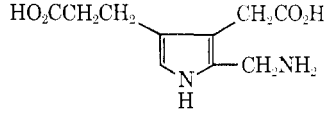
Chemical and Enzymatic Polymerization of 2-Aminomethyl-3,3′-carboxymethyl-4,4′-(β-carboxyethyl)dipyrrylmethane†

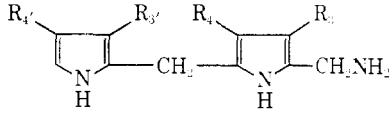
Rosalia B. Frydman, Aldonia Valasinas, and Benjamin Frydman*

ABSTRACT: The enzymatic incorporation of 2-aminomethyl-3,-3′-carboxymethyl-4,4′-(β-carboxyethyl)dipyrrylmethane into uroporphyrinogens was examined. The dipyrrylmethane was found not to be a substrate of the enzymatic system in the absence of porphobilinogen. The dipyrrylmethane-^{14}C was incorporated only into uroporphyrinogen I, when incubated in the presence of porphobilinogen with porphobilinogen deaminase and uroporphyrinogen III cosynthetase. No incorporation into uroporphyrinogen III could be detected. The dipyrrylmethane inhibited uroporphyrinogen III formation and diverted the enzymatic reaction to the formation of uroporphyrinogen I. The incorporation of the dipyrrylmethane into uroporphyrinogen I, which also took place when only porphobilinogen deaminase was used, oscillated between 5 and 30% of the total uroporphyrinogen formed. The dipyrrylmethane dimerized chemically by heating at 37° during 60 min, affording uroporphyrinogens in about 10% yield. An analysis of the isomer composition of the formed products indicated that equal amounts of uroporphyrinogen I and uroporphyrinogen III (IV) were formed. These results were compared with the isomer distribution among the products formed in the chemical dimerization of isomeric 2-aminomethyldipyrrylmethanes, and it was concluded that no randomization of the side chains took place during the process.

More than 20 hypotheses have been put forward to explain the enzymatic conversion of porphobilinogen **1** into



1



	R_3	R_4	R_3'	R_4'
2, A		P	A	P
3, A		P	P	A
4, P		A	A	P

A = CH_2CO_2H; P = $CH_2CH_2CO_2H$

uroporphyrinogen III, the basic skeleton of all the natural porphyrins, chlorins, and corrin derivatives (Margoliash, 1961).[1] The interest that this biochemical mechanism has aroused is understandable, since a repetitive head-to-tail condensation of a porphobilinogen unit (an α-Mannich base) with a second porphobilinogen unit would give rise, after four units have reacted, to the cyclic uroporphyrinogen I and 4 mol of ammonia. This is indeed the stoichiometry of the enzymatic reaction but the final product is not uroporphyrinogen I but uroporphyrinogen III (Lascelles, 1964).

No discrete intermediates (pyrrylmethanes) were isolated during the enzymatic reaction that could help explain the inversion of the side chains in the ring D of uroporphyrinogen III. The hypotheses that sought to explain the biochemical mechanism of this inversion (Margoliash, 1961)[1] proposed different rearrangement reactions at the various stages of an intermediate pyrrylmethane formation or at the final cyclization step. To put some of these proposals to test we prepared by a general synthetic method (Frydman *et al.*, 1971) a series of 2-aminomethyldipyrrylmethanes of which one, 2-aminomethyl-3,3′-carboxymethyl-4,4′-(β-carboxyethyl)dipyrrylmethane (**2**), resulted from the formal head-to-tail condensation of two units of porphobilinogen. The other two dipyrrylmethanes, **3** and **4**, were pepared to be used as references. They were examined as substrates of the enzymatic system that transformed porphobilinogen into uroporphyrinogen. This enzymatic system consists of two different components: a heat-stable protein called porphobilinogen deaminase which consumes porphobilinogen but forms only uroporphyrinogen I, and a heat-labile protein called uroporphyrinogen III cosynthetase which does not consume porphobilinogen but steers the reaction, when present, toward the formation of uroporphyrinogen III (Lascelles, 1964). The system was separated nondestructively and both proteins could be isolated and recombined (Stevens and Frydman, 1968; Stevens *et al.*, 1968). The relative proportions of uroporphyrinogens III and I formed at the expense of porphobilinogen depended on the relative amounts of both enzymes present in the incubation mixture.

