Biosynthesis of Uroporphyrinogens. Interaction among 2-(Aminomethyl)bilanes and the Enzymatic System†

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ABSTRACT: The biosynthesis of uroporphyrinogens III and I from 2-(aminomethyl)bilanes formally derived from porphobilinogen was examined with the help of deaminase–cosynthase systems from wheat germ, *Rhodopseudomonas sphaeroides*, rat blood, and rat spleen. 2-(Aminomethyl)bilane **4**, formally derived from the "head-to-tail" condensation of four units of porphobilinogen, gave uroporphyrinogen I by chemical cyclization. When **4** was incubated with large amounts of the aforementioned enzymes (300–400 units), an increase in uroporphyrinogen formation was observed. The deaminase–cosynthase systems from rat blood and spleen formed only uroporphyrinogen I, while the systems from wheat germ and *R. sphaeroides* formed relatively small amounts (13–27%) of uroporphyrinogen III. This formation of uroporphyrinogen III was not closely related to the enzymatic uroporphyrinogen formation from bilane **4** or to the amount of uroporphyrinogen III formed by the systems from porphobilinogen. 2-(Aminomethyl)bilane **5**, formally derived from the "head-to-head" polymerization of porphobilinogen, gave uroporphyrinogen II by chemical cyclization. When incubated with the above-mentioned enzymatic systems from wheat germ, rat blood, and *R. sphaeroides*, no increase in the formation of uroporphyrinogen was detected. These systems, however, formed large amounts of uroporphyrinogen III from **5** (45–60%). The rat spleen system, although it increased total uroporphyrinogen formation, did not form uroporphyrinogen III. When the four enzymatic systems were incubated with 2-(aminomethyl)bilane **6**, which formed uroporphyrinogen III by chemical cyclization, no enzymatic increase in uroporphyrinogen formation was detected. However, the systems from *R. sphaeroides*, wheat germ, and rat spleen formed uroporphyrinogen I in good yields (47–85%) from **6**. These changes in isomer type produced by the interaction of the deaminase–cosynthase systems with the 2-(aminomethyl)bilanes are due to a rearrangement that is dependent of the amount of enzyme used and the source of the deaminase–cosynthase system. It is apparently independent of the cyclization process of the 2-(aminomethyl)-bilanes to uroporphyrinogens and of the uroporphyrinogen III forming capacity of the deaminase–cosynthase system from porphobilinogen.

Porphyrins are formed in nature by the enzymatic polymerization of porphobilinogen (**1**) (PBG).[1] The latter is converted into the cyclic tetramer uroporphyrinogen III (**2**) by the concerted action of porphobilinogen deaminase and uroporphyrinogen III cosynthase while in the absence of the cosynthase, only uroporphyrinogen I (**3**) is formed (Scheme I). Uroporphyrinogen III (**2**) is the biosynthetic precursor of all the natural porphyrins, chlorins, and corrinoids (heme, chlorophylls, vitamin B_{12}, and their derivatives). The mechanism of this enzymatic polymerization has been extensively reviewed elsewhere (Frydman et al., 1979), and we will therefore briefly summarize some of its most outstanding features. It has been long known (Bogorad, 1958a,b) that PBG deaminase is the substrate consuming enzyme, while uroporphyrinogen III cosynthase does not consume PBG but in its presence the end product is not uroporphyrinogen I (**3**) but uroporphyrinogen III (**2**). The formation of uroporphyrinogen I (**3**) can be easily visualized as the "head-to-tail" condensation of four units of PBG [the 2-(aminomethyl) residue of one unit attacking the free C-5 position of the next unit] to form the linear "type I" 2-(aminomethyl)bilane **4** (Scheme II), followed by the thermodynamically favored cyclization of the latter. The puzzle in the structure of uroporphyrinogen III (**2**) is the inversion of the β-substituents of ring D (Scheme I) with respect to those of the other three rings. This question was examined by us with the help of synthetic type I 2-(aminomethyl)dipyrrylmethanes and tripyrranes (Frydman et al., 1973, 1978a,b). From the interaction of the latter with the deaminase and the deaminase–cosynthase system, it was concluded that the deaminase catalyzes the formation of a bilane of type I (such as **4**), which remains bound to the enzyme and finally cyclizes to uroporphyrinogen I. On the other hand, dipyrrylmethanes and tripyrranes derived from a "head-to-head" polymerization of PBG were found to be incorporated into uroporphyrinogen III (**2**) but not into uroporphyrinogen I (**3**) under the same experimental conditions (Frydman et al., 1973, 1978a,b; Scott et al., 1976; Franck et al., 1980). On the basis of these results and on the fact that deaminase and cosynthase can associate to form an enzymatic complex (Frydman & Feinstein, 1974; Higuchi & Bogorad, 1975), it was proposed that cosynthase acts as a "specifier" protein of the deaminase [see also, Shemin (1975)], changing the mode of porphobilinogen condensation. Independent results by Battersby et al. (1981a) indicated however that the 2-(aminomethyl)bilane **4** was incorporated into uroporphyrinogen III (**2**) by a mechanism involving ring A reversal of **4**. An analysis of the interaction of bilane **4** and of isomeric 2-(aminomethyl)bilanes with the deaminase–cosynthase system from *Euglena gracilis* indicated that ring A reversal was essentially quantitative in the case of bilane **4** while in some of the other isomeric bilanes the terminal ring A reversal took place only to a small extent (Battersby et al., 1982a). Similar results were reported in studies with isomeric 2-(hydroxymethyl)bilanes, where the latter were assayed as substrates of *E. gracilis* cosynthase (Battersby et al., 1981b).

When we examined the interaction of bilane **4** with a deaminase and a deaminase–cosynthase system from wheat germ

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[1] Abbreviations: PBG, porphobilinogen; uro'gen, uroporphyrinogen; HODPM, 2-(hydroxymethyl)dipyrrylmethane **9**; HPLC, high-pressure liquid chromatography; EDTA, ethylenediaminetetraacetic acid.

