71. Biosynthesis of Betalaines. On the Cleavage of the Aromatic Ring during the Enzymatic Transformation of Dopa into Betalamic Acid

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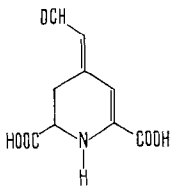
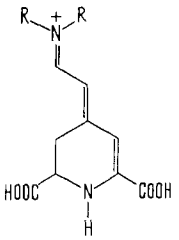
(3. X. 70)

Zusammenfassung. Bei der enzymatischen Umwandlung von 3′,4′-Dihydroxyphenylalanin (Dopa) in Betalaminsäure, in Betalain-haltigen Centrospermen, wird der aromatische Ring an der Bindung neben den beiden Hydroxylgruppen (C(4′)—C(5′)) und nicht zwischen den Hydroxyl gruppen (C(3′)—C(4′)) gespalten. Dies ergab sich aus dem Einbau von L-Tyrosin-[3′,5′-^{3}H] in Betanin mit Kaktusfruchtmaterial. Das [^{3}H]-Betanin und das daraus durch Hydrolyse erhaltene Betanidin enthielten ~90% des Tritiums am C(11), denn nach Behandlung des Betanins mit Prolin fanden sich ~90% der Radioaktivität im Indicaxanthin und nur ~10% im Cyclodopaderivat, und Behandlung des Betanidins mit CF_3COOH bewirkte keine Radioaktivitätsverminderung durch ^{1}H-^{3}H-Austausch.

Previous work [1] [2] has shown that 3′,4′-dihydroxyphenylalanine (dopa) and tyrosine are good precursors for the biosynthesis of the betalamic acid portion of betalaines[2]) [3], the red and yellow nitrogenous pigments of most *Centrospermeae*.

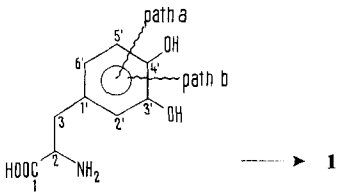


1, betalamic acid



2, general formula for the betalaines

It was concluded that the aromatic ring of dopa is cleaved enzymatically and that the hydropyridine ring system results from a subsequent cyclization involving the amino group.



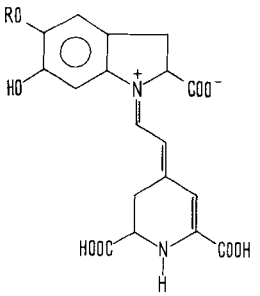
⟶ **1**

[1]) On leave of absence from the Department of Chemistry, Louisiana State University, Baton Rouge, Louisiana, 1968–1970.

[2]) In order to conform with a nomenclature rule of the I.U.P.A.C., from now on we are modifying our previous English orthography of the names betalain, betanin, betanidin, etc. by the addition of a terminal e.

In principle, dopa can form the betalamic acid skeleton by cleavage of the aromatic ring either at the bond *next to* the two hydroxyl groups (C(4')–C(5'), path a) or *between* the hydroxyl groups (C(3')–C(4'), path b). A number of enzymes which catalyse either of these cleavages in several phenols or catechols have been found in and isolated from plants other than *Centrospermeae* (for reviews, see [4]). In the present paper we show that the enzymatic cleavage in the betalaine-producing *Centrospermeae* proceeds by path a.

For the incorporation experiments we used the pulp (including the seeds) freshly taken out from the soft center of young cactus fruits (*Opuntia decumbens*). It was allowed to stand for 3 to 5 hours in a vial with an aqueous solution of the precursor. After controlled dilution the betanine (**3**)[2]) produced was isolated by adsorption on Al_2O_3 and then on polycaprolactam, followed by crystallization. At this stage, radiopurity had been achieved in several previous experiments [2]. The present samples, however, exhibited some loss of radioactivity (50–70%) after hydrolysis to betanidine (**4**)[2]), which was isolated as the crystalline hydrochloride. The radioactive impurity in these betanine samples could not be identified (see below).

