cation[8]. Considering the results obtained in the previous communication[1], however, all experimental data can be explained most reasonably through a mechanism shown in the Scheme. Thus, the reaction is believed to involve the intermediacy of the cyclized, hydrindene cation (V), which would be followed competitively[9] by the rearrangement (a) or (b). In spite of many valuable reports on the conjugated polyenylic cations[10], little is known on the properties and chemical structures of the quenched products derived from the protonated, conjugated trienoic or tetraenoic acid. It has been disclosed by our studies that the conjugated trienoic acid undergoes double-cyclization in H_2SO_4 to yield almost exclusively saturated lactones, whereas the conjugated tetraenoic acid affords the cyclized and subsequently rearranged, unconjugated acid isomers almost quantitatively, both via the same type of key intermediate. Thus, our finding offers an additional contribution to the chemistry of conjugated polyenoic acids of biological interest, including vitamin A acid.

Zusammenfassung. Es wird eine neuartige, säurekatalysierte Zyklisierung und Umlagerung an einer konjugierten Tetraencarbonsäure beschrieben.

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[8] Coloration of (II) in H_2SO_4: 368 nm (80% H_2SO_4), 364 (85%), 360 and 462 (90%; two peaks turn into a single absorption band at 364 nm in a few min). These values strongly suggest a formation of certain dienylic cyclized cation as a main species. The coloration can be regenerated either from the compound A (369 nm in 80% H_2SO_4) or from B (361 nm, 80%).

[9] The competition seems to be dependent on the concentration of H_2SO_4 used. Glc analysis[4] indicates that the formation ratio of A to B is 7:2 (80% H_2SO_4), 2:1 (85%), and 1:4 (90%).

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Rabbit Kidney Alkaline Phosphatase: Role of Sialic Acid in the Heterogeneity

Heterogeneity of alkaline phosphatase has been demonstrated in a number of tissues[1–3]. The kinetic properties of the resolved activities from either liver or kidney were shown to be very nearly the same[4]. In addition, the anodic migration of fast-moving enzymes in human serum[5], liver[6] and kidney[7] were reduced by previous treatment with neuraminidase, suggesting that they were one and the same protein with differences in the carbohydrate moiety[7]. However, the sheep brain alkaline phosphatase had been resolved into 2 activities which were shown to differ in their kinetic properties[8]. It was also shown that the neuraminidase susceptible enzyme retained its original kinetic behaviour after the removal of the terminal sialic acid[9].

We wish to report that, of the 3 isoenzymes in rabbit kidney, only the slow-moving component in acrylamide gel was found to be a sialoprotein. Further, the kinetic properties remain unaffected after the treatment with neuraminidase.

Experimental. Rabbits, of both sexes aged about 45 days, were sacrificed and the kidneys (weighing 5 to 6 g) were homogenized in 0.01 *M tris*-HCl buffer, pH 7.5. Subsequently, the material was extracted with butanol[10], dialyzed and then loaded on DEAE-cellulose column, previously washed and equilibrated with 0.01 *M tris*-HCl buffer, pH 7.5, according to the method of PETERSON and SOBER[11]. Protein in the enzyme solution was estimated by the method of LOWRY et al.[12] using bovine serum albumin as standard.

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Table I. a) Kinetics of normal and neuraminidase treated Enz I[a]

Substrate	$K_m \times 10^{-3}$	
	Normal	Neuraminidase treated
Phenylphosphate	3.3	2.5
β-Glycerophosphate	5.0	5.5
α-Glycerophosphate	4.0	4.0
3′-AMP	6.6	6.5
5′-AMP	6.0	5.5

b) Effect of activators and inhibitors

Modifiers	Concentration (*M*)	Original activity (%)	
		Normal	Neuraminidase treated
None	–	100	100
L-Phenylalanine	6.6×10^{-3}	110	110
Phosphate	6.6×10^{-3}	80	85
Mg^{++}	3.3×10^{-3}	300	300
Zn^{++}	6.6×10^{-4}	0	0
Be^{++}	6.6×10^{-4}	0	0