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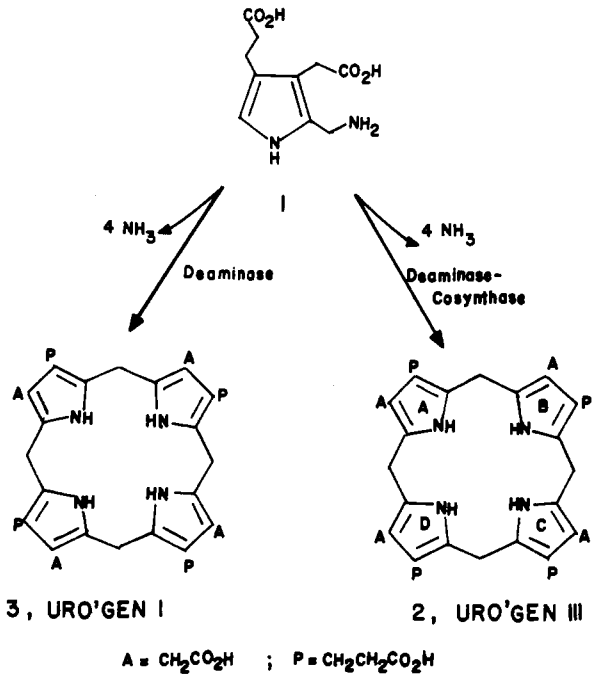
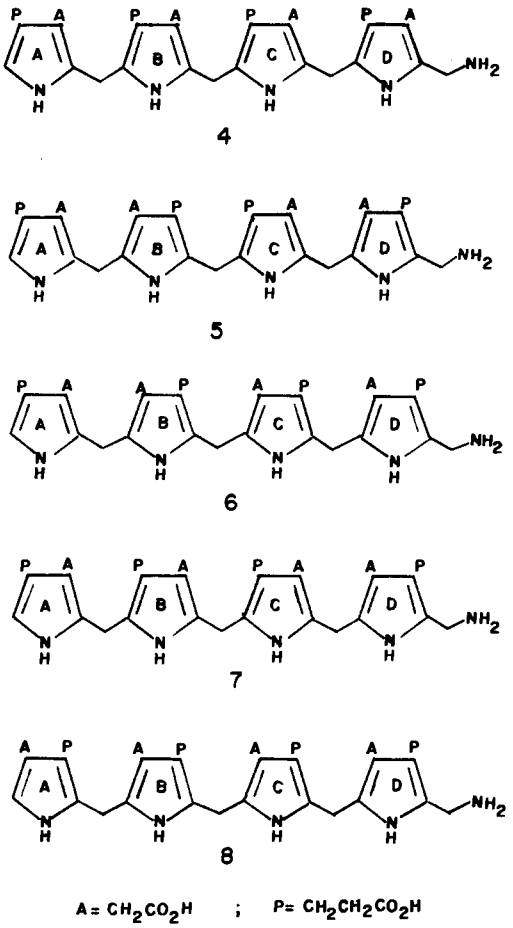
Scheme I



Scheme II



and from human erythrocytes, we found no enzymatic incorporation of bilane **4** into either uroporphyrinogen I (**3**) or uroporphyrinogen III (**2**) (Díaz et al., 1979). A similar lack of interaction was also observed by Radmer & Bogorad (1972). To find out the reason for the discrepancies among these results and those reported by Battersby and co-workers, the interaction of isomeric bilanes with deaminase, deaminase–cosynthase, and cosynthase obtained from different sources was examined.

Experimental Procedures

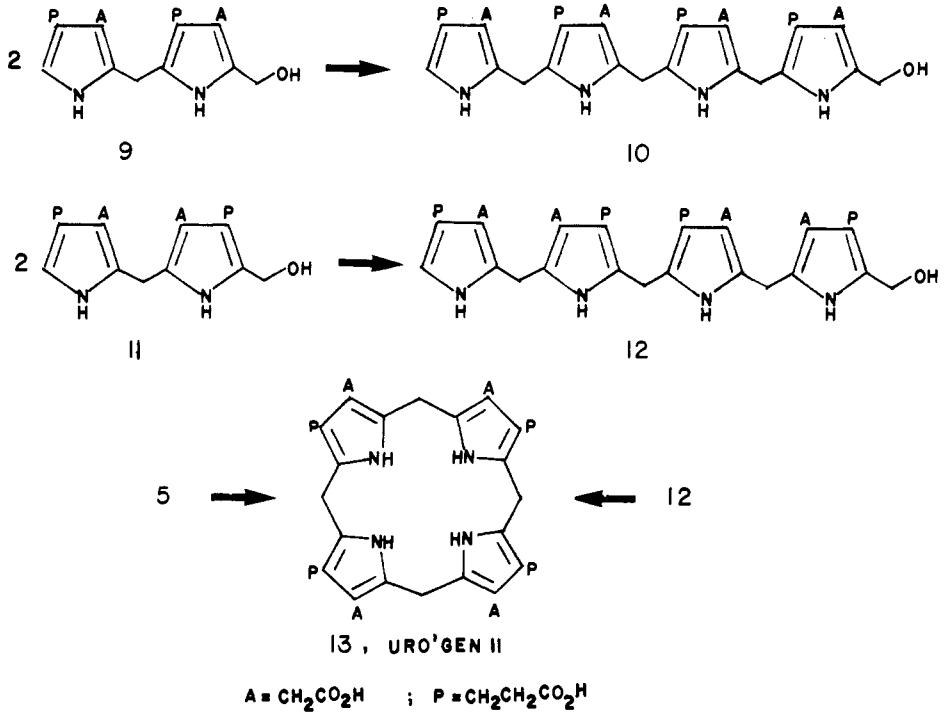
Synthetic Substrates. Porphobilinogen (**1**) was prepared by synthesis (Frydman et al., 1969). 2-(Aminomethyl)bilane **4** was prepared by synthesis as described in detail elsewhere (Díaz et al., 1979). 2-(Aminomethyl)bilanes **5** and **6** were prepared as described previously (Díaz et al., 1981). Bilanes **7** and **8** were obtained following the synthetic procedures described for the above-mentioned bilanes. 2-(Hydroxymethyl)dipyrrylmethane **9** (Scheme III) was obtained from benzyl 3,4′-bis[(methoxycarbonyl)methyl]-4,3′-bis[β-(methoxycarbonyl)ethyl]-5′-formyldipyrrylmethane-5-carboxylate (Díaz et al., 1979) after hydrogenolysis of the benzyl ester, thermal decarboxylation of the acid, reduction of the formyl group with sodium borohydride, and, finally, saponification of the methyl ester residues. 2-(Hydroxymethyl)dipyrrylmethane **11** was prepared in analogous fashion. The chemicals and solvents used were of the highest analytical grade.

