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Inhibition of Sterol and DNA Syntheses in Phytohemagglutinin-Stimulated Human Lymphocytes by 7α-Hydroxycholesterol

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HEMMI, H., KITAME, F. and ISHIDA, N. *Inhibition of Sterol and DNA Syntheses in Phytohemagglutinin-Stimulated Human Lymphocytes by 7α-Hydroxycholesterol.* Tohoku J. exp. Med., 1985, **145**(2), 105-113 —— Sterol and DNA syntheses were induced in human peripheral blood lymphocytes stimulated with phytohemagglutinin (PHA). DNA synthesis in the PHA-stimulated lymphocytes was suppressed by 7α-hydroxycholesterol (7α-HC). The maximum suppression of DNA synthesis was observed when 7α-HC was added in the culture within 6 hr of PHA stimulation to the lymphocytes. However, as the concentration of fetal bovine serum (FBS) in the culture medium was increased, the inhibitory effect of 7α-HC on the syntheses of sterol and DNA were decreased. Furthermore, low and high density lipoproteins completely reversed the inhibition of DNA synthesis by 7α-HC. These results suggest that cholesterol is an essential requirement of lymphocyte blastogenesis regardless of whether the source of the sterol is exogenous or endogenous. ———— lymphocyte blastogenesis; 7α-hydroxycholesterol; sterol synthesis; DNA synthesis; lipoproteins

The activation of sequential metabolic pathways in lymphocytes is stimulated by phytohemagglutinin (PHA), concanavalin A, and lipopolysaccarides (Ling and Kay 1975). Sterol and DNA synthesis during blastogenesis of lymphocytes are suppressed by the addition of 25-hydroxycholesterol and 20α-hydroxycholesterol (Chen et al. 1975; Pratt et al. 1977). Oxygenated cholesterol derivatives such as 7α-hydroxycholesterol (7α-HC), 7β-hydroxycholesterol, 7-ketocholesterol, 20α-hydroxycholesterol, and 25-hydroxycholesterol regulate sterol synthesis in normal and malignant mammalian cell lines at the site of the reaction catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG

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This work was supported in part by a grant from the Ministry of Health and welfare, Japan. Abbreviations used are: 7α-HC, 7α-hydroxycholesterol; PHA, phytohemagglutinin; FBS, fetal calf serum; LDL, low density lipoprotein; HDL, high density lipoprotein.

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CoA reductase ; EC 1. 1. 1. 34 ; Kandutsch and Chen 1973, 1977 ; Bell et al. 1976 ; Pratt et al. 1977 ; Kandutsch et al. 1978). However, the inhibitory effect of these sterols on HMG CoA reductase activity is not mediated by low density lipoprotein (LDL)-receptor system which is the feedback regulative mechanism of cholesterol synthesis on the cell surface in normal human cells and established malignant human cell lines (Brown and Goldstein 1976 ; Ho et al. 1977). Recently we reported that three 7-position oxygenated cholesterols isolated from the Cohn fraction IV-I of normal human sera suppressed human lymphocyte proliferation and prolonged the rejection time of rat allogenic skin graft. Moreover, the concentrations of 7-ketocholesterol in the sera of cancer patients are 2 to 3 times higher than in normal sera (Saito et al. 1983).

In this study, the relationship between sterol and DNA syntheses in PHA-stimulated human peripheral blood lymphocytes was studied using inhibitors of sterol synthesis such as 7α-HC, citrinin, clofibrate, and chlofibric acid. These inhibitors of blastogenesis were also examined under culture conditions of various cholesterol contents.

Materials and Methods

Materials

The [methyl-^{3}H]-thymidine (50 Ci/mmol) and [2-^{14}C] -acetic acid sodium salt (54 mCi/mmol) were purchased from New England Nuclear (Boston, MA, USA). Clofibrate [ethyl-2-(*p*-chlorophenoxy)-2-methylpropionate] and citrinin were donated by Dr. A. Endo (Fermentation Research Lab., Sankyo Co., Ltd., Tokyo). Cholesterol and heamatoporphyrin dihydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium borohydride and antilipoprotein sera were obtained from Nakarai Chemical Co. (Kyoto) and Hoechst Co. (Tokyo), respectively. All other chemicals used were described in a previous paper (Hemmi et al. 1979).

