"As I Remember It"

Research on Biosynthesis of Fatty Acids, Triglycerides, Squalene, and Cholesterol

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ABSTRACT AND SUMMARY

Dr. George Popják, recipient of the 1977 Award in Lipid Chemistry, presented a chronological account of his career in lipid biochemistry during his address to the American Oil Chemists' Society on May 9, 1977, in New York City during the Society's 68th Annual Meeting. The address covers his early work in fetal lipids, through his pioneering studies of cholesterol and squalene biosynthesis, and his present work on mevalonate metabolism. "On reading the citation for this award," Dr. Popják said, "I had difficulty in deciding the topics for this address. I chose, rightly or wrongly, an autobiographical approach and to relate, as best I can remember, how I started and progressed in the study of the biochemistry of lipids."

It is with much pleasure and humility that I accept the Society's award. The Society in honoring me has honored many of my young, or younger, associates without whose contributions to my career I would not be standing before you today. I am happy to say that all the young men and women listed on my first slide[1] have achieved during the past 10, 15, or 25 years distinguished careers of their own; to them I express my deepest gratitude. There is one young person's name missing from this slide who has supported me with much devotion for 36 years: the name of my wife. May I express publicly my loving thanks to her for having put up for so many years—I dare say not without occasional grumbles—with the hardships of a scientist's wife.

THE BEGINNING

There were three great men who profoundly influenced my career. The first one was my teacher and mentor in Hungary: Professor Joseph Baló, best known in this part of the world as the discoverer of elastase, one of the earliest explorers of the viral origin of cancer after Rous, who I am happy to say is still active. From him I learned that a pathologist need not be confined to the postmortem room, and he first aroused in me interest in problems of atherosclerosis.

After I moved to England in December 1939 as a British Council Postdoctoral Fellow, I was fortunate to have been assigned to the Postgraduate Medical School in London where Professor Henry Dible, my second mentor, was probing an old theory of pathologists concerning the nature of fatty degeneration of heart, liver, and kidneys. According to the theory of fat phanerosis (the term derived from the Greek *φανεροσ* = visible), fatty degeneration resulted from a dissociation of protein-lipid complexes and consequently the lipids became stainable by fat-soluble dyes, such as Sudan, Scharlach R, or osmium. I was assigned to carry out the experimental work testing this hypothesis. We were able to dispose at short shrift of fat phanerosis, as we could demonstrate that in fatty degeneration mainly triglycerides accumulated in the organs with a composition characteristic of adipose tissue fat (1,2). It was during that period of about a year and a half that I first learned methods of lipid fractionation before the advent of chromatography.

After my appointment in 1941 as a demonstrator in pathology to St. Thomas's Hospital Medical School in London, I was introduced to Sir Joseph Barcroft, the eminent physiologist, who had just retired from the Chair of Physiology at Cambridge and who had set out on a new career investigating fetal physiology. When he learned that I was interested in lipids, he fired two questions at me: "What is the origin of fetal lipids? Are they made in the fetus or does the fetus depend on maternal supply of preformed substances?" There was no answer to these questions in the early 1940s.

Although in those days I did not read regularly the *Journal of Biological Chemistry*, I came across, fortunately, Schoenheimer's monograph, "The Dynamic State of Body Constituents" (3), and I realized that Barcroft's questions could be answered with the help of isotopes. Schoenheimer's monograph directed me back to the original papers in the *Journal of Biological Chemistry* and—although I was still working in a department of pathology—I started to make my plans for the first experiments with heavy water and ^{32}P. My chief, Professor William George Barnard, was a most enlightened man and allowed me complete freedom in research; besides, St. Thomas's Hospital was at that time a rich establishment endowed by private donors, and I did not need to write research grant applications.

Although I had been given complete freedom in research at St. Thomas's, I encountered heavy obstacles, not because of shortness of funds, but because instruments and glassware were difficult to come by during and immediately after the war in England. Undaunted by such trifles as lack of equipment, I set about to make my own instruments and succeeded, with the help of a carpenter, a sheet-metal worker, and a glass-blower, in constructing a water bath, the temperature of which was controlled to about 2 millidegrees C for determination of deuterium in water by the "falling drop" method (4).

The home-construction of the second vital piece of apparatus needed for determination of deuterium in organic substances, a combustion furnace, led to a near disaster. On returning one evening to the laboratory, I found smoke rising from the teak-topped bench where this furnace stood. To my horror, I saw a lava of molten copper oxide and quartz flowing from the furnace and burning a large hole into the bench. The simple on-off thermoregulator ('Simmerstat') that I used to keep the furnace at around 650 C had stuck in the on-position. That accident taught me that it was better to rely on professionally made apparatus than on skillful amateurism.

By 1946 deuterium oxide became once again available in England, also ^{32}P arrived from the MIT cyclotron and a

[1]The names of twenty-eight colleagues were listed on the slide; they can be identified from the list of references and from the text, or from the list of references given in article (64).

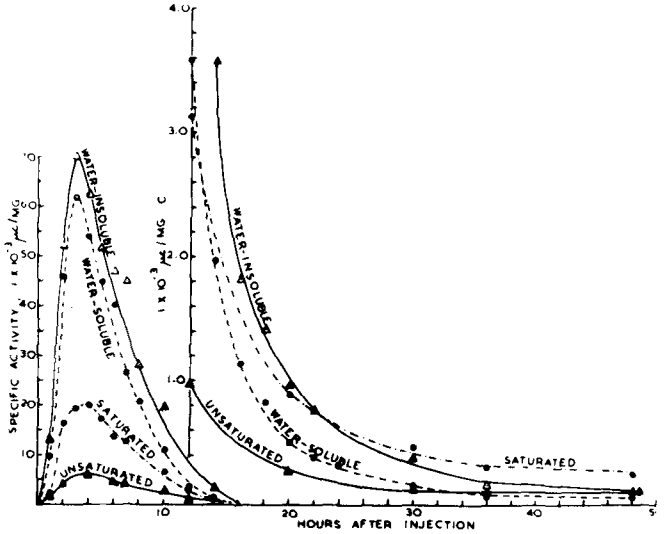


FIG. 1. Specific activity-time curves of milk fatty acids of goat after intravenous injection of [1-^{14}C]acetate (14). (Reproduced with the permission of the Editor, *Biochemical Journal.)*

