Cholesteryl esterase and cholesteryl ester pools in corpus luteum

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ABSTRACT Cholesteryl esterase activity has been demonstrated in the corpus luteum of the rat and the cow. The hydrolytic activity in bovine corpora lutea shows two pH optima, and is distributed throughout the particulate and supernatant fractions of the tissue. The greatest activity is present in the 5000 *g* pellet. The size of the available endogenous cholesteryl ester pools is also estimated. Some properties of the bovine luteal enzyme are different from those of pancreatic cholesteryl esterase from the same species.

KEY WORDS corpus luteum · bovine · rat · cholesteryl esterase · cholesteryl ester · pool size · radioactivity dilution method

THE CORPUS LUTEUM of a number of species is known to contain esterified cholesterol in amounts which vary during the estrous cycle (1–4) and during pregnancy (5). Conn, Vogel, Louis, and Fajans (6) suggested that the sterol esters might serve as precursors of steroid hormones, and it has been shown that luteinizing hormone in vivo will induce depletion of cholesteryl esters in the corpus luteum of rat (7, 8) and rabbit (9) and the interstitial tissue of rabbit (10). In the adrenal cortex cholesteryl ester depletion is a consequence of treatment of the animal with ACTH (11), and Dailey, Swell, and Treadwell (12) have shown that cholesteryl esters can act as steroid hormone precursors in this tissue.

A considerable amount of evidence supports the existence of cholesteryl esterase (sterol-ester hydrolase, E.C. 3.1.1.13) in adrenal tissue (12–14). There is also evidence for the existence in adrenal cortex of a cholesteryl ester-synthesizing enzyme that requires a number of cofactors for maximal activity (14–16).

This report concerns the presence in corpus luteum of cow and rat of cholesteryl esterase activity, together with a partial characterization of the bovine enzyme, and an estimate of the size of the pool of endogenous cholesteryl ester available to the enzyme in tissue homogenates.

MATERIALS AND METHODS

All organic solvents were either of A.R. grade or were purified according to Fieser (17). All other reagents were of A.R. grade. Cholesteryl-4-^{14}C oleate (20 mc/mmole) was purchased from the Radiochemical Centre, Amersham, Bucks, England. DFP was purchased from Koch–Light Laboratories, Ltd., Colnbrook, Bucks, England. "Mipafox" was a gift from Dr. W. Hartley of Fisons Pest Control Ltd., Harston, near Cambridge, England. PHMB was obtained from Sigma Chemical Co., London, England. Human chorionic gonadotropin (Pregnyl) and pregnant mare's serum gonadotropin (Gestyl) were purchased from Organon Laboratories, Newhouse, Scotland.

Tissues

Bovine corpora lutea were obtained fresh from the local slaughterhouse and were transported to the laboratory on ice before being frozen for storage. Rat corpora lutea were obtained from animals of the Wistar strain (A. Tuck & Sons, Rayleigh, Essex, England) made pseudopregnant by subcutaneous injections of pregnant mare's serum gonadotropin and human chorionic gonadotropin according to Parlow (18). The animals were killed by cervical dislocation 5–9 days after the luteinizing treatment, and the superovulated ovaries were removed by the dorsal approach. After connective tissue had been trimmed away, the ovaries were deep frozen until required.

A preliminary report of this work has been presented to the Biochemical Society.

Abbreviations: DFP, diisopropyl fluorophosphate; "Mipafox," *N,N'*-diisopropyl phosphodiamidic fluoride; PHMB, *p*-hydroxy mercuribenzoate; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

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Homogenates were prepared in buffer with the aid of a high-speed homogenizer (Virtis 23) (rat tissue) or a glass–Teflon Potter type homogenizer (bovine tissue), except for the pH–activity experiment (when the initial homogenate was prepared in distilled water) and the centrifugal fractionation experiment (when the homogenate was prepared in 0.25 M sucrose). In the pH range 5.0–5.5, 0.1 M acetate buffers were used; above pH 5.5 the buffers were 0.1 M phosphate (12).

All homogenates were cleared of cell debris by centrifugation at 500 *g* for 10 min in the cold, and the 500 *g* supernatant fraction was used as the source of enzyme in all experiments other than the one involving fractional centrifugation.