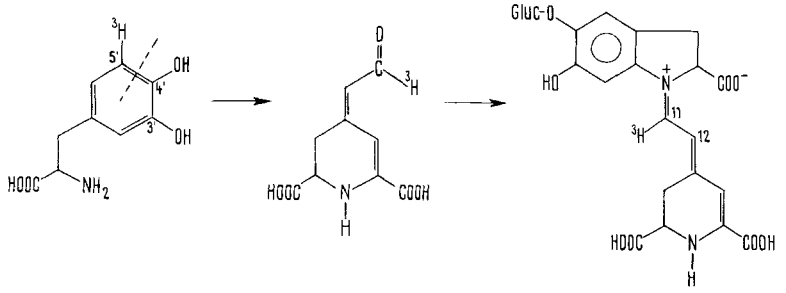


3, R = glucose, betanine

4, R = H, betanidine

Tyrosine was used as precursor since its 3',5'-ditritiated derivative is readily available. The following argumentation is based on the assumption that tyrosine is first converted to dopa. This assumption is made more probable by our observation that – under comparable conditions – L-dopa is incorporated at a rate (2.6%) about 5 times higher than L-tyrosine (0.53%). The radioactive dopa, presumably first formed from tyrosine-[3',5'-^{3}H] in the biomaterial, must be dopa-[5'-^{3}H] (**5**), half of the tritium being lost statistically (see below). Undoubtedly this contributed to the lower

Path a:

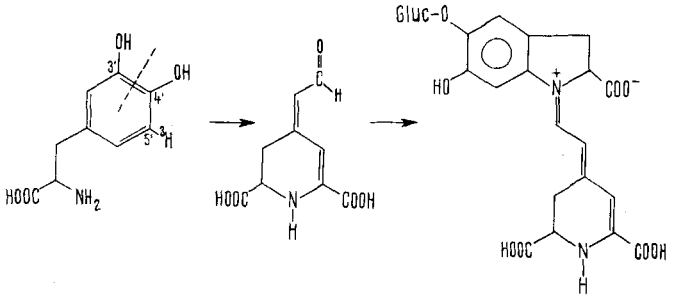


5 **6** **7**

incorporation rate with L-tyrosine-[3′,5′-^{3}H] (0.03–0.08%) than with L-tyrosine-[1-^{14}C] (0.53%).

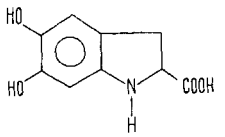
If the aromatic ring of dopa is cleaved between C(4′) and C(5′) (*path a*) the tritium must appear at the aldehyde carbon of betalamic acid (**6**) and thus at C(11) of betanine (**7**). On the other hand, with cleavage of the C(3′)–C(4′) bond (*path b*) the tritium would be lost (**5** → **1** → **3**).

Path b:



5 **1** **3**

The betanine obtained from the incorporation of L-tyrosine-[3′,5′-^{3}H] was indeed radioactive (0.03–0.08% incorporation). This radioactivity could, however, be solely due to tritium in the cyclodopa (**8**) portion of betanine. Even though in most of our



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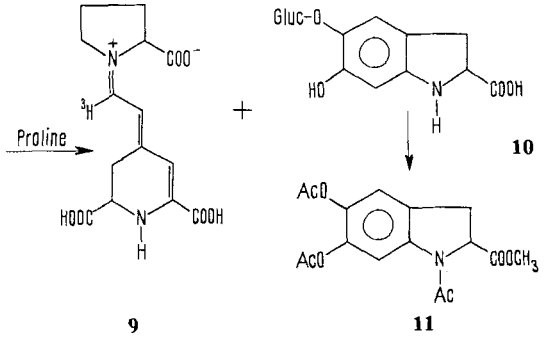
previous experiments the incorporation into the cyclodopa portion was only about 10–20% of that into the betalamic acid portion, occasionally as much as 50% was observed. We therefore subjected the tritiated betanine (**7**) to a base exchange reaction [5] with proline, and isolated the indicaxanthine (**9**) and the cyclodopa (as the



9 **11**

methyl ester of its triacetyl derivative, **11**) produced. The ratio of the specific activities of the indicaxanthine (**9**) to that of the cyclodopa derivative **11** ranged from 94:6 to 88:12. The same ratio was obtained when comparing the specific activity of betanidine

(**4**, isolated as the hydrochloride after hydrolysis of betanine) with that of the cyclodopa derivative **11**[3]). It is thus clear that the biosynthesized betalamic acid (***1***) contained the tritium and that therefore the aromatic ring is cleaved between C(4′) and C(5′) (*path a*).

