systemic arterial pressure. The multiple hemodynamic changes induced by the three prostaglandins are caused, not only by their direct effect on the peripheral vasculatures, but also by their effects on the myocardium.

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Regulation of Cell Lipid Metabolism and Accumulation. VII. Increase by Glycerol of the Polar Lipid and Triglyceride Content of Cultured Cells* (32938)

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The discovery of Polge *et al.* (1) that spermatozoa are preserved by freezing in 5% glycerol was extended to cultured mammalian cells in 1954 by Scherer and Hoogasian (2). They found that mouse fibroblast (L) and HeLa cells suspended in a glycerol–serum solution remained viable for months when frozen either rapidly or slowly and stored at —70°C. Subsequently, Stulberg, *et al.* (3) reported that human fibroblast cells were killed by rapid freezing but survived when the temperature was reduced in a programmed apparatus at 1°C per min.

Several years ago we noticed the presence of perinuclear particles in the cytoplasm of cultured mammalian cells that have been subjected to either the rapid or slow freezing procedure in glycerol (3). Although the particles can be detected in suspended cells immediately after thawing, they are more conspicuous after the cells have attached and spread out on a glass surface. In living cells viewed under the phase contrast microscope, the particles appear as dark bodies ranging from 0.3 to 1.0 μ in diameter. In fixed cells, the particles stain brilliantly with lipid soluble dyes such as oil red O.

The formation of these lipophilic particles is unrelated to the time of cell storage at —80° C, and they disappear after several cell divisions in glycerol-free medium. This latter observation led us to test the effect of glycerol on unfrozen cells and it was found that the addition of glycerol to a medium containing horse serum caused the formation of lipophilic particles in less than 24 hours. Furthermore, incorporation of the higher and lower homologues of glycerol in the medium had a

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similar effect. This paper reports the lipid analysis of human liver (Chang) and mouse fibroblast (L) cells exposed to glycerol, ethylene glycol, and erythritol.

Methods. The basal medium consisted of our modification of Eagle's medium (4) plus 10% horse serum (Microbiological Associates, Inc., Bethesda, Md.). Experimental media were made up by substituting aqueous solutions of the polyhydric alcohols for an equal volume of water in the basal medium. All media were sterilized by filtering through a 03 porosity Selas candle (Selas Flotronics, Spring House, Pa.). Cells were plated in eight or ten 6-cm Petri dishes containing 5 ml of basal media, pH 7.4, and grown at 37.5°C in a constant atmosphere of 5% CO_2 in air (4). The number of cells added per dish was calculated to yield approximately 100 μg of cell protein after 18 hours. At that time, the zero time of the experiment, cell protein was measured in two dishes, and the remaining dishes were divided into two groups, one of which received fresh basal medium and the other an experimental medium. Both media were changed daily throughout the experiment.

At the end of the experiment, the cells were washed free of medium and their total lipid was quantitatively extracted and weighed to $\pm$ 5 μg by the procedure described previously (5). Protein was measured in the extracted cell residue by the method of Oyama and Eagle (6).This procedure gives protein values that agree with those obtained on unextracted cells (5). The isolated cell lipid was separated into five fractions on a column of silicic acid by the procedure of Barron and Hanahan (7) as modified and adapted to microquantities of material (5).

The human liver cell of Chang (8) was obtained from Microbiological Associates, Inc. The mouse fibroblast cell, clone L of strain NCTC 2071 (9), was kindly supplied by Dr. Virginia Evans (National Cancer Institute). Erythritol, ribotol, and sorbitol were obtained from Calbiochem, Los Angeles, Calif. The glycerol was Mallinckrodt glycerine, analytical reagent, and the ethylene glycol, Baker analyzed reagent.

Results. Increase in total cell lipid by glycerol. As shown in Table I, the addition of 5% glycerol to the basal medium almost doubled the μg of cell lipid per μg of cell protein in the mouse fibroblast (L) cell in 3 days. The difference in the mean values of the lipid to protein ratios was highly significant as shown by the t test (10). The calculated value of t is 21 as compared with the value of 3 required for $p<0.01$ at 15 degrees of freedom. Since an increase in the cell lipid to protein ratio can be due either to an increase in lipid or a decrease in protein, the mean protein content of cells grown in the presence and absence of glycerol was determined. The former cells contained 4.6×10^{-4} μg of protein per cell and the latter cells 3.8×10^{-4} μg of protein per cell. Consequently, the increase in the lipid to protein ratio caused by glycerol was not due to a decrease in cell protein, but to a true increase in the lipid content of the cells. Furthermore, acidification of the medium (due to the metabolic production of acid) was eliminated as a cause of the lipid increase (11) because the external pH was 7.3 for both groups of cells at the end of the experiment.