Preparations of chemicals

Clofibric acid [2-(*p*-chlorophenoxy)-2-methyl propionic acid ; m.p. 118-119°C] was derived from clofibrate by hydrolysis in 2N HCl-ethanol. The resultant compound was detected as a single spot by thin layer chromatography.

7α-HC was synthesized from cholesterol in our laboratory according to the method of Mitropoulos and Balasubramanian (1972). Cholesterol in pyridine was photo-oxygenated in the presence of heamatoporphyrin dihydrochloride, the resultant 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide was isolated from the reaction mixture and recrystallized with ethyl acetate-petroleum ether (m.p. 146 - 149°C). Crystallized 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide was then dissolved in chloroform and left at room temperature for 24 hr to isomerize to 3β-hydroxycholest-5-ine-7α-hydroperoxide (m.p. 153-155°C). The isomerized peroxide was crystallized, redissolved in methanol and reduced with sodium-borohydride to 7α-hydroxycholesterol (m.p. 182-184°C), which was then recrystallized from the aqueous methanol. The intermediate compounds and the final product were checked by thin layer chromatography on Kieselgel 60 F 254 (Merck, Art. 5517).

Lymphocyte preparation and stimulation

Human peripheral blood lymphocytes were obtained from healthy volunteers. The lymphocytes were isolated from heparinized blood using the method of gelatin sedimenta-

tion (Saito et al. 1975). PHA-stimulated lymphocyte blastformation was estimated in microplates as described previously (Hemmi et al. 1979). The culture medium of RPMI 1640 was supplemented with 20% fetal bovine serum (FBS).

Preparation of chylomicron, LDL, and HDL

Chylomicron ($d<1.006$ g/ml), LDL ($d=1.006$-1.073 g/ml) and high density lipoproteins (HDL; HDL_2 and HDL_3; $d=1.073$-1.21 g/ml) were isolated from fresh pooled human plasma by preparative ultracentrifugation according to standard techniques (Alaupovic et al. 1972). Each preparation was standardized against normal human sera by acetate-cellulose electrophoresis and the bands were identified by the immunochemical method using anti-α-lipoprotein and anti-β-lipoprotein sera. The amount of protein and cholesterol was determined by the method of Lowry et al. (1951) and with the Total Cholesterol Measurement Kit (Wako, Code 274-46401), respectively.

Preparation of lipoprotein-deficient FBS

Lipoprotein-deficient fetal bovine serum ($d>1.21$ g/ml) was prepared by ultrafiltration (Fogelman et al. 1977). No cholesterol was detected in this preparation.

Assay for sterol synthesis

The sterol synthetic capacity of PHA-stimulated lymphocytes was measured by the digitonin-precipitative method (Knaus et al. 1959; Chen et al. 1975). PHA-stimulated lymphocytes in the culture medium were cultured in a glass test tube with a Molton cap. Two hours before the end of incubation, [^{14}C]-acetate (10 μCi) was added to the culture. After the addition of concentrated H_2SO_4, the culture was saponified with 95% KOH and was heated at 120°C for 60 min under 2 atmospheres of pressure. After cooling, nonsaponified compounds were extracted with three 5-ml portions of petroleum ether. The combined extracts of each sample were dryed by blowing nitrogen gas across the samples. The residue was dissolved in acetone-diethyl ether (1 : 1) and 0.5% digitonin in 50% ethanol was added to the acidified solution containing acetic acid. The acidified solution was left at room temperature for 24 hr and the resulting precipitate was collected on a glass-wool filter. The trapped radioactivity of the filter was measured by a liquid scintillation system described previously (Hemmi et al. 1979).

Results

Inhibition of DNA synthesis by sterol synthesis inhibitors in PHA-stimulated lymphocytes

When 7α-HC, citrinin, clofibrate, and clofibric acid, were added at the initiation of the culture, PHA-induced DNA synthesis was strongly inhibited. 7α-HC was the most potent inhibitor (Fig. 1). The fifty percent inhibitory doses were approximately 1.5, 7, 60 and 100 μg/ml for 7α-HC, citrinin, clofibrate, and clofibric acid, respectively. The inhibitors were not cytotoxic at the doses used as examined by trypan blue dye exclusion at the end of 36 hr of culture (data not shown), indicating that the inhibition of thymidine uptake is not secondary to cytotoxic effects of the inhibitors.

