Noncovalent Interactions between Amino Acid Side Chains and a Coenzyme Model

SIDNEY SHIFRIN

From the National Cancer Institute, National Institutes of Health, Bethesda, Md.

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A series of compounds has been synthesized in which phenol, imidazole, benzene, thiomethyl ether, or a quaternary ammonium ion is joined to a nicotinamide ring by two methylene groups. The first four functional groups mentioned have been shown to interact with the pyridinium ion as measured by the appearance of an absorption band which is associated with the intramolecular complex. The intensity of the transition is associated with the electron affinity of the pyridinium ion and the wavelength is correlated with the donor ability (energy of the highest occupied molecular orbital) of the amino acid side chain. The quaternary ammonium ion was the only substituent among those which were examined to produce a significant shift in the absorption maximum of the dihydronicotinamide moiety. The results of this study are compared with the spectral properties of some enzyme-coenzyme systems.

The one-to-one complex which is formed between NAD^+ or its model, *N*-benzylnicotinamide, and indole is characterized by a broad, featureless absorption band which extends into a region far removed from the absorption maximum of either component (Cilento and Tedeschi, 1961). The similarity between the absorption properties of this complex and the spectrum of NAD^+–glyceraldehyde-3-phosphate dehydrogenase (Racker and Krimsky, 1952) prompted the suggestion that the coenzyme is bound to its apoenzyme in the vicinity of a tryptophan residue (Alivisatos *et al.*, 1961; Cilento and Tedeschi, 1961; Shifrin, 1964).

A more detailed examination of the electron donor–electron acceptor pair in the region where the two chromophores have their absorption maxima was made possible by incorporating the indole and pyridinium ion into the same molecule which was designed to permit intramolecular interaction (Shifrin, 1964). Substitution of the indole by other amino acid side chains has been effected and their absorption and emission properties as a measure of interaction are reported here. The functional groups of the following amino acids have been included in this study: phenylalanine, tyrosine, histidine, methionine, and lysine.

MATERIALS AND METHODS

Synthesis of β-Substituted 1-Ethyl-3-carbamoylpyridinium Chloride Derivatives

N-(β-Indolylethyl)-3-carbamoylpyridinium Chloride.—The preparation and properties of this compound have been described previously (Shifrin, 1964).

N-(β-p-Hydroxyphenylethyl)-3-carbamoylpyridinium chloride was synthesized by treatment of a methanolic solution of *N*-(2,4-dinitrophenyl)-3-carbamoylpyridinium chloride (1.25 g in 25 ml of methanol) with 1.0 g of tyramine in 10 ml of methanol. The 2,4-dinitrophenylnicotinamide salt was prepared from 1-chloro-2,4-dinitrobenzene and nicotinamide by the procedure described by Lettre *et al.* (1953). The reddish color which was produced on mixing the reactants gradually faded to light yellow at which point the product was precipitated by the addition of ether. The product was recrystallized several times from methanol-ether. Physical properties are summarized in Table I.

N-(β-Phenylethyl)-3-carbamoylpyridinium chloride was prepared as above except that β-phenylethylamine replaced tyramine. Recrystallization was from methanol-ether.

N-(β-4'-Imidazolylethyl)-3-carbamoylpyridinium chloride was prepared from histamine and 2,4-dinitrophenylnicotinamide chloride.

N-(β-Methylthioethyl)-3-carbamoylpyridinium chloride was prepared from β-chloroethyl methyl sulfide (Aldrich Chemical Co.) and nicotinamide in refluxing methanol. Purification was by several recrystallizations from methanol-ether solvent mixture.

N-(β-Trimethylaminoethyl)-3-carbamoylpyridinium dichloride was prepared by refluxing 12 g of nicotinamide with 16 g of β-chloroethyltrimethylammonium chloride in ethanol for 24 hours. The yield of product was very low but no further attempts were made to improve the yield.

Molecular weights, melting points, and elemental analyses for all the compounds are given in Table I.

