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*

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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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phosphate (IPP)[1] to squalene and sterols by testicular homogenates.

Methods

Materials.—D,L-[2-^{14}C]mevalonic acid, specific activity 1.24 mc/mmole was purchased from New England Nuclear Corp. β-Diphosphopyridine nucleotide, triphosphopyridine nucleotide monosodium salt, glucose-6-phosphate dipotassium salt, reduced glutathione, and adenosine-5′-triphosphate disodium salt were obtained from Sigma Chemical Co. Squalene, lanosterol, and cholesterol were purchased from Mann Research Laboratories. Lanosterol acetate was a gift of Dr. K. Bloch. Silica gel, a product of the Davison Co., was reduced to the proper mesh size by grinding in a Waring Blendor for 30 seconds and was screened to obtain the fraction between 200 and 350 mesh. The gel was equilibrated at the relative humidity of 7%, which was provided by a desiccator containing a saturated solution of sodium hydroxide (Klein and Szczepanik, 1962). Skellysolve B (Skelly Oil Co.) was distilled from sodium. Ether (Mallinckrodt), analytical reagent, was used without further purification. Benzene, analytical reagent, was redistilled.

Preparation of Enzymes from Baker's Yeast.—Dry baker's yeast was a source of enzymes to convert mevalonic acid to IPP. Fifty g of yeast powder was dissolved in 75 ml of water and 7.5 ml toluene was added. The thick paste was autolyzed at 37° for 1 hour and diluted with 100 ml of water. The *p*H was adjusted to 7.4 with 5 N KOH and the adjustment was repeated every half hour for 5 hours. The yeast extract was then centrifuged at 30,000 × *g* for 10 minutes, and the supernatant was decanted and dialyzed against 0.02 M potassium phosphate buffer, *p*H 7.4, for 12 hours.

Preparation of [2-^{14}C]Isopentenyl Pyrophosphate.—Radioactive IPP was prepared by incubating 1 ml yeast enzyme with 1 mg DL-[2-^{14}C]mevalonic acid; Tris, *p*H 7.4, 1.6 mmoles; iodoacetamide, 0.24 mmole; sodium ethylenediaminetetraacetate, 0.32 mmole; $MgCl_2$, 0.48 mmole; ATP, 0.24 mmole; NaF, 2.4 mmoles, in a final volume of 50 ml at 37° for 1 hour. After boiling, the incubation mixture was chromatographed on a 1.4 × 10–cm column of Dowex-1-formate (Bloch *et al.*, 1959). IPP was eluted with increasing concentrations (0.4–0.8 M) of ammonium formate in 4 N formic acid. The fractions containing IPP were combined and lyophilized free of water and ammonium formate. IPP was further characterized by paper chromatography in the system *t*-butanol–formic acid–water (40:10:16). The R_F value found was 0.53–0.57 while Bloch *et al.* (1959) reported an R_F value of 0.53–0.61 for IPP. On incubation with snake venom (Naja Naja) phosphatase [^{14}C]IPP was converted to a hexane-soluble volatile material (presumably [^{14}C] isopentenol).

Purification of Squalene.—Commercial fish squalene was chromatographed in 150-mg quantities on 40 g Woelm alumina, grade I, and 25-ml fractions were collected. Fractions 1–4 were eluted with Skellysolve B, 5–8 with Skellysolve B containing 1% benzene, and 9–12 with Skellysolve B containing 2% benzene. Squalene appeared in fractions 6–9 and was further purified twice via the thiourea adduct (Goodman and Popjak, 1960). Squalene hexahydrochloride was prepared as described by Loud and Bucher (1958).

[1] Abbreviations used in this work: IPP, isopentenyl pyrophosphate; ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; Tris, tris(hydroxymethyl)aminomethane.

Purification of Lanosterol and Cholesterol.—Commercial lanosterol was purified by bromination followed by regeneration with zinc (Johnston *et al.*, 1957). After two crystallizations from methanol–methylene chloride the lanosterol melted at 133–134°. Cholesterol was crystallized twice from ethanol and once from glacial acetic acid. The melting point was 147–148° (Radin and Gramza, 1963).