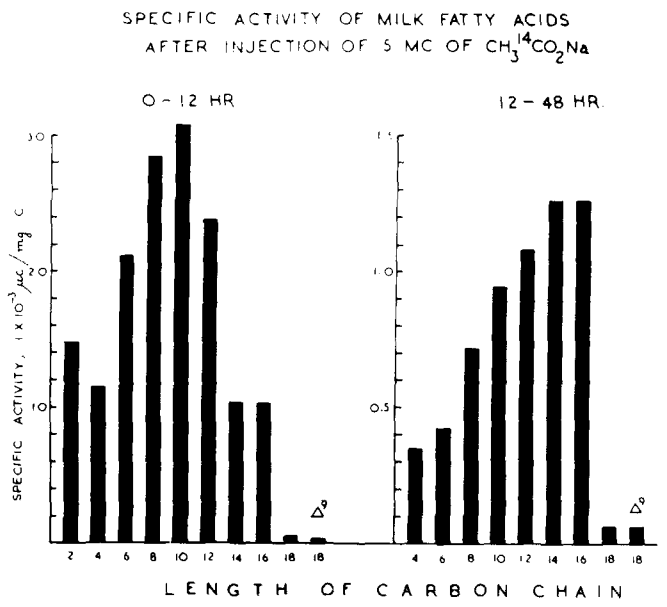


FIG. 2. Specific activities of individual fatty acids of triglycerides from goat milk.

little later from Oak Ridge, and 1948 witnessed the introduction of ^{14}C to Britain from the U.S. Atomic Energy Commission and from Chalk River in Canada [for an account of the introduction of isotopes to Britain cf. ref. (5)]. With the aid of these three isotopes, I did experiments on pregnant rats, rabbits, and guinea pigs (6-8), and, by 1950, I was able to answer Barcroft's questions: most of the fetal lipids, phospholipids, triglycerides, and cholesterol were made in the fetus rather than transported from the mother.

BIOSYNTHESIS OF MILK TRIGLYCERIDES

The experiments on the pregnant rabbits, in addition to having provided answers to questions of fetal physiology and biochemistry, gave us a great bonus. We noted that the mammary gland of nonlactating pregnant rabbits synthesized, very actively, triglyceride fatty acids and cholesterol at least one week before parturition. This was shown by the incorporation of deuterium from the body water into these substances in the mammary gland at a rate greater than that which occurred in liver lipids and by the avid utilization of [^{14}C]acetate for both fatty acid and cholesterol synthesis in the gland (9,10). The results obtained on the mammary gland triglycerides of the nonlactating pregnant rabbits had one particularly interesting feature: the short-chain fatty acids, C_4 to C_8, contained, after administration of [^{14}C]acetate, some ten to twenty times more ^{14}C than the long-chain saturated and unsaturated fatty acids. This made it most unlikely that they were derived by the degradation of the long-chain fatty acids, particularly of oleic, as suggested by Hilditch (11), and indicated rather their direct synthesis in the mammary gland.

These observations, coupled with those of Folley and French (12,13) on the respiratory quotients of mammary gland slices of lactating animals (cow, goat, rat, rabbit), encouraged us to design specific experiments for the study of milk fat synthesis in lactating animals. The lactating goat and rabbit became our cherished experimental animals.

The experiment on the goat provided convincing evidence that the fatty acids of milk triglycerides were largely synthesized in the udder and not derived from plasma lipids. Figure 1 shows the rapid rise of the specific activity of the milk fatty acids peaking at 3-4 hr after injection of [1-^{14}C]acetate, in contrast to the plasma fatty acids which plateaued at about 24 hr. The labeling of the plasma fatty acids was so weak in comparison with the milk fatty acids, that if their specific activity-time curve were superimposed in Figure 1 it would not be above the level of the abscissa by more than the thickness of the line (14). We realized that in order to obtain the maximum information from this experiment we needed (a) to fractionate the triglyceride fatty acids and to determine their specific activity individually and (b) to locate the position of the label in the molecules.

It was most fortunate for me that at that time A.J.P. Martin's laboratory was opposite mine, and he had just developed one of the earliest chromatographic techniques for the separation of fatty acids (15). So the fractionation became possible even on a small scale, although I also used vacuum distillation of the methyl esters in a "Podbielniak" high-efficiency still and in one of my own making.

Figure 2 shows the specific activities of the individual fatty acids of the milk glycerides pooled from two periods of the experiment (16). The stepwise increases in the specific activities of the fatty acids were at first puzzling, but the chemical degradation of some of the acids (17) solved the mystery. First we found that only the odd-numbered carbon atoms contained ^{14}C (Table I). In butyric acid the carboxyl carbon and C-3 contained equal amounts of isotope. However, in caproic (C_6) and caprylic (C_8) acid, while the four terminal carbon atoms were labeled as in butyric acid, the C_2 units nearer the carboxyl end contained successively increasing amounts of ^{14}C (16,18). The observations gave clear evidence of the overall mechanism of fatty acid synthesis by the successive elongation of a shorter acid by the addition to the carboxyl end of a C-2 unit.

ASYMMETRY OF GLYCEROL IN ENZYMIC REACTIONS

We had a special bonus from the experiment on the goat. We found that [1-^{14}C]acetate labeled not only the fatty acids of milk triglycerides, but also the lactose in the milk and also the glycerol of the triglycerides. We showed that the galactose moiety of lactose must have been formed by the isomerization of glucose in the mammary gland (19) (Fig. 3) and found that the specific activity-time curves of the milk lactose and the glycerol of triglycerides bore a relationship characteristic of a precursor and product (20) as predicted by the theory of Zilversmit, Entenman, and Fishler (21). Chemical degradation of the glycerol (20) indicated that the label in this substance was in positions 1 and

TABLE I

Specific Activities of C-Atoms in Goat Milk Fatty Acids after Injection of [1-^{14}C] Acetate

Fatty acid	Carbon atom number 8	7	6	5	4	3	2	1
	10^{-3} μCi/mg carbon							
Acetic[a]	---	---	---	---	---	---	0.0	23.6
Butyric[a]	---	---	---	---	0.0	15.3	0.0	15.3
Caproic[a]	---	---	0.0	13.2	0.0	13.2	0.0	32.6
Caprylic[b]	0.0	30.0	0.0	30.0	0.0	59.6	0.0	92.6

[a]From samples pooled between 1 and 48 hr after injection.

[b]From samples pooled between 1 and 12 hr after injection. Data of Popják, French, Hunter, and Martin (1951); and Popják (1952).

