may indicate less selectivity than in the perchloric acid method, but the O-acetyl analysis and isoionic points indicate at least equal selectivity.

The O-acetyl and Van Slyke nitrogen analyses permit the conclusion that, within experimental error of about 5%, acetylation by both methods has taken place selectively at the hydroxyl groups. Although no analyses for guanidino groups were made, it is assumed that they were not attacked, for theoretical reasons which apply as well to the amino groups, discussed below, and because of the correspondence between the total acetyl and the O-acetyl values.

Partial degradation appeared to take place in both acetylation procedures as indicated by the reduction of intrinsic viscosity (Table I). Attempts to reduce degradation by reducing the reaction time by 50% gave, in both procedures, products that were about 50–60% acetylated.

Acetylation with trifluoroacetic acid and acetic anhydride is the preferred procedure as it occurs in a homogeneous reaction mixture. The heterogeneous perchloric acid method gave, in some cases, products of low acetyl content, *e.g.*, run 3, Table I, probably owing to inability of the reagents to penetrate the gelatin thoroughly in the short reaction time employed. In addition, while the use of perchloric acid in acetic acid at 0° appears to be a safe procedure, explosion hazards may be involved if it is used in some other acids. The presence of acetic acid is necessary to swell the gelatin and carry the reagents into it. Since the function of the perchloric acid is to inactivate the amino groups through salt formation and to catalyze the reaction of the hydroxyl groups with the anhydride,[1] it may be possible to replace it with other strong acids known to catalyze esterifications.

Since trifluoroacetic acid is also a very strong acid, it will prevent acetylation of amino and guanidino groups by salt formation and catalyze the acetylation of the hydroxyl groups, possibly through the formation of a mixed trifluoroacetic–acetic anhydride.[3]

Since the trifluoroacetic acid method was superior, it was investigated in greater detail. It was found that trifluoroacetic acid alone does not cause a decrease of viscosity. The effect of increasing amounts of acetic anhydride on the viscosity and extent of acetylation was investigated, and the results are shown in Table I. Runs 4 and 6, in which a larger amount of acetic anhydride was used than in run 5, have lower viscosities than run 5. The lower viscosities of the acetylated gelatins probably represent lower molecular weights due to chain degradation. Whether the degradation is due to acetic anhydride itself, to the mixed anhydride or to acetyl carbonium formed by ionization of the mixed anhydride[3] is not clear. When gelatin swelled in acetic acid was treated with a quantity of mixed acetic–trifluoroacetic anhydride (prepared from trifluoroacetic anhydride and excess acetic acid) equivalent to that of run 5, Table I, there was only a 10% decrease of intrinsic viscosity, in spite of tripled reaction time and a 20° higher temperature. (The product was partially and non-selectively acetylated.) It seems therefore that the mixed anhydride alone does not cause degradation although it might do so in such a highly acidic medium as trifluoroacetic acid.

Acknowledgment.—This work was supported by the Office of the Surgeon General, Department of the Army, under Contract No. DA-49-007-MD-298. We wish to thank Miss Helene Riese for technical assistance.

PASADENA, CALIFORNIA

[CONTRIBUTION FROM THE DEPARTMENTS OF PHYSIOLOGICAL CHEMISTRY AND OF MICROBIOLOGY, WAYNE UNIVERSITY COLLEGE OF MEDICINE]

The Metabolism of Squalene by Cell-free Yeast Extracts[1,2]

BY LAURENCE M. CORWIN,[3] L. J. SCHROEDER AND WILLARD G. MCCULLOUGH

RECEIVED AUGUST 31, 1955

A cell-free yeast extract capable of synthesizing sterols from acetate was developed. On addition of the cell-free extract to a medium in which 1-C^{14}-acetate was present, squalene was isolated and it was found to have a lower specific activity than the sterols, a result contrary to that obtained with whole yeast cells. C^{14}-Labeled squalene obtained from whole yeast cells, when incubated with the cell-free yeast extract, was mainly oxidized to the fatty acids with a lesser conversion to sterols. The reduction of 1-C^{14}-acetate conversion to the sterols by addition of non-radioactive squalene was not considered significant in the light of other data. It was concluded that in yeast, squalene is not an obligatory intermediate in the synthesis of sterols.