Sterol and DNA synthesis in PHA-stimulated lymphocytes

After the addition of PHA to the lymphocyte cultures, the rate of sterol synthesis in the blast cells had increased at 6 hr, reaching a maximum by 24 hr,

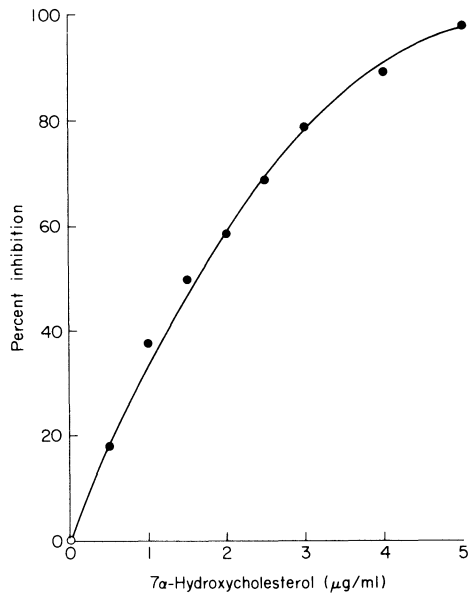

Fig. 1. Inhibition of DNA synthesis in PHA-stimulated lymphocytes by 7α-hydroxychloesterol.
[^{3}H]-Thymidine (0.02 μCi/culture) was added to the lymphocyte cultures (2×10^5 cells/culture) with PHA 10 hr before the end of incubation as described in Materials and Methods.

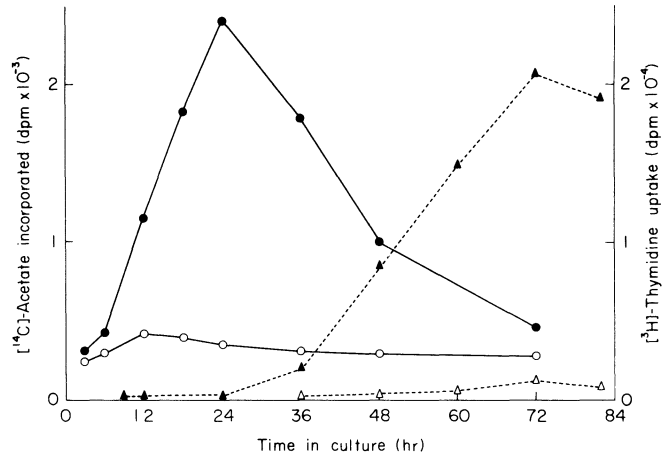

Fig. 2. Time course of PHA-induced syntheses of sterol and DNA
[^{14}C]-Acetate (1 μCi) and [^{3}H]-thymidine (5 μCi) were added to each culture (1×10^6cells/culture) 2 hr before the end of cultivation. Sterol synthesis rate was estimated by the incorporation rate of [^{14}C]-acetate into the digitonin-precipitative fraction (cultures with PHA, ●; without PHA, ○). DNA synthesis was determined by the rate of [^{3}H]-thymidine uptake in the lymphocyte cultures with PHA (▲) and without PHA (△).

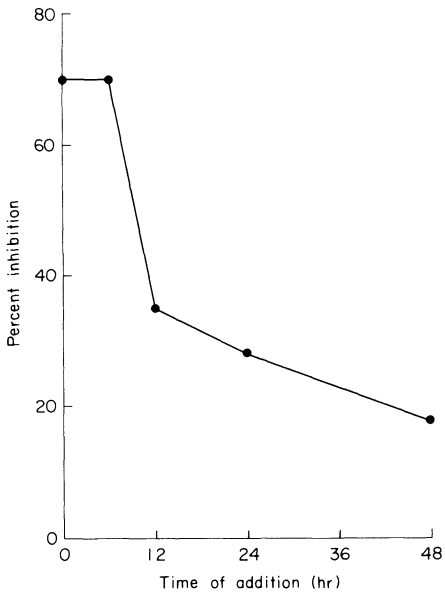

Fig. 3. Time of addition of 7α-hydroxycholesterol to PHA-stimulated lymphocyte cultures.
At indicated times, 7α-hydroxycholesterol (3 μg/ml) was added to the cultures (2×10^5cells/culture) with PHA. Thymidine uptake was estimated as described in Fig. 1 and Materials and Methods.