Incubation

2 ml of tissue homogenate was mixed with 4 ml of appropriate buffer at 0°C in 25-ml conical flasks. In all experiments, except those concerned with the estimation of endogenous substrate pools, 0.04 μc of cholesteryl-4-^{14}C oleate (1.3 μg) was then added in 0.1 ml of acetone.

In the experiments concerned with estimation of endogenous substrate the radioactive cholesteryl oleate (0.06 μc; 2.11 μg) and the nonradioactive cholesteryl oleate used to reduce the specific activity were added in 0.3 ml of dimethyl formamide (17).

Unless otherwise stated, all incubations were carried out in triplicate, and the figures quoted are mean values.

Incubations were for 2 hr at 37°C. The gaseous atmosphere is indicated in the text and figures.

The incubations were terminated by the addition of acetone–ethanol 1:1 (10 ml), and protein precipitates were centrifuged down after 30 min. The supernatant solution was decanted and the precipitates were re-extracted three times with acetone–ethanol (15 ml) and finally with ether (15 ml). The five supernatant fractions were combined and evaporated to dryness on a rotary evaporator at temperatures not exceeding 50°C.

Chromatography and Estimation of Substrate Hydrolysis

The total lipid extracts were applied to thin-layer plates, 20 cm × 20 cm, spread with Silica Gel GF_{254} (Merck) 250 μm thick. The chromatograms were developed upwards in closed tanks in three successive solvent systems: petroleum ether (60–80°C); ethyl acetate–benzene 95:5; and ethyl acetate–benzene 95:5, with air drying between each development.

Standards of cholesteryl oleate, cholesterol, and progesterone were applied to the same plates as the lipid extracts and made visible after chromatography with phosphomolybdic acid spray (10% w/v in ethanol). The chromatographic standards had the following R_f values after the three developments: progesterone, 0.37; cholesterol, 0.57; cholesteryl oleate, 0.95.

The zones of silica gel corresponding to free cholesterol were removed and eluted successively with 3 ml of acetone, of ethyl acetate, and of benzene. The extracts were combined into silvered scintillation vials and the solvents evaporated off under nitrogen at temperatures below 50°C. The radioactivity associated with the cholesterol was then counted four times for 600 sec in a scintillation fluid containing 5 g of 2,5-diphenyloxazole and 50 mg of POPOP per liter of toluene, in a Nuclear Enterprises automatic liquid scintillation spectrometer (NE 8305). Counting efficiency was estimated at 89.5% and the efficiency of elution of cholesterol-^{14}C from the silica gel was consistently 99%.

Sterol-ester hydrolase activity is expressed as the percentage of the radioactivity added as cholesteryl-4-^{14}C oleate which appeared in the free cholesterol.

RESULTS

Table 1 shows that the 500 *g* supernatant fraction of homogenates of both bovine and rat corpora lutea can cause the hydrolysis of cholesteryl-4-^{14}C oleate; for both animals, the hydrolytic agent is thermolabile. The gaseous atmosphere was not a decisive factor in the production of free cholesterol-^{14}C; O_2–CO_2 95:5 was used usually but could be replaced by N_2 without altering the degree of substrate hydrolysis. Less than 5% of the recovered radioactive material was more polar than cholesterol even under the atmosphere enriched in oxygen. Fig. 1 shows that the rate of hydrolysis of the labeled substrate was constant up to at least 3 hr. All other incubations were terminated after 2 hr.

Over the pH range 5.0–8.5 two pH optima, 6.0 and 7.5, were found for bovine corpora lutea (Fig. 2). All other incubations were carried out at pH 7.5.

The cholesteryl esterase activity was inhibited by organophosphorus compounds at final concentrations of 2×10^{-4} M. Table 2 shows that both "Mipafox" and

TABLE 1 HYDROLYSIS OF CHOLESTERYL-4-^{14}C OLEATE BY HOMOGENATES OF RAT AND BOVINE CORPORA LUTEA

Tissue and Preparation*	Atmosphere	Hydrolysis
		%
Bovine	O_2–CO_2	22.8
" (boiled control)	O_2–CO_2	1.4
"	N_2	23.8
" (boiled control)	N_2	1.0
Rat	O_2–CO_2	45.9
" (boiled control)	O_2–CO_2	9.4

* Homogenates prepared from deep-frozen tissue at 250 mg/ml in 0.1 M phosphate buffer, pH 7.5, in glass–Teflon (bovine) or Virtis (rat) homogenizers. 2 ml of the 500 *g* supernate of the homogenates plus 4 ml of buffer were incubated at 37°C, pH 7.5, for 2 hr with 0.89 × 10^6 cpm cholesteryl-4-^{14}C oleate added to each flask in 0.1 ml of acetone.

