Effects of Detergents on the Properties of 4-Hydroxybenzoate. Polyprenyl Transferase and the Specificity of the Polyprenyl Pyrophosphate Synthetic System in Mitochondria†

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ABSTRACT: The properties of 4-hydroxybenzoate:polyprenyl transferase and the system synthesizing polyprenyl pyrophosphate have been studied in mitochondria from rat and guinea pig livers. With solanesyl pyrophosphate and 4-hydroxybenzoate as substrates the formation of 3-nonaprenyl 4-hydroxybenzoate was linear with time, concentration of protein, and concentration of solanesyl pyrophosphate. Solanesyl monophosphate is inactive as a substrate and is noninhibitory. Conversion of solanesyl monophosphate to the pyrophosphate could not be detected. Detergents such as Triton X-100, Tween-80, and sodium deoxycholate activated the enzyme in mitochondria which were aged by freezing at −20 °C for periods ranging from 1 h to several days. Maximum activation also required Mg^{2+}. In agreement with previous observations the effect of Mg^{2+} and Triton X-100 on fresh mitochondria was quite variable; however, activation with aged preparations was very consistent. Treatment with Triton X-100 causes an alteration in the biosynthetic pattern of rat liver mitochondria so that rather than nonaprenyl, decaprenyl pyrophosphate is preferentially made in the presence of solanesyl pyrophosphate and isopentenyl pyrophosphate. In the presence of Triton X-100 an added pool of solanesyl pyrophosphate appears to exert a feedback inhibition on the incorporation of isopentenyl pyrophosphate into solanesyl pyrophosphate. In the case of guinea pig liver mitochondria a different pattern is observed with Triton X-100 in contrast to the rat. The de novo formation of decaprenyl pyrophosphate from isopentenyl pyrophosphate appears to be inhibited by Triton X-100, but the synthesis of decaprenyl pyrophosphate from isopentenyl pyrophosphate and nonaprenyl pyrophosphate is not inhibited. The data also indicate that in guinea pig liver in a system synthesizing decaprenyl pyrophosphate from isopentenyl pyrophosphate, there does not appear to be a detectable pool of nonaprenyl pyrophosphate. These results show that detergents can affect the specificity of the mitochondrial system synthesizing polyprenyl pyrophosphates.

This laboratory has presented evidence that prenylation of *p*-hydroxybenzoic acid (PHB)[1] is the first step in the synthesis of ubiquinone from PHB in rat liver mitochondria and results in the formation of 3-nonaprenyl 4-hydroxybenzoate (Momose and Rudney, 1972). The synthesis of this molecule takes place in the inner mitochondrial membrane and is catalyzed by the enzyme PHB:polyprenyl transferase. The total synthesis of ubiquinone from PHB has been shown in rat liver mitochondria (Trumpower et al., 1974). Further studies from our laboratory have shown that the enzyme requires Mg^{2+} for activity and is inhibited by Ca^{2+}, and that Mg^{2+} can overcome the inhibition by Ca^{2+} (Schechter et al., 1972, 1973). Bacitracin, an antibiotic known to complex with polyprenyl pyrophosphates and metal ions (Stone and Strominger, 1971, 1972), was also strongly inhibitory.

In this work we report further studies on the formation of nonaprenyl-PHB in mitochondria which are designed to examine factors which determine the final chain length of polyprenyl pyrophosphate derivatives. Previous studies indicated that there was broad specificity associated with the length of the polyprenyl pyrophosphate chain which could be handled by the enzyme PHB:polyprenyl transferase (Momose and Rudney, 1972). The prevailing polyprenyl analogue of ubiquinone was dependent more on the characteristics of the polyprenyl pyrophosphate synthetic system. Thus, if the synthetic system made predominantly a solanesyl pyrophosphate (nonaprenyl pyrophosphate) this would be the determining factor in the observation that the rat made primarily ubiquinone with nine isoprenoid units in the side chain. The specificity of this system which leads to the synthesis of defined polysoprenoid analogues of ubiquinone in different tissues, e.g. nonaprenyl ubiquinone in rat tissues and decaprenyl ubiquinone in humans and guinea pigs, appears to reside in the system for forming polyisoprenoid chains. The foregoing considerations have been essentially verified (Thomas and Threlfall, 1973). In this work we report some characteristics of the PHB:polyprenyl transferase system using nonaprenyl pyrophosphate and PHB as the substrates. The observations reported indicate that the specificity of the polyprenyl pyrophosphate synthetic system can be altered by agents which affect membrane conformation.

