The Effect of Estradiol-17β and Vegetable Oil on the Hydroxylation of Protocollagen[1] (35545)

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Various investigators have studied the effect of estrogenic hormones upon the synthesis of collagen. Woessner (1) reported that the amount of uterine collagen (measured as hydroxyproline) remaining 4 days after parturition was significantly greater in estrogen-treated than in nontreated rats. Smith and Allison (2) showed that estradiol benzoate increased the amount of collagen in the femur of rats. Hamilton *et al.* (3) demonstrated that 20 min following administration of estrogen to ovariectomized rats, there is an increase of several humdred percent over the control value in the rate of uterine nuclear RNA synthesis. Recent studies by Halme and Jaaskelainen (4) showed that an increase in the amount of collagen in mouse uterus late in pregnancy was due to an increase of the activity of protocollagen proline hydroxylase.

The experiments reported below were undertaken to study the effect of estrogen upon the activity of protocollagen proline hydroxylase *in vitro*. In these experiments, we added estrogen dissolved in sesame seed oil to the enzyme system. Increases in enzyme activity were observed, part of which was due to the estrogen and part to the sesame seed oil used as a carrier. This report deals with these results with special reference to a study of the effect produced by the oil itself.

Materials and Methods. Enzyme preparation. Proline hydroxylase was prepared from chick embryo homogenates by an adaption of the procedure of Kivirrikko and Prockop (5). Ten-day-old chick embryos were excised, decapitated, and 10 g of tissue were homogenized in 10 ml of 0.01 *M* KCl, in a Waring blender. The homogenate was centrifuged at 15,000*g* for 30 min and the pellet was discarded. Ammonium sulfate was added to the supernatant to 30% saturation. The mixture was centrifuged as before, and the pellet again, was discarded. The supernatant was brought to 65% saturation with ammonium sulfate and again centrifuged. The pellet was dissolved in 3 ml of a solution of 0.1 *M* KCl and 0.02 *M* Tris–HCl buffer, pH 7.5. This mixture was dialyzed overnight against 4 liters of a 0.05 *M* KCl; 0.02 *M* Tris–HCl buffer, pH 7.5; and 0.5 m*M* ascorbic acid solution. The dialyzed solution was stored in aliquots at —20°.

Substrate preparation. The tritiated substrate for proline hydroxylase was prepared according to the method of Hutton *et al.* (6) with some modification. About 7–8 g of 10- to 11-day-old-chick embryos were homogenized briefly in 10 ml of Krebs–Ringer buffer (7) with a Craftsman homogenizer. The mixture was incubated aerobically at 37° with 1 m*M* α, α'-dipyridyl and 500 μCi of proline-3,4-^{3}H (sp act 5.86 Ci/mmole, New England Nuclear) for 90 min. The whole mixture was then centrifuged for 20 min at 27,000*g*. The supernatant was discarded, and the pellet was extracted with 14 ml of 0.5 *N* acetic acid for 120 min at 0°, and the mixture was centrifuged. The supernatant was dialyzed for 18 hr against 2 changes of 2 liters of water at 4°. Specific activity of the substrate ranged from 100,000 to 750,000 dpm/mg of protein for each batch prepared. A preliminary incubation study was done with each batch of substrate to determine an optimum time. That time was selected in which adequate radioactive product could be detected and in

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which the reaction had not yet attained completion in order that a stimulatory effect could be observed.

Measurement of enzyme activity. The components of the incubation mixture included (μ mole): Tris–HCl (pH 7.5), 200; α-ketoglutaric acid, 0.2; ascorbic acid, 1.0; ferrous ammonium sulfate, 0.2; substrate preparation; and enzyme preparation. The cofactors, α-ketoglutaric acid, ascorbic acid, and ferrous ammonium sulfate were each dissolved in 15% Tween 80 (Nutritional Biochem. Corp.), and 0.25 ml of each of these detergent solutions was used for these studies. Various concentrations of estradiol-17β (spectrophotometric grade, Cal. Biochem.) from 0.1 to 30 μg/tube were dissolved in 0.1 ml of sesame seed oil (Hain Pure Food Corp.). The methylenedioxyphenyl compound and sesamol (4-hydroxy-methylenedioxyphenyl) were gifts from the Dept. of Entomology, Univ. of California at Berkeley. Methylenedioxyphenyl was dissolved in acetone (1:3), and various quantities ranging from 10 to 100 μl were used for each tube. Various concentrations of sesamol (from 40 to 200 μg/tube) were also used. Methyl esters of oleate, linoleate, and linolenate were gifts from Dr. M. A. Williams. Methyl esters of myristate, palmitoleate, and triolein were purchased from Applied Science Lab., Inc.