N-Methylnicotinamide perchlorate was prepared from the corresponding iodide by the method described by Kosower and Bauer (1960).

Chemical reduction was carried out in sodium carbonate solution (at 0° and in a helium atmosphere) by gradual addition of sodium dithionite. In the case of the indole, phenol, and benzene derivatives the reduction product precipitated from the reaction mixture, and was filtered and washed with cold water and dried under vacuum. The reduction products of the other amino acid–nicotinamide salts were isolated by extraction of the reaction mixtures with dichloromethane followed by removal of the solvent *in vacuo*.

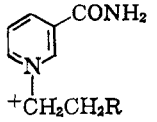
Absorption spectra were taken on a Cary recording spectrophotometer, Model 15, at a slow scan speed to permit accurate determination of absorption maxima and extinction coefficients. Difference spectra were taken in tandem double cells such as the ones described by Herskovits and Laskowski (1962) in which a solution of the synthetic compound in water or methanol was placed in one half of the sample cuvet (closest to the phototube) and the solvent in the other half of the cuvet; one half of the blank cuvet contained an equimolar solution of *N*-methylnicotinamide perchlorate and the appropriate amine hydrochloride was in the other half.

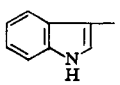
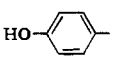
All organic solvents were Spectroquality Grade purchased from Matheson, Coleman and Bell. All organic reagents were Eastman White Label chemicals.

Fluorescence excitation spectra were taken on the Aminco-Bowman spectrophotofluorometer having a xenon arc as the exciting source and an RCA 1P 28 photomultiplier as the detector. Spectra were corrected for variations in intensity of the light source as a function of wavelength by the thermopile method described by White *et al.* (1960).

Determination of *pK* values was made potentiometrically using a Metrohm *p*H meter (Compensator E 322).

TABLE I
PROPERTIES OF β-SUBSTITUTED 1-ETHYL-3-CARBAMOYLPYRIDINIUM CHLORIDES



R	Empirical Formula	Mol Wt	Melting Point (deg C)	Calcd. (%) C	Calcd. (%) H	Calcd. (%) N	Found (%) C	Found (%) H	Found (%) N
	$C_{17}H_{20}N_3O_2Cl$[a]	333.82	238	61.16	6.03	12.59	60.53	5.88	
	$C_{14}H_{15}N_2O_2Cl$[a]	310.78	199.5	57.97	6.16	9.02	58.19	5.98	9.48
	$C_{14}H_{15}N_2OCl$	262.74	239.5	63.99	5.75	10.66	64.55	5.80	10.69
	$C_{11}H_{13}N_4OCl$	252.70	200–201	52.28	5.18	22.17	52.23	5.20	21.62
CH_3S—	$C_9H_{13}N_2OSCl$	232.74	205.0–205.5	46.44	5.63	12.04	45.87	5.67	11.68
$(CH_3)_3\overset{+}{N}$—	$C_{11}H_{19}N_3OCl_2$	280.21	240–241	47.15	6.84	15.00	46.48	6.39	14.73

[a] Contains 1 mole of methanol of crystallization.

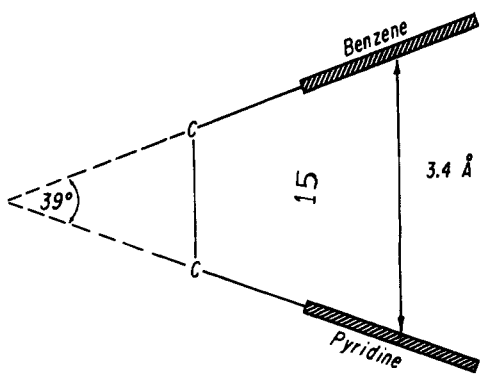


FIG. 1.—Diagrammatic representation of the angles and distances of the two aromatic rings in *N*-(β-phenylethyl)-pyridinium chloride (eclipsed conformation).