Since no free dipyrrylmethanes were liberated into the reaction medium during the enzymatic polymerization of porphobilinogen it must be concluded that the possible intermediates are bound to the enzymes. Hence, the use of dipyrrylmethanes to examine the course of the enzymatic reaction is based on the assumption that they are equal (or that they resemble) to the first enzyme-bound intermediate formed in

† Facultad de Farmacia y Bioquimica, University of Buenos Aires, Buenos Aires, Argentina. *Received June 5, 1972.* Supported by grants from the National Institutes of Health (GM-11973) and the Consejo Nacional de Investigaciones (Argentina). Dedicated to Professor C. E. Cardini on his sixtieth birthday.

[1] Recent hypotheses are: (a) selective bilane cyclization (Mathewson and Corwin, 1961); (b) interconversion of uroporphyrinogens (Bullock, 1965); (c) condensation of porphobilinogen and a pyrrylmethyldipyrrylmethane (Conford, 1964; Dalton and Dougherty, 1969); (d) condensation of porphobilinogen and a dipyrrylmethane (Llambias and Battle, 1971).

the process. The possibility then exists that if the natural enzyme–dipyrrylmethane association is very stable, no exchange with the reaction medium will take place, and no incorporation into the porphyrins of an external added dipyrrylmethane will be observed. An alternative possibility is that the added dipyrrylmethane will bind to the enzyme competing with porphobilinogen at the first binding site. In this case it must be expected that the enzyme will have a much greater affinity for the natural substrate (the monomer) than for the added external dimer, and the incorporations of the latter will necessarily be low. However, if the enzymatic polymerization takes place in various stages and the product of the first stage is the substrate of the second one, then an added free dimer identical with the natural one will be incorporated in a higher proportion than the starting monomer into the final tetramer (uroporphyrin).

Our preliminary results (Frydman *et al.*, 1971) indicated that the dipyrrylmethane **2** was incorporated in the presence of porphobilinogen **1** into uroporphyrinogens in low yields (average 10–15% incorporation), even when it was present in a fivefold excess over the latter. Pluscec and Bogorad (1970), who also examined the possibility that dipyrrylmethane **2** would be a substrate of porphobilinogen deaminase in the presence of porphobilinogen, found, using isotopic dilution data, that 50–76.5% of the added dipyrrylmethane **2** was incorporated into uroporphyrinogen I. Such high incorporation values indicated that either the free dipyrrylmethane **2** competes very favorably with the natural substrate (porphobilinogen) for the first binding site, or else that it functions as a second substrate together with porphobilinogen at a latter stage of the enzymatic polymerization. This would be interesting in view of the many hypotheses advanced to explain the biosynthesis of uroporphyrinogen III (Margoliash, 1961),[1] which proposed that dipyrrylmethanes or tripyrrylmethanes formed by porphobilinogen deaminase serve as substrates together with porphobilinogen of the uroporphyrinogen III cosynthetase system at the subsequent stages of the process. The isotopic dilution data obtained by us (Frydman *et al.*, 1971) indicated that the dipyrrylmethane **2** competed unfavorably with porphobilinogen during the enzymatic polymerization process, as could be expected from an added intermediate very similar (but probably not identical) to the natural enzyme-bound intermediate. It was also observed (Frydman *et al.*, 1971) that the dipyrrylmethane **2** was incorporated only into uroporphyrinogen I and not into uroporphyrinogen III, a result that could help explain the mechanism of the inversion that takes place during the biosynthesis of uroporphyrinogen III. The experiments described below will attempt to clarify these problems by examining the enzymatic incorporation into uroporphyrinogens of dipyrrylmethane-^{14}C **2**, compared with reference dipyrrylmethanes.

