Biophysical Chemistry 27 (1987) 183–189
Elsevier

BPC 01169

Potassium transport through lipid bilayer membranes facilitated by tentoxin dimers

A new mechanism of ion carrier transport?

Martin G. Klotz, Eberhard Müller and Bernd Liebermann

Section of Biology, Departments of Plant Biophysics and Microbial Biochemistry, Friedrich Schiller University, Jena DDR-6900, G.D.R.

Received 7 November 1986
Revised manuscript received 16 March 1987
Accepted 6 April 1987

Tentoxin; Lipid bilayer membrane; Ion carrier transport

The cyclic tetrapeptide tentoxin at concentrations greater than 5×10^{-7} M selectively increases the ion conductivity for potassium of lipid bilayer membranes, while the naturally occurring derivative dihydrotentoxin has no influence on this property. Current-voltage curves, zero-current potential and charge-pulse measurements were used to characterize the action of tentoxin. The results suggest that a new mechanism of facilitated ion transport operates. The model of tentoxin dimerization and tentoxin-K^+ association developed is in contradiction to the model of tentoxin pore formation described recently by Heitz et al. (Biophys. Chem. 23 (1986) 245).

1. Introduction

It has been previously reported [2,3] that the cyclic tetrapeptide tentoxin increases the ion conductivity of lipid bilayer membranes. This phytotoxic substance is a product of the phytopathogenic fungus *Alternaria alternata* (FR.) Keissler and produces disease in the seedlings of several plant species by irreversible chlorosis [4]. In several papers it was investigated whether the property of tentoxin affecting ion transport is in any sense connected with known tentoxin induced phenomena such as chlorosis (not tested), photosynthetic electron-transport inhibition [5,6] and closure of stomata (Dahse and Schnabl, manuscript in preparation) or with as yet unelucidated properties such as its effect on potassium uptake by plant roots (Klotz and Erdei, manuscript in preparation). Tentoxin has also been used to model peptide-lipid interactions with respect to the permeation process [7].

The model of tentoxin interaction with membrane lipid components developed here, concluded from partitioning measurements and tested by means of NMR experiments [7], is based on our conductivity measurements which have not previously been published. Because of the contradiction between our results and those recently obtained in ref. 1, we describe further data concerning the action of tentoxin on the ion conductivity of lipid bilayer membranes in support of our model of tentoxin dimerization.

Correspondence address: M.G. Klotz, Section of Biology, Departments of Plant Biophysics and Microbial Biochemistry, Friedrich Schiller University, Jena DDR-6900, G.D.R.

2. Experimental

Tentoxin was isolated from a culture filtrate of *A. alternata* as described elsewhere [8]. The bilayer

membranes used here were formed from a dispersion of egg yolk lecithin (EYL) from both Merck (F.R.G.) and Koch Light (U.K.) as well as of dioleoylphosphatidylcholine (DOPC) in *n*-decane across a 1 mm circular hole in a Teflon septum, separating two identical electrolyte solutions with a pH of 7.2. KCl, NaCl, RbCl, NH_4Cl, $MgCl_2$ and $CaCl_2$ were used at 1 M for conductivity measurements, KCl also being employed for determining the zero-current potential (Ag/AgCl electrodes). The charge-pulse measurements were performed as described previously [9]. A voltage of approx. 10 mV was applied to membranes preformed from DOPC, (Serva, F.R.G.).

3. Results

Fig. 1 shows a plot of the conductivity of EYL bilayer membranes used as a function of the total applied tentoxin concentration in the presence of KCl. Linear regression analysis of the data ($n = 45$) yielded a slope of $a = 2.04$, the intersection with the axis being at $b = 5.4$ (correlation coefficient $r = 0.895$). A tentoxin concentration of 6×10^{-7} M was the lowest one to lead to a conductivity increase in the presence of K^+. Up to 10^{-5} M, tentoxin did not increase the membrane ion conductivity in the presence of NH_4^+, Rb^+, Na^+, Li^+, Ca^{2+} or Mg^{2+}. This strong selectivity is quite different from that of valinomycin-type carrier molecules and, of course from that of pore-forming substances, which have a rather low selectivity. Additionally, the effectivity of tentoxin action in this sense is much lower compared to valinomycin.