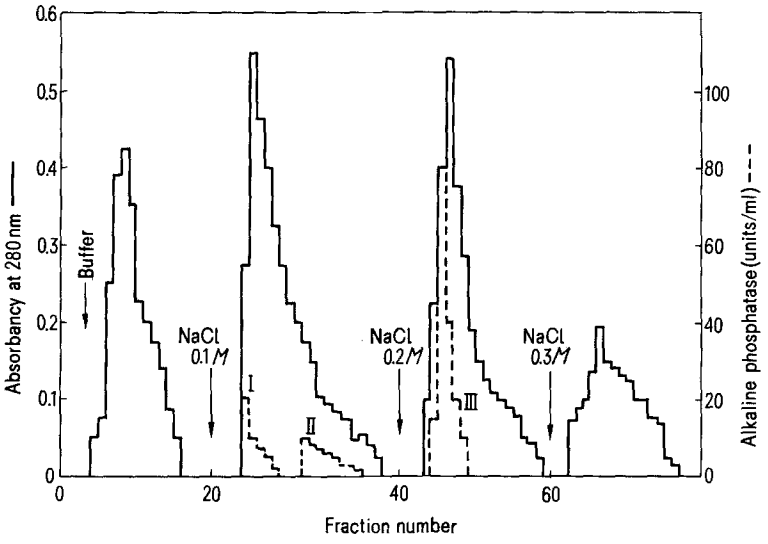

Fig. 1. DEAE-cellulose chromatographic profile. The column (1.8 cm × 22 cm) was loaded with 2755 units of enzyme and eluted with 0.01 *M tris*-buffer (pH 7.5) and then stepwise elution with 0.1 *M*, 0.2 *M*, 0.3 *M*, NaCl in *tris* buffer (0.01 *M*, OH 7.5) respectively. The flow rate was 10 ml per 10 min and 10 ml fractions were collected. The enzyme activity is expressed in units/mg protein (---) and protein is determined by absorbance at 280 nm (—) About 85% of activity applied to the column was recovered. Peak I, designated as Enz Ia (fraction 24–28) contained 19% of the activity recovered; peak II, designated as Enz Ib (fractions 31–36) contained 13% and peak III, designated as Enz II (fractions 44–48) contained 58%.

Disc electrophoresis of the resolved activities was performed on polyacrylamide gel by a slight modification of the procedure of DAVIS[13] with the use of 7.5% cyanogum-41 separating gel, prepared in 0.1 *M tris*-glycine buffer, pH 8.6. Initially, 2 mA per tube of 10 min was given, followed by 5 mA per tube for 150 min at 4–6 °C. The isoenzyme activities were visualized when incubated with β-naphthyl acid phosphate followed by coupling with *O*-dianisidine as described by CANAPA-ANSON and ROWE[14].

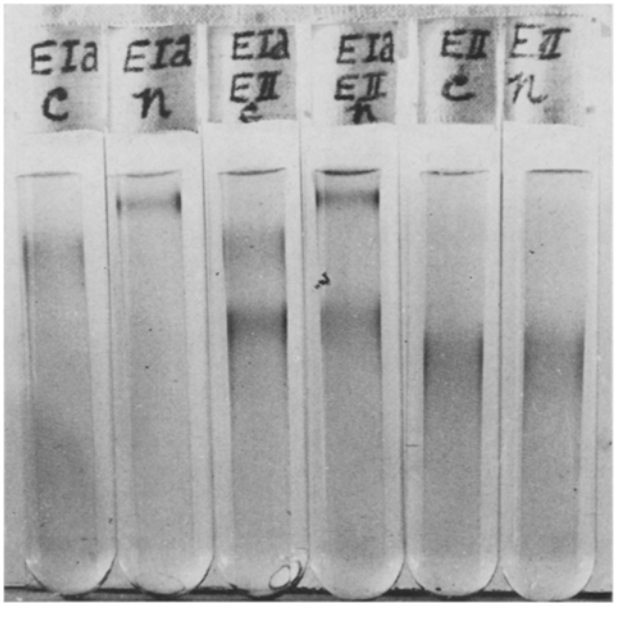

Fig. 2. Polyacrylamide gel electrophoresis. L to R: 1. Normal Enz I_a. 2. Enz I_a after neuraminidase treatment. 3. Normal Enz I_a and Enz II. 4. Neuraminidase treated Enz I_a and Enz II. 5. Normal Enz II and 6. Neuraminidase Enz II. Enz I_b is not shown in the picture. The enzyme activity was visualized by the method described in the text.

Neuraminidase treatment. The enzyme samples were first dialyzed against 0.1 *M* sodium acetate buffer, pH 5.5, for 8 h at 4–6 °C. To 1 ml of these dialyzed enzyme solutions (30 units/ml) were added the following: 2 μmoles $CaCl_2$; 5 μmoles $Mg(CH_3COO)_2$; 60 μmoles NaCl; and 0.44 unit neuraminidase (*Cl. perfringens*, type V Sigma). These mixtures were then incubated at 37 °C for 12 h. Aliquots were directly taken for electrophoresis and then the remaining enzyme was used for kinetic studies after dialysis against *tris*-HCl buffer.

Alkaline phosphatase activity in the extracts and in the eluates of DEAE-cellulose column was determined by the method of KING as modified by MOOG[15] using phenyl phosphate as the substrate. 1 unit of alkaline phosphatase activity is defined as the amount of enzyme that liberates 1 μmole of phenol or P_i (including for other substrates) in 15 min.

Results and discussion. The rabbit kidney alkaline phosphatase was resolved into 3 activities on DEAE-cellulose column (Figure 1), designated as Enz I_a, Enz I_b and Enz II. Of these, Enz I_b and Enz II had identical faster mobility in polyacrylamide gel electrophoresis. It was of interest to study whether the heterogeneity was due to the sialoprotein nature of the fast moving components. Contrary to the observations already reported, we find that only the slow-moving component (Enz I_a) contained the terminal sialic acid residue (Figure 2), and the fastmoving components (Enz I_b and Enz II) remained unaffected by neuraminidase treatment.