Materials and Methods

Porphobilinogen and porphobilinogen-^{14}C were prepared by synthesis (Frydman *et al.*, 1969). Dipyrrylmethanes **2**, **3**, and **4** were prepared according to the general synthetic method described (Frydman *et al.*, 1971). Dipyrrylmethane-^{14}C **2** was prepared as described for the unlabeled substance but using labeled porphobilinogen lactam methyl ester during the synthesis (Frydman *et al.*, 1969). The label was at C-2 of the propionic acid side-chain R_3. Other chemicals were Reagent grade. Porphobilinogen deaminase and uroporphyrinogen III cosynthetase were isolated and purified from wheat germ (Frydman and Frydman, 1970; Stevens and Frydman, 1968) and were recombined to afford the desired isomer composition in the reaction products. Chemical dimerizations of dipyrrylmethanes were carried out by heating during 60 min at 37° a dipyrrylmethane solution at pH 7.4 (buffer phosphate 0.1 M) in a final volume of 100 μl. When the incorporation of dipyrrylmethane-^{14}C **2** into isomer I was measured (either as uroporphyrin I (Table II) or as coproporphyrin I (Table III)) the complete system contained (in a final volume of 100 μl) 10 μmol of phosphate buffer (pH 7.4), 4 nmol of porphobilinogen, 20 μl of porphobilinogen deaminase, and 8 nmol of dipyrrylmethane-^{14}C (42,000 cpm). The incubations were carried out at 37° during 60 min. The reaction was stopped by addition of a 1% iodine solution and evaporated to dryness, the resulting product was esterified with a 5% sulfuric acid solution of methanol, and the resulting uroporphyrin octamethyl esters were isolated and analyzed for isomer composition with the Falk and Benson paper chromatographic method as described elsewhere (Stevens and Frydman, 1968). When the isomers of the resulting porphyrin mixture were separated and measured as coproporphyrins (coproporphyrin method), the uroporphyrin octamethyl esters were decarboxylated with acid to coproporphyrins, and the latter separated by paper chromatography as described (Eriksen, 1958). The porphyrin isomers were located by their fluorescence, and eluted from the paper with a 0.7 N ammonium hydroxide solution. The porphyrin concentration in the eluates was determined by spectrophotometric methods (Frydman and Frydman, 1970) using as reference a calibration curve prepared with authentic samples.

The preparation of the control systems for the incorporation experiments (Tables II and III) was done by preparing two separate incubation mixtures. One contained (in a final

TABLE II: Enzymatic Incorporation of Dipyrrylmethane-^{14}C **2** into Uroporphyrinogen I (Estimated as Uroporphyrin I).[a]

System	Addition	Uroporphyrin Formed (nmol)	Dipyrrylmethane-^{14}C Incorporated (cpm)	Specific Activity of Uroporphyrin Formed (cpm/nmol)	Specific Activity (Δ)
Complete	None	0.4	1270	3170	1700
Control	None	0.53	772	1456	
Complete	Dipyrrylmethane **2**	0.52	1220	2300	1100
Control[b]		0.64	760	1200	
Complete	Dipyrrylmethane **3**	0.50	1256	2500	1700
Control[c]		0.70	552	800	

[a] The incubation mixtures and the controls were prepared as described in Materials and Methods. [b] The control contained, in addition to dipyrrylmethane-^{14}C **2**, an equivalent amount of unlabeled dipyrrylmethane **2**. [c] The control contained in addition to dipyrrylmethane-^{14}C **2**, an equivalent amount of dipyrrylmethane **3**.

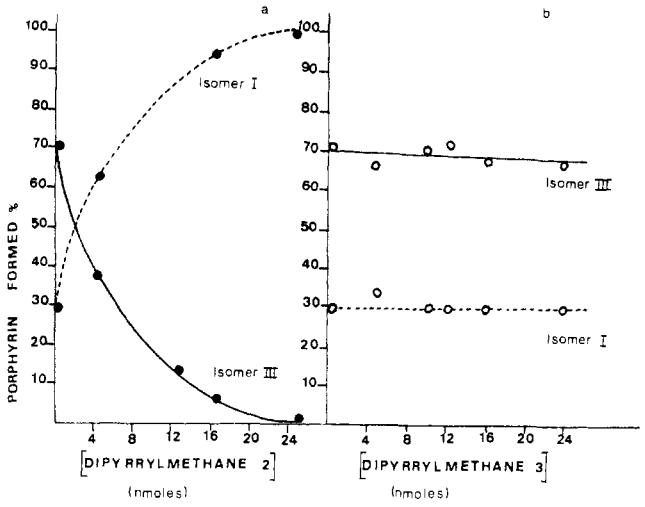


FIGURE 1: Effect of dipyrrylmethanes **2** and **3** on the enzymatic formation of uroporphyrinogens I and III. The incubation mixture contained, in a final volume of 100 μl, 10 μmol of buffer phosphate (pH 7.4), 4.5 nmol of porphobilinogen-^{14}C (specific activity 2500 cpm/nmol), and enzyme (deaminase–cosynthetase) from wheat germ (20 μl). The isomers were separated and estimated as coproporphyrins. The isomer composition was expressed as the percentage of the total amount of prophyrins formed in each case: (a) ●, dipyrrylmethane **2**; (b) ○, dipyrrylmethane **3**.