This conclusion is unequivocal if no NIH. shift[4]) has taken place in the conversion of tyrosine to dopa. The absence of such a shift during this transformation has, in fact, been shown in other systems [7]. Our experiments offer the possibility of an independent proof of the absence of an NIH. shift during the oxidation of tyrosine to dopa in this biosystem; for, had such a shift taken place, then roughly half (neglecting isotope effect) of the intermediate dopa should be tritiated at C(2′) (formula **12**), leading by

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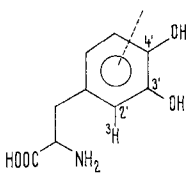

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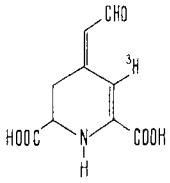

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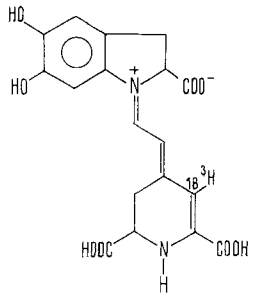

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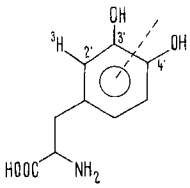
Path b:



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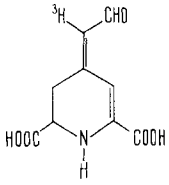

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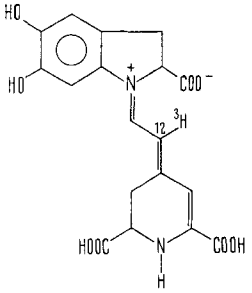

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path a to betanidine-[18-^{3}H] (**14**) or by path b to betanidine-[12-^{3}H] (**16**). Both centers 12 and 18 in betanidine (**4**) are nucleophilic and have been shown [8] to suffer deuterium exchange in CF_3COOD solution. The tritium in both **14** and **16** should thus be removable by CF_3COOH treatment.

The tritiated betanidine obtained by hydrolysis of the tritiated betanine (see above) was therefore repeatedly treated with CF_3COOH for a total time up to tenfold

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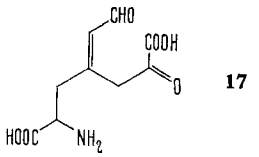
[3]) These results parallel our previous observation [2] that the cactus fruit system incorporates radioactivity from dopa much more efficiently into the betalamic acid than into the cyclodopa portion of betanine.

[4]) The shift of hydrogen which occasionally takes place from the position of (enzymatic) hydroxylation on a benzenoid ring to the neighbouring position is named after the National Institutes of Health (NIH.), where this was discovered [6].

that required for nearly complete deuteration with CF_3COOD. Very little loss of radioactivity was observed after this treatment; thus no tritium was located at C(12) or at C(18) and no NIH. shift had occurred. The possibility that the tritium at C(12) and/or at C(18) had already been washed out during the hydrochloric acid hydrolysis of betanine to betanidine (in which 50–70% of the radioactivity had been lost; see above) was eliminated by showing that the water, obtained by the low temperature distillation of the mother liquor from the hydrolysis-crystallization of betanidine hydrochloride, contained only 5% of the radioactivity.

It is still uncertain whether the cyclization of the ring-cleaved intermediate **17** to the hydropyridine system takes place before or after the condensation with cyclodopa (**8**) (or its glucoside **10**) and whether it is enzymatically catalysed or spontaneous.



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Experimental Part

1. *Materials.* – a) *Plant system:* Relatively young fruits from one plant of *Opuntia decumbens* were used in all incorporation experiments. We are indebted to Mr. *H. Krainz*, Director of the Succulent Plant Collection of the City of Zurich, for kindly providing the fruits.

b) *Radioactive precursors:* L-Tyrosine-[1-^{14}C] and L-tyrosine-[3′,5′-^{3}H] were obtained from *New England Nuclear Corporation*. L-Dopa-[2-^{14}C] was kindly provided by *F. Hoffmann-La Roche & Co. AG*, Basle, Switzerland.