The volume of the incubation mixture was 2.0 ml. Incubation was carried out in test tubes submerged in a 30° water bath for a time period dependent upon the particular substrate preparation used. The tubes were gently agitated during the incubation period. The reaction was stopped by adding 0.2 ml of 50% TCA to each incubation tube.

The conversion of protocollagen proline-3,-4-^{3}H to hydroxyproline-^{3}H involves the loss of one tritium atom to form the product plus tritiated water. The progress of this conversion was followed by distilling the tritiated water from the reaction mixture (8), and counting a 0.5-ml aliquot of the distillate in Bray's solution (9) in a Beckman CPM 100 liquid scintillation counter.

Results and Discussion. Table I summarizes the results of our 3 initial experiments. It is apparent that both estradiol-17β (dissolved in 0.1 ml of sesame seed oil) and its carrier, sesame seed oil alone, stimulate the hydroxylation of protocollagen. However, estradiol-17β produced marginally significant increases in enzyme activity in some experiments. No effect was observed in others. The effect of estradiol-17β on proline hydroxylase activity must therefore be regarded as minor.

The possibility existed that estrogen and sesame seed oil affected the transhydrogenase activity in the crude preparation of protocol-

TABLE I. The Effect of Estradiol-17β Dissolved in Sesame Seed Oil and Sesame Seed Oil Alone on the Hydroxylation of Tritiated Acetic Acid-Extractable Protocollagen.[a]

Sesame seed oil (ml/tube)	Estradiol[b] (μg/tube)	Expt.:	Relative hydroxylation rate 1	2	3
0.0	0.0		100[c]	100[c]	100[cd]
0.1	0.0		159	118	147
0.1	0.1		149	122	145
0.1	0.3		196	124	—
0.1	1.0		199	132	165
0.1	3.0		160	131	147
0.1	10.0		162	125	146
0.1	30.0		162	—	151

[a] The tritiated substrates used for each tube for the 3 experiments were 700,000 dpm; 99,800 dpm; and 361,800 dpm; respectively.

[b] Estradiol was dissolved in 0.1 ml of sesame oil for each tube.

[c] The dpm/0.5-ml aliquot for the control of 3 experiments were 1426, 680, and 588, respectively.

[d] Indicates an average value of 4 tubes; other results are the average value of 2 tubes.

TABLE II. The *in Vitro* Effect of Sesame Seed Oil and α,α'-Dipyridyl on the Hydroxylation of Tritiated Acetic Acid-Extractable Protocollagen.

Expt. no.	Enzyme preparation	Sesame seed oil (ml/tube)	Estradiol (μg/tube)	α,α'-Dipyridyl (mmoles/incubation)	dpm/0.5-ml aliquot
1[a]	—				144
	+	0.25	5.0	1.0	155
2[b]	—	0.10			172
	—	0.30			194
	+	0.00			1856[c]
	+	0.10			2142
	+	0.30			2360

[a] Tritiated substrate used for each incubation tube was: 117,400 dpm; [b] 323,500 dpm.
[c] An average value of 4 tubes; others are the average value of 2 tubes.

lagen proline hydroxylase. Transhydrogenase activity could be responsible for transfer of tritium from the substrate to water. To test the transhydrogenase activity in our crude enzyme preparation, α, α'-dipyridyl (1 m*M*), an inhibitor of proline hydroxylase was added to a reaction mixture containing enzyme, substrate, estradiol, and cofactors. The radioactivity recovered in the aliquot of this incubation mixture was as low as that in the incubation without enzyme (Table II). Therefore, transhydrogenase activity in this preparation was negligible. It was concluded, therefore, that the stimulation of hydroxylation of protocollagen by estradiol-17β and sesame seed oil resulted from an increase in proline hydroxylase activity. Also, it was shown that detergent was not responsible for exchange of substrate tritium with water of the incubation mixture.