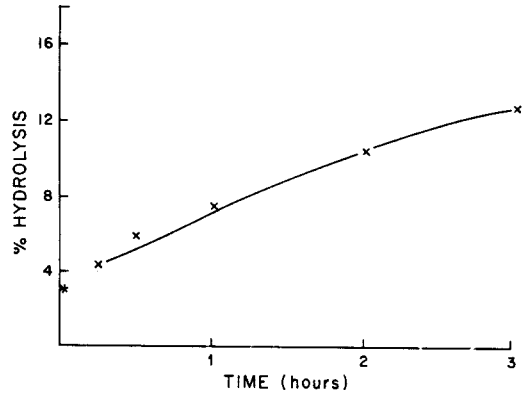

FIG. 1. Cholesteryl esterase activity of bovine corpus luteum as a function of time. 2-ml aliquots of the 500 *g* supernate of the luteal homogenate were incubated with 4 ml of buffer and 89,000 cpm of cholesteryl-4-^{14}C oleate at 37°C for the times indicated. Atmosphere O_2–CO_2 95:5.

* Boiled enzyme control.

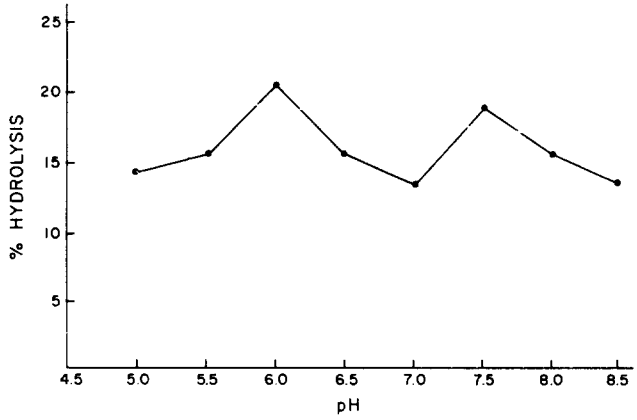

FIG. 2. Effect of pH on cholesteryl esterase activity of bovine corpus luteum. The homogenate was prepared in distilled water at 250 mg/ml; 2 ml of the 500 *g* supernate was mixed with 4 ml of buffer. Buffers used: pH 5.0–5.5, 0.1 M acetate; pH 6.0–8.5, 0.1 M phosphate. Each flask received 89,000 cpm of cholesteryl-4-^{14}C oleate in 0.1 ml of acetone. Incubation for 2 hr under O_2–CO_2 95:5 at 37°C.

DFP caused 90% inhibition of the activity when incubated with the 500 *g* supernate of a bovine luteal homogenate for 15 min before the radioactive substrate was added. Similar preincubation with PHMB did not cause appreciable inhibition.

When the radioactive cholesteryl oleate was incorporated into the lithium oleate-stabilized complex of Korzenovsky, Diller, Marshall, and Auda (19), in the absence of cholic acid, there was less hydrolysis of the ester than when the same amount of it was introduced in 0.1 ml of acetone (Table 3). The hydrolysis of the Korzenovsky-type substrate was decreased even further by the presence of cholic acid at a concentration of 0.014 M with respect to the substrate emulsion.

Preliminary experiments designed to demonstrate the subcellular localization of the enzyme showed that it was

TABLE 2 EFFECT OF ORGANOPHOSPHORUS AND ORGANOMERCURIAL INHIBITORS ON THE BOVINE LUTEAL CHOLESTERYL ESTERASE

Incubation*			Hydrolysis of Ester	Inhibition
			%	
Luteal homogenate			51.8	—
"	"	+ DFP†	6.6	87.0
"	"	+ "Mipafox"†	6.9	86.5
"	"	+ PHMB†	49.7	4.1
"	"	(boiled control)	2.4	95.0

* Bovine luteal homogenate was prepared in a glass–Teflon homogenizer at 250 mg/ml of 0.1 M phosphate buffer, pH 7.5. 2 ml of 500 *g* supernatant solution was first incubated at 37°C for 15 min with 4 ml of buffer, or 3 ml of buffer plus 1 ml of inhibitor in buffer. Then 0.1 ml of acetone, containing cholesteryl-4-^{14}C oleate (0.8×10^5 cpm), was added and the mixture was incubated for 2 hr at 37°C under O_2–CO_2 95:5.