2. *Methods.* The methods and equipment used for measuring the composition and the amounts of betalaines as well as the ^{14}C-radioactivities are described in [2]. The ^{3}H-radioactivities of the colourless triacetyl-cyclodopa methyl ester (**11**) were measured by direct scintillation counting, whereas those of the coloured betalaine samples (**3**, **4** and **9**) were obtained by combustion at 900–1000° and scintillation counting of the water formed. The radioactivities were measured in our Microanalytical Laboratory by Mr. *H. Frohofer* and his staff [9].

3. *Incorporation.* Portions from the commercial (in the case of L-dopa-[2-^{14}C] freshly prepared) solutions of the labeled tyrosine containing the indicated (see table 1) radioactivities were placed in a vial and freed from excess hydrochloric acid by evaporation over KOH in an evacuated desiccator. To the residue, dissolved in 1 ml H_2O, was added the gelatinous pulp without removing the seeds (together 5 to 15 g) taken out from the soft center of three young cactus fruits (skin still green) with a flattened glass rod. The vial was closed with a plastic cap through a hole of which the glass rod was kept in the material for occasional stirring. The mixture was allowed to stand at room temperature for 2 to 3.5 h and then diluted with about 10 ml of 80% ethanol. The betanine was isolated and purified as described in [2]. For data pertaining to 5 such experiments, see Table 1.

4. *Hydrolysis of Labeled Betanine* (**3**) *to Betanidine* (**4**) *Hydrochloride.* The crystallized betanine samples from experiments II and III (Table 1) were hydrolysed by the procedure described in [2]. The crystals of betanidine hydrochloride were filtered off and washed with several drops of 7.5 N HCl (results, see Table 2, sections 1 and 2). The combined mother liquor and washings were brought to dryness by high vacuum low temperature distillation and the distilled aqueous acid was collected in a flask at liquid air temperature. An aliquot (0.2 ml) of this distillate was counted by the scintillation method (results, see Table 2, section 3).

Table 1. *Data on the Incorporation of* L-*Tyrosine-[3′,5′-*^{3}*H], L-Tyrosine-[1-*^{14}*C] and L-Dopa-[2-*^{14}*C] into Betanine by the Pulp from* Opuntia decumbens *fruits*

Step	Experiment Units	I	II	III	IV	V
Pulp from three cactus fruits:						
pigmentation	color	deep red-violet	light red-violet	deep red-violet	deep red-violet	deep red-violet
amount of pulp	g	16	5	7.5	~15	16
Precursor		L-tyrosine-[3′,5′-^{3}H]	L-tyrosine-[3′,5′-^{3}H]	L-tyrosine-[3′,5′-^{3}H]	L-tyrosine-[1-^{14}C]	L-dopa-[2-^{14}C]
Specific activity of precursor	μCi/mole	2.65×10^{10}	4.48×10^{10}	4.48×10^{10}	1.91×10^{7}	2.70×10^{7}
Radioactivity supplied with precursor (precursor radioactivity)	μCi	25	50	50	5.0	7.14
Incorporation time	hours	2	3	3.5	2	2
Amount of betanine extracted from the fruits (plant-derived betanine)	moles $\times 10^{-5}$	0.333	0.01	0.289	0.415	0.398
Amount of betanine used for dilution at this stage	moles $\times 10^{-5}$	1.65	5.75	6.60	3.13	3.43
Amount of betanine taken for the Al_2O_3-adsorption procedure	moles $\times 10^{-5}$	1.95	5.57	6.89	3.55	3.83
Amount of betanine eluted from Al_2O_3	moles $\times 10^{-5}$	1.70	4.89	6.15	2.27	2.94
Amount of betanine used for dilution at this stage	moles $\times 10^{-5}$	0.873	–	–	–	–
Amount of betanine taken for the first polycaprolactam-adsorption procedure	moles $\times 10^{-5}$	2.573	4.89	6.15	–	2.94