Methods and Materials

Rat and Guinea Pig Liver Mitochondrial Preparations. Preparation of mitochondrial suspensions has been described previously (Momose and Rudney, 1972). A minor modification was introduced after the final centrifugation. The pellet was centrifuged again at 20 000*g* for 5 min and the Tris-sucrose-EDTA supernatant solution was very carefully and completely aspirated. The mitochondrial pellet was resuspended in 50 mM potassium phosphate buffer (pH 7.3) containing 0.02% bovine plasma albumin unless otherwise described. Where fresh mitochondria are referred to, this preparation was immediately

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[1] Abbreviations used are: IPP, isopentenyl pyrophosphate; SPP, solanesyl pyrophosphate; EDTA, ethylenediaminetetraacetic acid; PHB, *p*-hydroxybenzoic acid; NPHB, 3-nonaprenyl-4-hydroxybenzoic acid.

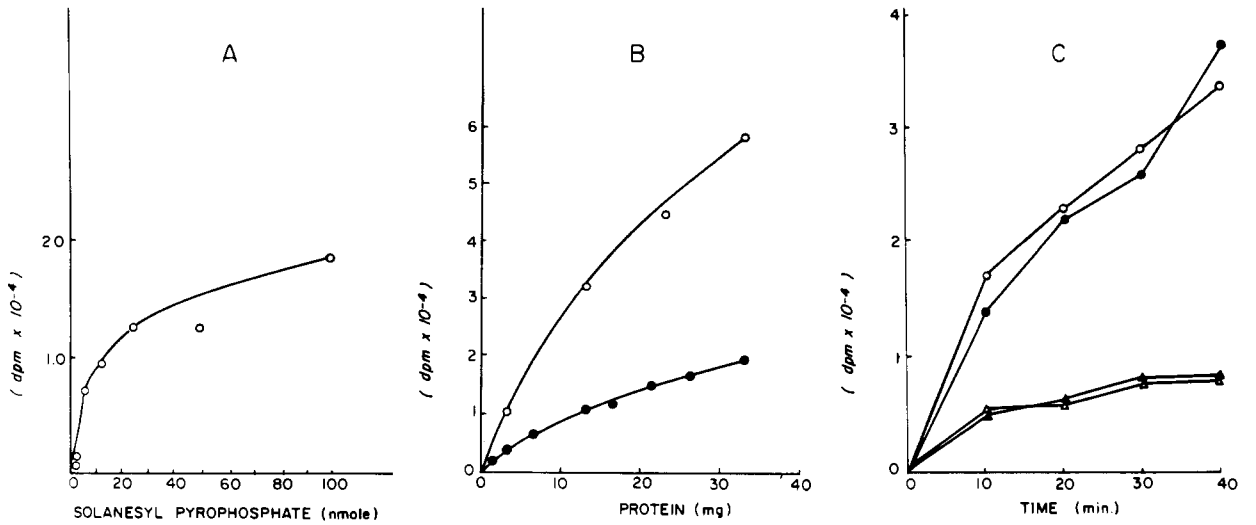

FIGURE 1: Effect of concentrations of substrate and protein and time on the formation of nonaprenyl-PHB; ordinates represent radioactivity (disintegrations per minute) incorporated into nonaprenyl-PHB from [7-^{14}C]PHB. (A) Aged mitochondria were incubated as indicated in the text for 10 min at 37 °C. Each assay contained 10 mg of mitochondrial protein. Reaction velocity represents radioactivity of [7-^{14}C]PHB incorporated into NPHB. (B) Mitochondria incubated for 20 min at 37 °C: (○) 5 nmol of SPP; (●) 1 nmol of SPP added to incubation flask. (C) Effect of ATP on the formation of nonaprenyl-PHB with SPP and solanesyl monophosphate as substrates; 8.2 mg of mitochondrial protein was incubated with: (○) 5 nmol of SPP; (●) 5 nmol of SPP + 50 nmol of ATP; (△) 5 nmol of solanesyl monophosphate; (▲) 5 nmol of solanesyl monophosphate + 50 nmol of ATP.

incubated, otherwise the suspension was kept at −20 °C and activity was maintained for several months.