† Present at 2×10^{-4} M in preincubation mixture.

TABLE 3 EFFECT OF MODE OF PRESENTATION OF SUBSTRATE, AND OF CHOLIC ACID, ON THE ACTIVITY OF THE BOVINE LUTEAL CHOLESTERYL ESTERASE

Luteal Homogenate	Substrate in	Hydrolysis
		%
Not boiled	Acetone*	31.4
Boiled	"	2.8
Not boiled	Oleate complex†	8.1
Not boiled	Oleate complex with cholic acid (0.014 M)	3.8
Boiled	Oleate complex	0.4

Bovine luteal homogenate was prepared in a glass–Teflon homogenizer at 250 mg/ml in 0.1 M phosphate buffer, pH 7.5. Incubations were for 2 hr at 37°C under O_2–CO_2 95:5.

* 79,000 cpm of cholesteryl-4-^{14}C oleate in 0.1 ml of acetone; 4.0 ml of buffer; and 2.0 ml of 500 *g* supernate of luteal homogenate.

† 73,000 cpm of cholesteryl-4-^{14}C oleate in 1.0 ml of lithium oleate–albumin emulsion; 3.0 ml of buffer; and 2.0 ml of 500 *g* supernate of luteal homogenate.

distributed in all the fractions of a fractional centrifugation (500 *g* pellet, 5000 *g* pellet, 100,000 *g* pellet, and 100,000 *g* supernatant fraction from 0.25 M sucrose) and that the three fractions derived from the 500 *g* supernate (5000 *g* pellet, 100,000 *g* pellet, and 100,000 *g* supernatant fractions) hydrolyzed more cholesteryl oleate than the 500 *g* supernate from which they were derived.

The experiments were then repeated according to the technique used by Ichii, Kobayashi, and Matsuba (20) to determine the size of cholesterol pools in adrenal cortex. Table 4 shows the pool sizes of available cholesteryl ester and the cholesteryl esterase activity in each of four fractions derived from two different bovine corpora lutea. In experiment 1, the diluting (nonradioactive) cholesteryl oleate was added at levels of 100 and 200 μg/incubation; in experiment 2 the diluent was added at levels of 25 and 50 μg. In both experiments the fractions taken were 500 *g* pellet, 5000 *g* pellet, 200,000 *g* pellet, and 200,000 *g* supernate.

TABLE 4 SUBCELLULAR DISTRIBUTION OF CHOLESTERYL ESTERASE AND CHOLESTERYL ESTERS IN BOVINE LUTEAL HOMOGENATE FRACTIONS

Fraction	Ester Pool Size		Ester Hydrolyzed		Mean Enzyme Distribution	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
			$\mu g/g$		%	
500 g × 10 min pellet	15	60	6.35	29.4	14	25
	20	41	7.8	20.8		
5000 g × 30 min pellet	236	137	21.0	52.8	46	43
	272	84	24.4	33.0		
200,000 g × 30 min pellet	130	39	15.7	18.1	33	16
	—	29	—	14.0		
200,000 g × 30 min supernatant fraction	9	42	2.4	20.5	7	16
	18	10	4.0	11.0		

Luteal homogenates prepared in 0.25 M sucrose (expt. 1, 175 mg of tissue per ml; expt. 2, 190 mg of tissue per ml) were centrifuged at 4°C in an M.S.E. Superspeed 50 (angle head No. 2410). Pellets were resuspended in the original volume of 0.25 M sucrose. 2 ml of tissue fraction plus 4 ml of 0.1 M phosphate buffer, pH 7.5, were incubated for 2 hr at 37°C with cholesteryl-4-^{14}C oleate (0.066 μc; 2.11 μg) with or without unlabeled cholesteryl oleate. The esters were dissolved in 0.3 ml of dimethyl formamide. Each tissue fraction received unlabeled ester as follows. Expt. 1:0, 100, or 200 μg; expt. 2:0, 25, or 50 μg.

The results for each fraction are calculated from three nonduplicated experiments.