Preparation of Testis Homogenate and Incubation.—Testes from 2-month-old rats were removed, decapsulated, and homogenized with a Ten-Broeck glass tissue grinder at 0° in 0.15 M Tris-KCl buffer, *p*H 7.4, containing 0.04 M nicotinamide, 2 ml/g of tissue. Unbroken cells, nuclei, and large cell debris were removed by centrifugation at 700 × *g* for 10 minutes, at 4°. After addition of the indicated substrates and cofactors incubations were conducted at 37° for 1 hour in a shaking incubator.

Saponification and Extraction.—At the end of the incubation the flask contents were transferred to tubes containing 7 ml of 15% KOH, and the flasks were rinsed with 5 ml of methanol. The samples were saponified at 70° for 30 minutes under nitrogen. The nonsaponifiable fraction was extracted four times with equal volumes of ether-chloroform (4:1). The organic solvent was washed with small volumes of water until neutral and dried over anhydrous Na_2SO_4, and the extract was evaporated to dryness. The nonsaponifiable material was fractionated on a 1 × 4–cm column of Woelm neutral alumina deactivated by addition of 7% water.

Gas Chromatography.—A 1% XE-60–coated column was used for analysis of squalene and sterols. The column temperature for squalene was 192° and the pressure (argon) 20 psi; for lanosterol and cholesterol 214°, 20 psi; and for lanosterol acetate 215°, and 25 psi. The individual compounds were identified and measurements of mass were made by comparison with the peaks produced by known amounts of authentic squalene and sterols. The areas under the peaks were measured by triangulation. Individual compounds were collected in ethanol after gas chromatography and transferred to counting vials for radioactive estimation. Counting of radioactivity in the samples was performed in a Packard Tri-Carb scintillation counter using 5–10 ml of scintillation fluid prepared by dissolving 4 g 2,5-diphenyloxazole and 40 mg 1,4-bis-2-(5-phenyloxazolyl)benzene in 1 liter of toluene.

Results

Numerous attempts to convert either radioactive acetate or mevalonate to sterols or steroids in testicular homogenates yielded only a small amount of radioactivity in the sterol fraction. Incubation of testicular homogenates with [^{14}C]IPP as a substrate, however, resulted in marked incorporation of radioactivity into nonsaponifiable lipids. Chromatography of these lipids on deactivated alumina showed three radioactive peaks (Fig. 1). The major peak behaved chromatographically like squalene, while the second and third peaks resembled those of lanosterol and cholesterol.

Identification of Radioactive Squalene.—The radioactive material found in peak I from several incubations was combined to give a total of 50,000 cpm and was added to 156 mg of authentic squalene. This mixture was chromatographed on a 2 × 16–cm column of Woelm neutral alumina, grade I. As is shown in Figure 2 the radioactivity coincided with the mass of authentic squalene and the specific activity of the squalene remained constant throughout the elution of the radioactive material.

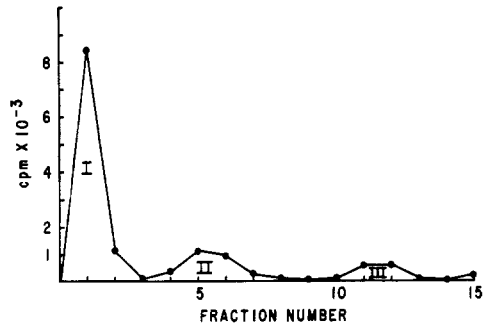

FIG. 1.—Fractionation of nonsaponifiable material. 20-ml fractions were collected. Fractions 1 and 2 were eluted with Skellysolve B, fractions 3–9 with Skellysolve B containing 5% benzene, and fractions 10–14 with Skellysolve B containing 20% benzene. Fraction 15 was eluted with 50% ethanol in benzene.

A second sample of the radioactive hydrocarbon isolated from incubations (50,000 cpm) was analyzed by gas chromatography. The retention time of the main peak was the same as for the standard squalene and this peak contained 89% of the total radioactivity recovered from the gas-liquid column.

A sample of pure nonradioactive squalene, 336 mg, was added to another radioactive sample of material from peak I giving a specific activity of 149 cpm/mg. Three successive formations of squalene thiourea complex gave specific activities for squalene of 137, 142, and 142 cpm/mg, respectively.

A portion of radioactive material from peak I (150,000 cpm) and 410 mg of pure squalene were combined and dissolved in acetone, and the hexahydrochloride was formed. The specific activity of the hexahydrochloride was 363 cpm/mg. Two further recrystallizations gave specific activities of 370 and 374 cpm/mg. The squalene hexahydrochloride had partial melting points of 109.5° and 136° owing to two isomers of hexahydrochloride. Reported melting points are for the first hexahydrochloride 109–112° and for the second 136–143° (Capstack *et al.*, 1962).