Enzyme Preparations. Wheat germ deaminase was prepared as described elsewhere (Frydman & Frydman, 1970). The DEAE-cellulose purified enzyme was concentrated by ammonium sulfate precipitation. The wheat germ cosynthase was obtained from the fraction that precipitated between 50–70% ammonium sulfate. This fraction was dialyzed, centrifuged, and again reprecipitated with ammonium sulfate. The fraction precipitating between 40–80% was dialyzed, centrifuged, and used as the cosynthase source for the reconstruction of the wheat germ deaminase–cosynthase system.

Deaminase–cosynthase from spleen and blood was prepared from phenylhydrazine treated rats. Female Wistar rats (160–180 g) were injected with a 2% phenylhydrazine solution (adjusted to pH 7). Injections of 0.3 mL of the solution were given subcutaneously every other day during 5 days (three doses). After the last dose, the rats were rested for 60 h before being used. The blood was collected from the abdominal aorta into a heparinized needle containing 0.2 mL of heparin (5000 units/mL). Pooled blood fractions from 10 animals were centrifuged for 10 min at 10000*g*. The red cells were washed with an ice-cold 0.9% NaCl solution, and the cell pellet was diluted to the original blood volume by addition of water and stored at −20 °C for 24 h before use. The hemolysate was centrifuged at 20000*g* for 15 min, and the supernatant (50 mL) was applied on a DEAE-cellulose column (3.5 × 25 cm) previously equilibrated with 3 mM phosphate buffer (pH 7.4). The column was then exhaustively washed with 10 mM phosphate buffer (pH 7.4, 1000 mL), followed by 10 mM phosphate buffer (pH 6.8, 2000 mL). The deaminase–cosynthase was eluted with 100 mM potassium phosphate buffer (pH 7.4). The active fractions were pooled and concentrated by ultrafiltration.

The rat spleens removed from the anesthesized animals were washed with an isotonic saline solution. They were then cut into small pieces and homogenized in a Potter-Elvehjem with 20 mM potassium phosphate buffer (pH 7.4). The suspension was then centrifuged at 20000*g* for 20 min, and the supernatant was used for further fractionation with ammonium sulfate. The fraction that precipitated between 25–60% ammonium sulfate was dissolved in 20 mM phosphate buffer, dialyzed overnight against the same buffer, centrifuged, and used as enzyme source. *Rhodopseudomonas sphaeroides* (ATCC 17024) were grown in medium S of Lascelles (1956) semianaerobically in the light at 30 °C for 4 days in 1-L Erlenmeyer flasks. The cells were harvested and washed thrice with a saline solution. The cells were resuspended in 50 mM

Scheme III



phosphate buffer (pH 7.4) and disrupted by ultrasonication. The suspension was centrifuged for 4 h at 20000*g* or for 1.5 h at 105000*g*. The supernatant was precipitated with ammonium sulfate, and the fraction that precipitated between 35 and 60% ammonium sulfate was used as the *R. sphaeroides* deaminase–cosynthase after dialysis overnight against 50 mM phosphate buffer (pH 7.4). When the deaminase of *R. sphaeroides* was needed, the above-described enzyme was heated at 65 °C for 30 min. The PBG-consuming and uroporphyrinogen-forming activities remained the same, except for the isomer formed that was exclusively uroporphyrinogen I.

Enzyme Assays. The incubations of the deaminase–cosynthase and deaminase of different origins with the bilanes, 2-(hydroxymethyl)dipyrrylmethanes, or PBG were performed in a final volume of 1.1 mL. The incubation mixtures contained 50 μmol of potassium phosphate (pH 7.4), enzyme (350–400 units), and the synthetic bilanes (200 nmol) or PBG (320 nmol). A unit of enzyme was the amount of enzyme that produced 1 nmol of uroporphyrinogen/h from PBG. When the synthetic substrates were the 2-(hydroxymethyl)dipyrrylmethanes **9** and **11**, 400 nmol of each was used. When other substrate concentrations were used, they are indicated in the text. Incubations were carried out at 37 °C. Each run included simultaneous incubations of the enzymes with PBG, of the enzymes with the synthetic substrates [either the 2-(aminomethyl)bilanes or the 2-(hydroxymethyl)dipyrrylmethanes], of the synthetic substrates in the absence of enzyme (chemical blanks), and of the synthetic substrates plus PBG and enzyme. Uroporphyrinogen formation and isomer analysis were carried out on aliquots (20 μL for the former and 150 μL for the latter) withdrawn at the times indicated in the text. PBG consumption and uroporphyrinogen formation were measured as described previously (Frydman et al., 1978a,b).

Analysis of Uroporphyrinogen Isomers. The four uroporphyrinogen isomers (I, II, III, and IV) were separated as the corresponding free-acid uroporphyrins following the HPLC procedure described by Wayne et al. (1979). A 150-μL aliquot (see above) was treated with a 1% aqueous iodine solution to oxidize the uroporphyrinogens to uroporphyrins. The excess of iodine was destroyed with a 2% sodium thiosulfate solution, the total volume was diluted to 1 mL, and the solution was filtered through a SEP-PAK/C_{18} cartridge for rapid sample preparation (Waters). The cartridge was washed with water to eliminate the contaminants, and the uroporphyrins were eluted in a 20% acetonitrile solution. The porphyrin eluate was adjusted to pH 3 with 4 M HCl, and the porphyrins were extracted with ethyl acetate. The extracts were evaporated to dryness, and the residue was dissolved in the HPLC solvent (7% acetonitrile in phosphate buffer, pH 7) to which 10 mM EDTA was added. The isomer separation was carried out on a Lichrosorb RP-18 column of 5 μm (250 × 4.6 mm).