Introduction

The question of the role of squalene as a precursor of the sterols has been the subject of much recent experimentation. Langdon and Bloch[4] were able to isolate radioactive squalene from rats fed 1-C^{14}-acetate and carrier squalene. Upon feeding of this radioactive squalene to rats, radioactive cholesterol was isolated from the rat liver and little activity was demonstrated in the fatty acid fraction. They were also able to show that feeding of natural squalene to rats, for several days previous to giving radioac-

(1) Abstracted from a dissertation submitted by Laurence M. Corwin to Wayne University in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1956.

(2) A preliminary report of this work was presented at the 128th National American Chemical Society Meeting at Minneapolis, September, 1955.

(3) United States Public Health Pre-doctoral Fellow; present address, Division of Biology, Cal. Inst. of Tech., Pasadena, Cal.

(4) R. G. Langdon and K. Bloch, *J. Biol. Chem.*, **200**, 129, 135 (1953).

tive acetate, resulted in a marked reduction of conversion of acetate to cholesterol, a result to be expected from an intermediate in cholesterol synthesis. In experiments with ovarian membranes and liver slices, however, Popjak[5] found that with C^{14}-acetate in the medium, the incorporation of acetate into squalene was one-hundredth of that into cholesterol. The fact that the specific activity of the squalene was also lower negates the explanation of a simple high turnover of squalene. Schwenk, on the other hand, isolated squalene and sterols from pig liver[6a] and yeast cells[6b] and in both cases found the specific activity of squalene slightly higher than that of the sterols, a result commensurate with the theory that squalene cyclizes to form the sterols. Nicolaides, Reiss and Langdon[7] found that the specific activity of scalp squalene was ten times that of the sterols upon incubation of scalp tissue with C^{14}-acetate. Such great variance in the specific activities of squalene and of the sterols obtained from various sources leads one to consider the possibility that although squalene and the sterols may stem in part from a common pathway, they may be on different branches of this pathway. To explore this possibility, a cell-free yeast extract capable of synthesizing ergosterol from acetate was used in three sets of experiments similar to those used by the above-mentioned workers.

Experimental

Preparation of Cell-free Yeast Extract.—To break down the extremely hardy cell walls of the yeast cells, a bacterial disintegrator was made, patterned to some extent after the model built by Nossal.[8] Essentially the disintegrator is a reciprocal shaking machine working at a speed of approximately 2500 cycles per minute. With the amount of yeast used in these experiments, 60 seconds was enough time to yield a potent extract.

An extract was made by shaking for one minute a mixture of 2.5 g. of fresh Red Star Bakers yeast, 5.0 ml. of 0.1 *M* phosphate buffer at *p*H 6.2, 10 g. of small glass beads and 2.0 g. of Superbrite.[9] The homogenized mixture was decanted from the beads. The beads were washed with 1 ml. of buffer and the washing was added to the mixture. The latter was then centrifuged for three minutes at 5,000 r.p.m. A milky supernatant was decanted and used as the extract. Microscopic examination revealed no whole cells, but an abundance of mitochondria and other sub-cellular particles was present.

To 5 ml. of the extract were added 0.3 ml. of each of the following: 0.0008 *M* ATP, 0.007 *M* $MgCl_2$, 10 mg. % CoA and 50 mg. % DPN.[10] When these cofactors were present an active preparation capable of converting acetate to sterols was obtained.

This incubation mixture, contained in a 50-ml. erlenmeyer flask, was shaken on a wrist-action shaker for four hours in a warm room maintained at 30°.