Sesame seed oil alone can also stimulate the hydroxylation of protocollagen. The enhanced hydroxylation of protocollagen by sesame seed oil was not related to an ascorbic acid-dependent nonenzymatic hydroxylating system (10, 11), since hydroxylation was not increased by sesame seed oil in the absence of enzyme (Table II).

Sesame oil is known to possess approximately 0.2% of the methylenedioxyphenyl compound which is an insecticide synergist. Detoxification of insecticide or related compounds involves hydroxylation (12). However, methylenedioxyphenyl had little effect on the hydroxylation of protocollagen by proline hydroxylase. Sesamol also had no effect on the hydroxylation of protocollagen.

To determine whether any of the fatty acids in sesame seed oil[3] might affect proline hydroxylase activity, the effect of various methyl esters of saturated and unsaturated fatty acids on the hydroxylation of protocollagen was studied. From results in Table III, it is obvious that all of the methyl esters of unsaturated fatty acids tested stimulated the hydroxylation of protocollagen. The stimulation did not appear to be related to the degree of unsaturation. Saturated fatty acid, *e.g.*, myristate, seemed to be inactive. Rancid sesame seed oil was also shown to be inactive. This might be due to the destruction of unsaturation of fatty acids. Mineral oil was inactive, also.

Summary. Estradiol-17β dissolved in sesame oil increased the activity of proline hydroxylase as measured by the release of tritium from protocollagen containing tritiated proline. Sesame seed oil alone was responsible for the major part of this effect. The increases produced by the addition of estradiol to sesame seed oil were marginally significant. Studies with constituents of sesame seed oil showed that unsaturated fatty acids, such as oleic, palmitoleic, and linoleic acids, were active. Myristic acid was inactive.

[3] The fatty acid composition of sesame seed oil is: (%) linoleic acid, 42.5; oleic acid, 39.3; palmitic acid, 9.8; stearic acid, 6.4; linolenic acid, 0.9; palmitoleic acid, 0.2.

TABLE III. The *in Vitro* Effect of Fats and Oil on the Hydroxylation of Tritiated Acetic Acid-Extractable Protocollagen.

Addition	Amount used (ml/tube)	Relative hydroxylation rate Test: 1	2
None[a]	—	100	100
Mineral oil	0.10	91 (4)[b]	—
	0.30	100 (4)	—
Sesame seed oil	0.10	119 (1)	123 (2)
	0.30	154 (3)	158 (4)
Rancid sesame seed oil[c]	0.10	83 (1)	100 (2)
Methyl oleate	0.15	116 (3)	—
	0.30	149 (3)	—
Methyl linoleate	0.10	115 (2)	—
	0.15	123 (3)	—
	0.30	123 (2)	136 (3)
Methyl linolenate	0.15	123 (3)	—
	0.30	133 (3)	—
Methyl palmitoleate	0.25	125 (5)	—
Methyl myristate	0.10	100 (4)	—
	0.30	101 (4)	—
Triolein	0.10	106 (1)	—
	0.30	121 (1)	—

[a] The dpm/0.5-ml aliquot for the control of 5 experiments were: 1020, 1060, 1040, 824, and 2000, respectively.

[b] Numbers in parentheses indicate the series number of experiments in which the test was done.

[c] Aerated sesame seed oil.

1. Woessner, J. F., Biochem. J. **112**, 637 (1969).
2. Smith, Q. T., and Allison, D. J., Endocrinology **79**, 486 (1966).
3. Hamilton, T. H., Widnell, C. C., and Tata, J. R., Biochim. Biophys. Acta **108**, 168 (1965).
4. Halme, J., and Jaaskelainen, M., Biochem. J. **116**, 367 (1970).
5. Kivirrikko, K. I., and Prockop, D. J., Arch. Biochem. Biophys. **118**, 611 (1967).
6. Hutton, J. J., Jr., Tappel, A. L., and Udenfriend, S., Arch. Biochem. Biophys. **118**, 231 (1967).
7. Stone, N., and Meister, A., Nature (London) **194**, 555 (1962).
8. Hutton, J. J., Jr., Tappel, A. L., and Udenfriend, S., Anal. Biochem. **16**, 384 (1966).
9. Bray, G. A., Anal. Biochem. **1**, 279 (1960).
10. Udenfriend, S., Clark, C. T., Axelrod, J., and Brodie, B. B., J. Biol. Chem. **208**, 731 (1954).
11. Breslow, R., and Lukens, L. N., J. Biol. Chem. **235**, 292 (1960).
12. Esaac, E. G., PhD thesis, Dept. of Entomology, Univ. of California, Berkeley (1967).