TABLE III: Enzymatic Incorporation of Labeled Dipyrrylmethane into Uroporphyrinogen I (Estimated as Coproporphyrin I).[a]

System	Addition	Coproporphyrin Formed (nmol)	Dipyrrylmethane incorporated (cpm)	Specific Activity of Coproporphyrin I Formed (cpm/nmol)	Specific Activity (Δ)
Complete		0.6	1467	2447	1847
Control		0.58	347	600	
Complete	Dipyrrylmethane **2**	0.70	1817	2595	445
Control		0.96	2070	2150	
Complete	Dipyrrylmethane **3**	0.18	374	2077	1520
Control		0.36	200	555	

[a] The incubation mixtures and controls were the same as described for Table II. Uroporphyrinogen was determined by the coproporphyrin method.

volume of 100 μl) 10 μmol of phosphate buffer (pH 7.4), 4 nmol of porphobilinogen, and 20 μl of porphobilinogen deaminase. The second one contained dipyrrylmethane-^{14}C **2** (8 nmol, 42,000 cpm), porphobilinogen (4 nmol), and phosphate buffer, pH 7.4 (10 μmol), in a final volume of 100 μl. The mixtures were incubated at 37° during 60 min and stopped by addition of a 1% iodine solution, and the resulting uroporphyrin octamethyl esters obtained as described above were mixed and the mixture was used as controls (Table II). When the incorporation of dipyrrylmethane-^{14}C **2** was measured by the coproporphyrin method (Table III) the ester mixture was decarboxylated with acid as described. The controls prepared in this way contained the uroporphyrinogens formed enzymatically from porphobilinogen and the uroporphyrinogens formed by the chemical dimerization of the dipyrrylmethane-^{14}C **2**. When the incorporation of dipyrrylmethane-^{14}C was measured in the presence of unlabeled dipyrrylmethane **2** (8 nmol) or dipyrrylmethane **3** (8 nmol) the latter were added to the complete incubation system. The corresponding controls were then prepared by adding the unlabeled dipyrrylmethanes to the second incubation mixture of the control system. When the incorporation of dipyrrylmethane-^{14}C into isomer I and isomer III was measured (Table IV) the complete system was composed of two separate incubation mixtures. One incubation mixture contained (in a final volume of 100 μl) 10 μmol of phosphate buffer (pH 7.4), 4 nmol of porphobilinogen, 20 μl of porphobilinogen deaminase–uroporphyrinogen III cosynthetase, and dipyrrylmethane-^{14}C **2** in the indicated concentrations (Table IV). The second incubation mixture contained (in a final volume of 100 μl) 10 μmol of phosphate buffer (pH 7.4), porphobilinogen (4 nmol), and unlabeled dipyrrylmethane **2** in equivalent concentrations to those of dipyrrylmethane-^{14}C used in the former mixture. Both were worked up as described and the resulting uroporphyrin octamethyl esters were mixed and the mixture decarboxylated to the free coproporphyrin mixture. The control system was also composed of two separate incubation mixtures. The first mixture contained (in a final volume of 100 μl) 10 μmol of phosphate buffer (pH 7.4), 4 nmol of porphobilinogen, 20 μl of deaminase–cosynthetase, and unlabeled dipyrrylmethane **2** at the same concentration of the dipyrrylmethane-^{14}C **2** used in the complete system. The second incubation mixture contained (in a final volume of 100 μl) porphobilinogen (4 nmol), phosphate buffer (pH 7.4), and dipyrrylmethane-^{14}C **2** at the indicated concentrations. After the usual work-up the resulting uroporphyrinogen octamethyl esters were mixed and decarboxylated to free coproporphyrins. All the incubations were carried out at 37° during 60 min. The reasons for preparing the above described mixtures are the following. The first incubation mixture of the control system contained unlabeled dipyrrylmethane **2**, to account for the effect of the dipyrrylmethane **2** on the isomer composition of the enzymatically formed products (Figure 1a). The second incubation mixture of the control system accounts for the uroporphyrinogen formed during the chemical dimerization of dipyrrylmethane-^{14}C **2**, which must be deducted from the enzymatic incorporation data (Table IV). A further correction in the complete system is then necessary to account for the chemical dimerization of the unlabeled dipyrrylmethane present in the first incubation mixture of the control system, Hence, a second identical incubation mixture containing porphobilinogen and unlabeled dipyrrylmethane **2** had to be prepared and added to the complete system. In this way the different sources of error were balanced and the net effect of the enzymatic incorporation of dipyrrylmethane-^{14}C **2** into isomers I and III could be measured.