RESULTS

General Considerations of the Models.—Optimum conditions for interaction between the amino acid side chain and the pyridine ring were presumed for the two groups lying in the same plane, in close proximity and in a somewhat rigid orientation. Although not all these conditions were completely fulfilled, they were all partially satisfied by separating the two species under consideration by two methylene groups. As an example let us consider the phenylalanine side chain (benzene) as being the functional group whose π-electron system might interact with the pyridinium ring. If the disubstituted ethane is considered to be in the eclipsed form, the two rings make an angle of 39° with each other as shown by the dashed lines in Figure 1, and the distance from the center of the benzene ring to the center of the pyridinium ring was measured as 3.4 A from Dreiding molecular models.

Interaction is defined here as (1) the appearance of a transition in the absorption spectrum of the *N*-(β-substituted ethyl) pyridinium salt which is absent in the sum of the spectra of the individual components, (2) a shift in the maximum of the dihydronicotinamide absorption band in *N*-(β-substituted ethyl)-1,4-dihydronicotinamide, and (3) in the case of indole and phenol, transfer of energy of excitation from the amino acid side chain to the dihydronicotinamide moiety.

Tryptophan Side Chain (Indole).—Although a detailed report of the spectral properties of indolylethylnicotinamide has been published elsewhere (Shifrin, 1964), a brief summary of the results will be given here so that a comparison may be made with the other amino acid side chains examined in this study. Interaction of the indole and nicotinamide rings resulted in the appearance of a broad absorption band extending from 300 to 450 mμ with an apparent maximum at 325 mμ (ε 1000), as determined from the difference spectrum, and negative absorption having maxima at 277.5 and 290.5 mμ. The normally intense emission of indole is quenched in the intramolecular complex.

The absorption spectrum of indolylethyldihydronicotinamide is simply the sum of the individual absorbancies of 1-methyl-1,4-dihydronicotinamide and tryptamine, but intramolecular interaction was detected by fluorescence spectroscopy. Although more than 90% of the light intensity at 280 mμ is absorbed by the indole moiety, only emission characteristic of the dihydronicotinamide ring at 460 mμ could be detected. From fluorescence-excitation spectra it can be seen that there is quantitative transfer of excitation energy from the amino acid side chain to the emitting species.

Tyrosine Side Chain (Phenol).—*N*-(β-*p*-Hydroxyphenylethyl)-3-carbamoylpyridinium Chloride.—The absorption spectrum of either a methanolic or an aqueous solution of this compound is shown by the unbroken curve in Figure 2. In an effort to determine the spectral changes which may have resulted from intramolecular interaction the spectrum of an equimolar mixture (*ca.* 10^{-4} M) of tyramine hydrochloride and *N*-methylnicotinamide perchlorate was examined. The resultant spectrum is given by the dashed curve in Figure 2. Subtraction of the two curves (unbroken line minus dashed line) gives the difference spectrum in Figure 3 (λ_{max} 296.5 mμ; ε 1000).

If a charge-transfer transition is assigned to the

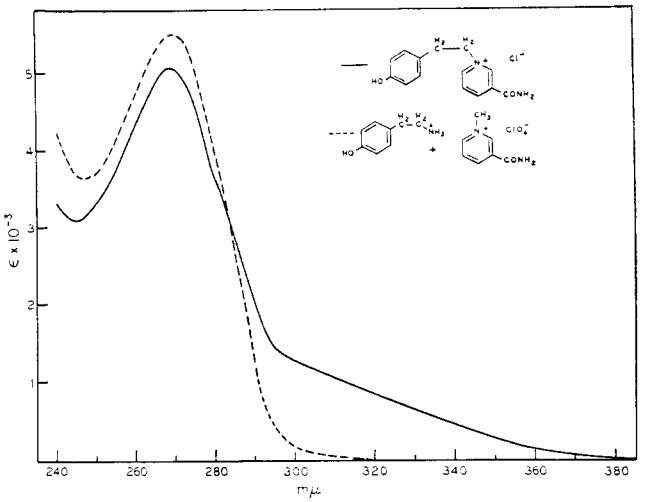


FIG. 2.—Absorption spectrum of *N*-(β-*p*-hydroxyphenylethyl)-3-carbamoylpyridinium chloride in water (solid curve); absorption spectrum of an equimolar mixture of tyramine hydrochloride and *N*-methylnicotinamide perchlorate in water (dashed curve).