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New Antigens in Lactose (35546)

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Bleumink and Young (1) reported over a 100-fold increase in skin reactivity on milk-sensitive persons of relatively inactive, crystalline β-lactoglobulin after prolonged heating with lactose at 50° at pH 7.0. They attributed the increased skin reactivity to a browning reaction condensation product of lactose with the ε-amino group of lysine in the β-lactoglobulin. They used 15 g of lactose/500 mg of β-lactoglobulin. After heating, the lactose was removed by dialysis and the undialyzed residue was recovered by lyophilization. The fact that such a large quantity of lactose was used and that no control test for nondialyzable antigens in the lactose was done prompted us to examine two reagent-grade, commercial samples of lactose for nondialyzable antigens which could account for the increased skin reactivity observed or for potential allergens *per se*. We found 4 unidentified antigens in the lactose as well as some β-lactoglobulin and α-lactalbumin. Less highly purified lactose would probably contain more of these antigens than were found in our two reagent-grade samples.

Materials and Methods. The α-lactose monohydrate had the following certificate of analysis (percentage basis) according to the supplier: dextrose 0.05; sucrose, 0.02; iron, 0.0001; insoluble matter, 0.001; residue on ignition, 0.016; dextrin, starch, 0.000; heavy metals (as Cu), 0.0003. Two properties of β-lactose by the supplier were: $(\alpha)_D$ +55° ± 1° (C = 6); mp, 224° dec. The dialyzer tubing retained materials with a molecular weight of 12,000 and higher. Total milk protein (TMP) was prepared from fresh skim milk by prolonged dialysis against many changes of 1 *M* sodium chloride. Sodium chloride was then removed by dialysis against distilled water until free from chloride ions. The proteins inside the membrane were recovered by lyophilization. The residue contained 13.7% nitrogen on an air-dried basis.

A 500-g sample of α-lactose monohydrate or anhydrous β-lactose was stirred up with 500 ml of water. The suspension, with toluene preservative, was dialyzed for about 8 days against running water or frequent changes of water until the dialysates gave negative or very faintly positive test for reducing sugar. The endo solution was filtered and lyophilized. The yield of brownish-colored residue, designated lactose-endo (LE) was 0.016 and 0.009% from α-lactose (α-LE) and β-lactose (β-LE), respectively. The nitrogen contents of various preparations ranged from 1.3 to 2.4%. Since α-LE and β-LE were both antigenic, a mixture containing 26% β-LE and 74% α-LE was used for this work. Unless otherwise indicated, LE refers to this mixture. The nitrogen content of LE was 1.8%.

Carbohydrate analysis of both unhydrolyzed and hydrolyzed LE was done using fractionation on a column of Type S Chromo-Beads (a resin by Technicon)[1] by an unpublished modification by Groves and Friedman of the method of Lee *et al.* (2). Hexosamine was determined on LE by the Elson–Morgan method (3) on the hydrochloric acid hydrolysate which was purified by Dowex-50(H^+) adsorption and elution. Losses were monitored by an internal radioactive control.

Antigenicity was determined by the Schultz-Dale technique (4) and by gel-diffusion analysis (5) using rabbit anti-LE. Vir-

[1] The use of a trade name, distributor or manufacturer is for identification only and implies no endorsement of the product or its manufacturer.

TABLE I. Summary of Results of Schultz-Dale Tests with α-LE, β-LE, LE, α-Lactalbumin, β-Lactoglobulin, Casein, and Bovine Serum Albumin.