Results

Interaction of Unrearranged Bilane **4** *with Deaminase–Cosynthase Systems from Different Sources.* Bilane **4** cyclizes when heated at 37 °C and pH 7.4 to give uroporphyrinogen I (**3**) in about 10% yield (Diaz et al., 1979). When incubated under the same conditions with large amounts of deaminase or deaminase–cosynthase (at least 300 units of enzyme or more), there was an increase in uroporphyrinogen formation over that formed by the chemical cyclization of **4**. The amount of uroporphyrinogen formed was strongly dependent on the enzyme's origin (Figure 1). The rates for uroporphyrinogen formation from bilane **4** were different although enzymes with similar PBG-consuming activities were used. Even different enzymatic preparations from the same origin showed different uroporphyrinogen formation rates when bilane **4** was used as a substrate. The product formed by the deaminase–cosynthase system from rat blood and rat spleen was always uroporphyrinogen I (**3**) while that formed by the deaminase–cosynthase system from wheat germ and *R. sphaeroides* was mainly uroporphyrinogen I (**3**) with a smaller amount of uroporphyrinogen III (**2**) (Table I). The possibility that the deaminase converted the 2-(aminomethyl)bilane **4** into the 2-(hydroxymethyl)bilane **10** (Scheme III) and that the latter was the substrate of cosynthase (Battersby et al., 1982b) is not supported by the rates obtained with the deaminase–cosynthase system as compared to the rates obtained with de-

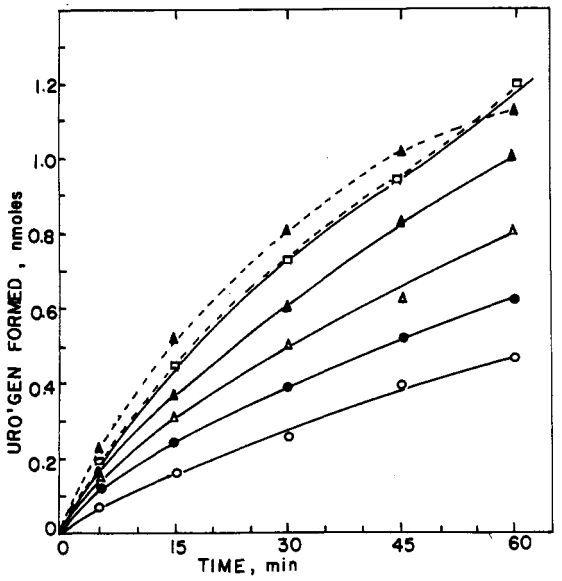


FIGURE 1: Time course of uroporphyrinogen formation by chemical and enzymatic cyclization of 2-(aminomethyl)bilane **4**. The incubation mixtures and conditions were as described under Experimental Procedures. Aliquots were taken at the indicated times, and the formed uro'gen was determined as uroporphyrin after oxidation with iodine. Deaminase (--) and deaminase–cosynthase (—) from different origins were used: (●) *R. sphaeroides*; (△) rat spleen; (▲) wheat germ; and (□) rat blood. (○) Chemical cyclization.

Table I: Interaction of Deaminase–Cosynthase from Different Sources with 2-(Aminomethyl)bilane **4**[a]

enzyme source	system	isomers formed (%) I	III
	bilane	100	
R. sphaeroides	enzyme + bilane	73	27
	enzyme + PBG	30	70
wheat germ	enzyme + bilane	87	13
	enzyme + PBG	50	50
spleen	enzyme + bilane	100	
	enzyme + PBG	5	95
blood	enzyme + bilane	100	
	enzyme + PBG		100

[a] The incubation mixtures and conditions were as described under Experimental Procedures. Isomer analysis was performed on aliquots withdrawn at 5 min after the start of the incubation.

aminase when the enzymes from rat blood and wheat germ were used (Figure 1). The rate of uroporphyrinogen formation by the deaminase–cosynthase system from wheat germ was lower than that from the deaminase, while both enzymes from rat blood showed the same uroporphyrinogen formation rate when bilane **4** was used as a substrate. If **10** would be formed from **4** and then consumed by the cosynthase, higher uroporphyrinogen formation rates should be expected for the deaminase–cosynthase systems since the enzymatic cyclization of **10** to uroporphyrinogen III (**2**) should be faster than its chemical cyclization to uroporphyrinogen I (**3**). No lag in the enzymatic formation of uroporphyrinogen was observed when 2-(aminomethyl)bilane **4** was incubated with either deaminase or deaminase–cosynthase.

Enzymatic uroporphyrinogen formation from bilane **4** by the deaminase–cosynthase system was always very low as compared to the natural substrate (PBG). Even when a 4-fold stoichiometric excess of **4** compared to that of PBG was used; the amount of uroporphyrinogen formed from **4** was only 30% of the amount of uroporphyrinogen formed from PBG.

The amounts of uroporphyrinogen III (**2**) present in the uroporphyrinogen formed by the four different deaminase–

Table II: Effect of 2-(Aminomethyl)bilane **4** Concentration on Isomer III Formation by Deaminase–Cosynthase from *R. sphaeroides*[a]

system	bilane (nmol)	isomers formed (%) I	III
enzyme + PBG		25	75
	40	100	
enzyme	40	84	16
enzyme	80	75	25
enzyme	160	90	10
enzyme	320	94	6
	320	100	

[a] The incubation mixtures and conditions were as described under Experimental Procedures, except for the 2-(aminomethyl)-bilane concentrations that were as indicated. Samples for isomer analysis were withdrawn after 10 min of incubation.

cosynthase systems (Figure 1) were entirely unrelated to the amount of uroporphyrinogen formed by the enzymes, as well as to the amount of uroporphyrinogen III formed by the same systems from the natural substrate (Table I). The deaminase–cosynthase systems from rat blood and rat spleen that gave the highest yields of uroporphyrinogen III (**2**) when PBG was used as a substrate did not form uroporphyrinogen III (**2**) from bilane **4**. All the uroporphyrinogen formed enzymatically by the rat blood and spleen systems at the expense of **4** was uroporphyrinogen I (**3**). The deaminase–cosynthase system from wheat germ formed a small amount of uroporphyrinogen III (**2**) while the same system from *R. sphaeroides* gave the highest yields of uroporphyrinogen III (Table I). The latter enzyme had the slowest rate of uroporphyrinogen formation from **4** (Figure 1). The enzymatic cyclization of **4** is therefore unrelated to the amount of uroporphyrinogen III (**2**) formed. The amount of uroporphyrinogen III formed from **4** by the deaminase–cosynthase system from *R. sphaeroides* was dependent on the concentration of bilane **4** used. At higher bilane concentrations, the percentage of uroporphyrinogen III (**2**) in the product mixture of uroporphyrinogen I (**3**) and III (**2**) decreased, due to the enhanced uroporphyrinogen I formation by the chemical cyclization of **4** and the inefficient enzymatic uroporphyrinogen III (**2**) formation (Table II). Longer incubation times at a constant bilane **4** concentration also resulted in a sharp decrease in the percentage of uroporphyrinogen III (**2**) present in the product mixture. This indicated again an inefficient enzymatic uroporphyrinogen III formation as compared to the chemical cyclization of bilane **4**.