long broad absorption band, an increase in the electron density of the donor partner (phenol) of the complex might be reflected by the new transition. The difference spectrum of *p*-hydroxyphenylethylnicotinamide versus equimolar solutions of tyramine and *N*-methylnicotinamide perchlorate all in 0.1 N sodium hydroxide is shown by the dashed curve of Figure 3. The red shift of the apparent maximum (320 mμ) is accompanied by a slight decrease in extinction coefficient (ε 700).

Interaction of the phenolic residue with the pyridinium ion in the excited state was also studied fluorometrically. Tyrosine or tyramine generally emits maximally around 303 mμ with a quantum yield of 0.21 (Teale and Weber, 1957; White, 1959). There was no detectable fluorescence from solutions of *p*-hydroxyphenylethylnicotinamide although an equimolar mixture of the separate chromophores did exhibit fluorescence characteristic of the aromatic amino acid side chain.

The absorption spectrum of the chemically reduced compound, *p*-hydroxyphenylethyldihydronicotinamide, was simply the sum of the tyramine and 1-methyl-1,4-dihydronicotinamide spectra. As was the case with the indole derivative, fluorescence proved to be a more useful method for detecting interactions in the reduced compound. Direct excitation of the dihydronicotinamide moiety at 355 mμ produced a fluorescence band with a maximum at 450 mμ (quantum yield, 0.056) which is identical with the properties of 1-methyl-1,4-dihydronicotinamide (Weber, 1958). Excitation at 275 mμ where nearly all the energy is absorbed by the phenol moiety also resulted in dihydronicotinamide emission at 450 mμ with no detectable fluorescence by the phenol itself at 303 mμ. This quantitative transfer of excitation energy was also found in indolylethyldihydronicotinamide as reviewed (*vide supra*).

Phenylalanine Side Chain (Benzene).—*N*-(β-Phenylethyl)-3-carbamoylpyridinium Chloride.—Interaction of an unsubstituted benzene (i.e., the π-aromatic system alone) with the pyridinium ion was evident from a comparison of the absorption spectrum of *N*-phenylethylnicotinamide (oscillator strength, $f \sim 0.189$) with that of its next lower homolog, *N*-benzylnicotinamide ($f \sim 0.151$). Subtraction of the two spectra, as reported for the phenol derivative, revealed a new band with an apparent maximum at 282.5 mμ (ε 1000).

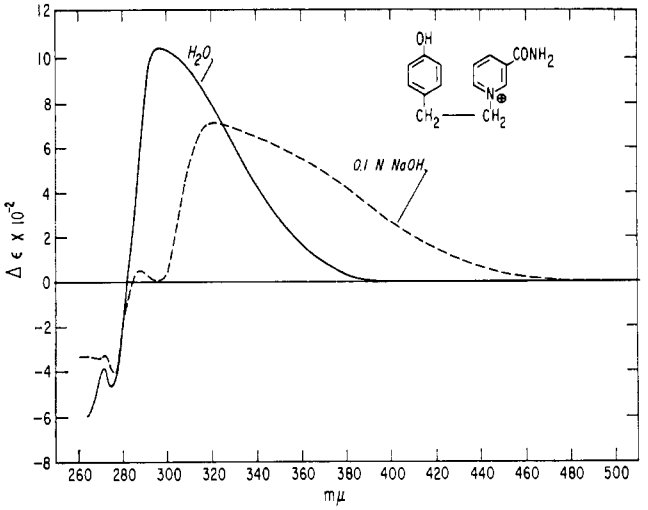


FIG. 3.—Difference spectrum of *N*-(β-*p*-hydroxyphenylethyl)-3-carbamoylpyridinium chloride (sample cuvet) versus an equimolar mixture of tyramine hydrochloride and *N*-methylnicotinamide perchlorate (blank cuvet). Solid curve, in water; dashed curve, in 0.1 N sodium hydroxide.