3 (Table II). The chemical degradation by periodate oxidation could not, of course, tell whether the label was confined to either position 1 or 3, or whether both positions were equally labeled. The Ciba Foundation in London organized in 1951 a symposium on the biochemical uses of isotopes and at this symposium I met for the first time Harland G. Wood, who by that time had followed and elucidated a number of intermediary pathways of metabolism from the labeling pattern of glucose after administration of variously labeled metabolites to animals. It was known specifically that [1-^{14}C]acetate, or any metabolite giving rise to [1-^{14}C]acetate in the body, labeled the glucose of liver glycogen exclusively on positions 3 and 4 (22). If similar reactions prevailed in the mammary gland and if the glycerol was derived from a [3,4-$^{14}C_2$]glucose as we surmised, there was a distinct possibility that the glycerol we isolated from the milk triglycerides was labeled only on one of its outside carbon atoms. Since glycerol has the same symmetry properties as citric acid, and since Ogston's hypothesis (23) of the possible asymmetric treatment of a *Caa'bd* molecule in enzymic reactions was proved correct in respect of citric acid only a little earlier (24,25), I offered to Harland Wood a specimen of the glycerol derived from the milk triglycerides of the goat injected with [1-^{14}C]acetate for testing. That glycerol was converted by rats almost exclusively to [3,4-$^{14}C_2$]glucose with predominant labeling in position 3. Since the specific activity of the glucose was as high as 75-85% of the glycerol fed, there could be no doubt that the carbon atoms of glycerol entered the glucose as a single unit and that the glycerol derived from the goat was asymmetrically labeled (26).

This experiment, corroborated by the independent work of Swick and Nakao (27), provided another example of an asymmetric reaction around a prochiral center and led to the postulation of phosphorylation of glycerol to L-α-glycerophosphate only one year before the discovery of glycerol-kinase by Bublitz and Kennedy (28). By today's assignments of absolute configuration the [^{14}C]glycerol we obtained from the triglycerides of goat milk was labeled on the pro-*S* hydroxymethylene group. We proposed that this position should be numbered as No. 1; this proposal subsequently has been universally accepted. Glycerol is phosphorylated by glycerol kinase on the pro-*R* hydroxymethylene group (position 3) (cf. Fig. 4). The memory of the experiment with glycerol served me well some years later when I was studying the mechanism of the biosynthesis of squalene from mevalonate and farnesyl pyrophosphate.

Let me not neglect the experiments with lactating rabbits; I can summarize the outcome of those studies very briefly. We turned from the lactating goat to lactating rabbits to examine the mechanism of the conversion of glucose into fat; this was still uncertain in the early 1950s. The experiments showed convincingly that glucose donated carbon to triglycerides in two ways: it was the direct source

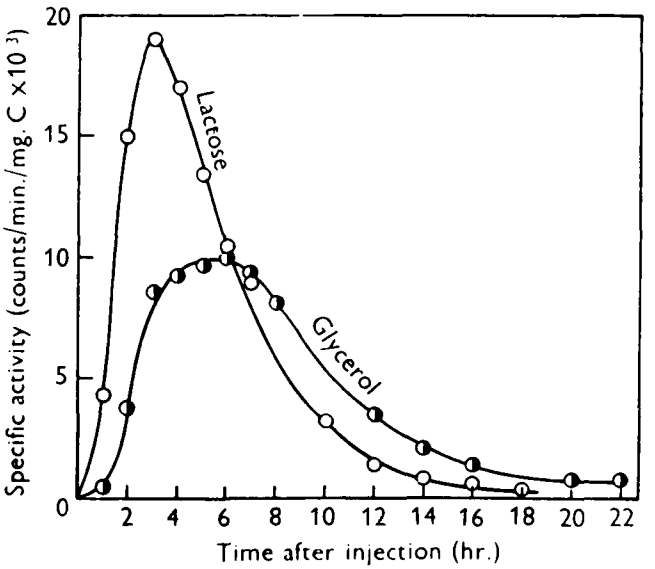


FIG. 3. Specific activity-time curves of lactose and glycerol of triglycerides from milk of goat injected with [1-^{14}C]acetate. (Reproduced with the permission of the Editor of the *Biochemical Journal.)*

TABLE II

Distribution of ^{14}C in Carbon Atoms of Glycerol

	Counts/min/mg C
Carbon 1 and 3	5750
Carbon 2	552
All carbons as determined	4050
All carbons as calculated from degradation	4017

of glycerol and of the C_2-units, via pyruvate, for the synthesis of fatty acids (29).

BIOSYNTHESIS OF FATTY ACIDS IN A SOLUBLE ENZYME SYSTEM OF THE MAMMARY GLAND

It was held in the early 1940s almost as a dogma that for the metabolism of fat (synthesis and degradation) the integrated structure of cells or tissues was needed. That dogma was shattered by the early 1950s when it became clear from the experiments of Lardy, D.E. Green, Seubert, and Lynen that the reactions of β-oxidation of fatty acids could be dissected into well-defined steps by individual enzymes isolated from mitochondria. One part of the dogma having been eroded, it seemed to me likely that a second part of it, relating to fatty acid synthesis, hung also on the thin thread of faith. Since the mammary gland of lactating animals proved to be an organ par excellence of

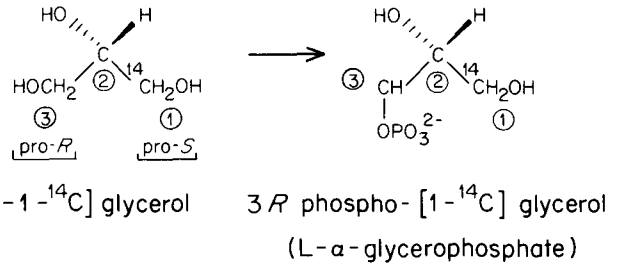


FIG. 4. Asymmetric labeling of glycerol from [1-^{14}C]acetate, on the pro-*S* hydroxymethylene group and its phosphorylation on the pro-*R* hydroxymethylene group to (L)α-glycerophosphate.