and then decreased steadily to 72 hr (Fig. 2). When radioactive acetate incorporation into digitonin-precipitable sterols was compared, five times as much radioactive acetate incorporation occured at 24 hr in PHA-stimulated lymphocytes than in the unstimulated controls. The incorporation of radioactive thymidine into activated cells was first detected 36 hr after the addition of PHA, at which time sterol synthesis had already begun to decrease. The rate of DNA synthesis increased steadily by 72 hr and then declined gradually (Fig. 2).

Time of addition of 7α-hydroxycholesterol determines DNA synthesis

DNA synthesis of lymphocytes was determined after the addition of 7α-HC (3 μg/ml) to the culture at different times throughout the course of a 72-hr incubation period (Fig. 3). When the inhibitor was added at zero time, almost 70% inhibition was obtained. When the inhibitor was added 12 hr after the addition of PHA, the percentage of inhibition was decreased. These results suggest that the degree of sterol synthesis determines the subsequent amount of DNA synthesis in these lymphocytes. DNA synthesis was markedly reduced when the inhibitor was added prior to the initiation of sterol synthesis (Fig. 3).

FBS and cholesterol suppress the inhibition of sterol and DNA syntheses by 7α-hydroxycholesterol

In the culture medium containing 5 or 20% FBS, 7α-HC inhibited sterol synthesis (Table 1). As the FBS concentration increased, inhibition of DNA synthesis decreased (data not shown). In a culture medium composed of 50% FBS and RPMI 1640, sterol synthesis was inhibited to about 50% of the control, while no significant inhibition of DNA synthesis was detected (Table 1). However, clofibrate inhibition of sterol and DNA synthesis was independent of FBS concentration. The mode of action of these two inhibitors on lymphocyte blastogenesis might be different. These observations suggest that sterol synthesis is an important component of blastogenesis. PHA-stimulated lymphocytes inhibited by 7α-HC respond to cholesterol in the medium by undergoing blastotransformation. To determine the effectiveness of the cholesterol carrier, lipoprotein fractions, chylomicron, and cholesterol were added to lipoprotein-free FBS medium. With a constant cholesterol concentration (21 μg/ml), the inhibitory activity of 7α-HC was completely suppressed by the addition of LDL and HDL (Table 2). Chylomicron and free cholesterol resulting in 50% of inhibition were comparably ineffective to 20% FBS. These findings suggest that both LDL and HDL are unique in restoring the blastogenic activity of lymphocytes even when sterol synthesis has been inhibited by 7α-HC.

TABLE 1. *Effect of FBS concentration on inhibitory activity of 7α-hydroxycholesterol in PHA-stimulated lymphocyte cultures*

FBS (%)	7α-Hydroxycholesterol (μg/ml)	Sterol synthesis	DNA synthesis
		dpm[a] (%)	dpm[b] (%)
5	None	2,064±106 (100)	13,800± 804 (100)
5	1.5	68± 7 (3)	4,981± 259 (36)
20	None	2,213± 81 (100)	16,112±1,154 (100)
20	1.5	73± 5 (3)	8,265± 692 (51)
50	None	1,324± 69 (100)	11,040± 573 (100)
50	1.5	634± 15 (48)	9,991± 109 (90)

[a]Each number is the mean of 3 experiments±S.E.; Experimental procedure was performed as described in "Materials and Method" and Fig. 2.

[b]Each number is the mean of 6 experiments±S.E.; [^{3}H]-thymidine (0.02 μCi) was added to each culture (2×10^5 cells) 10 hr before the end of cultivation.