Assay for Enzyme Activity. The reaction tubes contained, in a final volume of 1.0 ml, the following additions unless otherwise indicated. One nanomole of [7-^{14}C]PHB, 10 μmol of $MgCl_2$, 5 nmol of SPP, 0.01% final concentration of Triton X-100, and mitochondrial protein. The mixture was incubated at 37 °C for 30 min with shaking. The incubation was terminated by placing the tubes in ice water and rapidly adding 4 ml of chloroform–methanol (1:2) containing nonradioactive PHB (2.5 mg/ml). Lipids were extracted (Galliard et al., 1965) and an aliquot (3.0 ml) of the lower phase was evaporated on a steam bath and was assayed for radioactivity via liquid scintillation spectrometry in a toluene scintillator containing Triton X-100 (3:1). Protein concentration was estimated (Lowry et al., 1951) with bovine plasma albumin as a standard.

Phosphorylation of Solanesol. Solanesol was phosphorylated by application of the procedure of Cramer (Cramer and Böhm, 1959) for the synthesis of prenyl pyrophosphates as modified by Kandutsch et al. (1964). Identification of the products was made on the basis of the comparison of the infrared (IR) absorptions at 1160 and 1030 cm^{-1} for the monophosphate and at 1120, 935, and 730 cm^{-1} for the pyrophosphate (Nishino et al., 1972). Purity of the products was checked by thin-layer chromatography (TLC) on a silica gel G plate developed in 1-propanol–ammonia–water (6:3:1). Phosphorus-containing components were detected with the phosphomolybdic acid reagent (Hanes and Isherwood, 1949). The R_f values for the mono- and pyrophosphate derivatives of solanesol were 0.65 and 0.41, respectively. There was no inorganic pyrophosphate, but the solanesyl pyrophosphate contained approximately 5% solanesyl monophosphate. The solanesyl monophosphate on the other hand contained 20–25% solanesyl pyrophosphate.

Product Analysis. The radioactive extract was subjected to TLC on Chromar 1000 silica gel sheets (Mallinckrodt Chemical Company) with 15% acetone in petroleum ether. The radioactive areas were cut out and extracted four times with acetone and used for further analysis by reverse phase TLC. This consisted of a plate coated with Kieselguhr G (Merck) and impregnated with 5% liquid paraffin oil in *n*-hexane. The plate was developed with acetone–water–acetic acid (80:20:1) saturated with paraffin oil (Momose and Rudney, 1972). Radioactive areas on the TLC plate were detected with a Packard radiochromatogram scanner, Model 7201. Identification of these areas as the decaprenyl, nonaprenyl, and octaprenyl analogues of 3-polyprenyl derivatives of PHB was as described (Momose and Rudney, 1972).

Other Materials. [1-^{14}C]IPP trilithium salt (5 mCi/mmol) was obtained from Amersham Searle Company. Samples of solansol were provided as gifts from Dr. Otto Isler and Dr. Karl Folkers to whom we are deeply grateful. Triton X-100 and Tween 80 were obtained from Sigma Chemical Company. [^{14}C]Nonaprenyl-PHB and [^{14}C]decaprenyl-PHB were made biosynthetically (Momose and Rudney, 1972). Nonradioactive trilithium IPP, [7-^{14}C]-*p*-hydroxybenzoic acid (55 mCi/mmol), and nonradioactive chemicals were commercial products.

Results

Figure 1 shows that, under the conditions adopted, the incorporation of SPP into nonaprenyl-PHB apparently followed the normal saturation curve (Figure 1A). The effects of increasing concentration of mitochondrial protein and time are shown in Figures 1B and 1C. Furthermore, Figure 1C also shows that solanesyl monophosphate is inactive as a substrate for the transferase. The small activity observed can be accounted for by the known amount of SPP present in the preparation. Addition of ATP did not affect the rate with solanesyl monophosphate, indicating the absence in this preparation of a kinase to rephosphorylate solanesyl monophosphate to the pyrophosphate.

In other experiments when solanesyl monophosphate was added in addition to SPP no inhibition of formation of nonaprenyl-PHB could be detected (data not presented); only when extremely high concentrations of solanesyl monophosphate relative to SPP were present could inhibition be detected.