Kinetic studies of the control and neuraminidase-treated Enz I_a show that there was no alteration in the properties of the latter, characteristic of affinity for substrates and response to activators and inhibitors (Table I, a and b). It has also been observed that purified Enz I_a differed from purified Enz I_b and Enz II in many properties such as

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thermal stability, metal ion effect and affinity towards some substrates (unpublished observations).

These results point to the fact that at least in some tissues the heterogeneity observed is not solely due to the presence of terminal sialic acid residue. It may be mentioned here that the intestinal alkaline phosphatase isoenzymes are devoid of sialic acid[16,17]. The possibility of minor differences in the protein architecture itself seems to be gaining ground.

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Zusammenfassung. Es wurde gefunden, dass von den 3 Alkali-Phosphatase-isoenzymen nur das bei der Gelelektrophorese auf Polyacrylamid sich langsam bewegende Isoenzym ein Sialoprotein war. Die kinetischen Eigenschaften der normalen und der mit Neuraminidase bearbeiteten Enzyme waren ähnlich.

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Autolysis of *Coprinus comatus* Sporophores

The release of basiodiospores from species of the genus *Coprinus* is accompanied by a rapid autolysis of the cap of the fruiting body leaving only the stipe intact. This phenomenon has been examined in detail with respect to *Coprinus lagopus* (*C. cinereus*)[1,2]. From this work it was concluded that the main agents responsible for the autolysis of the sporophores of *C. lagopus* were chitinolytic enzymes located in the vacuoles of the fungus. These enzymes appeared shortly before the autolysis of the gills.

In view of the speed at which the autolysis of the gills of *Coprinus lagopus* occurs (4–5 h)[2], it is surprising that chitinases play such an important part in this process. Chitin is a substance notable for its resistance to degradation[3]. In Ascomycetes and Basidiomycetes it forms part of the wall, together with glucan, though it is usually present in smaller quantities than the latter[4]. ITEN and MATILE[2] assayed chitinase by measuring the release of reducing groups from purified *C. lagopus* walls, and chitobiase by the release of N-acetyl glucosamine from the same substrate. They found 30 times more reducing groups than N-acetyl glucosamine and interpreted this as meaning that chitinase was present in much greater quantities than chitobiase. The possiblity that the release of reducing groups was due to other carbohydrases (e.g. glucanase) does not seem to have been considered. As β-glucan is normally present as a prominent wall constituent of filamentous fungi[4], it is proposed that β-glucanase may have a significant part to play in the autolysis of *Coprinus* sporophores.

Enzyme activities detected in the autolysate of *Coprinus comatus* sporophores

Substrate	Enzyme	Activity/ml autolysate
Laminarin	β-(1 → 3) glucanase	1.30
Pustulan	β-(1 → 6) glucanase	0
Carboxymethyl Cellulose	β-(1 → 4) glucanase (cellulase)	0
Lichenin	β-(1 → 4), β-(1 → 3) glucanase	0.15
Colloidal Chitin	Chitinase	0.05
Azocoll	Protease	+

In order to test the validity of this idea, several sporophores of *Coprinus comatus* were collected from a wood near Montreux, Switzerland. Two of the best specimens, weighing 140 g each, were allowed to autolyse overnight at room temperature. After this time the sporophores were completely liquified. Microscopic examination showed the presence of spores and some fragments of hyphae, which were removed by centrifugation at 10,000 rpm for 30 min. The pellet was washed with water and the supernatant and washings were made up to 200 ml. This was tested for activity against the following substrates: laminarin and lichenin (Koch Light), pustulan and Azocoll (Calbiochem), carboxymethyl cellulose (Fluka) and colloidall chitin prepared from chitin (Koch Light) by the method of HOWARD and GLAZER[5]. All substrates except the Azocoll were dissolved or suspended in 0.05 *M* acetate buffer pH 5.0, and 10 ml were mixed with 1 ml of autolysate. The release of reducing groups from these substrates was followed at 30 °C by the NELSON SOMOGYI method[6], using the appropriate standard curve. One unit of activity was defined as that amount of enzyme which caused the release of 1 µMole reducing group/min. The Azocoll was used according to the manufacturer's instructions, though quantitative measurements could not be made due to interference from the colour of the autolysate. The results of these assays are shown in the Table.

No release of reducing groups was detected from pustulan or carboxymethyl cellulose, even after 24 h. The reducing groups liberated from lichenin must therefore have been due to the β-(1–3) linkages cleaved in this polymer. There appeared to be about 26 times as much β-(1–3) glucanase as chitinase in the 24 h autolysate, a figure similar to that of ITEN and MATILE[2] for the release of reducing groups and N-acetyl glucosamine from *Coprinus lagopus* walls. However, if the frozen residue was thawed, suspended in water and allowed to stand for a further 48 h, the ratio of β-(1–3) glucanase to chitinase dropped to 5 to 1. It thus appears that the β-(1–3)-glucanase plays a major role in the part of autolysis

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