TABLE 2. *Effects of serum lipoprotein on inhibitory activity of 7α-Hydroxycholesterol in PHA-induced DNA synthesis*

Condition	Cholesterol content (μg/ml)	7α-Hydroxy-cholesterol (μg/ml)	Thymidine uptake dpm[c] (%)
20% FCS	21	None	14,000±1,443 (100)
		1.5	6,860± 692 (49)
20% Lipoprotein-free FCS	0	None	10,400±1,358 (100)
		1.5	1,872± 107 (18)
Plus: Chylomicron[a]	21	None	8,376± 404 (100)
		1.5	4,531± 225 (54)
LDL[a]	21	None	8,527± 404 (100)
		1.5	8,612± 355 (101)
HDL[a]	21	None	7,087± 664 (100)
		1.5	7,444± 520 (105)
Cholesterol[b]	21	None	9,078± 404 (100)
		1.5	4,266± 309 (47)
	42	None	11,255± 779 (100)
		1.5	9,870± 923 (88)

[a]The amount of cholesterol/protein (w/w) used in these preparations was 0.12 (chylomicron), 1.42 (LDL) and 0.59 (HDL).

[b]Cholesterol (5 mg/ml) was suspended with culture medium containing 1% bovine serum albumin by sonication, and the solution diluted by culture medium was added to the cultures.

[c]Each number is the mean of 3 experiments±S.E.

DISCUSSION

Our observation demonstrated that cholesterol, whether it is supplied from *de novo* synthesis of sterol or from circumstance by LDL or HDL, is an essential requirement for blastogenesis of lymphocytes. Brown et al. (1975) reported that growth inhibition of human fibroblasts by 7-ketocholesterol was reversed by the addition of free cholesterol, LDL-cholesterol, and HDL-cholesterol. Furthermore, concanavalin-A induced lymphocyte blastogenesis inhibited by 7-ketocholesterol was reversed by liposomes containing cholesterol (Ip et al. 1980). Oxygenated cholesterol derivatives including 7α-HC bind to all density classes of human serum lipoproteins in the incubation media (Streuli et al. 1981). This lead us to speculate that recovery from 7α-HC inhibition of blastogenesis by LDL and HDL simply occurs by deletion of free 7α-HC in the culture medium and if

lipoprotein-bound 7α-HC shows no longer inhibitory activity. But this possibility is unlikely for the following reasons; First, chylomicron can also bind to 7α-HC, but showed no ability to recover in our results (Table 2). Secondly, oxygenated cholesterol-bound LDL is supposed to be incorporated by cells more efficiently and rapidly than the free form via specific cell surface receptors of lymphocytes (Brown et al. 1975; Ho et al. 1979).

LDL and HDL showed the ability to suppress PHA-induced DNA synthesis in lymphocytes cultured in lipoprotein-free serum (Table 2). It has been reported that immunoregulatory LDL (LDL-In) shows immunosuppressive activity in vitro and in vivo (Curtiss and Edgington 1976; Curtiss et al. 1977). The inhibitory mechanism of PHA-induced DNA synthesis by LDL-In differs from that by 25-hydroxycholesterol (Curtiss and Edgington 1980). It is not clear whether the preparations of lipoproteins used here possess some special activity of immunosuppression like LDL-In.

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References

1) Alaupovic, P., Lee, D.M. & McConathy, W.J. (1972) Studies on the composition and structure of plasma lipoproteins: Distribution of lipoprotein families in major density classes of normal human plasma lipoproteins. *Biochem. Biophys. Acta*, **260**, 689-707.
2) Bell, J.J., Sargeant, T.T. & Watson, J.A. (1976) Inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in hepatoma tissue culture cells by pure cholesterol and several cholesterol derivatives: Evidence supporting two distinct mechanisms. *J. Biol. Chem.*, **251**, 1745-1758.
3) Brown, M.S. & Goldstein, J.L. (1976) Receptor-mediated control of cholesterol metabolism: Study of human mutants has disclosed how cells regulate a substance that is both vital and lethal. *Science*, **191**, 150-154.
4) Brown, M.S., Faust, J.R. & Goldstein, J.L. (1975) Role of low density lipoprotein receptor in regulating the content of free and esterified cholesterol in human fibroblasts. *J. Clin. Invest.*, **55**, 783-793.
5) Chen, H.W., Heiniger, H.-J. & Kandutsch, A.A. (1975) Relationship between sterol synthesis and DNA synthesis in phytohemagglutinin-stimulated mouse lymphocytes. *Proc. Natl. Acad. Sci. (USA)*, **72**, 1950-1954.
6) Curtiss, L.K. & Edgington, T.S. (1976) Regulatory serum lipoproteins: Regulation of lymphocyte stimulation by a species of low density lipoprotein. *J. Immunol.*, **116**, 1452-1458.
7) Curtiss, L.K. & Edgington, T.S. (1980) Differences in the characteristics of inhibition of lymphocyte stimulation by 25-hydroxychloesterol and by the immunoregulatory serum lipoprotein LDL-In. *J. Immunol.*, **125**, 1470-1474.
8) Curtiss, L.K., DeHeer, D.H. & Edgington, T.S. (1977) *In Vivo* Suppression of the primary immune response by a species of low density serum lipoprotein. *J. Immunol.*, **118**, 648-652.