DISCUSSION

The presence of a sterol-ester hydrolase has been demonstrated in bovine and rat corpora lutea by labeling the endogenous ester pool with a tracer quantity of cholesteryl-4-^{14}C oleate. The pH-stat technique, which we have used in this laboratory for the estimation of pancreatic cholesteryl esterase (21), proved to be too insensitive for the low levels of activity present in corpus luteum.

Cholesteryl oleate was chosen as substrate on the basis of Shackleton's observation (3) that oleic acid was a major constituent of the fatty acid esterified to cholesterol in bovine corpus luteum. There are many precedents for our use of superovulated rat ovaries as rat corpora lutea (7, 22–26).

Table 1 and Fig. 1 show that deep-frozen bovine or rat corpora lutea can be used to prepare a 500 g supernatant fraction which contains a thermolabile cholesteryl esterase activity. Whether the atmosphere is aerobic or anaerobic makes no difference to the production of free cholesterol, which indicates that there is little oxidative breakdown of the cholesterol under our conditions. Only 5% of the ^{14}C added as cholesteryl oleate appeared in fractions more polar than cholesterol.

The double-peaked pH–activity curve (Fig. 2) suggests that the bovine preparation may contain more than one sterol-ester hydrolase; this possibility gains strength from the presence of hydrolase in all particulate and supernatant fractions of the homogenate (Table 4). Although two buffer systems were used (acetate for pH 5.0–5.5 and phosphate for 6.0 and above), both pH optima were found in the phosphate system; they are not the result of changing from one buffer system to another.

Some differences in behavior of the bovine luteal enzyme and the bovine pancreatic enzyme have emerged: the pancreatic enzyme was most active in the presence of substrate incorporated into a lithium oleate–albumin complex (21), and was activated by cholic acid (27), whereas Table 3 shows that the luteal enzyme was less active when the substrate was presented as the lithium oleate–albumin complex, and that this activity was decreased in the presence of cholic acid at a concentration optimal for the pancreatic enzyme. The luteal esterase, moreover, was not inhibited by PHMB but was inhibited by organophosphate compounds (Table 2). Murthy and Ganguly (28) have shown organophosphorus inhibition of pancreatic cholesteryl esterase and have confirmed the report of Hernandez and Chaikoff (29) that organomercurials are also inhibitory.

All of the results discussed above have been obtained by estimating the percentage breakdown of labeled substrate and have been expresssed only in terms of the radioactivity found in the free cholesterol fraction. Thus what absolute activity (in terms of μmoles of substrate hydrolyzed) is present is not known.

Ichii et al. (20) have used an isotope dilution method to estimate the size of available cholesterol pools in adrenal tissue before and after ACTH stimulation. The expression they used is,

$$P = [C_a(A_r + A_c) - C_o \cdot A_r]/(C_o - C_a)$$

where P = endogenous pool (μg), C_a = cpm in product in the presence of "cold" dilution, C_o = cpm in product in the absence of "cold" dilution, Ar = μg of ^{14}C substrate, and Ac = μg of ^{12}C substrate ("cold" diluent).

Application of this approach to determine the available endogenous cholesteryl ester pools in various tissue subfractions also allowed a calculation to be made of the absolute amount of substrate hydrolyzed during the in vitro incubation.

Table 4 shows that all fractions of the homogenate were able to hydrolyze the labeled substrate and that almost 50% of the enzyme activity was present in the 5000 *g* pellet. The sterol-ester hydrolase of bovine corpus luteum is capable of hydrolyzing up to about 120 μg of cholesteryl ester per g of tissue during 2-hr incubations at 37° C.

The maximum amounts of substrate available were 440 μg/g of tissue in experiment 1 and 278 μg/g of tissue in experiment 2. Zimbelman, Loy, and Casida (4) have found 260 μg of cholesteryl ester/g of bovine corpus luteum at day 14 of the estrous cycle and Shackleton (3) found 720–1740 μg/g of the same tissue. If the former figures are correct, then all of the sterol ester present is available for hydrolysis, whereas if Shackleton's figures hold, only 25% of the ester is readily hydrolyzed. Rat corpus luteum contains much more esterified cholesterol than does bovine (8), but preliminary investigations have shown that a 30% hydrolysis of radioactive tracer was not accompanied by any major change in the total sterol ester during in vitro hydrolysis (as estimated by Liebermann–Burchard reaction before and after hydrolysis), indicating that the endogenous pool of substrate cholesteryl ester must be fairly small (30). The size of ester pools in the rat is currently being investigated by the technique of Ichii et al. (20).