Chemical Separation and Isolation of Lipids.—The incubation mixture was refluxed with an equal volume of 10% potassium hydroxide in 70% methanol for three to four hours. The insoluble residue was filtered and washed twice with boiling methanol. The washings and the filtrate were combined. The alcohol was distilled from the latter and it was then extracted four times with equal volumes of ethyl ether. The ether was removed from the ether extract under reduced pressure and the residue taken up in a volume of petroleum ether (30–60°) 10 to 15 times the volume of the incubation mixture. The petroleum ether solution was extracted four times with 25 ml. of 10% aqueous potassium hydroxide. The potassium hydroxide extracts were acidified with hydrochloric acid and extracted three times with 50 ml. of ether. The ether was evaporated under reduced pressure and the fatty acids were weighed. An aliquot portion was plated on an aluminum planchet and counted in a gas-flow counter.

The petroleum ether was evaporated from the unsaponifiable fraction and the residue taken up in 6 ml. of a 1:1 ethanol–acetone mixture. To this was added 4 ml of 0.5% digitonin in 80% ethanol. After incubation of the mixture for 1.5 hours at 40° the digitonides were centrifuged, washed with 1:1 acetone–ethanol mixture, dried and weighed. An aliquot was plated on an aluminum planchet and counted. The mother liquor from the digitonides was then concentrated to 2 ml. and chromatographed over 10 g. of alumina with 200 ml. of petroleum ether as eluent. The petroleum ether was evaporated and the residue weighed. An aliquot portion was plated on an aluminum planchet and counted in a gas-flow counter.

This petroleum ether eluate constitutes to a large extent the squalene moiety of the yeast. The identification of squalene was based on three lines of evidence. Incubation of 1-C^{14}-acetate with a cell-free extract to which carrier squalene had been added yielded a squalene fraction which was radioactive. The mixed hexahydrochlorides derived from this fraction had a m.p. of 126–129° and retained almost the same specific activity (corrected for 6 HCl) as that of the original squalene fraction (squalene 438 cts./mg.; squalene–6 HCl 423 cts./mg.). Squalene isolated from a yeast extract to which no carrier squalene had been added formed a thiourea complex, a typical property of squalene. Infrared spectra of this fraction showed a strong carbon–hydrogen band and were devoid of any oxygen-containing functional groups. They were identical to the spectrum of a known sample purified from squalene obtained from Distillation Products, Inc.

In a given experiment, all aliquots of the several lipid fractions measured for radioactivity were of equal weight and amounted to less than 0.5 mg. of sample per square centimeter of planchet area.

Preparation of C^{14}-Squalene.—Since whole yeast cells produce squalene of a higher specific activity than does the cell-free extract, it was decided to use the whole cells as the source of the radioactive squalene used in these experiments. The procedure involved simply the incubation of 1-C^{14}-acetate, 500 ml. of Difco Yeast Nitrogen Base, 20 μg. of calcium pantothenate, and 10 g. of fresh Red Star Yeast for four hours. The isolation procedure was the same as stated above.

When the squalene was added to the incubation mixture in these experiments, 0.1 ml. of Tween 80[11] always accompanied it to ensure proper homogenization of the squalene in the mixture. Squalene in petroleum ether solution was always added to the flask first. The petroleum ether was evaporated off completely and then the Tween 80 added. The rest of the mixture followed. The squalene added to each had an activity of 2×10^5 counts per minute, measured by a gas-flow counter. To control flasks equal amounts of Tween 80 were always added, so that any observed differences could only be attributable to the squalene.

Experiments with C^{14}-Squalene.—The experiments performed with biologically synthesized C^{14}-squalene were carried out under the same conditions as those used with the acetate incubation. One purification precaution was added, however. To diminish any possibility of radioactive squalene appearing in the saponified fatty acid fraction, the water layer containing the potassium salts was carefully washed three times with a 1% solution of non-radioactive squalene in petroleum ether and three times with petroleum ether.