9) Fogelman, A.L., Seager, J., Edwards, P.A. & Popjak, G. (1977) Mechanism of induction of 3-hydroxy-3-methylglutaryl coenzyme A reductase in human leukocytes. *J. Biol. Chem.*, **252**, 644-651.
10) Hemmi, H., Kitame, F., Ishida, N., Kusano, G. & Nosoe, S. (1979) Inhibition of thymidine transport into phytohemagglutinin-stimulated lymphocytes by triterpenoids from *Cimicifuga* species. *J. Pharm. Dyn.*, **2**, 339-349.
11) Ho, Y.K., Faust, J.R., Bilheimer, D.W., Brown, M.S. & Goldstein, J.L. (1977) Regulation of cholesterol synthesis by low density lipoprotein in isolated human lymphocytes: Comparison of cells from normal subjects and patients with homozygous familial hypercholesterolemia and abetalipoproteinemia. *J. Exp. Med.*, **145**, 1531-1549.
12) Ip, S.H.C., Abrahm, J. & Cooper, R.A. (1980) Enhancement of blastogenesis in cholesterol-enhanced lymphocytes. *J. Immunol.*, **124**, 87-93.
13) Kandutsch, A.A. & Chen, H.W. (1973) Inhibition of sterol synthesis in cultured mouse cells by 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 7-ketocholesterol. *J. Biol. Chem.*, **248**, 8408-8417.
14) Kandutsch, A.A. & Chen, H.W. (1977) Consequences of blocked sterol synthesis in cultured cells: DNA synthesis and membrane composition. *J. Biol. Chem.*, **252**, 409-415.
15) Kandutsch, A.A., Chen, H.W. & Heiniger, H.-J. (1978) Biological activity of some oxygenated sterols. *Science*, **201**, 498-501.
16) Knaus, H.J., Porter, J.W. & Wasson, G. (1959) The biosynthesis of mevalonic acid from 1-^{14} C-acetate by a rat liver enzyme system. *J. Biol. Chem.*, **234**, 2835-2840.
17) Ling., N.R. & Kay, J.E. (1975) "Lymphocyte stimulation". North-Holland Publication Co., Amsterdam-Oxford
18) Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265-275.
19) Mitropoulos, K.A. & Balasubramaniam, S. (1972) Cholesterol 7-hydroxylase in rat liver microsomal preparations. *Biochem. J.*, **128**, 1-9.
20) Pratt, H.P.M., Fitzgerald, P.A. & Saxon, A. (1977) Synthesis of sterol and phospholipid induced by the interaction of phytohemagglutinin and other mitogens with human lymphocytes and their relation to blastogenesis and DNA synthesis. *Cell. Immunol.*, **32**, 160-170.
21) Satio, H., Kitame, F., Uemura, Y. & Ishida, N. (1983) The regulating effect of cholesterol derivatives isolated from human sera on lymphocyte response to phytohemagglutinin. *Tohoku J. exp. Med.*, **140**, 245-258.
22) Saito, T., Takada, M. & Ishida, N. (1975) Inhibition of PHA-induced lymphocyte stimulation by factors in maternal serum during late pregnancy. *Tohoku J. exp. Med.*, **116**, 77-80.
23) Streuli, R.A., Chung, J., Scanu, A.M. & Yachnin, S. (1981) Serum lipoproteins modulate oxygenated sterol insertion into human red cell membranes. *Science*, **212**, 1294-1296.