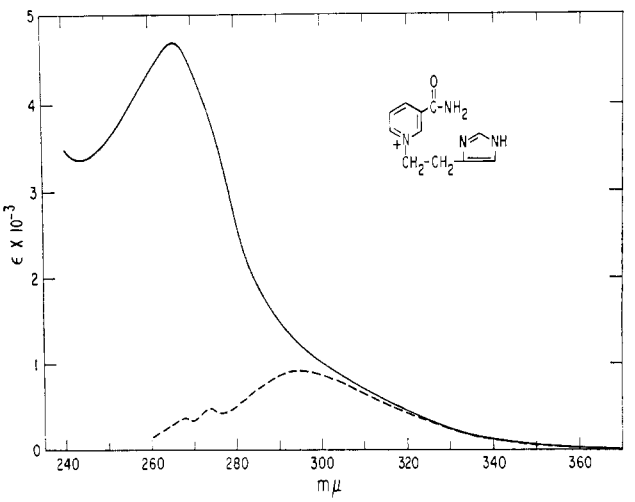


FIG. 4.—Absorption spectrum of *N*-(β-4′-imidazolylethyl)-3-carbamoylpyridinium chloride in methanol (solid curve). The dashed curve is the spectrum obtained after subtraction of the absorption by *N*-methylnicotinamide perchlorate.

Histidine Side Chain (Imidazole).—*N*-(β-4′-Imidazolylethyl)-3-carbamoylpyridinium Chloride.—The nonaromatic imidazole side chain was also found to give a long-wavelength band on interaction with the pyridinium ion (unbroken curve, Fig. 4). Since imidazole does not absorb above 240 mμ at the concentrations being used in these studies, only the absorption of the pyridinium ion was subtracted to produce the difference spectrum shown by the dashed curve in Figure 4 (λ_{max} 294.5 mμ, ε 900).

The long-wavelength band disappeared in 0.1 N hydrochloric acid which suggests that the lone pair of electrons on the imidazole nitrogen is the electron donor of the intramolecular complex.

Thus far interaction between the amino acid side chain and the coenzyme model has been examined in the *excited state*. If such nonbonding interaction is to be considered as an active force in biological systems, such intramolecular complexes should be demonstrated for the *ground state*. The ionization constant of the imidazole moiety was chosen as a crude method for examining possible interaction with the pyridinium

TABLE II

ABSORPTION AND EMISSION PROPERTIES OF β-SUBSTITUTED 1-ETHYL-3-CARBAMOYLPYRIDINIUM CHLORIDES

	Difference Spectrum		Cyanide Adduct		Dithionite Reduction		
Side Chains	λ_{max} (mμ)	(ε)	λ_{max} (mμ)	(ε)	λ_{max} (mμ)	(ε)	Emission Intensity at 450 mμ[a]
Indole	325	(1000)	340	(7000)	356	(7000)	1.00
Thiomethyl	300	(900)	337	(6100)	355	(7000)	0.95
Phenol	296.5	(1000)	341	(6800)	356.5	(7000)	1.10
Imidazole	294.5	(900)	337.5	(7900)	355		1.00
Benzene	282.5	(1000)	338	(7000)	355	(7100)	1.12
Trimethylamino			330	(6000)	340		0.02

[a] The emission intensity of a methanolic solution of 1-methyl-1,4-dihydronicotinamide (A_s = 0.100 at 355 mμ) was arbitrarily chosen as 1.00. All solutions were adjusted to have an absorbancy of 0.100 at the long-wavelength maximum for comparison of their fluorescence intensity.

ion. The pK_a of the imidazole nitrogen of imidazolylethylnicotinamide was found to be 5.5 as measured potentiometrically. Under identical conditions of ionic strength, the imidazole nitrogen of histamine hydrochloride had a pK_a of 6.0. The slight lowering of the pK_a may result from participation of the imidazole nitrogen in an electron donor–acceptor complex in the ground state. More sensitive methods of examining interaction are currently under investigation.