TABLE I. Increase in Cell Lipid Caused by Glycerol.[a]

Glycerol in medium (%)	(μg of cell lipid/μg of cell protein)	
	Mouse fibroblast (L)	Human liver (Chang)
0	0.167 $\pm$ 0.003[b]	0.188 $\pm$ 0.003
2.5		0.214 $\pm$ 0.002
5	0.323 $\pm$ 0.007	0.266 $\pm$ 0.002
8	0.404 $\pm$ 0.012	dead

[a] Cells were grown for 3 days in medium containing 10% horse serum.

[b] SE of the mean.

Although growth, as measured by cell protein, was reduced by glycerol, it was by no means abolished. Thus, cell protein was increased 5.3-fold over a 3-day period in the presence of glycerol. Moreover, many of the cells contained mitotic figures at the end of this time.

Human liver cells (Chang) exposed to glycerol also showed an increase in their lipid content (Table I). Though smaller than the

TABLE II. Silicic Acid Column Chromatography of Lipid of Cells Grown in Presence of Glycerol.[a]

Lipid fraction	Mouse fibroblast (L)			Human liver (Chang)	
	Control	5% glycerol	8% glycerol	Control	5% glycerol
Total lipid	0.169	0.276	0.430	0.183	0.261
Cholesterol esters plus hydrocarbons	0.003	0.006	0.018	0.004	0.010
Triglycerides	0.008	0.052	0.056	0.003	0.037
Free cholesterol plus diglycerides	0.016	0.022	0.031	0.021	0.021
Monoglycerides	0.002	0.008	0.008	0.009	0.003
Polar lipids	0.139	0.188	0.310	0.142	0.195

[a] Results are expressed as μg of lipid in fraction per μg of total cell protein. Fractions listed in the order of their emergence from the column. Recovery of the applied lipid ranged from 98 to 102%.

TABLE III. Increase in Cell Lipid Caused by Polyhydric Alcohols.[a]

Alcohol	Mouse fibroblast (L)		Human liver (Chang)	
	μg cell lipid/ μg cell protein	Increase in cell protein[b]	μg cell lipid/ μg cell protein	Increase in cell protein[b]
None	0.168	9.2	0.180	8.0
Ethylene glycol	0.218	3.7	0.213	3.2
Glycerol	0.320	4.5	0.258	4.0
Erythritol	0.449	2.3	0.293	2.0
Ribitol	lipophilic particles[c]	0		
D-Sorbitol	dead	0		

[a] Cells grown for 3 days in medium containing alcohols at the molar equivalent of 5% glycerol.

[b] Final cell protein divided by initial cell protein.

[c] The few cells alive after 3 days contained many cytoplasmic particles that stained with oil red O.

increase observed with the mouse fibroblast cell, it was nevertheless significant at the 0.01 level for both 2.5 and 5.0% glycerol. Increasing the glycerol to 8% killed the liver cells in 2 days.

Composition of cell lipid. Approximately 1-mg quantities of lipid isolated from cells grown in the presence and absence of glycerol were fractionated by column chromatography on silicic acid. Recovery of the lipid applied to the column was complete. The lipid present in the various eluate fractions was identified from the distribution of known compounds which are separated sharply by the solvents employed (5). Table II shows the lipid present in each fraction expressed as μg of lipid per μg of total cell protein. In both mouse fibroblast and human liver cells the increase in total cell lipid was due almost entirely to increases in the triglyceride and polar lipid fractions.

Increase in total cell lipid by polyhydric alcohols. Polyhydric alcohols containing two to six carbon atoms were added to the medium at the molar equivalent of 5% glycerol. In both cell lines total lipid was increased by ethylene glycol and erythritol, the former being less active, and the latter more active than glycerol (Table III). In the case of ribitol and sorbitol the number of cells that survived was insufficient for chemical analysis. However, both of these compounds produced lipophilic particles within 24 hours.