The amount of total uroporphyrinogens formed in an incubation mixture was determined directly by oxidizing it to uroporphyrins with a 1% iodine solution and then estimating the latter by spectrophotometric methods (Frydman and Frydman, 1970).

TABLE IV: Specific Enzymatic Incorporation of Dipyrrylmethane-^{14}C into Isomer I.[a]

System	Dipyrrylmethane-^{14}C (nmol)	Isomers Formed: I nmol	I Cpm	I Specific Activity (cpm/nmol)	I Δ Specific Activity	III nmol	III Cpm	III Specific Activity (cpm/nmol)	III Δ Specific Activity
Complete	4	0.24	350	1450	250	0.154	283	1840	—
Control	4	0.25	300	1200		0.152	281	1850	
Complete	8	0.23	630	2730	1200	0.14	515	3680	—
Control	8	0.35	530	1520		0.16	600	3750	

[a] The incubation mixtures and controls were prepared as described in Materials and Methods. Isomers were estimated by the coproporphyrin method.

Results

Chemical Dimerization of 2-Aminomethyldipyrrylmethanes. Porphobilinogen **1**, a 2-aminomethylpyrrole, was stable at 37° (pH 7.4) under incubation conditions similar to those described in Materials and Methods and did not polymerize chemically to form uroporphyrinogens. 2-Aminomethyldipyrrylmethanes, however, were more sensitive and dimerized at 37° and 60 min (pH 7.4), affording uroporphyrinogens in about 9–12% yield (Table I). No oxidation to dipyrrylmethenes or bilenes took place during the process and strict anaerobiosis conditions were not required. While the chemical dimerization of dipyrrylmethane **2** resulted in the formation of approximately equal amounts of uroporphyrinogen I and uroporphyrinogen III (or IV), the dimerization of dipyrrylmethanes **3** and **4** afforded exclusively isomer II, measured either as uroporphyrinogen II or coproporphyrinogen II (Table I). It is very difficult to distinguish between isomer III (either as uroporphyrin III or coproporphyrin III) and isomer IV (either as uroporphyrin IV or coproporphyrin IV), since both series of isomers were identical when examined with the different chromatographic methods (Falk, 1964) and since both coproporphyrinogens were enzymatically transformed into the equally unseparable protoporphyrin IX and the equivalent isomer derived from coproporphyrinogen IV (Granick and Levere, 1964; Porra and Falk, 1964). The dimerizations could proceed through two mechanisms exemplified for dipyrrylmethane **2** in Scheme I: either a head-to-tail condensation of two units which will afford a 2-aminomethylbilane after elimination of 1 mol of ammonia (reaction A), or, alternatively, a head-to-head condensation of two units of dipyrrylmethane which will afford an α,α'-unsubstituted bilane and liberate formaldehyde and ammonia (reaction B).

Recondensation of the formaldehyde with the bilane in the latter reaction followed by ring cyclization will afford uroporphyrinogen IV. In the case of reaction A which affords a 2-aminomethylbilane, two types of ring closure can be expected. A classical ring closure through the free α position (a–c), with elimination of 1 mol of ammonia, will afford uroporphyrinogen I. Mathewson and Corwin (1961) proposed an alternative ring closure for the 2-aminomethylbilane depicted in Scheme I, starting with an attack of (a) on the occupied position (b), followed by bond breaking at (b) and recondensation of the resulting diene at (c), which results in the formation of uroporphyrinogen III. The chemical dimerizations of the dipyrrymethanes **2** and **3** will afford exclusively uroporphyrinogen II by either reaction A or reaction B, and uroporphyrinogen III by a Mathewson and Corwin type of cyclization. The absence of other isomers besides uroporphyrinogen II among the dimerization products of the dipyrrylmethanes **3** and **4** is an indication that the latter mechanism is not contributing to the reaction. Since no special ring strain or hindrance would account for any specific ring cyclization of the 2-aminomethylbilane formed by reaction A, it must be concluded that this mechanism is not operative during the cyclization of dipyrrylmethane **2** either. The 100% formation of uroporphyrinogen II in the dimerization of dipyrrylmethanes **3** and **4** excluded also any randomization process.

When the chemical dimerizations were carried out in the presence of porphobilinogen-^{14}C almost no radioactivity was incorporated into the uroporphyrinogens, indicating that at 37° (pH 7.4) no chemical condensation took place between the dipyrrylmethane and porphobilinogen.