Identification of Radioactive Lanosterol.—About 50,000 cpm of biological material from incubations chromatographing like lanosterol (peak II) was added to 550 mg of nonradioactive lanosterol. The specific activity of the sample was measured and was found to be 52 cpm/mg. The dibromide derivative gave a specific activity of 34 cpm/mg corrected for bromine. After debromination the specific activity dropped to 27 cpm/mg. Three further recrystallizations from methylene chloride–methanol (1:10) gave specific activities of 22, 22, and 17 cpm/mg. Mother liquors were 55, 34, and 22 cpm/mg, respectively. Lanosterol was then acetylated (Johnston *et al.*, 1957) and the acetate was subjected to chromatography on a silica gel column, 1 × 45 cm (Klein and Szczepanik, 1962). The material was resolved into two peaks, A and B (Fig. 3), the radioactivity coinciding with the second peak. It was apparent from these data that the commercial lanosterol contained an impurity. The melting points of the material recovered from peaks A and B were 115–117° and 127–128°, respectively. The melting point of the authentic lanosterol acetate was 127–128°. Both peaks were then examined by gas-liquid chromatography and the retention time of the peak B was found to be identical with the retention time of the authentic lanosterol acetate.

Eighty per cent of the radioactivity of acetylated peak II (Fig. 1) material recovered from gas-liquid chromatography was associated with lanosterol acetate.

Identification of Radioactive Cholesterol.—About 17,000 cpm of material from incubations chromatographing like cholesterol (peak III) was added to 345 mg of nonradioactive cholesterol. Specific activity of the mixture was 29 cpm/mg. The first recrystallization from ethanol gave a specific activity of 26 cpm/mg. Mother liquor was 182 cpm/mg. Crystallizations from glacial acetic acid gave 10 cpm/mg (mother liquor 148 cpm/mg) and from ethanol 9 cpm/mg (mother liquor 15 cpm/mg). After bromination, debromination, and recrystallization from ethanol the specific activity was 10 cpm/mg. Mother liquor from this recrystallization was 11 cpm/mg.

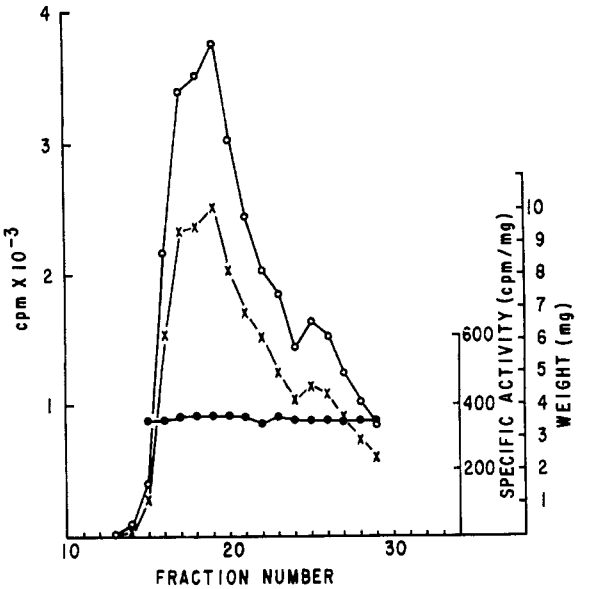

FIG. 2.—Chromatography of radioactive peak I material with authentic squalene. The column was eluted with 200 ml of 1% benzene in Skellysolve B followed by 200 ml of 2% benzene in Skellysolve B. 10-ml fractions were collected. O—O, radioactivity in cpm; ×—×, mg squalene; ●—●, specific activity in cpm/mg.

Gas-liquid chromatography of material from peak III showed only one major peak. This and the large drop in specific activity in recrystallizations indicate that there is some radioactive material in this fraction which is not cholesterol.