Interaction of 2-(Hydroxymethyl)dipyrrylmethane **9** *and Deaminase–Cosynthase System.* When 2-(aminomethyl)-bilane **4** was used as a substrate of the deaminase–cosynthase system, there was always the possibility that **4** inhibited the cosynthase, since it was shown that it partially inhibited the formation of uroporphyrinogen III (**2**) from PBG (Díaz et al., 1979). On the other hand, Battersby et al. (1982b) defined 2-(hydroxymethyl)bilane **10** (Scheme III) as the substrate of cosynthase in the formation of uroporphyrinogen III (**2**). We therefore assayed the action of deaminase–cosynthase on 2-(hydroxymethyl)dipyrrylmethane **9**. This dipyrrylmethane is readily dimerized in solution to give 2-(hydroxymethyl)bilane **10** (Scheme III) and has been used as a substrate of the cosynthase (Battersby et al., 1982b). The chemical cyclization of bilane **10** formed 98% of uroporphyrinogen I at pH 7.4 and 37 °C. A small amount (2–5%) of uroporphyrinogen IV was formed during the dimerization of 2-(hydroxymethyl)dipyrrylmethane **9**, very likely by a head-to-head encounter similar to that described for the analogous 2-(amino-

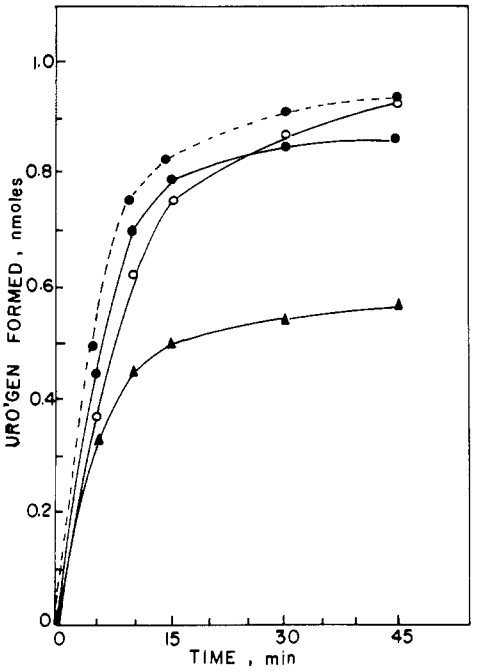


FIGURE 2: Rates of chemical and enzymatic polymerization of 2-(hydroxymethyl)dipyrrylmethane **9**. The incubation mixtures and conditions were as described under Experimental Procedures. (●—●) Deaminase and (●—●) deaminase–cosynthase of *R. sphaeroides*. (▲) Deaminase–cosynthase of wheat germ. (○) Chemical polymerization.

Table III: Interaction of 2-(Hydroxymethyl)dipyrrylmethane **9** with Deaminase and Deaminase–Cosynthase[a]

		isomers formed (%)			
		5 min		10 min	
enzyme source	system	I	III	I	III
	HODPM	97		94	
R. sphaeroides	deaminase–cosynthase + HODPM	78	22	81	9
	deaminase–cosynthase + PBG	25	75	25	75
	deaminase + HODPM	100		100	
wheat germ	deaminase–cosynthase + HODPM	90	10	94	6
	deaminase–cosynthase + PBG	30	70	30	70
	cosynthase	97			

[a] The incubation mixtures and conditions were as described under Experimental Procedures. Samples for isomer analysis were taken at the indicated times.

methyl)dipyrrylmethane (Frydman et al., 1973).

When 2-(hydroxymethyl)dipyrrylmethane **9** was assayed as a substrate of the deaminase and the deaminase–cosynthase system of *R. sphaeroides*, a small enzymatic formation of uroporphyrinogen was detected over that formed by the chemical cyclization of bilane **10** formed in situ (Figure 2). If bilane **10** would be the substrate of cosynthase, more uroporphyrinogen could be expected to be formed by the deaminase–cosynthase system than by deaminase alone. However, this effect was not observed (Figure 2). The amount of uroporphyrinogen III (**2**) formed by the deaminase–cosynthase system of *R. sphaeroides* at the expense of **9** was comparable to that formed from 2-(aminomethyl)bilane **4** (Table III). The amount of uroporphyrinogen III (**2**) decreased at longer incubation times due to the increase in the amount of the uroporphyrinogen I (**3**) isomer formed by the chemical cyclization of **10**. Parallel studies carried out with [^{14}C]PBG indicated that the presence of 2-(hydroxymethyl)dipyrrylmethane **9** in the incubation mixture was not inhibitory for uroporphyrinogen III (**2**) formation from PBG.

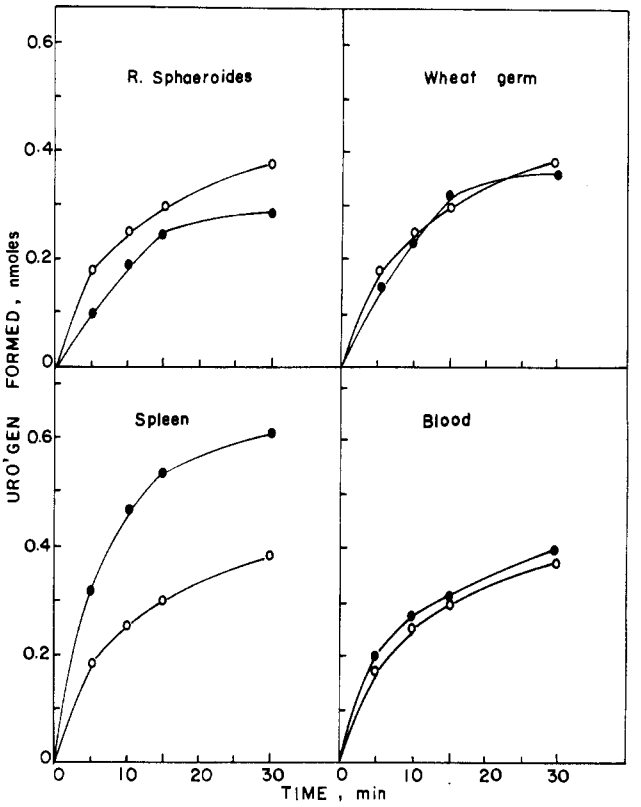


FIGURE 3: Time course of chemical cyclization of 2-(aminomethyl)bilane **5** and its interaction with deaminase–cosynthase of different sources. The incubation mixtures and conditions were as described under Experimental Procedures. (●) Incubated with deaminase–cosynthase. (○) Chemical cyclization. The enzyme sources are indicated in the figure.