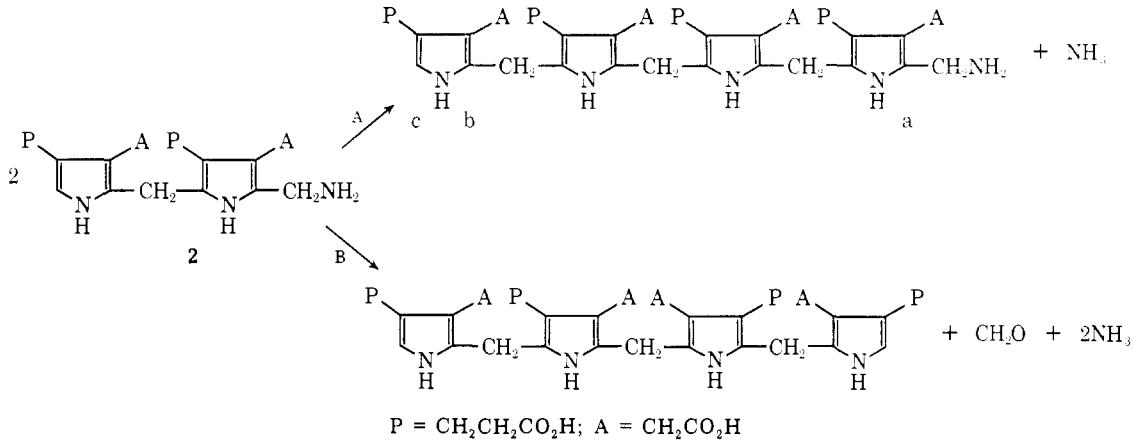
Enzymatic Incorporation of Dipyrrylmethane-^{14}C 2 into Uroporphyrinogens. The dipyrrylmethane **2** was not a substrate in the absence of porphobilinogen of either porphobilinogen deaminase, uroporphyrinogen III cosynthetase, or the combined enzymatic system. The addition of the enzymes did not affect to any extent the uroporphyrinogen pattern originated by the chemical dimerization of the dipyrrylmethane in the absence of porphobilinogen. The same was

TABLE I: Chemical Dimerization of Dipyrrylmethanes at 37°.[a]

Dipyrrylmethane	Uroporphyrinogen Formed: nmol	%	Isomer Composition: I	II	III (IV)
2, 14 nmol	0.62	9	50		50
3, 10 nmol	1.06	11.8		100	
4, 22.5 nmol	0.95	8.6		100	

[a] The dipyrrylmethane solutions were prepared and heated as indicated in Materials and Methods. The exact concentration of each is given in the table. Isomer distribution was determined by the coproporphyrin method, and is expressed as the percentage of the total porphyrin formed.

SCHEME I



P = $CH_2CH_2CO_2H$; A = CH_2CO_2H

also true of an incubation mixture which contained the dipyrrylmethane **2**, porphobilinogen, and uroporphyrinogen III cosynthetase, thus excluding the possibility that dipyrrylmethane **2** formed enzymatically by porphobilinogen deaminase is one of the substrates of cosynthetase. When dipyrrylmethane **2** was added to an enzymatic mixture forming both uroporphyrinogens I and III it had two different effects; it inhibited the formation of uroporphyrinogen III and enhanced in the same proportion the formation of uroporphyrinogen I, and was incorporated only into uroporphyrinogen I. The first effect can be followed in Figure 1a. Dipyrrylmethane **3**, a "nonsense dipyrrylmethane" from the metabolic standpoint, is devoid of this effect (Figure 1b). Dipyrrylmethane **2** acts probably by dissociating the deaminase–cosynthetase system since the inhibitory effect on the formation of uroporphyrinogen III was not enhanced by preincubation of the cosynthetase with dipyrrylmethane **2**.