Effect of Detergents on PHB:Polyprenyl Transferase. In a previous communication (Schechter et al., 1973) we reported

TABLE I: Effect of Aging on Formation on NPHB.[a]

Expt. No.	Mg^{2+}, 10 mM	Triton 0.01%	Phosphate Buffer (mM)	Time of Aging: 0	30 min	60 min	24 h	72 h	196 h
1	−	−	30	590	560	445	115		
	+	−	30	200	465	355	485		
	+	+	30	410		530	920		
2	−	−	50	470				500	
	+	−	50	190				630	
	+	+	50	385				1050	
3	−	−	25	280					165
	+	−	25	230					420
	+	+	25	465					935

[a] Conditions of incubation are the same as described under Methods and Materials. Data are expressed as dpm (mg of protein)$^{-1}$ (30-min incubation)$^{-1}$. In experiment 1, mitochondria were prepared as described and kept for 30 min and 60 min at 0 °C in 30 mM phosphate buffer and then incubated for 30 min at 37 °C. The 24-h sample was kept at −20 °C before incubation. In experiments 2 and 3, the aged samples were kept at −20 °C for the periods indicated before incubation for 30 min at 37 °C. The amounts of mitochondrial protein present in each experiment were 2.1, 2.7, and 3.7 mg in experiments 1, 2, and 3, respectively.

that detergents, especially Triton X-100, had a variable effect on the activity of PHB:polyprenyl transferase. We have examined this further, and find that the variability may be partially explained by the age and state of the mitochondrial preparations. As shown in Table I, when fresh mitochondria are examined with different concentrations of phosphate buffer, the effects of detergent and Mg^{2+} are variable, whereas in aged mitochondria the activation by Mg^{2+} and several-fold activation by detergents become clear and are consistently reproducible for periods as long as 196 h.

In aged mitochondria, we observed that other detergents in addition to Triton X-100 were effective in activating the PHB-polyprenyl transferase system. Tween 80 was almost as effective as Triton, whereas deoxycholate was least effective. All detergents in concentrations approaching 0.5% were inhibitory (Table II). Although detergents increased the activity of the enzyme, all attempts to solubilize the activity with detergents plus a variety of other standard methods were unsuccessful, indicating that the enzyme is strongly bound to the mitochondrial membrane.

Effects of Triton X-100 on the Synthesis of Polyprenyl Pyrophosphates. Normally in rat liver mitochondria when PHB is present as acceptor and IPP is the substrate for the polyprenyl pyrophosphate synthesizing system, the major product is nonaprenyl-PHB; however, a small amount of the decaprenyl analogue is also formed. Conversely, in guinea pig liver the sole product is the decaprenyl analogue (Momose and Rudney, 1972).

When [7-^{14}C]PHB is incubated with SPP, incorporation of PHB primarily into nonaprenyl-PHB is observed; however, some incorporation into octaprenyl-PHB is also present. The latter is accounted for by the fact that octaprenol is a known contaminant of the solanesol used to form SPP and therefore small amounts of octaprenyl pyrophosphate are also present (Table III, experiment 1A). When Triton X-100 is omitted, the extent of incorporation is smaller (experiment 1B). This is in agreement with the observations in Tables I and II. When IPP is present, one observes a small incorporation into decaprenyl-PHB in addition to the nonaprenyl form (experiments 1C and 1D). These results can be readily explained by the fact that the SPP is the major polyprenyl pyrophosphate substrate present, and small amounts of decaprenyl pyrophosphate are formed by lengthening the chain via prenyl transferase.

If ^{14}C-labeled IPP is the precursor, it is incorporated into both the nonaprenyl and decaprenyl moieties as previously observed, and there is always a preponderance of nonaprenyl compared to decaprenyl. This is shown in experiment 2E. If, however, unlabeled SPP is also present, one now observes incorporation of IPP only into decaprenyl-PHB and none into nonaprenyl-PHB (experiment 2F). This becomes more pronounced as the concentration of SPP is increased (experiment 2G). Thus, it appears that SPP is an inhibitor of the incorporation of IPP into SPP. If the conditions of experiments 2E, 2F, and 2G are duplicated with the exception that Triton X-100 is omitted (experiments 2H, 2I, and 2J) then one observes incorporation of IPP primarily into the nonaprenyl moiety and small amounts into the decaprenyl form. As SPP is added, however, an inhibition of incorporation of IPP into the nonaprenyl moiety is again observed (experiments 2I and 2J). Thus, it appears as if Triton X-100 has two major effects: (1) it facilitates inhibition by SPP of the incorporation of IPP into nonaprenyl-PHB; (2) it facilitates the addition of IPP to SPP to form decaprenyl pyrophosphate which can subse-

TABLE II: Effect of Detergents on the Formation of NPHB.[a]

Detergent	Concn (%)	Nonaprenyl-PHB Formation (dpm/mg of Protein)
None		241
Triton	0.005	1000
	0.01	1350
	0.02	1483
	0.05	1025
	0.10	841
	0.50	143
Tween 80	0.005	1183
	0.01	1291
	0.05	1150
	0.10	1133
	0.50	731
Na deoxycholate	0.01	415
	0.05	773
	0.10	696
	0.50	37

[a] Mitochondria aged for 24 h at −20 °C and then incubated for 20 min at 37 °C. Other conditions are the same as described in the text. Each incubation contained 12 mg of mitochondrial protein.