Amount of betanine eluted from the polycaprolactam column	moles $\times 10^{-5}$	1.93	4.00	5.20	0.285	1.84
Amount of betanine used for dilution at this stage	moles $\times 10^{-5}$	3.02	7.16	3.60	4.40	3.96
Amount of betanine taken for the second polycaprolactam-adsorption procedure	moles $\times 10^{-5}$	–	11.16	–	–	–
Amount of betanine eluted from the second polycaprolactam column	moles $\times 10^{-5}$	–	10.00	–	–	–
Amount of betanine used for crystallization	moles $\times 10^{-5}$	4.95	10.00	8.80	4.685	5.80
Amount of crystalline labelled betanine	mg moles $\times 10^{-5}$	18.2 2.50	47.2 7.05	43.0 5.9	6.0 0.69	16.6 2.09
Specific activity of crystalline labelled betanine sample	μCi/mole	226	89	353	50	1530
Controlled dilution factor		22.6	1613	41.4	127	30.6
Specific activity of plant-derived betanine	μCi/mole	5100	143.700	14.620	6350	46.800
Radioactivity associated with total betanine extracted from plant (bioproduct radioactivity)	μCi	0.017	0.0144	0.0423	0.0264	0.186
Incorporation rate	%	0.068	0.029	0.085	0.528	2.60
Biodilution factor		5.2×10^6	3.12×10^5	3.06×10^6	3.01×10^3	5.77×10^2

5. *Trifluoroacetic Acid Treatment of Labeled Betanidine.* Weighed samples (~2 mg) of [^{3}H]-betanidine hydrochloride (derived from incorporation experiments II and III) were allowed to stand with 1 ml of CF_3COOH at room temperature for 24 h. Each solution was evaporated to dryness. The residue was again dissolved in 1 ml of CF_3COOH, allowed to stand for 24 h, concentrated, redissolved and left standing for 72 h. The solution was transferred as quantitatively as possible to a cotton ball of approx. 5 mm diameter and the ball dried over KOH in a high vacuum. The ball was then burned and the radioactivity of the collected water measured by scintillation counting (results, see Table 2, section 4).

6. *Conversion of Labeled Betanine* (**3**) *to Indicaxanthine* (**9**) *and Triacetyl-cyclodopa Methyl Ester* (**11**). The base exchange reaction with weighed samples of [^{3}H]-betanine (~50 mg) and proline (~200 mg) was performed as described in [2] (results, see Table 3, sections 1, 2, 3, and 4; for the triacetyl-cyclodopa methyl ester an ε-value at 250 nm of 15100 was used; compare with [10]).

In experiment III the solution of the base exchange reaction was concentrated in an evacuated system permitting quantitative collection of the distillate. The radioactivity of this distillate was measured by scintillation counting (results, see Table 3, section 5).

Table 2. *Data on the Preparation of Crystalline Labeled Betanidine Samples and on their CF_3COOH Treatment*

Section	Step (Units)	Experiment	
		II	III
1	labeled crystalline betanine used for hydrolysis		
	a) amount of betanine used (moles $\times 10^{-5}$)	2.32	2.68
	b) radioactivity (μCi)	2.07×10^{-3}	9.22×10^{-3}
	c) specific activity (μCi/mole)	89	353
2	labeled crystalline betanidine hydrochloride from hydrolysis		
	a) amount obtained (yield) (moles $\times 10^{-5}$(%))	1.65 (71)	2.25 (84)
	b) radioactivity (μCi)	0.401×10^{-3}	3.52×10^{-3}
	c) specific activity (μCi/mole)	24.3	156.5
	d) loss of specific activity in the hydrolysis step (%)	72.7	55.2
3	mother liquor from the hydrolysis-crystallization of betanidine		
	a) amount of distillate from the mother liquor (ml)	2.81	1.41
	b) radioactivity associated with this distillate (μCi)	0.0	4.62×10^{-4}
	c) total radioactivity found in distillate (% of radioactivity of betanine; see 1b)	0	5.0
4	CF_3COOH treatment of betanidine samples		
	a) amount used (moles $\times 10^{-5}$)	0.49	0.433
	b) radioactivity of solid residue after treatment (μCi)	1.143×10^{-4}	6.28×10^{-4}
	c) specific activity of solid residue after treatment (μCi/mole)	23.4	145.5
	d) retention of specific activity (%)	96.0	93.0