When a deaminase–cosynthase system from wheat germ was assayed with **9**, uroporphyrinogen III (**2**) was also detected among the products at short incubation times (Table III). The amounts of uroporphyrinogen III (**2**) were again comparable to those obtained from bilane **4**. Incubation of **9** with wheat germ cosynthase did not result in the formation of uroporphyrinogen III (**2**).

Interaction of 2-(Aminomethyl)bilane **5** *with Deaminase–Cosynthase System from Different Sources.* Bilane **5** is the dimerization product of the 2-(aminomethyl)dipyrrylmethane that results from the head-to-head polymerization of PBG (Frydman et al., 1978a,b). The chemical cyclization of bilane **5** gave 100% of uroporphyrinogen II (**13**) (Scheme III). When bilane **5** was incubated with the same enzymatic systems and under the same conditions as those described for bilane **4**, no appreciable increase in the formation of uro'gen was detected. While the systems from wheat germ, rat blood, and *R. sphaeroides* formed less uroporphyrinogen than that formed by the chemical cyclization of **5**, the system from rat spleen gave an increase over the chemical blank (Figure 3). An analysis of the uroporphyrinogen isomers formed by the interaction of bilane **5** with the enzymatic systems revealed that important amounts of uroporphyrinogen III (**2**) were present (Table IV). The only system that failed to give uroporphyrinogen III (**2**) was the deaminase–cosynthease system from rat spleen. At short incubation times, the system from *R. sphaeroides* was the most efficient for uroporphyrinogen III (**2**) formation, followed by the rat blood enzyme, while the wheat germ deaminase–cosynthase was the least efficient. The increase in the amount of uroporphyrinogen III (**2**) was si-

Table IV: Interaction of Deaminase-Cosynthase from Different Sources with 2-(Aminomethyl)bilane **5**[a]

enzyme source	system	isomers formed (%) I	III	II
	bilane			100
R. sphaeroides	enzyme + bilane		60	40
	enzyme + PBG	30	70	
wheat germ	enzyme + bilane		20	80
	enzyme + PBG	50	50	
spleen	enzyme + bilane			100
	enzyme + PBG	4	96	
blood	enzyme + bilane		45	55
	enzyme + PBG	3	97	

[a] The incubation mixtures and conditions were as described under Experimental Procedures. The isomer analysis was performed on aliquots withdrawn at 5 min after the start of incubation.

multaneous with the decrease in the chemically formed uroporphyrinogen II (**13**). A comparison of the relative proportions of uroporphyrinogen III (**2**) formed from **4** and **5** by the same enzymatic system from *R. sphaeroides* can be seen in Figure 4.

The lack of an increase in uroporphyrinogen formation from bilane **5** by the concerted action of deaminase and cosynthase was independent of the enzymatic formation of isomer III. The presence of PBG increased the amount of uroporphyrinogen III (**2**) formed from bilane **5**. The bilane had no inhibitory effect on the deaminase–cosynthase system. As was the case with bilane **4**, the efficiency for uroporphyrinogen III (**2**) formation from bilane **5** had no relation with the capacity for uroporphyrinogen III (**2**) formation from PBG by the deaminase–cosynthase systems (Table IV). Uroporphyrinogen III (**2**) formation from **5** takes place very likely by an inversion of ring A before or during its cyclization to uroporphyrinogen.

Since it was proposed that the 2-(hydroxymethyl)bilanes are the intermediates in uroporphyrinogen III (**2**) formation, 2-(hydroxymethyl)dipyrrylmethane **11** (Scheme III) was assayed as a substrate of the enzymatic systems. Dimerization of **11** gave 2-(hydroxymethyl)bilane **12**, which cyclized chemically to give uroporphyrinogen II (**13**). If the deaminase transforms 2-(aminomethyl)bilane **5** into 2-(hydroxymethyl)bilane **12** and the latter is the substrate of cosynthase to give uroporphyrinogen III, a formation of uroporphyrinogen III (**2**) should be observed by incubation of 2-(hydroxymethyl)dipyrrylmethane **11** with the aforementioned enzymatic systems. This was not the case, and incubation of 2-(hydroxymethyl)dipyrrylmethane **11** with the four deaminase–cosynthase systems gave only uroporphyrinogen II (**13**). Therefore, it is very unlikely that uroporphyrinogen III (**2**) formation from bilane **5** involves a 2-(hydroxymethyl)bilane intermediate.

Interaction of 2-(Aminomethyl)bilane **6** *with Deaminase–Cosynthase System from Different Sources.* Bilane **6** (Scheme II) can formally be derived from the head-to-head polymerization of four PBG units. It cyclized at pH 7.4 and 37 °C to give 100% of uroporphyrinogen III (**2**). When incubated with the deaminase–cosynthase systems used in the former experiments, no increase in uroporphyrinogen formation due to the presence of the enzymes could be detected. However, when the isomer mixture was analyzed, it was found that the deaminase–cosynthase systems from *R. sphaeroides*, wheat germ, and rat spleen formed uroporphyrinogen I (**3**) in good yields. The efficiency of the change in the isomer type produced by the interaction of deaminase–cosynthase with **6** as compared to that produced with **4** and **5** can be seen in Figure 4. The only exception was the system from rat blood that did not affect the formation of uroporphyrinogen III (**2**) produced by the chemical cyclization of **6** (Table V). The formation of uroporphyrinogen I (**3**) can only be explained by an inversion of ring A of **6** during or before ring closure. Formation of uroporphyrinogen I (**3**) was not detected when bilane **6** was incubated with deaminase or cosynthase alone. The ring closure to uroporphyrinogen I (**3**) that takes place with inversion of ring A of **6** cannot be directly correlated with the uroporphyrinogen III forming capacity of the enzymes from PBG (Table V).