Sensitization		Challenge		Results		
Antigen	Dose (μg of antigen N/guinea pig)	Antigen	Dose (μg of antigen N)	No. of guinea pigs tested	No. giving 4+ response[a]	No. with negative responses
α-LE	20	LE	10	6	4	2
		β-Lactoglobulin	10	6	1	5
		α-Lactalbumin	10	4	0	4
		Casein	10	1	0	1
		κ-Casein	10	1	0	1
		BSA	10	2	0	2
β-LE	10	LE	10	5	2	3
		β-Lactoglobulin	10	5	0	5
LE	200	LE	10	3	3	0
		β-Lactoglobulin	10	2	0	2
		α-Lactalbumin	10	3	1	2
		Total milk protein	100	1	0	1
None	—	LE	10	3	0	3

[a] 4+ reaction was a response 80 to 100% that of histamine.

gin female guinea pigs (about 225 g) were sensitized by subcutaneous injection (nuchal area) with two simultaneous 0.5-ml volumes of LE in physiological salt solution emulsified with Freund's complete adjuvant (1:1). Incubation was for at least 28 days. Uterine strips of the sensitized guinea pigs were used for testing. Rabbits were immunized by injection of 0.25 ml of LE emulsified with Freund's complete adjuvant in each of the footpads. The dosage was 2.5 mg of LE (45 μg of LE nitrogen). A booster dose of 3 mg of LE (54 μg of LE nitrogen) was administered intra-abdominally in 1 ml of solution 28 days later. Rabbits were bled out in 7 days after administration of the booster dose. Anti-LE was concentrated by filtration through a filter which retained material with a molecular weight of 20,000 or over.

Results and Discussion. Antigenicity of LE, α-LE, and β-LE and their antigenic relationships with some known milk proteins as determined by Schultz-Dale technique are shown in Table I and Fig. I. The β-LE (10 μg of nitrogen, each) sensitized 2 of 5 guinea pigs to LE and none of these 5 were sensitive to β-lactoglobulin. α-LE (20 μg of nitrogen, each) sensitized 4 of 6 guinea pigs to LE, 1 of 5 of these was sensitive to β-lactoglobulin, none of the 4 tested was sensitive to α-lactalbumin, none of the 2 was sensitive to BSA and none of the 1 each tested was sensitive to soluble casein or to κ-casein. LE (200 μg of nitrogen each) sensitized 3 of 3 guinea pigs to LE, none of 2 of these was sensitive to

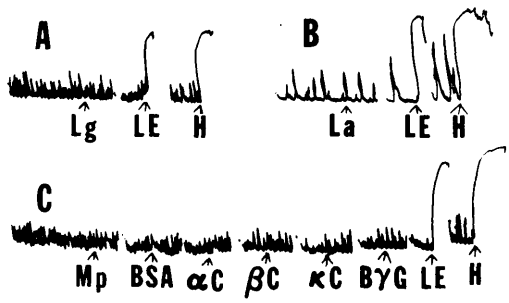

FIG. 1. Demonstration of antigenicity of LE and nonidentity of LE with several milk proteins by the Schultz-Dale technique using 3 segments of uterine horns from a guinea pig sensitized with 200 μg of LE nitrogen: challenge dose (μg of N) of respective substance (H = histamine for maximum response): (A) Lg (β-lactoglobulin), 10; LE, 10. (B) La (α-lactalbumin), 10; LE, 10. (C) Mp (mucoprotein), 1; BSA (bovine serum albumin), 1; C (α-casein), 1; βC (β-casein), 1; κC (κ-casein), 1; BγG (bovine gamma globulin), 1; LE, 1.

β-lactoglobulin, and 1 of 3 tested was sensitive to α-lactalbumin. LE gave no nonspecific reactions with nonsensitized guinea pig uteri. The nonidentity of LE antigen with several milk proteins is apparent from the Schultz-Dale test shown in Fig. 1 using 3 segments of uterine horns from a guinea pig sensitized with LE (200 μg of nitrogen). Figure 1A and B show that 10 μg of LE nitrogen produced 4+ responses (80 to 100% the reaction to histamine) following negative responses with 10 μg of β-lactoglobulin and α-lactalbumin nitrogen, respectively. Fig. 1C shows that 1 μg of LE nitrogen produced a 4+ response following negative responses to successive 1 μg nitrogen of each mucoprotein (6, 7), BSA, α-casein, β-casein, κ-casein, and bovine γ-globulin. In another experiment 10 μg of LE nitrogen produced a 4+ response following a negative response with 100 μg of TMP nitrogen in the uterine strip of a guinea pig sensitized with 200 μg of LE nitrogen. This result shows that the TMP preparation either contains none or an undetectably small amount of LE antigens. The proportions of β-lactoglobulin and α-lactalbumin present in LE were estimated by the Schultz-Dale test. Titration with β-lactoglobulin and LE of uterine strips of guinea pig sensitized with β-lactoglobulin showed that LE contained less than 10% β-lactoglobulin. Likewise, titration with α-lactalbumin and LE of uterine strips of guinea pigs sensitized with α-lactalbumin showed that LE contained less than 1% α-lactalbumin.