Accumulation of Radioactive Squalene during Incubations.—Figure 4 shows that the conversion of [^{14}C]IPP to both squalene and total nonsaponifiable lipids is maximal at the end of 1 hour's incubation. It can also be seen from this figure that about 70% of the nonsaponifiable lipid is accounted for as squalene. Table I shows a series of experiments in which the amount of [^{14}C]IPP was varied and again squalene represented the major portion of the radioactivity in the nonsaponifiable lipid. Usually less than 20% of this radioactivity was associated with the lanosterol and cholesterol fractions. This is in contrast with our unpublished finding that with rat liver homogenate, under similar conditions, between 40 and 50% of the radioactivity from IPP incorporated into nonsaponifiable material is associated with lanosterol and cholesterol. In these experiments squalene accounted for 30–40% of the nonsaponifiable lipid. The following experiments were performed in an attempt to explain the accumulation of squalene in rat testis homogenates.

It has been shown that the cyclization of squalene to sterols requires oxygen and reduced pyridine nucleotide, TPNH being more effective than DPNH (Tchen and Bloch, 1957; Goodman, 1961). Consequently the accumulation of squalene could be accounted for by an inadequate supply of TPNH. To evaluate

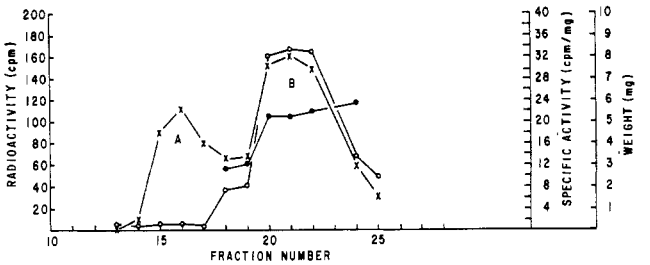

FIG. 3.—Chromatography of radioactive peak II material with nonradioactive lanosterol. The material was eluted with 20% benzene in hexane. 20-ml fractions were collected. O—O, radioactivity in cpm; ×—×, mg lanosterol; ●—●, specific activity in cpm/mg.

TABLE I
CONVERSION OF [^{14}C]IPP TO NONSAPONIFIABLE LIPID AND SQUALENE[a]

Experiment	[2-^{14}C]IPP Added (cpm)	Recovered Radioactivity: Total Nonsaponifiable Material (cpm)	Recovered Radioactivity: Squalene (cpm)	Squalene in Total Nonsaponifiable Material (% ^{14}C)
1	22,000	10,548	6,820	65
	22,000	8,808	5,550	63
2	45,000	19,160	9,640	50
	45,000	23,296	11,955	51
3	157,000	78,952	55,249	70
	471,400	138,168	101,418	73
	628,500	151,488	96,888	64

[a] Each incubation flask contained: Tris buffer, *p*H 7.4, 285 μmoles; nicotinamide, 76 μmoles; sodium ethylenediaminetetraacetate, 3 μmoles; $MgCl_2$, 12 μmoles; TPN, 3 μmoles; DPN, 1.5 μmoles; reduced glutathione, 40 μmoles; glucose-6-phosphate, 14 μmoles; and about 50 μg of glucose-6-phosphate dehydrogenase. Homogenate (0.5 ml) was used in each flask in total volume of 3 ml. The specific activity of [2-^{14}C]IPP was 10^6 cpm/μmole in the counting system used in this experiment. Time of incubation was 1 hour.

TABLE II
CONCENTRATION OF TPNH DURING INCUBATIONS[a]

Time of Incubation of Testis Homogenate (minutes)	Estimated Amounts of TPNH (μmoles/ml)
10	0.27
30	0.16
50	0.14
70	0.13

[a] Samples of incubation mixture, 0.5 ml, prepared as described under Methods, were taken at various time intervals, boiled, and centrifuged, and oxidized glutathione was added. Samples were made to 3 ml with phosphate buffer, 0.1 M, *p*H 7.5, and their absorbancy was measured at 340 mμ; 0.02 ml of dialyzed pea TPNH-glutathione reductase (Kaplan *et al.*, 1953) was then added and absorbancy measured at time intervals. The decrease in optical density at 340 mμ from the initial to the final reading indicates the amount of TPNH present.

this possibility, the reduced pyridine nucleotide content of the homogenate was assayed for TPNH by TPNH-glutathione reductase (Mapson and Goddard, 1951). The data presented in Table II indicate that TPNH was present throughout the incubation. Thus the accumulation of squalene cannot be explained by the absence of TPNH.