The dithionite reduction product, imidazolylethyldihydronicotinamide, was examined spectrophotometrically and fluorometrically from 250 to 600 mμ with no evidence of any interaction between the two moieties. Had the dihydronicotinamide formed a charge transfer complex with the imidazole as has been suggested (Estabrook *et al.*, 1963), the fluorescence intensity of the reduced coenzyme model would have diminished (e.g., indole and phenol in this report).

Methionine Side Chain (Methyl Thioether).—*N*-(β-Methylthioethyl)-3-carbamoylpyridinium Chloride.—Interaction between the methyl thioether and the pyridinium ion was again demonstrated by the appearance of a new band (λ_{max} 300 mμ; ε 900) as measured by difference spectroscopy.

There was no detectable interaction between the two moieties as measured by fluorescence and absorption methods.

Lysine Side Chain (Ammonium Ion).—*N*-(β-Trimethylaminoethyl)-3-carbamoylpyridinium Dichloride.—In order to study the effect of a neighboring positive charge on the spectral properties of dihydronicotinamide, protonation of an amine to the corresponding ammonium ion might result in the simultaneous acid decomposition of the dihydropyridine. Therefore the quaternary ammonium salt, ethyltrimethylammonium chloride, was used as a model for the lysine side chain. The absorption spectrum of an aqueous solution of this nicotinamide derivative was almost identical with that of *N*-methylnicotinamide perchlorate.

The most pronounced effect of the positive charge is on the position of the absorption maximum of the cyanide adduct or of the dithionite-reduction product. The dihydronicotinamide band in the neighborhood of the positively charged side chain is at 340 mμ in methanol, or a shift of 15 mμ to shorter wavelengths compared with 1-methyl-1,4-dihydronicotinamide in the same solvent (Cilento *et al.*, 1958). This shift is comparable to the value obtained by Kosower and Remy (1959) for a system in which a quaternary nitrogen atom is located 3.1 A from an α,β-unsaturated ketone. In the present study the distance of the quaternary ammonium ion from the ring nitrogen of the dihydropyridine is 2.7 A when the substituents are *cis* and 3.9 A when they are *trans*. (Values obtained from molecular models.)

The absorption properties of this model compound are similar to those of NADH when complexed with liver alcohol dehydrogenase (Theorell and Bonnichsen, 1951). However, the fluorescence properties of the trimethylaminoethyldihydronicotinamide were not at all similar to the enzyme-NADH system. The intensity of the fluorescence from the dihydronicotinamide moiety of the model system was $^1/_{50}$ the value found with 1-methyl-1,4-dihydronicotinamide. These results are completely opposite to the enhanced fluorescence of NADH when it is bound to liver alcohol dehydrogenase (Boyer and Theorell, 1955).

DISCUSSION

In this study pyridinium ions have been shown to interact with a number of amino acid side chains giving rise to absorption bands which were not observed in the separate substituents. The absorption and emission properties of the synthetic compounds are summarized in Table II.

Column 1 demonstrates the variability of the wavelength of the new transition arising from intramolecular interaction and the constancy of the intensity of this transition. The first five amino acid side chains may be considered as electron donors (Lewis bases) and the pyridinium ion as an electron acceptor (Lewis acid), resulting in the formation of an intramolecular charge transfer complex. The energy of the new band to a first approximation is dependent upon the ease with which an electron may be removed from the donor partner (ionization potential), the electron affinity of the acceptor molecule, and a term which accounts for the mutual electrostatic energy of the molecule in the excited state relative to that in the ground state. The electron affinity is a constant factor in this study (pyridinium ion) so that the variation in wavelength is dependent upon the donor abilities of the side chains.