Four antigens which were not identifiable with any of the milk proteins tested were demonstrated by gel diffusion analysis using 5-fold concentrated rabbit anti-LE as shown in Fig. 2. A qualitatively similar precipitate pattern was obtained using unconcentrated rabbit anti-LE and no precipitate was obtained in this test using the following antigens, β-lactoglobulin, α-lactalbumin, soluble casein, and BSA. LE gave no precipitate with normal rabbit serum.

LE contained 33% lactose, whereas the acid hydrolyzate of LE contained 24 and 21% glucose and galactose, respectively, along with traces of mannose and of two unidentified substances. Although 100% composition of LE has not been determined, the fact that

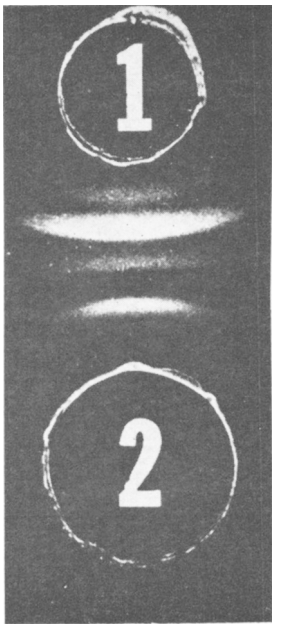

FIG. 2. Demonstration of 4 unidentified antigens in LE by gel double diffusion analysis: Well 1, 0.1 ml of LE, 0.2 mg of LE nitrogen/ml; Well 2, 0.2 ml of rabbit anti-LE, concentrated 5-fold. Photographed 5 days after test started.

the sum of glucose and galactose significantly exceeds that of the unhydrolyzed lactose, indicates that some lactose may be combined with antigenic nitrogenous components. This could explain the retention by the dialysis membrane of lactose-containing antigens. LE contained 0.68% hexosamine.

Every antigen is a potential allergen. Consequently, a possibly significant allergenic role of these unidentified antigens must be considered not only in the experiment of Bleumink and Young (1), but also in all cases of adverse response to ingestion of lactose until clinical evaluation of these antigens can be made. Whether the four unidentified antigens are chemically combined with lactose or present as a contaminant is not known. In either case, their association with lactose has provided a means for their first detection and only further studies will show whether or not these antigens can be more readily obtained in greater yields directly from milk. Further work on the nature of these antigens is contemplated.

Summary. Four unidentified antigens,

which are distinct from known milk proteins, are contained in commercial, reagent-grade α-lactose and β-lactose. These unidentified antigens, rather than the condensation product of lactose with the ε-amino group of lysine of β-lactoglobulin, may cause, or partially cause, the reported increase in allergenicity of crystallized β-lactoglobulin on heating with lactose. The unidentified antigens present in lactose have possible, hitherto unrecognized, allergenic significance.

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1. Bleumink, E., and Young, E., Int. Arch. Allergy Appl. Immunol. **34**, 521 (1968).

2. Lee, Y. C., McKelvey, J. F., and Lang, D., Anal. Biochem. **27**, 567 (1969).

3. Elson, L. A., and Morgan, W. T. J., Biochem. J. **27**, 1824 (1933).

4. Coulson, E. J., J. Allergy **24**, 458 (1953).

5. Ouchterlony, O., Progr. Allergy **6**, 30 (1962).

6. Jackson, R. H., Coulson, E. J., and Clark, W. R., Arch. Biochem. Biophys. **97**, 373 (1962).

7. Coulson, E. J., and Jackson, R. H., Arch. Biochem. Biophys. **97**, 378 (1962).

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