Results

Specific Activity Studies.—Since Schwenk and co-workers[6b] had worked with the whole yeast cell, it was decided first to conduct similar experiments. Five grams of fresh Red Star Yeast was placed in

(5) G. Popjak, *Arch. Biochem. Biophys.*, **48**, 102 (1954).

(6) (a) E. Schwenk, D. Todd and C. A. Fish, *ibid.*, **49**, 187 (1954). (b) E. Schwenk, G. J. Alexander, T. H. Stoudt and C. A. Fish, *ibid.*, **55**, 274 (1955).

(7) N. Nicolaides, O. K. Reiss and R. G. Langdon, THIS JOURNAL, **77**, 1535 (1955).

(8) P. M. Nossal, *Austral. J. Exp. Biol.*, **31**, 583 (1953).

(9) Obtained from Minnesota Mining and Manufacturing Company.

(10) The following abbreviations are used in the text: ATP = adenosine triphosphate, CoA = coenzyme A, and DPN = diphosphopyridine nucleotide.

(11) Atlas Powder Company, Wilmington, Delaware.

each of six flasks containing 50 ml. of Difco Nitrogen Base for yeast, 10 μg. calcium pantothenate and 17 μc. of 1-C^{14}-acetate and this mixture was incubated for three hours. Isolation procedures were as described in the Experimental section with the use of correspondingly increased quantities of materials.

The results in Table I agree essentially with those of Schwenk in that the specific activity of the squalene is higher than that of the sterols.

TABLE I

EXPERIMENT WITH YEAST INCUBATED WITH $CH_3C^{14}OONa$

Fraction	Wt. in mg.	Total counts	Specific activity
Lipids	341.6	5.01×10^5	1468
Non-saponifiable	115.4	2.07×10^5	1800
Digitonide	62.2	1.95×10^4	314
Mother liquor from digitonide	120.0	1.90×10^5	1570
Squalene	27.6	4.10×10^4	1478
Sterols[a]	15.6	1.95×10^4	1256
Fatty acids	257.0	2.13×10^5	827

[a] Calculated from digitonide as ergosterol.

Similar experiments were tried with the cell-free extracts. The results of a typical experiment appear in Table II. In this case 20 μc. of acetate was used in total.

TABLE II

CELL-FREE YEAST EXTRACT INCUBATED WITH $CH_3C^{14}OONa$

Fraction	Wt. in mg.	Total counts	Specific activity
Lipids	153.5	34,500	226
Non-saponifiable	76.0	3,600	48
Digitonide	24.5	1,320	54
Mother liquor from digitonide	68.7	1,140	17
Squalene	17.7	560	32
Sterols[a]	6.1	1,320	216
Fatty acids	69.0	26,300	380

[a] Calculated from digitonide as ergosterol.

Results of other experiments with the cell-free yeast extracts appear in Table III.

TABLE III

CONVERSION OF 1-C^{14}-ACETATE TO SQUALENE AND STEROLS BY CELL-FREE EXTRACTS

1-C^{14}-Acetate added, μc.	Squalene Total counts	Squalene Specific activity	Sterols Total counts	Sterols Specific activity
20	560	31	1320	215
20	665	416	2290	6920
5	54	34	311	940

Incubation with C^{14}-Squalene.—These experiments were performed to ascertain some of the metabolic pathways open to squalene with regard to other members of the lipid fraction. The results of these experiments are to be found in Table IV.

TABLE IV

INCUBATION WITH C^{14}-SQUALENE

	Sterols Total counts	Fatty acids Total counts
Whole cell	210	900
Cell-free extract	100	1020
Cell-free extract	22	1740
Cell-free extract	33	1145

C^{14}-Squalene added = 2×10^5 c.p.m.