The enzymatic incorporation of dipyrrylmethane-^{14}C **2** into uroporphyrinogen I was measured by deducting in each run the amount of uroporphyrinogen I formed by the chemical dimerization of the dipyrrylmethane and the amount of uroporphyrinogen I formed exclusively by the enzymatic polymerization of porphobilinogen (Table II). The addition of unlabeled dipyrrylmethane **2** diluted the incorporation of labeled **2** into uroporphyrinogen I, while the addition of dipyrrylmethane **3** did not affect the incorporation. When the incorporation was measured at the coproporphyrin stage by decarboxylating the formed uroporphyrin I to coproporphyrin I, essentially the same results were obtained (Table III). Dilution of the incubation mixture with unlabeled **2** resulted in a decrease of the incorporation, while addition of the biologically "nonsense dipyrrylmethane" **3** did not affect the extent of the enzymatic incorporation of **2**. When dipyrrylmethane-^{14}C **2** was added to an enzymatic system that formed uroporphyrinogens I and III it was incorporated only into isomer I (Table IV). Since the incorporation of the dipyrrylmethane-^{14}C **2** into uroporphyrinogen I was simultaneous with its inhibitory effect on the formation of uroporphyrinogen III (Figure 1a), control reactions must be carefully prepared to account and discriminate between both effects, and this was done as described under Materials and Methods.

Discussion

According to the data summarized in Tables II–IV, the amounts of uroporphyrinogen I formed at the expense of the dipyrrylmethane-^{14}C **2** oscillate between 5 and 36%. When the dipyrrylmethane **2** and porphobilinogen were present in equimolar amounts in the incubation mixture, the amount of uroporphyrinogen formed at the expense of **2** was only 5% of the total uroporphyrinogen formed (Table IV). These low incorporation values indicate the necessity of accounting for the amount of uroporphyrinogen formed by the chemical dimerization of the dipyrrylmethane **2**. Higher amounts of uroporphyrinogen were formed at the expense of the dipyrrylmethane **2**, when a large excess of the latter (four times the stoichiometric amount needed to combine with all the porphobilinogen present) was used (Tables II and III). These data together with the former isotopic dilution results (Frydman *et al.*, 1971) indicate that free dipyrrylmethane **2** competes very unfavorably with porphobilinogen or with the enzyme-bound dimer in the enzymatic formation of uroporphyrinogen. Nevertheless, it is incorporated into uroporphyrinogen I, while the very similar analog **3** is not. Thus, the dipyrrylmethane **2** is very similar, but probably not identical, with the natural intermediate which is enzyme bound. When porphobilinogen deaminase was extensively purified it was found that it behaved as a single enzyme (Frydman and Frydman, 1970), and it was then concluded that the porphobilinogen polymerization must take place on the enzyme's surface. This agrees with the results presented above, and with the very low absolute incorporations of the dipyrrylmethane **2** into uroporphyrin I (500–1100 cpm of an expected total of 10,500 cpm; Tables II and III). From the previous isotopic dilution data we have concluded (Frydman *et al.*, 1971) that higher free polymers of porphobilinogen (pyrrylmethyldipyrrylmethanes and bilanes) would be incorporated to a very low extent, if any, into uroporphyrinogens, and this seems to have been confirmed recently (Radmer and Bogorad, 1972).

Dipyrrylmethane-^{14}C **2** was not enzymatically incorporated into uroporphyrinogen III in the presence of porphobilinogen. It also exerted an inhibitory effect on the formation of uroporphyrinogen III (Figure 1a), which indicated an interaction with the deaminase–cosynthetase system. As a consequence, higher relative amounts of uroporphyrinogen I were formed, and since the cosynthetase was not inhibited by preincubation with **2**, it may be concluded that it affected the interaction of both enzymes. The specific incorporation of dipyrrylmethane **2** into uroporphyrinogen I argues against the proposals that the biosynthesis of both uroporphyrinogens I and III share a common biosynthetic pathway. Since dipyrrylmethane **2** led exclusively to uroporphyrinogen I, a different dipyrryl-

methane must be the first intermediate in the biosynthesis of uroporphyrinogen III. Both uroporphyrinogens must proceed through different pathways after the first porphobilinogen units became bound to the deaminase.

