In vitro hydroxyproline release from bone of rachitic rats killed 4–24 h after the i.v. administration of 2.5 μg 25-HCC or 2.5 μg D_3, or the solvent

Time lag between injection and killing (h)	Hydroxyproline release (ng/mg dry bone)		
	Group R (solvent)	Group 25-HCC	Group D_3
4	340 ± 99 (4)	355 ± 181 (5)	299 ± 86 (4)
6	374 ± 105 (5)	629 ± 173 (5)	360 ± 139 (5)
10	381 ± 70 (5)	790 ± 242 (4)	329 ± 73 (4)
13	321 ± 45 (5)	705 ± 138 (5)	379 ± 39 (6)
15	366 ± 68 (4)	998 ± 142 (4)	540 ± 180 (4)
19	340 ± 73 (4)	1065 ± 220 (4)	874 ± 153 (4)
24	329 ± 54 (4)	993 ± 212 (4)	982 ± 129 (5)

Means and standard deviations. The number of rats is given in brackets.

in 3 ml of modified Krebs-Ringer bicarbonate solution. It contained in 100 ml: 2.6 mg Ca, 10 mg inorganic P, 100 mg glucose, 15 mg ascorbic acid, 500 IU Penicillin, 0.1 mg Streptomycin and 6 ml serum from vitamin D deficient rats. The vessels were gassed with 95 % O_2 + 5% CO_2 and shaken in a Warburg apparatus. The hydroxyproline content of the incubation medium at the end of the incubation period was considered as hydroxyproline release from bone. The samples were hydrolysed in 6 *N* HCl at 120 °C for 3 h, and their hydroxyproline content was estimated[12].

Results and discussion. The mean hydroxyproline release of normal bone during the incubation period was 97 ± 27 ng/mg dry bone tissue. The other results are summarized in the Table. The hydroxyproline release of rachitic rat bone (R group) was more than 200 % higher than that of the normal control group. Compared to the R groups an increase of hydroxyproline release was found in all the groups killed 6–24 h after the 25-HCC supplementation and 13–24 h after the injection of vitamin D_3. For the groups killed 6, 10 and 13 h after the injection, the hydroxyproline liberation of the 25-HCC groups was significantly greater than that of the D_3 groups. At 24 h this difference had disappeared.

The observed 7 h difference in the lag period of action on bone collagen metabolism between 25-HCC and D_3 is similar to the findings of BLUNT et al.[2] for intestinal Ca transport and for serum Ca level. According to BLUNT et al. 25-HCC exhibits 140 % of the antirachitic activity of cholecalciferol[13]. KODICEK et al.[14] did not observe such difference for the intestinal ^{45}Ca transfer into the blood of rachitic chick. We administered larger doses, compared with the aforementioned studies, and observed a stronger effect of 25-HCC than that of D_3 at 13 and 15 h after the supplementation, but 24 h after the injection, when full effect of D_3 had developed, 25-HCC was not more active than D_3. Similar results were published earlier on intestinal transport of calcium[15].

Zusammenfassung. Nachweis, dass die Freisetzung von Hydroxyprolin bei Knochenfragmenten bereits 6 h nach i.v. Verabreichung von 2,5 μg 25-Hydroxycalciferol signifikant erhöht war, während eine entsprechend zugeführte Dosis von Vitamin D_3 erst 13 h später deutlich wirkte.

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Determination of the Binding Constant of Thiamine Diphosphate in Transketolase from Baker's Yeast by Circular Dichroism Titration

Transketolase from baker's yeast utilizes thiamine diphosphate and Mg^{++} as cofactors[1]. In order to understand the nature of coenzyme binding, transketolase reconstitution has been studied by activity and fluorescence quenching measurements[2]. The involvement of a tryptophan residue at the thiamine diphosphate binding site could be demonstrated by its chemical modification[3]. Furthermore, the appearance of a new circular dichroism band around 320 nm upon addition of thiamine diphosphate to apotransketolase was regarded as the result of a charge transfer interaction between the indole ring system of a tryptophan residue and the positively charged thiazolium ring system of thiamine diphosphate[4]. It is

