Polarographic curves recorded within 10 min after mixing the protein with b. e. Concentration of the protein in b. e. : 0.4 mg/ml. Other conditions of polarographic analysis the same as in Fig. 1.

For details of the polarographic analysis at 0 °C see Ruttkay-Nedecký *et al.* (1977). Before mixing with b. e., the viruses were dissolved in water and the denatured virus protein in 8 M urea. As ascertained in preliminary experiments, the presence of urea in b. e. at the concentrations used (1 M and less) did not appreciably influence the height and shape of the polarographic curve of the denatured virus protein.

Results

Comparison of DP and d. c. polarograms of TMV vulgare

TMV vulgare was studied at concentrations usual in d. c. polarographic experiments at standard conditions (see Materials and Methods). A freshly prepared test solution of TMV (measured within 20 min after mixing of the virus with b. e.) produced a single DPP peak (B) except the peak of cobalt

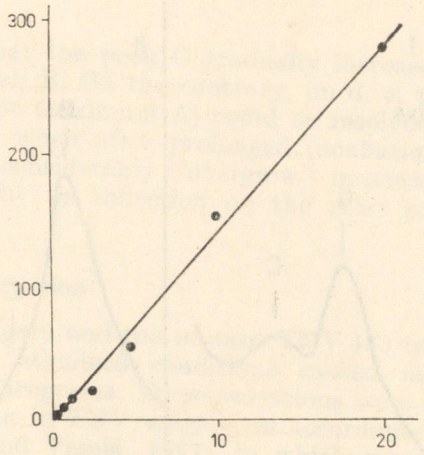

Fig. 3.
Plot of the height of the DPP peak C (ordinate: divisions) against the concentration (μg/ml) of the denatured TMV vulgare protein (abscissa)
A 3100 Pulse—polarograph; other conditions of the polarographic analysis as in Fig. 2.

(Fig. 1-I). This peak corresponded to the known d. c. polarographic maximum B (Fig. 1-II). Prolonged incubation of the TMV solution in the polarographic vessel at 0 °C resulted in the appearance of an inflection on the positive part of the DPP peak (25 min), then a shoulder (50 min; not shown) and finally a well-resolved peak (A) (150 min; Fig 1-III). On the negative part of the peak B there appeared an inflection (C) which has not changed into a well--resolved peak even after long incubation times (Fig. 1-III). Under the same conditions d. c. polarography produced a well-separated maximum C and an inflection A on the positive part of maximum B (Fig. 1-IV).

DPP of denatured protein of TMV vulgare

Under standard conditions, the denatured protein of TMV vulgare gave a single DPP peak (C) (Fig. 2-I) corresponding to the known d. c. polarographic maximum C (Fig. 2-II). The height of the DPP peak C depended linearly on the protein concentration in the range from 0.3 to 20 μg/ml (Fig. 3) and the limit of detection of the denatured protein was far below 0.3 μg/ml. Upon addition of the denatured protein to untreated TMV vulgare, an inflection (C) corresponding to the DPP peak C appeared on the negative part of the DPP peak B. This inflection increased with increasing concentration of the denatured protein in the solution (not shown).

Variation of polarograms in the course of TMV 483 disintegration

Prolonged incubation of TMV vulgare in the polarographic vessel under standard conditions (Fig. 1-III) caused the appearance of a well-defined peak A on the DPP curves. As shown previously (Ruttkay-Nedecký and Veselá, 1977), a similar treatment of the protein of TMV mutant 483 resulted in the appearance and rise of maximum C in the d. c. polarographic curves. Consequently, it was expected that by repeated recording of the DPP curve