TABLE III: Effect of Triton X-100 and SPP on the Incorporation of PHB and IPP into 3-Polyprenyl Analogues of PHB in Rat Liver Mitochondria.[a]

Expt. No.	Incubation	Compds Present				pmol of Labeled Substrate Incorpd		
		PHB	SPP	IPP	Triton X-100	Decaprenyl-PHB	Nonaprenyl-PHB	Octaprenyl-PHB
1	A	+*	+	−	+	0	19.9	3.6
	B	+*	+	−	−	0	10.7	1.6
	C	+*	+	+	+	1.6	37.9	3.3
	D	+*	+	+	−	0.7	12.5	1.1
2	E	+	−	+*	+	15.5	39.5	0
	F	+	+	+*	+	340	0	0
	G	+	+	+*	+	600	0	0
	H	+	−	+*	−	10.0	115	0
	I	+	+	+*	−	2.0	60.0	0
	J	+	+	+*	−	1.0	3.0	0

[a] Incorporation of [7-^{14}C]PHB and [1-^{14}C]IPP into prenyl derivatives of PHB was measured by determination of total radioactivity in each moiety by scanning of reversed-phase TLC chromatograms of various incubations with rat liver mitochondria. The location of decaprenyl, nonaprenyl, and octaprenyl analogues of PHB was determined as described under Methods and Materials. Mitochondrial preparations aged for 24 h as described were incubated for 1 h at 37 °C (experiments IA, IB, IC, and ID). Experiments 2E through 2J were incubated for 2.5 h. The amounts of substrate added to each incubation unless otherwise noted were: 1 nmol of [7-^{14}C]PHB, 5 nmol of SPP, 50 nmol of IPP, 20 nmol of [1-^{14}C]IPP, 100 nmol of PHB, and 0.01% Triton X-100 in 1 ml. Incubations in experiments 2G and 2J contained 12.5 nmol of SPP. Incubations A through D contained 12 mg of mitochondrial protein and 10 μmol of potassium fluoride to inhibit phosphatases. In experiment 1 the numbers represent total picomoles of [7-^{14}C]PHB incorporated. In experiment 2 the numbers represent total picomoles of [^{14}C]IPP incorporated. Zero means no radioactivity detectable, a plus sign indicates the presence of a compound, and a minus sign indicates its absence. An asterisk indicates the compound added is labeled with ^{14}C as described above.

TABLE IV: Effect of Triton X-100 and SPP on the Incorporation of PHB and IPP into 3-Polyprenyl Analogues of PHB in Guinea Pig Liver Mitochondria.[a]

Incubation	Substrates Present				pmol of Labeled Substrate Incorpd		
	PHB	SPP	IPP	Triton X-100	Decaprenyl-PHB	Nonaprenyl-PHB	Octaprenyl-PHB
A	+*	−	+	−	44.6	0	0
B	+*	+	+	−	35.7	35.7	13.4
t.7	357	3.4					
C	+*	+	−	+	0	78.1	11.2
D	+*	+	+	+	15.6	69.2	15.6
E	+	−	+*	−	142	0	0
F	+	+	+*	−	115	0	0
G	+	+	+*	+	44.2	0	0

[a] Incorporation of [7-^{14}C]PHB and [1-^{14}C]IPP into prenyl derivatives of PHB was measured by determination of total radioactivity in each moiety by scanning of reversed-phase TLC chromatograms of various incubations with guinea pig liver mitochondria. The location of decaprenyl, nonaprenyl, and octaprenyl analogues of PHB was determined as described under Methods and Materials. Guinea pig liver mitochondria aged for 24 h as described in the text were incubated with substrates for 1 h at 37 °C. The amounts of the substrate added to each incubation were 2 nmol of [7-^{14}C]PHB, 5 nmol of SPP, 40 nmol of IPP, 40 nmol of [1-^{14}C]IPP, 100 nmol of PHB, and 0.02% Triton X-100 in 1 ml. Each incubation contained 27 mg of mitochondrial protein. In incubations A through D the numbers represent total picomoles of [7-^{14}C]PHB incorporated. In incubations E through G the numbers represent total picomoles of [1-^{14}C]IPP incubated. Zero means no radioactivity detectable, a plus sign indicates the presence of a compound, and a minus sign indicates its absence. An asterisk indicates the compound added is labeled with ^{14}C as described above.

quently be transferred to PHB to form the decaprenyl derivative.