After addition of tentoxin (10^{-5} M) zero-current potentials were measured by increasing the salt concentration on one side of the membrane. Only in the presence of K^+, did the measured values differ from controls (without tentoxin), these potentials being shown in fig. 2. As in the case of valinomycin (data not shown, but see refs.

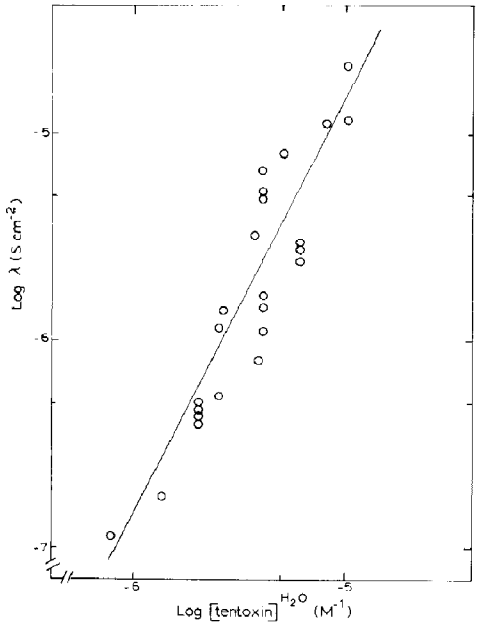

Fig. 1. Conductivity λ_m of EYL/*n*-decane bilayer membranes as a function of the total applied tentoxin concentration in the presence of 1 M KCl at $T = 295$ K and pH 7.1. Linear regression analysis yields: $n = 45$, slope = 2.04 and correlation coefficient = 0.895.

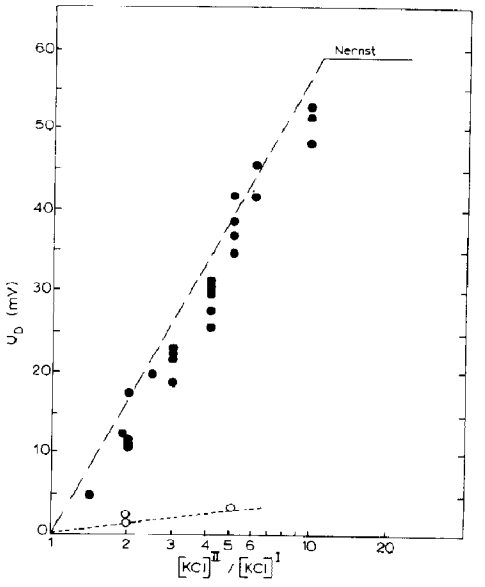

Fig. 2. Zero-current potentials: low K^+ concentration: 10^{-2} M. The concentration of KCl was progressively increased by the addition of small amounts from a 3 M KCl stock solution. $T = 295$ K. (○) Bilayer membrane; (●) bilayer membrane + 10^{-5} M tentoxin.

10 and 11), the data obtained on tentoxin satisfy the Nernst equation. These K^+ concentration potentials were observed when tentoxin was added to chambers containing either low or high K^+ concentrations. This led us to conclude that tentoxin is able to cross the lipid membrane interior without the cation obeying only the partitioning rules. This was also concluded from the partition coefficients measured for tentoxin [7].

Current-voltage curves were recorded in order to determine the rate-limiting step of the K^+ permeation caused by tentoxin. Following the theory in ref. 12, the conductivity ratio λ/λ_0 as a function of the reduced voltage $u = U_N F/RT$ can be expressed as

$$\lambda/\lambda_0 = \frac{(1+A)\,\sinh(k\cdot u/2)}{ku/2(1+A\,\cosh(ku/2))} \qquad (1)$$

where k is that part of the applied voltage which acts up on the charged complex (free K^+ experiences a fraction of the voltage drop for penetration of the head groups of the lipid molecules) and the parameter A is given by

$$A = 2k_{MS}\left[(k_R c_M + 2k_S + 2k_S^{ma})\right]\left[(k_D + k_{MS}^{ma})\cdot(k_R c_M + 2k_S + 2k_S^{ma}) - k_R c_M k_D\right]^{-1} \qquad (2)$$

which evaluates the influence of the membrane/water (ma) interface for the permeation process and is fixed by the kinetic rate constants of complex translocation (k_{MS}), carrier back transport (k_S), recombination (k_R) with respect to the ion concentration (c_M) and dissociation (k_D).