While the experimental values for the ionization potentials for all the amino acids used here are not directly available, energies of the highest occupied molecular orbital which give a relative value of the ionization potentials have been obtained from quantum mechanical calculations (Pullman and Pullman, 1958). When the energy of the highest occupied molecular orbital of indole, phenol, benzene, and imidazole was plotted against the apparent absorption maximum of the corresponding nicotinamide derivative, a straight line was obtained. These studies present another example of the regularities reported for the spectra of molecular complexes (McConnell *et al.*, 1953) in which the wavelength of the transition is related to the

ionization potential of the electron donor and the intensity of the transition is related to the electron-acceptor partner of the complex.

The model compounds reported in this study permit experimental evaluation of postulated interactions. For example, the suggestion by Estabrook *et al.* (1963) that imidazole might be an electron acceptor in a charge-transfer complex with the dihydronicotinamide moiety of NADH as the electron donor does not appear reasonable from the results obtained in the present report. Partial quenching of the dihydronicotinamide emission by imidazole would have been apparent in imidazolylethyldihydronicotinamide; yet the value shown in the last column of Table II indicates that such quenching does not take place.

The absorption maximum of the dihydronicotinamide moiety present in the first five compounds in Table II was found to be remarkably constant. The earlier suggestion that the shift in the maximum of NADH on binding to liver alcohol dehydrogenase is a result of the coenzyme's being in a hydrophobic environment (Shifrin and Kaplan, 1960) has not been verified. The postulated effect of a positive charge on the position of the absorption maximum of NADH (Kosower, 1962) has been corroborated experimentally. However, emission properties of the NADH–alcohol dehydrogenase complex are not simulated by trimethylaminoethyldihydronicotinamide. Nevertheless, the enhanced fluorescence of NADH on binding to a large number of dehydrogenases may be intimately related to the still unexplained fluorescence behavior of proteins.

Such nonbonding interactions as have been reported here for the molecules in the excited state might be operative in enzyme-substrate or enzyme-coenzyme systems where they would have to function in the ground state. Some of the compounds reported in this study are being examined by nuclear magnetic resonance spectroscopy which would permit examination of proton shielding in the neighborhood of an aromatic ring (Johnson and Bovey, 1958).

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Terpene Metabolism in the Rat Testis. I. The Conversion of Isopentenyl Pyrophosphate to Squalene and Sterols*

R. A. Salokangas, H. C. Rilling,† and Leo T. Samuels

From the Department of Biological Chemistry, University of Utah College of Medicine, Salt Lake City

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Certain aspects of the terpenoid biosynthetic pathway in testis have been investigated. It has been shown that [^{14}C]isopentenyl pyrophosphate is readily converted to squalene and to a much lesser extent to lanosterol and cholesterol. The accumulation of radioactive squalene in rat testis homogenates cannot be accounted for by either a lack of cofactors or the presence of a large endogenous pool of squalene. The squalene content of rat testis has been found to be 9 μg/g wet tissue.

The biosynthesis of sterols from acetate has been studied extensively in both liver and yeast (Wright, 1961). Mevalonic acid has proved to be a key intermediate in this sequence of reactions in that its formation is apparently irreversible and all of its metabolic products are terpenoid compounds. The principal fate of mevalonic acid in mammalian tissue is its conversion to cholesterol. Since cholesterol is a precursor of the steroids in the endocrine organs (Staple *et al.*, 1956) it follows that mevalonic acid should also serve as a precursor of these compounds.

While one group of workers has succeeded in demonstrating the conversion of mevalonic acid to steroids (Rabinowitz and Ragland, 1958) a number of others (Bryson and Sweat, 1962; Savard *et al.*, 1960) have failed to find this conversion.

With this background we have undertaken a study of terpene biosynthesis in testicular tissue. In this paper we report the conversion of isopentenyl pyro-

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