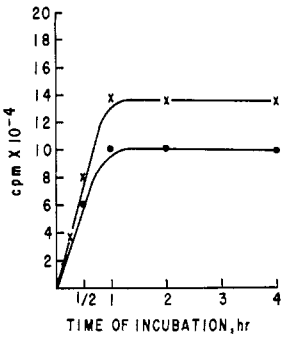

FIG. 4.—Time course of synthesis of nonsaponifiable lipids. Incubations were performed as described under Methods and cofactors were as indicated in Table I. 0.5 μmole (500,000 cpm) of [2-^{14}C]IPP was used as substrate. ×—×, nonsaponifiable lipid; ●—●, squalene.

TABLE III
SQUALENE CONTENT OF RAT TESTIS

Rat	Squalene (μg/g wet tissue)
1	11.3
2	9.5
3	6.4

The accumulation of radioactive squalene could also be explained by the presence of a large pool of endogenous squalene, which would dilute the concentration of radioactive material eventually converted to sterols. The importance of these pools has been shown (Loud and Bucher, 1958). The squalene content of testis was therefore estimated by the following experiment. The testes of three rats were removed, weighed, and homogenized. All three samples were divided into two aliquots and saponified. About 2000 cpm of pure radioactive squalene, specific activity 483 cpm/μg, was then added in order to determine the recovery of squalene during extraction and chromatographic procedures, and the saponified material was extracted into Skellysolve B. The extract was washed with small volumes of water, dried over anhydrous Na_2SO_4, and chromatographed on a small alumina column. The squalene fraction was collected and rerun on a 1 × 15–cm column of Woelm alumina, grade I. Squalene was eluted with 2–5% benzene in Skellysolve B and the radioactivity was counted. Mean recovery of squalene in six experiments was found to be 75.3%. The fractions containing squalene were combined and the amount of squalene was estimated by gas chromatography.

As shown in Table III, the average content of squalene in rat testis is 9 μg/g of wet tissue. In a typical experiment 170 mg of tissue was included in each flask. This amount of tissue would contain 3.7 mμmoles of squalene. During the incubation this quantity of tissue is able to convert up to 100 mμmoles of IPP to squalene (Table I). Thus in a typical experiment about 17 mμmoles of radioactive squalene are synthetized *de novo* during the incubation. The 3.7 mμmoles of squalene already present in the tissue would not be enough to produce the dilution that would be necessary to account for the small conversion of radioactive IPP to lanosterol and cholesterol.

DISCUSSION

The demonstration of the conversion of IPP to squalene by rat testis homogenates shows that this

tissue has at least part of the normal terpene biosynthetic pathway. It is apparent, however, that the relative activities of the various enzymes involved in the synthesis of sterols from mevalonic acid is different from that found in the liver. Rat liver homogenates will efficiently convert both mevalonic acid and IPP to sterols (Popjak, 1959; R. A. Salokangas, H. C. Rilling, and L. T. Samuels, unpublished data). We have also found that the rat testis homogenate, on the other hand, does not readily convert mevalonic acid to IPP and squalene is not converted to lanosterol to any great extent. In fact this accumulation of newly synthetized squalene and the relatively low endogenous content of squalene make this tissue useful for the preparation of highly labeled squalene.

The accumulation of radioactive squalene and the relatively meager synthesis of radioactive sterols could be explained in a number of ways. Squalene cyclooxidase requires both TPNH and oxygen. These studies have shown that TPNH is present throughout the incubation, thereby indicating that it cannot be a lack of cofactor necessary for proper enzymatic function. The accumulation of radioactive squalene cannot be accounted for by the presence of a large pool of endogenous squalene since it was determined that such a large pool does not exist in the testis. One is left then with the possibilities that the concentration of the enzyme in rat testis is quite low or that it does not survive homogenization and subsequent incubation. We have thus far not distinguished between these alternatives.

It should be noted, that the conversion of radioactive IPP to steroids was not detected in these preparations. This is not surprising since in similar preparations from the rabbit only about 0.2% conversion of radioactive cholesterol to testosterone was observed (Hall, 1961). If the small quantity of cholesterol synthesized from radioactive IPP in these preparations was being converted to steroids with a similar efficiency no detectable radioactivity would be expected in the steroid fractions.

Since the conversion of [^{14}C]IPP to squalene and sterols has been demonstrated in cell-free testicular preparations, we are now exploring the steps between mevalonic acid and IPP to determine the reasons for the inability of such preparations to convert mevalonic acid to squalene and sterols.

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