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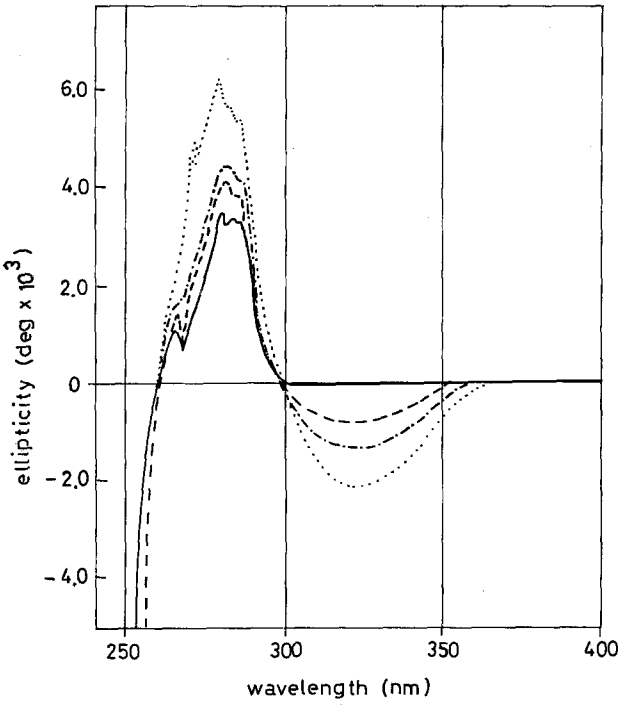
shown in the present communication that the measurement of the intensity of the new CD-band around 320 nm can be used for the determination of the binding constant.

Materials and methods. Crystalline apotransketolase (specific activity 16–21 units/mg protein) was obtained from Sigma Chemical Co. (St. Louis, USA). Transketolase activity was measured as described previously[2]. Transketolase protein was determined spectrophotometrically at 280 nm using $E^{1\%}_{1\,cm} = 14.5$[3].

Circular dichroism measurements were done on a Cary model 60 spectropolarimeter with Mod 6003 CD-attachment using cylindrical quartz cells of 0.5 cm pathlength.



Circular dichroism spectra of transketolase in 20 mM sodium phosphate buffer containing 10 mM Na_2SO_4, pH 7.2, 0.5 cm cuvettes were used. The reconstitution was carried out at 25° ± 0.1 °C. Conditions were: $7.9 \times 10^{-6} M$ apotransketolase, $3.7 \times 10^{-3} M$ $MgCl_2$ and $4 \times 10^{-6} M$ (---), $8 \times 10^{-6} M$ (-·-·), $24 \times 10^{-6} M$ (...) thiamine diphosphate.

Results and discussion. The Figure shows the CD-spectra obtained when apotransketolase/Mg^{++} is titrated with its coenzyme thiamine diphosphate. A CD-band around 320 nm is generated upon holotransketolase formation, which is due to charge transfer complex interaction between tryptophan as electron donor and thiamine diphosphate as electron acceptor. Assuming that all thiamine diphosphate molecules which bind to the tryptophan residues of apotransketolase give the same contribution to the intensity of the CD-band at 320 nm, it is possible to determine the apparent association constant for thiamine diphosphate from the following equation:

$$K_{app} = \frac{[\text{Holo-TK}]}{\{[\text{Apo-TK}] - [\text{Holo-TK}]\}\,\{[\text{TPP}] - [\text{Holo-TK}]\}}.$$

The initial concentrations of apotransketolase = [Apo-TK] and thiamine diphosphate = [TPP] are known, the concentration of holotransketolase = [Holo-TK] is obtained from the magnitude of the CD-band at 320 nm. The relation between the magnitude of the CD-band and the holotransketolase concentration is derived from the fact that the CD-band increases with increasing amounts of thiamine diphosphate up to a point where saturation is reached. Further increase of thiamine diphosphate concentration does not affect the intensity of the CD-band any more. We assume that at this point all apotransketolase has been converted to holoenzyme. An apparent association constant of $0.73 \times 10^6\ M^{-1}$ is obtained for thiamine diphosphate, which is in good agreement with the values obtained by other methods[2]. From activity and fluorescence quenching measurements $1.0 \times 10^6\ M^{-1}$ was found.

Zusammenfassung. Mit Hilfe des Circulardichroismus wurde die Bindungskonstante von Thiamindiphosphat in der Transketolase der Bäckerhefe bestimmt.

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The Number of Coenzyme Binding Sites in Transketolase from Baker's Yeast

Yeast transketolase has been found to consist of two identical subunits[1] suggesting two catalytic sites. In the present communication we report on the number of coenzyme binding sites.

Materials and methods. Crystalline apotransketolase (specific activity 16–21 U/mg protein) was obtained from Sigma Chemical Co. (St. Louis, USA). Transketolase activity was measured as described previously[2]. Transketolase protein was determined spectro-photometrically at 280 nm using $E^{1\%}_{1cm}$ 14.5[3]. The diphosphate of C^{14}-labelled thiamine (thiazole-2-C^{14}; specific activity 18.9 μC/μmole, obtained from the Radiochemical Centre, Amersham, England) was synthesized as previously described[2]. The enzyme preparation showed only a single band in the analytical acrylamide gel electrophoresis.

Results and discussion. For the determination of the number of coenzyme binding sites apotransketolase was incubated with an excess of Mg^{++} and C^{14}-thiamine diphosphate for 30 min at 25 °C. The reconstituted enzyme was gel-filtered over Sephadex G-25, the fractions were assayed for transketolase activity and pooled. Aliquots were taken for the determination of protein, thiamine diphosphate and transketolase activity. From the Table

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