Future work will be directed toward the possible action of luteinizing hormone on cholesteryl esterase activity and on cholesteryl ester pool sizes. This is of interest not only because cholesteryl esters may act as reserves of steroid hormone precursors but also because they inhibit cleavage of the side chain of free cholesterol (31) and might, therefore, be the means of controlling this cleavage under the influence of luteinizing hormone.

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References

1. Bloor, W. R., P. Okey, and G. W. Corner. 1930. *J. Biol Chem.* **86:** 291.
2. Everett, J. W. 1945. *Am. J. Anat.* **77:** 293.
3. Shackleton, C. H. L. 1964. B.Sc. (Hons.) Thesis. University of St. Andrews, Scotland.
4. Zimbelman, R. G., R. G. Loy, and L. E. Casida. 1961. *J. Animal Sci.* **20:** 99.
5. Boyd, E. M. 1935. *J. Biol. Chem.* **108:** 607.
6. Conn, J. W., W. C. Vogel, L. H. Louis, and S. S. Fajans. 1950. *J. Lab. Clin. Med.* **35:** 504.
7. Armstrong, D. T., and R. O. Greep. 1965. *Endocrinology.* **76:** 246.
8. Major, P. W., D. T. Armstrong, and R. O. Greep. 1967. *Endocrinology.* **81:** 19.
9. Claesson, L., N. A. Hillarp, B. Hogberg, and B. Hokfelt. 1949. *Acta Endocrinol.*, **2:** 249.
10. Solod, E. A., D. T. Armstrong, and R. O. Greep. 1966. *Steroids.* **7:** 607.
11. Sayers, G., M. A. Sayers, E. G. Fry, A. White, and C. N. H. Long. 1944. *Yale J. Biol. Med.* **16:** 361.
12. Dailey, R. E., L. Swell, and C. R. Treadwell. 1963. *Arch. Biochem. Biophys.* **100:** 360.
13. Brot, N., W. J. Lossow, and I. L. Chaikoff. 1963. *Proc. Soc. Exptl. Biol. Med.* **114:** 786.
14. Shyamala, G., W. J. Lossow, and I. L. Chaikoff. 1966. *Biochim. Biophys. Acta.* **116:** 543.
15. Longcope, C., and R. H. Williams. 1963. *Proc. Soc. Exptl. Biol. Med.* **113:** 754.
16. Dailey, R. E., L. Swell, and C. R. Treadwell. 1962. *Arch. Biochem. Biophys.* **99:** 334.
17. Fieser, L. F. 1955. Experiments in Organic Chemistry. 3rd edition. D. C. Heath and Company, Boston.
18. Parlow, A. F. 1958. *Federation Proc.* **17:** 402.
19. Korzenovsky, M., E. R. Diller, A. C. Marshall, and B. M. Auda. 1960. *Biochem. J.* **76:** 238.
20. Ichii, S., S. Kobayashi, and M. Matsuba. 1965. *Steroids.* **5:** 663.
21. Coutts, J. R. T., and D. A. Stansfield. 1967. *Biochem. J.* **103:** 55P.
22. Armstrong, D. T. 1962. *Endocrinology.* **72:** 908.
23. Armstrong, D. T., J. O'Brien, and R. O. Greep. 1964. *Endocrinology.* **75:** 488.
24. Huang, W. Y., and W. H. Pearlman. 1962. *J. Biol. Chem.* **237:** 1060.
25. Stansfield, D. A., and J. W. Robinson. 1965. *Endocrinology.* **76:** 390.
26. Stansfield, D. A., and A. P. Flint. 1967. *J. Endocrinol.* **39:** 27.
27. Coutts, J. R. T., and D. A. Stansfield. 1967. *Biochem. J.* **104:** 27P.
28. Murthy, S. K., and J. Ganguly. 1962. *Biochem. J.* **83:** 460.
29. Hernandez, H. H., and I. L. Chaikoff. 1957. *J. Biol. Chem.* **228:** 447.
30. Franks, D. J. 1967. B.Sc. (Hons.) Thesis. University of St. Andrews, Scotland.
31. Raggatt, P. R., and M. W. Whitehouse. 1966. *Biochem. J.* **101:** 819.