fat synthesis, I pinned my faith on the mammary gland to provide a cell-free enzyme system for the intimate study of fatty acid biosynthesis. It seems to me now almost incredible that the first experiment I made in 1952 with a homogenate of the mammary gland of a lactating rat gave a preparation synthesizing fatty acids from acetate at rates greater than I had seen in slices of such glands. Alisa Tietz, a graduate student from Israel, joined me at that time and we were able to work out the first essential requirements of a soluble fatty acid synthesizing system of the rat mammary gland (30-33). All the enzymes needed were in the 100,000 x g supernatant of the homogenates and CoA, ATP, DPNH and another cofactor, which later turned out to be TPNH, and Mn^{2+} ions were essential for fatty acid synthesis from acetate. We made three strange observations which we could not interpret at that time: (a) an atmosphere of air was better than pure O_2 in the incubations; (b) oxaloacetate, and especially α-ketoglutarate (but not citrate), stimulated fatty acid synthesis substantially and (c) malonate—which we added as a possible metabolic inhibitor—caused, particularly in combination with α-ketoglutarate, on occasion as much as a fiftyfold stimulation of fatty acid synthesis. After the discovery of the carboxylation of acetyl-CoA to malonyl-CoA by Wakil (34), Brady (35), Formica and Brady (36) and Lynen et al. (37) by a biotin-dependent enzyme and the participation of malonyl-CoA in the synthesis of fatty acids, the puzzle of the malonate effect in the mammary gland enzyme system became solved. On reinvestigating our earlier work we found that the properties of the mammary gland fatty acid synthesizing system were very similar to those of the chicken liver system studied by Wakil and others, i.e., it required CO_2, biotin, and Mn^{2+} ions in addition to ATP, CoA, and TPNH (38,39). However, malonate—even in the presence of CO_2—still caused a twentyfold stimulation of fatty acid synthesis, but oxaloacetate and α-ketoglutarate became ineffective. It was a little later that we realized that the effect of malonate in the mammary gland system was achieved by the activation of acetyl-CoA carboxylase (40).

There was one striking difference between the fatty acid synthesizing system of the mammary gland and that of the chicken liver in that the product of the synthesis in the latter was palmitic acid, but in the former every even-numbered fatty acid, short and long chain, up to stearic was synthesized, myristic acid being the main product (39). The idea was even entertained that perhaps in the mammary gland there existed two synthetases, one specific for the short and medium-chain and the other for the long-chain fatty acids, an arrangement that would have accounted for the presence of short-chain fatty acids in milk triglycerides especially in the milk of ruminants. However, when we tested acetyl-CoA + [2-^{14}C]malonyl-CoA in the mammary gland system, we found that the principal product was palmitic acid (40). It became evident that in the soluble enzyme system of the rat mammary gland, when the synthesis is initiated with acetate, CO_2, ATP, Mn^{2+}, and TPNH, acetyl-CoA and malonyl-CoA have to be generated first, and that malonyl-CoA is limiting under such conditions and accounts for chain termination before palmitic acid. Lynen and his colleagues (41) reported not very long ago that the purified fatty acid synthetase of yeast, which synthesizes palmityl-CoA and stearyl-CoA in equal amounts under standard conditions, also produced short-chain fatty acids when the supply of malonyl-CoA was restricted.

STUDIES OF CHOLESTEROL AND SQUALENE BIOSYNTHESIS

After 1961/62 I abandoned further study of fatty acid biosynthesis as my resources were too small to compete with other laboratories and because I was too deeply involved in experiments on the intermediary reactions of squalene and cholesterol biosynthesis. In any event, my experiments on fatty acid biosynthesis arose from the incidental observations made on pregnant rabbits, but to study cholesterol biosynthesis was my deliberate choice. I made this choice in the belief, perhaps naively, that elucidation of the reactions of cholesterol biosynthesis might one day be usefully exploited for the prevention of hypercholesterolemias and hence of atherosclerosis.

In 1947 I became a member of the scientific staff of the National Institute for Medical Research in London, where one of my duties was to receive, standardize, and distribute all radioactive isotopes used for biological research in Britain (5). Thus it happened that when in 1948 the first consignment of ^{14}C-labeled $BaCO_3$ arrived in Britain from Chalk River in Canada, I received for standardization the first specimen of 50 μCi's of [1-^{14}C]acetate, made from that $BaCO_3$ at the Amersham Radiochemical Centre. As I needed very little for standardization, most of the 50 μCi remained and, as it had a very low specific activity (50 μCi in 1.85 g of the Na salt), it was considered unsuitable for biological experiments. It was left to me to dispose of it as I pleased. On the principle that nothing ventured nothing gained, I injected a solution of 1.8 g of that acetate intravenously to a 28-days pregnant rabbit. That was the first experiment made in Britain with ^{14}C and gave gratifyingly good results. It confirmed my data obtained with ^{32}P and deuterium on the origin of fetal lipids, it led to the study of milk fat biosynthesis and also confirmed the report by Bloch and Rittenberg (42) that cholesterol was biosynthesized from acetate. It was at this point that I proposed to J.W. Cornforth, my colleague at the Institute, that it would be more profitable for him to work on the carbon-by-carbon degradation of [^{14}C]cholesterol biosynthesized from [^{14}C]acetates than on the total chemical synthesis of cholesterol on which he was then engaged. That proposal was the beginning of a 20-year collaboration, broken only by my move to the U.S.A. in 1968.

Looking back over the years, there were three distinct phases of our collaboration. During the first period, which lasted until the discovery of mevalonate in 1956, carbon-by-carbon degradation of the ring structure of [^{14}C]-cholesterol, biosynthesized by liver slices from either [1-^{14}C]- or [2-^{14}C]acetate, established a fixed pattern for the two acetate carbons in the sterol (43-47) and extended the pattern deduced by Würsch, Huang, and Bloch (48) for the side-chain of cholesterol. During the same period we also made a correlation with squalene biosynthesized from [^{14}C]acetates after I found in 1953 substantial synthesis of squalene in liver slices and certain structures of the hen's ovary (49,50). The pattern of the acetate carbons in squalene fitted perfectly the pattern found in cholesterol predicted by Woodward and Bloch (51; Fig. 5).

The second phase of our work began in the fall of 1956 after the discovery of mevalonate as an efficient precursor of cholesterol by Tavormina, Gibbs, and Huff at the Merck, Sharp and Dohme Research Laboratories (52). From that time onward Cornforth's wife, Dr. Rita H. Cornforth, joined us and carried out the many syntheses of variously labeled mevalonates we used in the elucidation of the biosynthesis

of squalene. Our concern was first to find out whether squalene was also synthesized from mevalonate and what were the individual enzymic reactions of the transformations of mevalonate. We soon learned that Konrad Bloch and Feodor Lynen were similarly engaged. I was most fortunate during that period to have had several most able visiting workers in my laboratory, such as Gordon Gould, Marjorie Horning, Nancy Bucher, Luke Gosselin, Adrian de Waard, DeWitt Goodman, and Jean Christophe, whose participation in our program made rapid progress possible.