Effect of Natural Squalene on Uptake of 1-C^{14}-Acetate into the Lipids.—It is to be expected that a compound intermediate in the biosynthetic path toward the steroids should be more readily utilized in the synthesis than one further removed from the final product. It would thus seem appropriate to determine whether squalene is utilized preferentially to acetate for sterol synthesis. Varying amounts of non-radioactive squalene were added to a medium containing 1-C^{14}-acetate. The sterols and fatty acids were isolated, their activity measured and compared with the activities when no additional squalene was added. The results of these experiments appear in Table V.

TABLE V

EFFECT OF NATURAL SQUALENE ON CONVERSION OF 1-C^{14}-ACETATE TO THE LIPIDS BY CELL-FREE EXTRACTS

Acetate, μc.	Squalene, mg.	Sterols[a] With squalene	Sterols[a] Without squalene	Fatty acids[a] With squalene	Fatty acids[a] Without squalene
1.0	950.00	50	708	41	860
1.4	150.00	578	1250	791	1138
1.4	0.95	875	1510	1560	1950
2.0	0.01	2100	2200	..	..

[a] Total counts.

Discussion

In the light of Schwenk's[6b] experiments with whole yeast cells, duplicated with our Red Star yeast, it is of especial interest to view the experiments with a cell-free yeast extract. The fact that from 1-C^{14}-acetate, the complete yeast cell synthesizes squalene with a greater specific activity than the sterols but that a cell-free extract of the same yeast reverses this result, raises a problem in interpreting squalene's relation to the sterols. The problem is intensified when the seemingly contradictory evidence of Popjak[5] and Nicolaides[7] is also considered. On theoretical grounds it would appear that any evidence demonstrating a lower specific activity for squalene than for the sterols would preclude the former as a precursor. In fact, the conflicting evidence from the whole cell and the extract would seem to indicate the loss of some of the factors required to make squalene. Were squalene a precursor, one would expect a corresponding decrease in the sterols. That this decrease is not found indicates either that squalene is not on the direct path toward the yeast sterols or that it may be on only one of two or more paths.

Experiments with squalene invariably raise the question of the penetrability of such an insoluble compound through the cell wall. It is for this reason that the cell-free extract was made. It is obvious from the experiments with C^{14}-squalene that the extract handles squalene quite differently from the animal organism in Langdon and Bloch's[4] experiments. Squalene in the yeast extract is more extensively converted to the fatty acids than to the sterols. There is also the possibility that the sterols are formed from the oxidation products of squalene. It is interesting to note that in the one experiment with whole yeast cells similar results (Table IV) were obtained, demonstrating that the cell is permeable to squalene.

The results obtained when natural squalene was

added to the medium together with 1-C^{14}-acetate offer little evidence to support the squalene-precursor hypothesis. It is true that squalene does lower the conversion of acetate to sterols, but it simultaneously affects the conversion of acetate to fatty acids. This may be explained by considering the formation of a common precursor to both sterols and fatty acids from oxidation of squalene. It should be realized too, that only when large, unphysiological amounts of squalene are added, can a marked reduction in acetate conversion be observed. A mere 50% reduction is no indication that a compound is an intermediate to the sterols, but that it is rather an equal competitor with acetate. A direct precursor should be utilized much more readily, although solubility differences between acetate and squalene may play some role in these results.

The evidence obtained from cell-free yeast extract experiments, especially when considered with results from whole yeast cells, lends no support to the hypothesis that squalene is an obligatory intermediate to the yeast sterols. It indicates, on the contrary, that squalene in yeast may be metabolized along different lines although the possibility that squalene and the sterols stem from the same precursors is not excluded.

Acknowledgment.—The authors are grateful to Dr. Adrian Kuyper for guidance in the radioactive tracer work, to the Red Star Yeast Company for supplying them with fresh yeast throughout the course of the experiments, and to the American Medical Association for a grant-in-aid to complete their work.