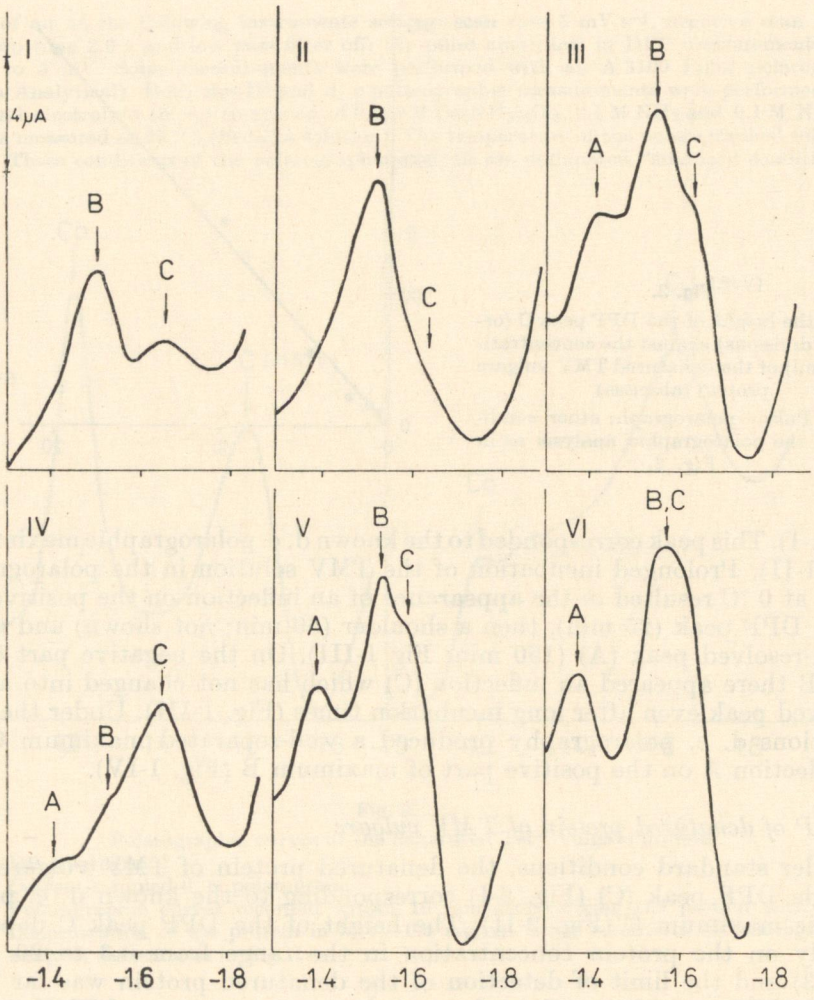

Fig. 4.
Time dependence of polarograms of TMV 483
I and IV: Current-sampled d. c. polarograms.
II, III, V, VI: DP polarograms.
I—VI: Time interval between mixing the virus with b. e. and the start of the recording of the polarographic curve: 4(I), 13(II), 63(III), 172(IV), 71(V) and 108(VI) min. Concentration of TMV 483 in b. e.: 3 mg/ml, other conditions of the polarographic analysis the same as in Fig. 1.

of TMV 483 in the course of its prolonged incubation in the polarographic vessel (under standard conditions) the simultaneous appearance and gradual rise of both peaks A and C could be achieved. Figs 4-II, III, V and VI show that under these conditions, the gradually developing DPP peak A was well

separated from peak B and also that the peak C gradually increased but remained poorly separated from peak B. On the contrary, on d. c. polarographic curves, maximum C (but not maximum A) could be resolved from maximum B (Fig. 4-I). Of course, when after prolonged incubation and repeated registration maximum C considerably "overgrew" maximum B, the latter manifested itself merely by an inflection on the more positive part of maximum C (Fig. 4-IV).

Discussion

Prolonged incubation ot TMV vulgare and the mutant TMV 483 in b. e. in the polarographic vessel under standard conditions caused marked variations of their DP and d. c. polarograms. These variations were much more pronounced in the mutant than in TMV vulgare, in accordance with previous results (Ruttkay-Nedecký and Veselá, 1977), in which report the differences in the d. c. polarographic behaviour of the two TMV strains are discussed in detail. In the present work our attention was focused on the capability of the DPP technique to resolve the polarographic catalytic maxima (peaks) A, B and C. We showed that DPP is more sensitive and that it resolves peak A from peak B much better than d. c. polarography (Fig. 1-III). The clear resolution of these two peaks under standard conditions is a prerequisite for the study of the changes in TMV structure causing the appearance and rise of maximum A in the course of TMV degradation. On the other hand d. c. polarography resolved much better maximum C from maximum B than DPP (Fig. 1-IV). Therefore, for the study of TMV protein denaturation, d. c. polarography is preferable, because this process is accompanied by the appearance and rise of maximum C. Thus the two polarographic techniques complement each other. Differences in the ability of these two techniques to resolve peaks (maxima) A, B and C are connected with slight differences in the potentials between DPP peaks and d. c. polarographic maxima. In DPP experients we used only a pulse amplitude of 5 mV. This is the lowest amplitude attainable with the PAR 174 instrument. Our preliminary data showed that at higher pulse amplitudes a higher sensitivity could be obtained for peaks B and C but not for peak A. The extremely high sensitivity of the DPP method is of great interest especially in the work with those viruses which unlike TMV are available only in very small amounts.

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