I should like to recall specifically from that period our proof that mevalonate provided six blocks of five-carbon units to the synthesis of squalene and that these blocks were derived in such a way that C-5 of one mevalonate molecule was joined to C-2 of another (53,54). We concluded that a nonsymmetrical intermediate must have been formed from mevalonate; this intermediate turned out to be isopentenyl pyrophosphate identified in 1958 first in Bloch's laboratory and then confirmed by Lynen's group [for review cf. Popják and Cornforth (55)]. We had also predicted the disposition of mevalonate carbon atoms in lanosterol and cholesterol (54) (Fig. 6). Partial proof of this disposition was presented by Isler et al. (56); its final proof—by the use of ^{13}C-labeled mevalonates and ^{13}C-NMR—was obtained very recently in my laboratory at UCLA in collaboration with Prof. Frank Anet [Popják et al. (57)].

Another significant observation Gordon Gould and I made during the first year of the "mevalonate era" was "that the physiological regulation of the rate of cholesterol synthesis is concerned with one or more of the steps between acetate and mevalonic lactone" (58). As is well known, this step turned out to be regulated by levels of 3-hydroxy-3-methylglutaryl-CoA reductase, the study of which is currently a very lively subject.

By 1960 all intermediates between acetyl-CoA and mevalonate and between mevalonate and squalene, except for one, had been identified [for review cf. Popják and Cornforth (55)]. The third phase of our investigations began after the identification of farnesyl pyrophosphate as a precursor of squalene in yeast by Lynen et al. (59) and in liver by Popják (60) and by Goodman and Popják (61). The head-to-head condensation of the two electrophilic farnesyl pyrophosphate molecules with the participation of DPNH and TPNH to form squalene was intriguing. We were still unaccustomed to a new mechanism of carbon-to-carbon bond formation distinct from the aldol and Claisen-type of condensations familiar from carbohydrate and fatty acid biochemistry. We therefore set out to examine whether there may be a change in the oxidation level of the farnesyl residues before or during condensation to squalene. For this purpose we used first [5-D_2] mevalonate in the multienzyme system of the 10,000 x g supernatant of liver homogenates in which mevalonate is converted efficiently into farnesyl pyrophosphate and squalene. We have shown that [5-D_2] mevalonate was converted into [D_6] farnesyl pyrophosphate, two of the deuterium atoms being at C-1 [Popják et al. (62)]. We were, therefore, surprised to find that the squalene biosynthesized from the [D_6] farnesyl pyrophosphate contained eleven atoms of deuterium [Popják et al. (63)] (Fig. 7). Degradation of the squalene by ozonolysis showed that the one deuterium atom lost was missing from one of the central carbon atoms of squalene presumably originating from C-1 of farnesyl pyrophosphate. Experiments with ^3H-labeled TPNH showed that the hydrogen atom (deuterium) lost from the farnesyl pyrophosphate was replaced by a hydride ion from the reduced coenzyme (63).

During 1960/61 I spent a 6-months' sabbatical leave at the National Heart Institute in Bethesda in Evan Horning's laboratory. This was a very happy event for me, not only because I could work undisturbed without administrative

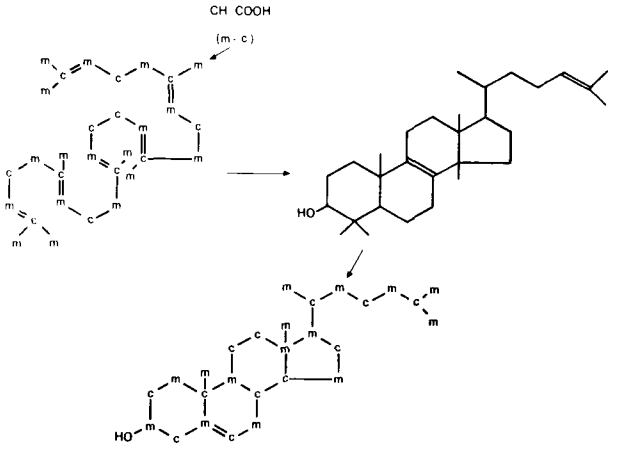


FIG. 5. Distribution of acetate carbons in squalene and in cholesterol.

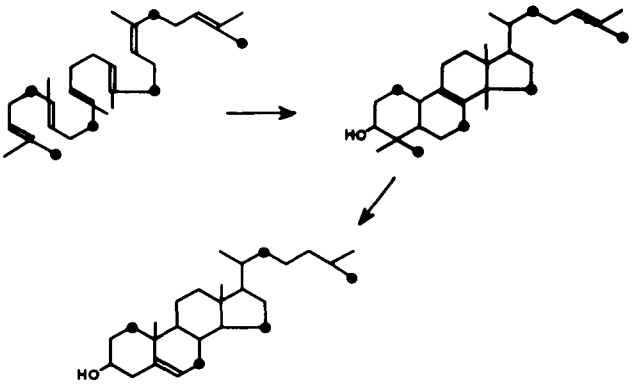


FIG. 6. Predicted disposition of C-2 of mevalonate in cholesterol based on the disposition found in squalene biosynthesized from [2-^{14}C] mevalonate.