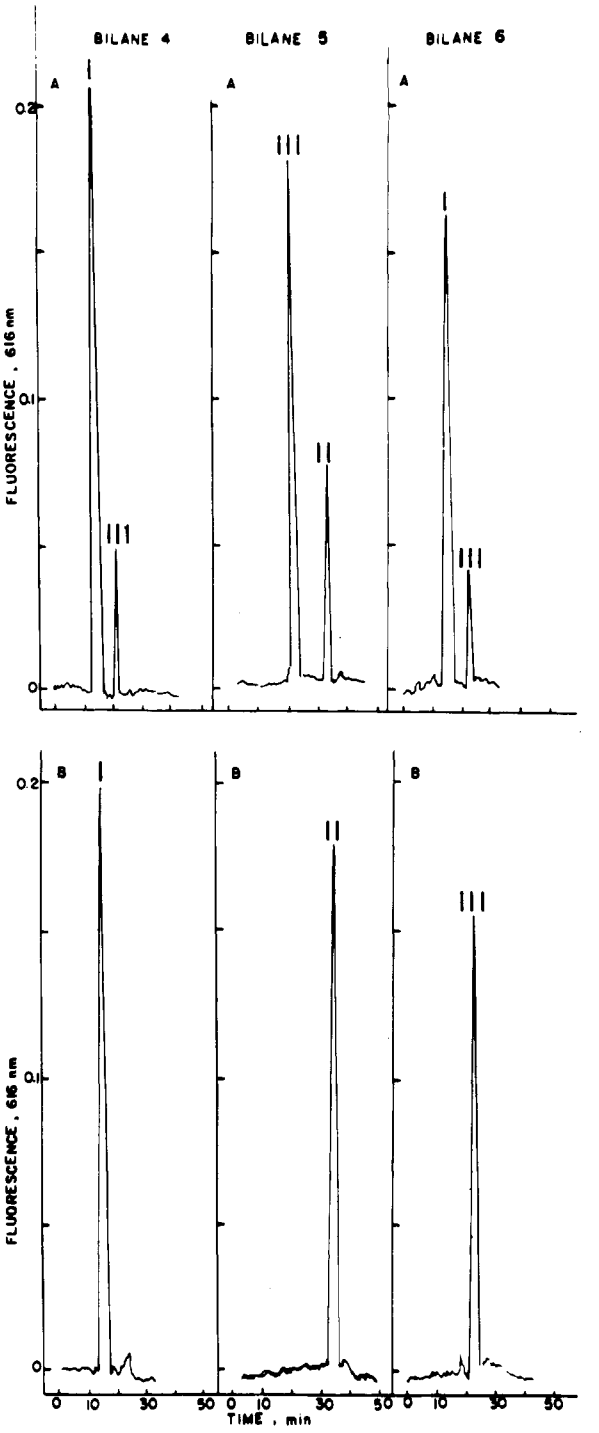


FIGURE 4: HPLC analysis of uro'gen isomers formed by (A) the incubation of the 2-(aminomethyl)bilanes with the deaminase–cosynthase system from *R. sphaeroides* and (B) the chemical cyclization of the 2-(aminomethyl)bilanes. 200 nmol of the bilanes was used, and the samples were taken after 5 min of incubation or heating at 37 °C at pH 7.4.

Table V: Interaction of Deaminase–Cosynthase from Different Sources with 2-(Aminomethyl)bilane **6**[a]

enzyme source	system	isomers formed (%) 5 min I	5 min III	15 min I	15 min III
	bilane		100		100
R. sphaeroides	enzyme + bilane	85	15	44	56
	enzyme + PBG	20	80	20	80
wheat germ	enzyme + bilane	47	53	23	77
	enzyme + PBG	58	42	50	50
spleen	enzyme + bilane	21	79	10	90
	enzyme + PBG	13	87	10	90
blood	enzyme + bilane		100		100
	enzyme + PBG		100		100

[a] The incubation mixtures and conditions were as described under Experimental Procedures. Isomers were analyzed and estimated from aliquots withdrawn at the indicated times.

Table VI: Interaction of Deaminase from *R. sphaeroides* and Cosynthase from Wheat Germ[a]

system	isomers formed (%) I	III
enzyme from *R. sphaeroides*	88	12
enzyme from *R. sphaeroides* + cosynthase (wheat germ)	80	20
heated enzyme (*R. sphaeroides*)	100	0
heated enzyme (*R. sphaeroides*) + cosynthase (wheat germ)	43	57

[a] *R. sphaeroides* enzyme (16 units) with a low uro'gen III forming capacity was incubated in a final volume of 100 μL with potassium phosphate buffer (pH 7.4, 10 nmol), PBG (32 nmol), and, when indicated, cosynthase from wheat germ. The incubations were carried out at 37 °C for 30 min. Isomer anlysis was performed as described. The activities for PBG consumption and uro'gen formation were identical for the heated and the unheated enzyme.

In the presence of bilane **6**, the formation of uroporphyrinogen III from [^{14}C]PBG was found to be partially inhibited, although the inhibition was not so pronounced as that detected for bilane **4**. The only system where bilane **6** did not inhibit uroporphyrinogen III (**2**) formation from PBG was the rat blood system. This system also failed to produce any uroporphyrinogen I (**3**) from bilane **6**. It can therefore be concluded that the absence of ring reversal during the cyclization of the bilane is due to the lack of interaction of the latter with this enzymatic system.

Assays of Bilanes **7** *and* **8** *with Deaminase–Cosynthase System.* Bilane **7** cyclized chemically at pH 7.4 and 37 °C to give uroporphyrinogen III (**2**). Bilane **8** formed uroporphyrinogen I (**3**) under the same conditions. Incubation of both bilanes with the deaminase–cosynthase systems of the different sources did not affect their chemical cyclization and did not result in the formation of uroporphyrinogen isomers derived from reversals in the ring chains.

Deaminase–Cosynthase Association in the Formation of Uroporphyrinogen III (**2**). An enzymatic system from *R. sphaeroides* with low cosynthase activity could not be transformed into an efficient uroporphyrinogen III (**2**) forming system by addition of cosynthase from wheat germ (Table VI). Heating the *R. sphaeroides* enzyme under conditions that were known to inactive the cosynthase and to change the conformation of the deaminase (Frydman & Frydman, 1973) afforded a *R. sphaeroides* deaminase system that could be efficiently reconstructed to a uroporphyrinogen III (**2**) forming system by addition of wheat germ cosynthase (Table VI). These results cannot be rationalized by assuming an independent role for both enzymes in uroporphyrinogen III (**2**) formation.