The best approximation of the data for tentoxin-mediated K^+ transport using eq. 1 yields $A = 0.08$ and $k = 0.73$ for $u \geq 3$ but $k = 1$ for $u \leq 2$ (shown in fig. 3). The charge-pulse measurements on DOPC/*n*-decane bilayer membranes shown in fig. 4 confirm the measured conductivity increase caused by tentoxin, however, the increase was only 30% compared to that of EYL/*n*-decane bilayer membranes. In this connection, it is interesting to note that the EYL used contains 34% DOPC.

For tentoxin, only one relaxation process was observed in charge-pulse measurements. This result has been described previously for beauvericin

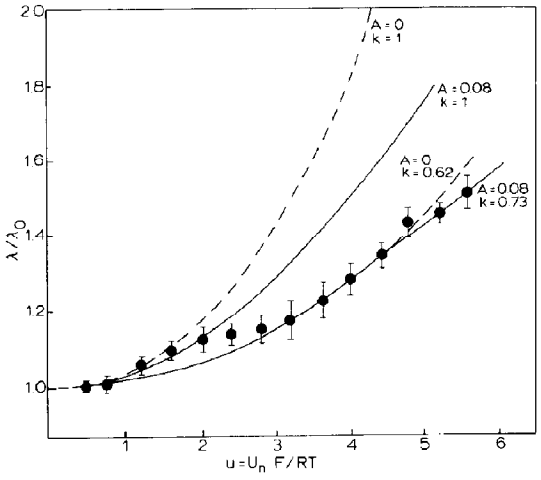

Fig. 3. Current-voltage curves: ratio of the voltage-dependent conductivity and low-voltage conductivity (U_N = 5 mV) as a function of the reduced voltage $u = U_N F/RT$. The parameters A and k used for data approximation provide a measure of the influence of the membrane interface on ion permeation and the voltage effectively drops through the membrane, respectively (see refs. 13 and 15). S.D. values were calculated for five independent experiments. Tentoxin concentration, 5×10^{-6} M; KCl concentration, 1 M.

[13] and the hydrophobic cations TPA^+ and TPP^+ [14,15]. The authors identified this single-relaxation process observed for the cations with the slower process of a two-step permeation mecha-

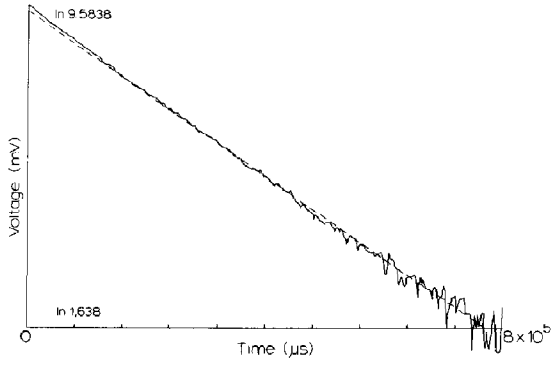

Fig. 4. Voltage-time plot of a charge pulse measurement at a DOPC/*n*-decane bilayer membrane under the influence of tentoxin. T = 295 K, pH 7.1. Tentoxin concentration, 10^{-5} M; KCl concentration, 1 M; $U_0 = 9.069$ mV; $\tau = 192970.56$ μs; $\lambda = 1.94\times10^{-6}$ S cm^{-2}, $r = -0.997$.

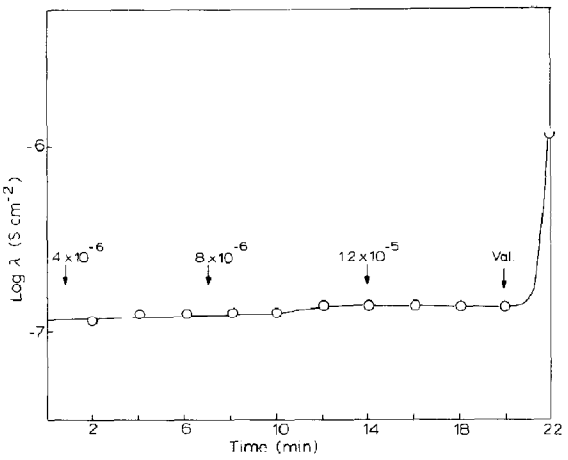

Fig. 5. Log ion conductivity of an EYL/*n*-decane bilayer membrane as a function of time. Arrows denote the addition of dihydrotentoxin to the given final concentrations in M. The system was tested by adding 10^{-8} M valinomycin (Val.). $T = 295$ K, pH 7.1; KCl concentration, 1 M.