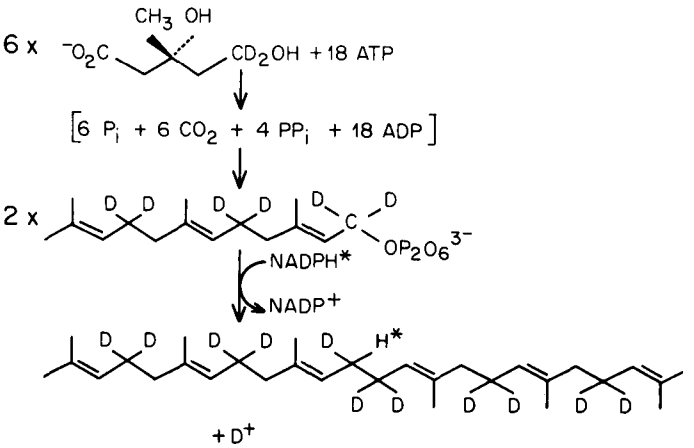
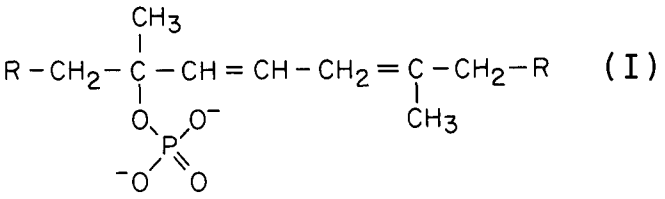


FIG. 7. Distribution of deuterium atoms in farnesyl pyrophosphate and squalene biosynthesized from [5-D_2] mevalonate.

responsibilities, but also because DeWitt Goodman, who had just returned to the U.S.A. after his postdoctoral year with me in England, was at NIH in a nearby laboratory and hence we could continue our collaboration that started a year earlier. It was during that time that I first synthesized *trans-trans*-[1-D_2-2-^{14}C]- and [1-$^3H_2$-2-^{14}C] farnesyl pyrophosphate from methyl [2-^{14}C] farnesoate that was made by Rita Cornforth previous to my departure for Bethesda (62). The labeled substrates were converted into squalene, again with the loss of one isotopic hydrogen from one of the two substrate molecules. Thus the [1-D_2] farnesyl-



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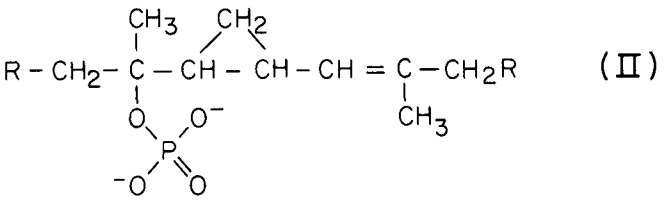

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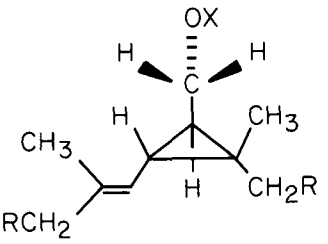

(III)

FIG. 8. Partial mass spectrum of normal squalene (at bottom) and of a mixture of natural squalene and [D_3]squalene biosynthesized from [1-D_2]-farnesyl pyrophosphate (62a). (Reproduced with the permission of The Royal Society.)

pyrophosphate gave [D_3]squalene (Fig. 8), the three deuterium atoms being in the center of the molecule as attested by chemical degradation of the molecule (62). We inferred from the fact that the loss of tritium from the [1-$^3H_2$-2-^{14}C]farnesyl pyrophosphate occurred without perceptible isotope effect that the removal must have been stereospecific and also that "the removal of the hydrogen cannot involve a molecule of farnesyl pyrophosphate but must occur in a later intermediate derived from it" [Popják et al. (62); cf. last paragraph left hand column on p. 61].

The problem now was how to prove our conjecture of the stereospecificity of squalene biosynthesis. One day late in 1961 it came to me, almost as a revelation, that if our surmise about the stereospecificity of the hydrogen exchange in farnesyl pyrophosphate during squalene synthesis was right, then either the [D_{11}]squalene biosynthesized from [5-D_2]mevalonate or the [D_3]squalene biosynthesized from [1-D_2]farnesyl pyrophosphate must contain an asymmetric carbon atom at one of its central carbon atoms. Consequently the succinic acid that we could obtain by ozonolysis from either specimen of squalene should be optically active. Within the hour that the idea hit me I drove, breaking all speed limits, to Mill Hill to share the idea with Cornforth. That idea was proved right and was the start of our investigations not only into the stereochemistry of squalene biosynthesis but also into the meaning of 'A'- and 'B'-side specificity of the dehydrogenases. That work, carried out with the participation of Cyril Donninger, a graduate student, and George Schroepfer, a postdoctoral fellow in my laboratory, and George Ryback, a postdoctoral fellow in Cornforth's laboratory, completed the third phase of our collaboration, the results of which we summarized in our Ciba Medal lecture in 1966 to the British Biochemical Society [Popják and Cornforth (64)].

In our first paper on the mechanism of the biosynthesis of squalene from farnesyl pyrophosphate (63), we have postulated on mechanistic grounds apart from enzyme-bound intermediates, the possible formation from farnesyl pyrophosphate of some stable intermediates such as (I) and (II). The notable features of both (I) and (II) are that they are asymmetric and that both have lost one hydrogen atom originally attached to C-1 of one of the two farnesyl pyrophosphate molecules from which they were hypothetically derived. Little did I know how close I was to the truth.

One of my serious weaknesses as a biochemist has been my unwillingness to make use of model enzyme systems not of animal origin. I suppose this reluctance, still with me, stems from my being an M.D. Besides, when a highly esteemed fellow scientist is studying sterol biosynthesis in yeast, I feel it incumbent on me to refrain from a similar undertaking. It was, therefore, with much reluctance – though admittedly with great curiosity – that I decided to check Rilling's claim (65) of the isolation, from incubation of yeast microsomes with farnesyl pyrophosphate in the absence of TPNH, of a substance that appeared to be an intermediate between farnesyl pyrophosphate and squalene and to which Rilling ascribed the structure of (II).

The structure of presqualene pyrophosphate, the name given by Epstein and Rilling (66) to the intermediate between farnesyl pyrophosphate and squalene, turned out not to be that of (II), but of another cyclopropane-containing structure (III), a structure that no biochemist could have predicted by any known reaction mechanism (66,67). We have shown (67) by NMR and mass spectrometry that presqualene alcohol biosynthesized from [1-D_2]farnesyl pyrophosphate contained only three deuterium atoms, two of these being on the carbinol carbon and the third at position 3 of the cyclopropane ring. Thus the elimination of the one pro-*S* proton from C-1 of one farnesyl pyrophosphate molecule, noted earlier during the overall synthesis of squalene (64), occurs during the synthesis of presqualene pyrophosphate and was first recognized by Rilling (65).