Discussion

The cyclization of 2-(aminomethyl)bilane **4** to uroporphyrinogens was accelerated by using large amounts of deaminase or deaminase–cosynthase. This effect was absent when smaller amounts of enzyme were used (Díaz et al., 1979). When the deaminase–cosynthase systems from *R. sphaeroides* and wheat germ were used, uroporphyrinogen III (**2**) was also formed together with the expected uroporphyrinogen I (**3**). The formation of uroporphyrinogen III (**2**) must be attributed to the ring A rearrangement of bilane **4** proposed by Battersby et al. (1981a). The latter workers reported that this rearrangement is highly specific for bilane **4** and that it therefore reflects the mechanism of uroporphyrinogen III (**2**) formation from PBG; i.e., the deaminase builds up a type I bilane **4** that is then ring A reversed by the cosynthase to give **2** (Battersby et al., 1982a). The results reported above (Tables I, IV, and V) indicate, however, that bilanes **5** and **6** undergo a much more efficient ring A reversal process by interaction with deaminase–cosynthase than bilane **4**.[2] Since bilanes **5** and **6** can be formally derived from head-to-head polymerizations of PBG, much of the strength of the argument that ring A reversal of **4** by deaminase–cosynthase reflects the unique mechanism of PBG polymerization is lost. The negative results obtained with bilanes **7** and **8** indicate that the interaction of the enzymes with bilanes **4**–**6** has a certain specificity. The enzymatic acceleration of ring closure of bilanes **4**–**6** (amount of uroporphyrinogen formation in excess of the chemical cyclization) was independent of the process of ring A rearrangement (compare Figures 1 and 3 and Tables I, IV, and V). The interaction of bilanes **4**–**6** with the deaminase–cosynthase system was also strongly dependent of the enzyme's source. The diverging results obtained for the same bilane with the enzymes from different origins (especially in what concerns the ring A reversal process) caution against the extrapolation of results obtained with the enzyme of a single source.

The reported results suggest that the process of ring A reversal is not simultaneous with the enzymatic ring closure. The inversion could take place on the enzyme's surface by fission of ring A, followed by its inversion and recombination with the remaining tripyrrane. Topological inversions of this type resulting from the interaction of enzymes (topoisomerases) and polymers are well-known (Gellert, 1981).

Acknowledgments

We thank Professor M. A. D'Aquino for help with the cultures of *R. sphaeroides*. We also thank E. Freixas (Molinos Morixe) for the samples of wheat germ.

Registry No. **2**, 1976-85-8; **3**, 1867-62-5; **4**, 36459-84-4; **5**, 75993-21-4; **6**, 65400-40-0; **9**, 73542-18-4; **11**, 86088-35-9; **13**,

[2] Ring A reversal of bilanes by interaction with a large excess of deaminase–cosynthase must also be the explanation of the long-standing discrepancy between our results (Frydman & Frydman, 1975; Frydman et al., 1976) and Battersby's (Battersby & McDonald, 1976) on the presence of protoporphyrin XIII in the protoporphyrin IX isolated by incubation of a "type I" dipyrrylmethane with duck blood hemolysates. Protoporphyrin XIII was formed from the 30% of uroporphyrinogen IV that arises from the chemical "head-to-head" dimerization of the dipyrrylmethane and cyclization of the resulting bilane (Frydman et al., 1973). Using relatively small amounts of enzyme, we detected the metabolic conversion of uroporphyrinogen IV into protoporphyrin XIII, while the very large amounts of enzyme used by Battersby very likely produced the ring A reversal of the bilane formed by the aforementioned chemical dimerization. This inversion resulted in the formation of uroporphyrinogen III (**2**) and, as a consequence, in the exclusive formation of protoporphyrin IX found by the latter authors.

53790-13-9; porphobilinogen deaminase, 9074-91-3; uroporphyrinogen III cosynthase, 37340-55-9.

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Photoaffinity Labeling and Partial Proteolysis of Wild-Type and Variant Glucocorticoid Receptors†

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ABSTRACT: Glucocorticoid receptors of wild-type lymphoid cells and of two classes of glucocorticoid-resistant variants of "nuclear transfer deficient" (nt^-) and "increased nuclear transfer" (nt^i) phenotypes, respectively, were investigated. Photoaffinity labeling of receptor complexes with a radiolabeled glucocorticoid of high affinity was used to analyze these receptor types by electrophoresis in sodium dodecyl sulfate containing gels. Wild-type and nt^--variant receptors yielded radiolabeled polypeptide bands of M_r 94 000 ± 5000 while nt^i-variant receptors had a molecular weight of 40 000 ± 2000. Partial proteolysis of wild-type and nt^- receptors with α-chymotrypsin resulted in steroid-labeled receptor fragments of M_r 37 000–38 000 while nt^i-variant receptors remained unchanged. In the case of wild-type receptors, the chymotryptic fragment had increased affinity for DNA indistinguishable from that of native nt^i-variant receptors. Depending on the nt^- cell clone, the chymotryptic receptor fragments containing the steroid binding site had either the same low affinity for DNA as the undigested receptors or a slightly increased affinity. Partial proteolysis with trypsin of wild-type, nt^-, and nt^i receptors resulted in steroid-labeled fragments of M_r 29 000 as major products and some fragments of M_r 27 000. These tryptic receptor fragments were devoid of DNA binding ability regardless of the original receptor types. With a lysine-specific protease, similar fragments were obtained from wild-type, nt^-, and nt^i receptors. In contrast, a protease specific for arginine residues did not produce receptor fragments detectable by our techniques. A model of the wild-type receptor is discussed.

Specific receptors play a pivotal role in the mechanism by which steroid hormones elicit physiological responses in target cells [for reviews, see Agarwal (1978), Higgins & Gehring (1978), Baxter & Rousseau (1979), and Katzenellenbogen (1980)]. Thus many cell culture lines of murine thymic lymphoid cells that contain functionally active glucocorticoid receptors respond to this class of steroids by growth inhibition and cell lysis. On the other hand, unresponsive cell variants are either deficient in receptors or contain defective receptors [for a review, see Gehring (1980a)].

Two types of receptor defects have thus far been identified in which hormone binding is roughly normal, but interaction of the receptor–glucocorticoid complexes with cell nuclei,

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0006-2960/83/0422-4013$01.50/0