References

Bullock, E. (1965), *Nature (London) 205*, 70.
Conford, P. (1964), *Biochem. J. 91*, 64.
Dalton, J., and Dougherty, R. C. (1969), *Nature (London) 223*, 1151.
Eriksen, L. (1958), *Scand. J. Clin. Lab. Invest. 10*, 319.
Falk, J. E. (1964), *Porphyrins Metalloporphyrins*, 193, 197.
Frydman, R. B., and Frydman, B. (1970), *Arch. Biochem. Biophys. 136*, 193.
Frydman, B., Reil, S., Despuy, M. E., and Rapoport, H. (1969), *J. Amer. Chem. Soc. 91*, 2738.
Frydman, B., Reil, S., Valasinas, A., Frydman, R. B., and Rapoport, H. (1971), *J. Amer. Chem. Soc. 93*, 2738.
Granick, S., and Levere, R. D. (1964), *Progr. Hematol. 4*, 1.
Lascelles, J. (1964), Tetrapyrrole Biosynthesis and Its Regulation, New York, N. Y., W. A. Benjamin, p 47.
Llambias, E. B. C., and Battle, A. M. del C. (1971), *Biochem. J. 121*, 327.
Margoliash, E. (1961), *Annu. Rev. Biochem. 30*, 549.
Mathewson, J. H., and Corwin, A. H. (1961), *J. Amer. Chem. Soc. 83*, 135.
Pluscec, J., and Bogorad, L. (1970), *Biochemistry 9*, 4736.
Porra, R. J., and Falk, J. E. (1964), *Biochem. J. 90*, 69.
Radmer, R., and Bogorad, L. (1972), *Biochemistry 11*, 904.
Stevens, E., and Frydman, B. (1968), *Biochim. Biophys. Acta 151*, 429.
Stevens, E., Frydman, R. B., and Frydman, B. (1968), *Biochim. Biophys. Acta 158*, 496.

Glycoproteins from the Surface of Metaphase Cells†

Mary Catherine Glick and Clayton A. Buck*

ABSTRACT: Several criteria related to carbohydrates of the cell surface were examined in baby hamster kidney fibroblasts, BHK_{21}/C_{13}, arrested in metaphase with vinblastin sulfate. Analysis by gel filtration of the glycopeptides from the surface of metaphase arrest cells revealed a pattern of fucose-containing glycopeptides similar to that seen previously for rapidly growing or virus-transformed cells. Surface glycopeptides from cells treated with vinblastin sulfate but not in metaphase have a distribution similar to surface glycopeptides derived from BHK_{21}/C_{13} cells in exponential growth. The results suggest that the glycopeptides expressed on the cell surfaces during mitosis are similar to those permanently expressed after viral transformation. The metaphase cells show a 43% decrease in the total sialic acid content when compared to cells treated with vinblastin sulfate but not in metaphase. Cells arrested by thymidine have a sialic acid content more similar to the cells in exponential growth, indicating that the fluctuations in silalic acid content of vinblastin treated cells are not merely the result of cessation of growth. In addition, a threefold increase was observed in the amount of radioactive fucose incorporated into metaphase-arrest cells when compared with vinblastin sulfate treated cells which had not yet entered metaphase. The activities of seven glycosidases in the metaphase cells show little or no deviation from the cells treated with vinblastin sulfate but not in metaphase or as previously shown from the cells in exponential growth.

Examination of the carbohydrate content of a synchronous population of KB cells has shown that the individual monosaccharides of the cell are markedly reduced as the cell population divides (Glick *et al.*, 1971). When the molar ratios of the monosaccharides are related to sialic acid throughout the cell cycle the fluctuations of the carbohydrates appear in an orderly fashion. For example, when the cell population is dividing, the molar ratio of sialic acid to fucose is 1; when the population is not dividing the molar ratio is 2.

Changes from the normal distribution of glycopeptides derived from cell surfaces have been described after transformation by RNA or DNA viruses (Buck *et al.*, 1970, 1971a). These changes are associated partially with the state of growth of the cells, that is, whether the cells are growing rapidly or have formed a confluent monolayer (Buck *et al.*, 1971b).

In order to more precisely localize the changes in the mitotic event of the cell cycle, baby hamster kidney fibroblasts (BHK_{21}/C_{13}) were arrested in metaphase with vinblastin sulfate. Cells in metaphase change to a round morphology and come off the monolayer making it possible to isolate a population which is 80–90% in metaphase. Furthermore, the cells which are not in metaphase remain attached to the monolayer and serve as control cells which have been treated with vinblastin. These two populations of cells from the same flask are similar with the exception that one population is arrested in metaphase. These two populations were compared with BHK_{21}/C_{13} fibroblasts which were growing exponentially. Three parameters concerning the carbohydrates of the cell surface were examined: (1) the distribution of glycopeptides of the cell surface, (2) the sialic acid content of the cells, and (3) the activities of a number of glycosidases.

† From the Department of Therapeutic Research, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, and the Division of Biology, Kansas State University, Manhattan, Kansas 66506. *Received July 12, 1972.* This work was supported by U. S. Public Health Service Grants 5 P01 A107005-06, CA 12426-01, and FR7036, and American Cancer Society Grant No. PRA-68.

* Address correspondence to this author at Kansas State University.