Presqualene pyrophosphate and its hydrolysis product, presqualene alcohol, are optically active (67,68). Thus, the symmetrical molecule of squalene is synthesized through the intermediacy of a truly asymmetric molecule, which is clearly a relative of chrysanthemum monocarboxylic acid that has been known since 1924 as a constituent of pyrethrin I. From a comparison of the NMR spectra of presqualene alcohol with those of the *trans*- and *cis*-chrysanthemols (made by $LiAlH_4$ reduction of the methyl esters of the respective acids), we established that the two large substituents on the cyclopropane ring were in an *anti*-position to the carbinol group whereas the methyl group on the ring was *syn* to it (67). Further, by an extension of Nakanishi's benzoate chirality rule (69), we were able to deduce that the absolute configuration of presqualene alcohol at all three asymmetric centers in the cyclopropane ring was *R* (70) as shown in (III), a configuration that brings it to an even closer relation to chrysanthemum monocarboxylic acid, the absolute configuration of which at its two asymmetric centers is known to be *R* (71).

The mechanism we proposed (67) for the synthesis of presqualene pyrophosphate, shown in Fig. 9, is similar to that presented by Epstein and Rilling (66). It assumes the

parallel alignment of two farnesyl pyrophosphate molecules on the enzyme and the initiation of the reaction by a nucleophilic group on the enzyme polarizing the allylic double bond of the farnesyl pyrophosphate lying at the top and resulting in the formation of C-C bond between C-2 of one farnesyl pyrophosphate and C-1 of the other, with elimination of the pyrophosphate anion from the second. Stereospecific elimination of the one proton that was originally the pro-*S* hydrogen atom at C-1 of this second substrate molecule, with closure of the cyclopropane ring, completes the sequence.

This mechanism accounts not only for the deduced absolute configuration of presqualene alcohol, but also for the observations I already mentioned that the carbinol carbon of presqualene pyrophosphate is derived from C-1 of one farnesyl pyrophosphate molecule and that C-1 of the second farnesyl pyrophosphate is at position 3 of the cyclopropane ring and carries one of the two hydrogen atoms originally at C-1 of that second farnesyl pyrophosphate. It also follows from this mechanism that the absolute configuration at the carbinol carbon of presqualene pyrophosphate or alcohol must be the same as at C-1 of farnesyl-PP and that the oxygen in the C-O-P bond of presqualene pyrophosphate is the one originally in farnesyl pyrophosphate.

Not long ago we carried out experiments with farnesyl pyrophosphate labeled stereospecifically with deuterium and also labeled with ^{18}O (72) and proved the hypothesis correct (Fig. 10). We could also show by NMR that the pro-*R* hydrogen atom (deuterium in our experiment) at C-1 of one of the farnesyl pyrophosphate molecules was at position 3 of the cyclopropane ring as we have surmised.

The mechanism of the conversion of presqualene pyrophosphate into squalene is still conjectural (Fig. 11). We have suggested a ring expansion and rearrangement to a cyclobutyl pyrophosphate which is then reductively cleaved with NADPH resulting in the introduction of the hydride ion, generation of a *trans*-double bond, and the elimination of the pyrophosphate anion (67). These mechanisms are in complete harmony with all the previously established stereochemical features of squalene biosynthesis (64). Now that squalene synthetase has been successfully detached from microsomes (73,74) and identified as a proteo-lipid (75), a more detailed study of these two intriguing reactions of squalene synthesis might become possible.

Let me close with some brief background and account of some of the current work in my laboratory pursued in collaboration with John Edmond, Jean Bardenheier, and Thomas Parker. I have said earlier that I made a deliberate choice in studying cholesterol biosynthesis in the hope that a knowledge of the intermediary reactions may one day be exploited usefully. This is one of our current objectives.

Christophe and I noted early (76) that in liver homogenates the allylic pyrophosphates synthesized from mevalonate were not only converted into squalene and sterols, but were also hydrolyzed to the free alcohols which were then converted irreversibly through the action of alcohol and aldehyde dehydrogenases to carboxylic acids. Liver alcohol dehydrogenase was particularly active with 3,3-dimethylallyl alcohol and with isopentenol. I had been brooding over those observations for several years and conceived the idea that if prenyl transferase, the enzyme which converts dimethylallyl and isopentenyl pyrophosphate into farnesyl pyrophosphate, were inhibited partially we could divert from the sterol synthetic pathway some intermediates that could be disposed of by an alternative pathway of metabolism. We have made, therefore, an intensive study of the properties of liver prenyl transferase and isopentenyl pyrophosphate isomerase (77,78). In the course of those studies we have found that analogues of geranyl pyrophosphate lacking the $\alpha\beta$-allylic double bond were powerful

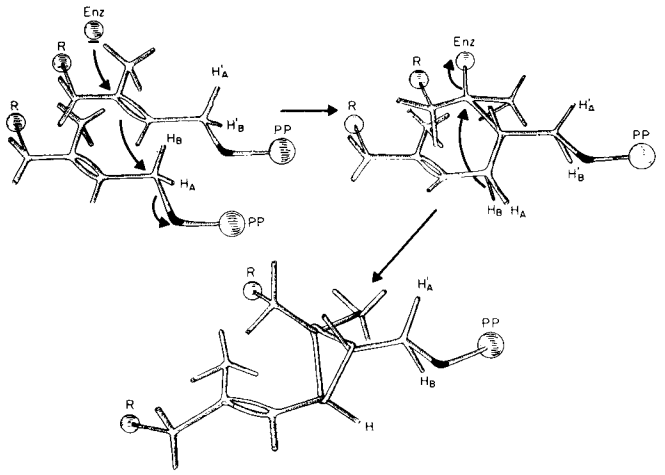

FIG. 9. Hypothetical mechanism of the biosynthesis of presqualene pyrophosphate (67). The pyrophosphate groups are represented by the large globes. (Reproduced with the permission of the Editor, *Journal of Biological Chemistry.)*

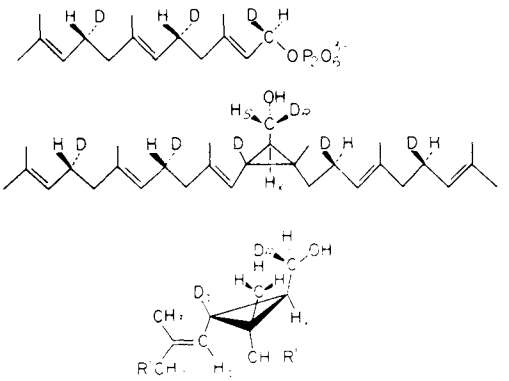

FIG. 10. Absolute configuration of presqualene alcohol biosynthesized from [1R,5R,9R-1,5,9-D_3]farnesyl pyrophosphate.