nism (fast, adsorption/desorption; slow, translocation). They found that in this case processes at the interface are not rate-limiting and therefore the relaxation process observed corresponds to the ion-translocation step. Thus, the rate constants cannot be determined using the theory in ref. 9 and only the product of the translocation rate constant and partition coefficient can be calculated. The measured relaxation time may, under these conditions ($k_{ma} \gg k_i$), be identified with the steady-state time constant of the membrane $1/k_i = C_m/\lambda_m = \tau$ (C_m, membrane capacity). In this way a translocation time for K^+ (1 M) of $t = 5.1$ s was calculated for the effect of 10^{-5} M tentoxin. Only for the hydrophobic cations mentioned above have times in the seconds range been described previously ($t = 5.18$ s at a DOPC/*n*-decane bilayer membrane [14]). With respect to the reported fact that the naturally occurring derivative dihydrotentoxin does not cause seedling disease in many plant species (which are affected by tentoxin via irreversible chlorosis), we investigated whether the small difference in conformation leads to different behaviour of the K^+ conductivity increase of lipid membranes. Surprisingly, we found that with the loss of the double bond the dihydrotentoxin molecule as compared to tentoxin is unable to alter the membrane ion conductivity. This is shown in fig. 5, the system being proved by the known effect of valinomycin.

4. Discussion

As reported previously [2,3], tentoxin increases the ion conductivity of EYL/*n*-decane bilayer membranes selectively for K^+. With further measurements presented below, the slope of 3 reported for the relation between ion conductivity and tentoxin concentration is corrected to a value of 2 by a linear regression analysis. This quadratic dependence of ion conductivity on tentoxin concentration, demonstrated in eq. 3, coincides with the tentoxin dimerization within the membrane interior observed in ref. 7.

$$\log \lambda = \log[T]^2 + b \qquad (3)$$

In ref. 7, it was shown by partitioning measurements that tentoxin forms dimers in *n*-alkanes and fluidization of the lipid bilayer membrane fatty acid chain region was established using NMR spectroscopy. Additionally, it was shown that K^+ increases the tentoxin partition coefficient between *n*-alkanes and water, however, stiffening of the fatty acid chains of a bilayer membrane is induced by both tentoxin and K^+. Ivanov et al. [17] investigated whether the second power dependence of ion conductivity vs. enniatine B concentration measured for bovine brain lipid bilayer membranes was caused by a carrier dimer formation by synthesizing a 'bis-enniatine B'. This artificial dimeric enniatine B indeed showed a linear effect, the dose response supporting the hypothesis that the slope of 2 in the plot for natural enniatine B reflects mediated K^+ transport during enniatine B dimer formation.

The similarity between the charge-pulse measurements (only one relaxation process was observed) in the presence of tentoxin with the action of the enniatine derivative beauvericin mentioned is lessened by the faster translocation time of beauvericin K^+ transport and the significant fact that, although beauvericin increases the ion conductivity of bilayer membranes dose-dependently with a slope of 3, no concentration

dependence of the beauvericin partition coefficient was found [13]. On the other hand, beauvericin shows a strong superlinear relation between λ/λ_0 and the reduced voltage as required by eq. 1 for $A = 0$ using the carrier concept. Such a superlinear relation between the conductivity ratio and voltage is evidence that the rate-limiting step should be the migration of ions through the membrane interior, the interface reaction being in the equilibrium domain [13–15,17]. The parameter A must then decrease, which is connected with the boundary conditions, $k_{MS}^{ma} \gg k_D$ and $k_{MS}^{ma} \gg k_{MS}$, describing a solution complexation mechanism.

The k value calculated for tentoxin ($u \geq 3$) is nearly the same as that for 10^{-5} M enniatine B at DOPC/*n*-decane bilayer membranes [13] and for nactine-cation transport at monoolein/*n*-decane membranes [17], but differs from that for 10^{-7} M valinomycin ($k = 0.6$) and beauvericin ($k = 1$) [13].