Table 3. *Data on the Preparation of Labeled Indicaxanthine and the Isolation of Triacetyl-cyclodopa Methyl Ester*

Section	Step (Units)	Experiment		
		I	II	III
1	Total betanine used for exchange:			
	a) labeled betanine:			
	amount (moles $\times 10^{-5}$)	1.135	3.89	2.265
	radioactivity (μCi)	2.45×10^{-3}	3.46×10^{-3}	8.0×10^{-3}
	b) unlabeled betanine added:			
	amount (moles $\times 10^{-5}$)	6.18	0	5.47
	c) total betanine:			
	amount (moles $\times 10^{-5}$)	7.315	3.89	7.735
	specific activity (μCi/mole)	33.5	89.0	103.5
2	Indicaxanthine obtained:			
	a) amount (moles $\times 10^{-5}$)	–[a]	0.520[b]	0.540[c]
	b) radioactivity (μCi)	–	4.65×10^{-4}	2.78×10^{-4}
	c) specific activity (μCi/mole)	–	89.5	177
	d) yield of indicaxanthine (%)	–	13.4	6.98
3	Triacetyl-cyclodopa methyl ester obtained:			
	a) crude distilled cyclodopa derivative:			
	amount (moles $\times 10^{-5}$)	3.43	2.73	6.04
	amount used for dilution (moles $\times 10^{-5}$)	3.39	2.67	5.97
	yield of cyclodopa derivative (%)	46.8	73.3	78.0
	b) unlabeled (crystalline) cyclodopa derivative added:			
	amount (moles $\times 10^{-5}$)	4.63	5.80	5.97
	c) total cyclodopa derivative:			
	amount (moles $\times 10^{-5}$)	6.72	5.91	7.02
	radioactivity (μCi)	1.33×10^{-5}	0.505×10^{-4}	2.38×10^{-4}
	specific activity (μCi/mole)	0.198	0.854	3.4
	d) cyclodopa derivative in 3c derived from betanine:			
	amount (moles $\times 10^{-5}$)	2.86	1.86	3.51
	specific activity (μCi/mole)	0.465	2.71	6.8
4	Ratio of specific activities in the cyclodopa and the betalamic acid portion of betanine:			
	a) ratio of the specific activity of cyclodopa derivative to that of indicaxanthine	0,99:99.01	2.93:97.07	3.70:96.30
	b) ratio of the specific activity of cyclodopa derivative to that of betanidine	–	11.3:88.7	4.35:95.65
5	Distillate of the exchange reaction solution:			
	a) amount of distillate (ml)	–	–	4.7
	b) radioactivity associated with the distillate (μCi)	–	–	6.0×10^{-4}
	c) total radioactivity in the distillate (%)	–	–	7.5

[a] The indicaxanthine in 0.05 N pyridinium formate buffer decomposed during the working-up.
[b] Amount measured spectroscopically in solution; radioactivity determined in the dried residue.
[c] Amount and radioactivity measured on the crystalline sample.

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72. Intramolekulare Übertragung von elektronischer Anregungsenergie in Spiroverbindungen

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(13. I. 72)

Summary. It is shown that the observation of the degree of polarization of the fluorescence of 9,9′-spirobifluorene VI and of 2,2′-diamino-7,7′-dinitro-9,9′-spirobifluorene VII allows a determination of the rate constant for energy transfer from the initially excited to the unexcited part. The rate constants are of the order of 10^8 s^{-1} for VI and $3 \cdot 10^9$ s^{-1} for VII. The corresponding electronic matrix elements of interaction are evaluated. They are rather due to the *Coulomb*-interaction of the transition densities than to spiroconjugation. In VI the coupling vanishes in the equilibrium conformation, vibronic effects must be evoked for explaining its existence.

1. Einleitung. – Die Übertragung von elektronischer Anregungsenergie zwischen zwei chromophoren Systemen einer Molekel war Gegenstand einer Reihe von Untersuchungen. *Schnepp & Levy* [1] beobachteten in Verbindungen vom Typ I, dass nach

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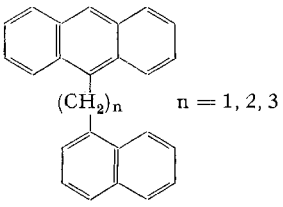

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