When a similar set of experiments is carried out with guinea pig liver mitochondria, a different set of data is obtained. As shown in Table IV, experiment A, when [^{14}C]PHB and IPP are incubated, the PHB is incorporated solely into decaprenyl-PHB. When SPP is present in addition to the IPP, incorporation of [^{14}C]PHB into nonaprenyl, decaprenyl, and octaprenyl moieties is observed (experiment B). If, however, Triton X-100 is present as in experiment C where only SPP is added, as expected only nonaprenyl- and octaprenyl-PHB are formed. However, when both IPP and SPP are present there is an inhibition of the incorporation of PHB into the decaprenyl derivative (experiment D). When similar experiments are performed with ^{14}C-labeled IPP as the sole labeled substrate, incorporation of radioactivity is observed only in the decaprenyl moiety and none in the nonaprenyl form (experiment E). If SPP is present the same result is obtained (experiment F). If Triton X-100 is present, inhibition of IPP incorporation into decaprenyl-PHB is again observed (experiment G). Thus, the activity of Triton is opposite to that observed in rat liver mitochondria, in that Triton X-100 appears to inhibit the formation of decaprenyl pyrophosphate from SPP, and IPP, whereas, in rat liver, this reaction is enhanced.

Discussion

In this report we have elaborated further properties of the polyprenyl pyrophosphate biosynthetic system in mitochondria. When IPP and SPP are both present, additional characteristics of the PHB:polyprenyl transferase system become evident. Since the system is located in the inner mitochondrial membrane, it might be expected that membrane conformation will play a major role in the characteristics and properties of the system. This is indeed borne out by the results reported here. In particular, the striking activation of the activity of the PHB:polyprenyl transferase in aged mitochondria with Mg^{2+} and detergents such as Triton X-100 and Tween 80 is of interest.

Previously, we have observed many variations in the effect of detergents. It appears that this effect is variable with freshly prepared mitochondria; however, activation with detergents becomes more consistent when mitochondria have been maintained at −20 °C for some time and then thawed for study purposes. Obviously, the membrane has been altered so that activation with detergents can occur.

Of further interest is the observation in the alteration of the relative specificity of the polyprenyl pyrophosphate synthetic system which occurs in the undamaged membranes. This becomes apparent when guinea pig and rat are compared. In the rat the major polyprenyl analogue of ubiquinone is the nonaprenyl moiety. Similarly, one also sees that the nonaprenyl-PHB derivative is a major product when PHB and IPP are incubated in mitochondrial preparations (Momose and Rudney, 1972).

The data reported here show that in the presence of Triton X-100, a preformed pool of SPP apparently inhibits the incorporation of IPP into SPP, i.e., a form of feedback inhibition may be occurring. Furthermore, the presence of Triton facilitates conversion of SPP to decaprenyl pyrophosphate when both SPP and IPP are present. This is especially seen in Table III, experiments 2F and 2G.

In the case of guinea pig the specificity in normal mitochondria is directed only to the synthesis of decaprenyl derivatives when IPP is the substrate. It appears that Triton X-100 has an inhibitory effect on this process. Furthermore, there is apparently no free pool of SPP because when SPP is present along with ^{14}C-labeled IPP no radioactivity is observed in the nonaprenyl-PHB (Table IV, experiments 3E, 3F, and 3G). Furthermore, in the guinea pig SPP does not seem to exert any inhibitory effect on the incorporation of IPP into decaprenyl-PHB. However, in the presence of Triton X-100, marked inhibition occurs.

The foregoing effects point out the close relationship of membrane integrity to the biosynthesis of ubiquinone and its precursors. This relationship takes on added interest because of observations on virus altered cells (Casey and Bliznakov, 1973). These workers found that mouse cells which normally contain ubiquinone primarily in the form of the octaprenyl and nonaprenyl ubiquinone, when infected with friend leukemia virus, shifted their biosynthetic pattern so that appreciable amounts of the decaprenyl analogue were formed. It is also generally recognized that one of the consequences of viral infection is often a transformation or alteration in the properties of the cell membranes. It would appear then that a change in the moiety of the isoprenoid chain of ubiquinone normally present to a longer or shorter form could possibly signal some change in conformation or properties of the membrane.

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