The apparent voltage independence of the conductivity in the voltage range between 50 and 75 mV must be caused by diminution of the voltage which acts on the charged complex through the membrane. Rather, the low A and k values measured for tentoxin ($u \leq 2$) indicate a beauvericin-like behaviour of tentoxin. The action of beauvericin as well as of Pro-valinomycin on ion transport has been described as a solution complexation mechanism ($A \approx 0$ [13,14]). In studies [14,15] of the transport mechanisms of larger hydrophobic ions very low A-values have also been found (e.g., $A = 0.02$ for 10^{-4} M tetraphenylarsonium ($TPhA^+$) and tetraphenylphosphonium ($TPhP^+$), respectively, at glyceromonooleate/*n*-hexadecane bilayer membranes), indicating that the adsorption/desorption rate (k_{ma}) is about two orders of magnitude larger than the rate of translocation (k_i). With respect to the rather long ion translocation time (in the seconds range), relatively low A value and stiffening of the fatty acid chains of bilayer membranes by tentoxin-K^+, localization of the tentoxin dimers associated with K^+ in the chain end region of the membrane could be expected. This is supported by the fact that, in experiments using DOPC, K^+ transport was less effective than in EYL bilayer membranes owing to the smaller available volume of the equal-chained DOPC membrane. Furthermore, support is also provided by the fact that tentoxin neither causes a conductivity increase nor induces a K^+ concentration potential on coloured membranes if 0.2 ml chloroform is used additionally as a lipid solvent (25%, v/v), but it does give rise to a small potential if chloroform is not employed (about 20% of the data shown in fig. 2). From partitioning measurements [7] it is known that tentoxin has a relatively high partition coefficient between chloroform and water and that it exists as a monomer in both solvents. It has also been reported [1] that up to 10^{-3} M, tentoxin exists in the monomeric form in chloroform as well as in water and methanol. Therefore, the electrochemical standard potential difference between *n*-decane, chloroform and the lipid inner layer should hinder dimerization.

Tentoxin neither influences the membrane capacity [7] nor leads to alteration of the membrane surface potential (A. Hattenbach, personal communication). Now, as a matter of fact, in addition to the capability of fast membrane permeation as has also been observed in vivo [18] by fluidization of the fatty acid chain region, tentoxin has the ability to associate as a dimer with K^+. K^+ as observed in conductivity measurements is able to use the tentoxin dimer as a vehicle to overcome the membrane energy barrier driven by an electrochemical gradient. The tentoxin dimer (with respect to the volume available within) should work as a cage coordinating the K^+ via the unbound carbonyl functions (of every pair of tentoxin molecules, one should be hydrogen-bonded with the two amide functions of the other to form the dimer), which has been tested successfully with the help of a CPK model.

As a summary of the results discussed here, we can confirm that the following facts are strong arguments against the channel concept for the action of tentoxin as proposed by Heitz et al. [1]: (1) low stoichiometry in connection with the small size of the molecule, (2) selectivity for K^+, (3) K^+ concentration potential measured in coloured membranes in the absence of chloroform as a lipid solvent, and particularly the facts that (4) tentoxin, depending on the temperature, fluidizes the membrane interior, and (5) in the presence of K^+ and tentoxin membrane stiffening is observed.

For a general discussion of the results using the parameters of the carrier model (A, k) developed only for 1 : 1 ion carrier ratios and dimerization of ion transport mediators, two assumptions appear to be necessary: (1) that the association of the dimer with the ion is independent of the dimerization process itself; and (2) that the concentration of the carrier molecule in the aqueous phase is about equal to the total carrier concentration used. From the partitioning experiments we know that tentoxin is able to form dimers in a hydrophobic environment in the absence of any cation [7]. Thus, we can introduce a stability constant for dimerization in the membrane:

$$K_T^m = [T_2^m]/[T_1^m]^2 \quad (4)$$

and an equilibrium constant for the association process:

$$K_{T_2K^+}^m = [T_2^m K^+]/[K^+][T_2^m]. \quad (5)$$

As mentioned before, the partition coefficient of tentoxin (Γ_T) is very low in comparison with other known carrier molecules (e.g., $\Gamma_{water}^{n\text{-decane}}$(tentoxin) $= 3 \times 10^{-3}$ [7]) because of its rather good solubility in water which allowed us then to assume:

$$\Gamma_T := [T_1^m]/[T_1^a] \cong [T_1^m]/[T^{total}]. \quad (6)$$

Combination of eqs. 4–6 gives

$$[T_2^m K^+] = K_T^m K_{T_2K^+}^m [K^+] \Gamma_T^2 [T^{total}]^2 \quad (7)$$

which should be equal to the expression for the concentration of the complex in the membrane used in the carrier model and, under the assumptions stated above, it seems possible to formulate an expression for ion conductivity as a function of the second power of the tentoxin concentration and partition coefficient using the equations developed in ref. 12:

$$\lambda = \frac{2F^2 k_{MS}}{dRT} f(u)[T_2^m K^+]. \quad (8)$$

On the other hand, a valinomycin-type interface complexation mechanism can easily be excluded from the discussion. If the action of tentoxin is described by a solution complexation mechanism, as reported for Pro-valinomycin-K^+ [14,19], tentoxin and K^+ would have to associate in the water phase. This possibility would appear to be excluded with respect to the increase in hydrophobicity on dimerization of tentoxin [7].

Eq. 8 should also be valid for the adsorption/desorption mechanism developed for permeation of large hydrophobic ions if k_{MS} is substituted by the translocation constant k_i.

However, especially in view of the lack of complete kinetic data, there is as yet no evidence available to help one to decide which of the mathematical models of carrier-mediated ion transport developed is useful to describe the action of tentoxin or whether one should determine if a possibly new ion carrier mechanism exists, with features of the translocation mechanism for large hydrophobic cations.

Further experiments and theoretical considerations are under way to resolve this problem.

Acknowledgements

The authors wish to thank Professor R. Benz (Würzburg) for performing the charge-pulse measurements, Dr. I. Dahse (Jena) for stimulating discussions and Dr. L. Erdei (Szeged) for critical reading of the manuscript.

References

1 F. Heitz, R. Jaquier, F. Kaddari and J. Verducci, Biophys. Chem. 23 (1986) 245.
2 M.G. Klotz, J. Gündel, E. Müller and B. Liebermann, Stud. Biophys. 90 (1982) 53.
3 E. Müller, M.G. Klotz, and I. Dahse, in: Membrane transport in plants, eds. W.J. Cram, K. Janácek, R. Rybová and K. Sigler (Academia Praha, Prague, 1984) p. 503.
4 N.D. Fulton, K. Bollenbacher and G.E. Templeton, Phytopathology 55 (1965) 49.
5 R. Beltzig, M.G. Klotz, E. Müller and B. Liebermann, Biochem. Physiol. Pflanzen 179 (1984) 707.
6 I. Dahse, D.N. Matorin and B. Liebermann, Biochem. Physiol. Pflanzen 181 (1986) 137.
7 M.G. Klotz, Thesis, University of Jena, GDR (1986).
8 B. Liebermann and B. Oertel, Z. Allg. Mikrobiol. 23 (1983) 503.
9 R. Benz and P. Läuger, J. Membrane Biol. 44 (1976) 171.
10 A.A. Lev and P.E. Bushinsky, Tsitologia 9 (1967) 102.
11 T.E. Andreoli, M. Tiefenberg and D.C. Tosteson, J. Gen. Physiol. 50 (1967) 2527.

12 G. Stark and R. Benz, J. Membrane Biol. 5 (1971) 133.
13 R. Benz, J. Membrane Biol. 43 (1978) 367.
14 A.D. Pickar and R. Benz, J. Membrane Biol. 44 (1978) 353.
15 J.P. Dilger and R. Benz, J. Membrane Biol. 85 (1985) 181.
16 V.T. Ivanov, A.V. Evstratov, L.V. Sumskaya, E.I. Melnik, T.S. Chumbridze, S.L. Portnova, T.A. Balashova and Yu.A. Ovchinnikov, FEBS Lett. 36 (1973) 65.
17 R. Laprade, F. Grenier, M. Page-Dansereau and J. Dansereau, Can. J. Biochem. Cell Biol. 62 (1984) 738.
18 I. Dahse, H. Felle, F.-W. Bentrup and B. Liebermann, J. Plant Physiol. 124 (1986) 87.
19 R. Benz, B.F. Gisin, H.P. Ting-Beall, D.C. Tosteson and P. Läuger, Biochim. Biophys. Acta 455 (1976) 665.