DETROIT, MICHIGAN

[CONTRIBUTION FROM THE DEPARTMENTS OF BOTANY AND BIOCHEMISTRY, UNIVERSITY OF WISCONSIN]

Isolation, Structure and Synthesis of Kinetin, a Substance Promoting Cell Division[1,2]

BY CARLOS O. MILLER, F. SKOOG, F. S. OKUMURA, M. H. VON SALTZA AND F. M. STRONG

RECEIVED SEPTEMBER 6, 1955

A substance which markedly promotes cell division in various plant tissue cultures in concentrations as low as one microgram per liter has been isolated in pure form from heated deoxyribonucleic acid preparations, and has been shown by degradation and synthesis to be 6-furfurylaminopurine. The specific name *kinetin* has been applied to this substance, and the generic term *kinin* is suggested for any substance which similarly stimulates cytokinesis.

Attempts in these laboratories to isolate from coconuts a factor promoting cell division in plant tissues resulted in some 4000-fold concentration of the active substance(s), but no isolation of any active pure compound was accomplished.[3,4] In related growth studies, it was observed that dried brewer's yeast similarly promoted cell division and that a small portion of the active material was extractable from aqueous solutions by *ether*. It seemed likely that this ether-soluble factor would be simpler in chemical composition and more readily purified than the highly water-soluble, ether-insoluble material in coconut concentrates.[3] A preliminary work-up of a yeast ether extract did, in fact, lead rather easily to high potency material. Activity was correlated with a substance which had a maximum absorption at 268 mμ and was precipitated from acid solutions by silver nitrate. This material was obtained in low yield, however, so other possible sources were investigated.

Since the active substance had purine-like properties, other purine-containing materials were tested, and eventually an old sample of deoxyribonucleic acid (DNA) was located which was extraordinarily potent. Its ether extract, without further purification, equalled the activity of the best concentrates previously obtained in our laboratories from any source. Fresh DNA samples were inactive, but could be activated by slurrying in water and autoclaving. Relatively large amounts of rich concentrates thus became available and, on further purification, mainly by ion-exchange chromatography, readily yielded a very highly active crystalline product, I.

The newly isolated compound proved to have the empirical formula $C_{10}H_9N_5O$. It was amphoteric (pK_a values approximately 4 and 10) and optically inactive. It dissolved easily in aqueous strong acids and alkalies and in glacial acetic acid, was slightly soluble in ethanol, butanol, acetone and ether, but practically insoluble in distilled water. It sublimed unchanged at 220° at atmospheric pressure and was unaffected by autoclaving either at *p*H 0.5 or 12.0. However, autoclaving in stronger acid solution, *e.g.*, 2.0 *N* sulfuric acid, did lead to loss in physiological activity.

The high nitrogen content, ultraviolet spectrum (single band near 268 mμ), amphoteric character, precipitation with silver, and isolation from DNA all suggested that I might be a purine derivative. The solubility in organic solvents pointed to a relatively non-polar grouping in the molecule. First guesses as to the nature of I, therefore, centered around dehydrated nucleosides. This hypothesis was strengthened when I was subjected to strong acid hydrolysis and the hydrolysate chromatographed on paper with solvent systems designed for purine separation. The original ultraviolet quenching spot characteristic of I disappeared after hydrolysis, and was replaced by another having the R_F value and, after elution, the ultraviolet spectrum of adenine. The presence of adenine in the hydrolysate was then confirmed by isolation of the

(1) Preliminary reports of this work have appeared in (a) THIS JOURNAL, **77**, 1392 (1955); (b) **77**, 2662 (1955).

(2) This work was in part supported by grants from the American Cancer Society, The National Science Foundation and the Wisconsin Alumni Research Foundation.

(3) J. R. Mauney, W. S. Hillman, C. O. Miller, F. Skoog, R. A. Clayton and F. M. Strong, *Physiol. Plantarum*, **5**, 485 (1952).

(4) D. A. Buyske, Ph.D. dissertation, University of Wisconsin, 1954.