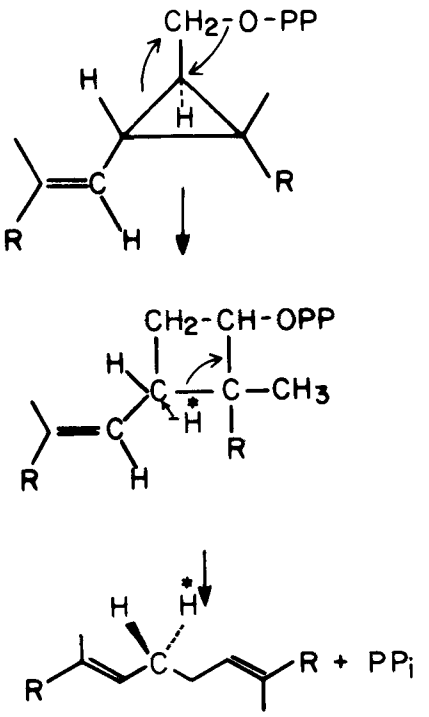

FIG. 11. Hypothetical mechanism of the conversion of presqualene pyrophosphate into squalene (67). (Reproduced with the permission of the Editor, *Journal of Biological Chemistry.)*

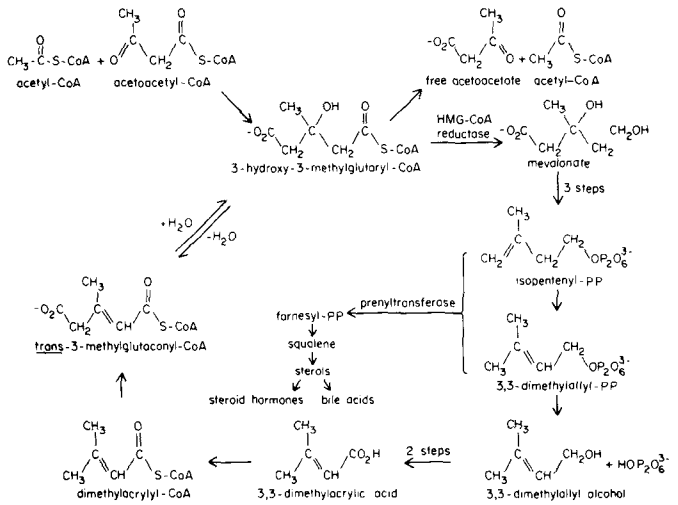

FIG. 12. Metabolic transformations of mevalonate.

inhibitors of prenyl transferase (79), but other analogues containing the αβ-double bond (such as 6,7-dihydrogeranyl-, *cis*- and *trans*-3-ethyl-3-methylallyl-, *trans*-3-propyl-3-methylallyl pyrophosphate) were artificial substrates for the enzyme (80-82). From these observations grew my hypothesis of the "*trans*-methylglutaconate shunt" of mevalonate metabolism (83) depicted in Fig. 12. The main idea of the hypothesis is that by the inhibition of prenyl transferase the accumulated dimethylallyl pyrophosphate will get hydrolyzed and the alcohol after two steps of dehydrogenation will merge with a path of leucine metabolism in which *trans*-3-methylglutaconyl-CoA is an intermediate. It was gratifying to find subsequently that mevalonate even under physiological conditions is not used exclusively for sterol biosynthesis as was thought formerly, but that some of its carbon atoms (C-2 to C-5) are transferred to the C-2 pool and blood ketone bodies (84,85). The shunt of mevalonate metabolism has been established not only in vivo in the rat, and in man (86), but also in vitro with tissue slices (87,88). It appears that approximately 25% of mevalonate is diverted in the rat onto the shunt pathway and that the kidneys, which are avid utilizers of mevalonate, account for over one-half of the shunt (89,90). Siperstein and his colleagues (91) recently have made the interesting observation that the extent of the mevalonate shunt is about twice as large in female as in male rats and that nephrectomy abolished the sex difference in this respect. It is tempting to speculate that perhaps the lower cholesterol content of the blood of women as compared with that of men is the result of greater shunt activity in females than in males.

It is too early to say whether the alkyl phosphonates and phosphonophosphates which are fairly specific inhibitors of liver prenyl transferase (92) and which we are currently studying, would increase the shunting of mevalonate in vivo and prove of clinical benefit in controlling hypercholesterolemia. However, it is better to travel hopefully than to arrive; I am still a traveller.

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Garrison licenses Wenger process

Wenger Manufacturing has announced that Garrison Products, Inc., has become a licensee of Wenger's Uni-Tex process to produce vegetable protein base meat analog.

Garrison's plant will be at 1471 Paddock Drive, Northbrook, IL, Wenger said. Wenger described Uni-Tex as a dense, untwisted, uniformly layered, meat analog made by extruding defatted, high protein vegetable flours such as defatted soy or peanut flours, concentrates, or grits. The analog, Wenger said, can be flavored, colored, and textured to resemble ham, beef, chicken, or seafoods. Garrison expected to have its product in market channels by June 15.

the latest in Lipids

JULY 1977

EMI system selected by Palmex Industries

Palmex Industries, Sdn. Bhd., Penang, Malaysia, has purchased a palm oil physical refining system from EMI Corp., Des Plaines, IL. The system is designed to produce a deodorized edible product from crude palm oil without caustic refining, EMI said. The new system will be installed alongside an existing physical refining system at Prai Penang. ●

Cottonseed oil mills exemption lifted

The Labor Department has lifted a two-year-old directive that exempted cottonseed oil mills from cotton-dust safety inspections imposed on textile mills.

The exemption was lifted in late June. The exemption originally was permitted because of a report that studies were needed to determine safe levels of dust in the oil mills, the *Wall Street Journal* said in quoting a former Labor Department assistant secretary. Conditions in oil mills and textile mills were judged sufficiently different to merit the exemption at that time.

"Brown lung," a debilitating condition is the target of the safety inspections. Brown lung is believed to be caused by cotton dust, but the precise irritant has not been identified. Cottonseed industry officials say the linters removed from cottonseed in oil mills have never been shown to be a factor. Consumer activist groups have been pressing for enforcement of the complete standards to cottonseed oil mills.

The Labor Department said removing the exemption does not mean there will be special emphasis on inspecting cottonseed oil mills, but that henceforth they will be inspected as part of the department's routine workload. ●