Discussion. Earlier experiments in our la-

boratory have shown that the presence of serum in the medium suppresses the synthesis of fatty acids and cholesterol from glucose-U-^{14}C in cultured mammalian cells (12). The cells studied included the human liver and mouse fibroblast clones employed in the present experiments. Similar results were obtained by Bailey (13) with glucose-U-^{14}C and acetate-1-^{14}C in mouse fibroblast and MBIII cells. Furthermore, we found that even when tritiated water was added to a medium containing serum, the incorporation of isotope into fatty acids and cholesterol was negligible (12). This was the case for the fatty acids of both the triglycerides and the polar lipids. It was also the case in cells whose triglyceride content was increased fivefold by the macromolecular factor(s) present in rabbit serum (12). These findings substantiated the results obtained with radioactive glucose and eliminated any appreciable synthesis of fatty acids from precursors containing either a carbonyl or an hydroxal function. It appears, therefore, that in a conventional tissue culture medium, the fatty acids of neutral and polar lipids alike are derived from an exogenous source; i.e., from the lipids of the serum.

In view of the foregoing observations, it seems unlikely that the polyhydric alcohols increase cell lipid solely by increasing the synthesis of fatty acids. Nor does it seem likely that they increase cell lipid only by providing a polyalcohol backbone for the synthesis of complex lipids in view of the greater activity of erythritol as compared with glycerol (Table III). In general, polyhydric alcohols are able to penetrate cells rapidly (14) and possibly their penetration facilitates the uptake of serum lipids and lipoproteins. In this connection the increase in both polar lipids and triglycerides caused by glycerol distinguishes it from the other agents hitherto found to increase total cell lipid. Thus, the macromoleuclar factor(s) of rabbit serum (12), high concentrations of heavy water (15), and high concentrations of H^+, pH 6.9 (11), all cause an increase in triglycerides without comparable increases in the polar lipid fraction. It appears therefore that at least two different mechanisms exist for increasing cell lipid by altering the chemical composition of the environment; one which causes an increase in both "structural lipids" and triglycerides and another which causes a selective increase in triglycerides.

Here a word should be said about the possible relationship between the inhibition of growth caused by the polyhydric alcohols and the increase in cell lipid. As pointed out earlier (5), growth retardation is not a necessary condition for an increase in cell lipid. For example, the lipogenic factor of rabbit serum produces a large increase in lipid in cells that continue to divide every 24 hours. Of even greater pertinence is the fact that we were unable to detect an increase in the lipid content of rat liver cells whose growth rate was reduced by one half by omitting threonine from the medium for 3 days.[1] Consequently, the accumulation of lipid by cells is not contingent on their rate of growth. It is primarily a function of the chemical composition of the environment and the genetic nature of the cell (12). The increase in polar lipids caused by glycerol, and presumably by the other polyhydric alcohols, should therefore be a useful adjunct to the study of lipid metabolism in viable cell systems.

Summary. Glycerol, at the concentrations used in cell freezing and storage procedures, causes a large increase in the lipid content of cultured mammalian cells. Column chromatography on silicic acid of the isolated lipid indicates that the increase is due primarily to increases in polar lipids and triglycerides. The homologues of glycerol, ethylene glycol and erythritol, also increase cell lipid.

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Intensive Nursing and Lactational Performance during Extended Lactation* (32939)

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Suckling stimulation and milk removal are important in maintenance of mammary structure and milk synthesis during lactation. Adjusting litter size of rats on the third day of lactation to six pups per six (6/6) intact abdominal-inguinal mammary glands caused significant progressive increases in mammary deoxyribonucleic acid (DNA) content to day 16 and in ribonucleic acid (RNA) to day 20 of lactation (1). However, the 6/6 suckling intensity of the original litter was not sufficiently strong to prevent eventual declines in both DNA and RNA (1). Fostering litters of rats has been used to extend lactation beyond its normal length (2,3), but maintenance of maximal milk secretion rates has not been achieved. Possible ways of providing a maximal nursing stimulus for maintenance of lactation which have not been investigated in previous studies are: frequent replacement of litters; use of litters 12 to 16 days of age (when nursing intensity is greatest); and maintenance of at least one pup per mammary gland. The objective of this experiment was to determine if intensive nursing would maintain mammary cell numbers (DNA) and secretory activity (RNA, RNA/DNA, and litter weight gain) during an extended lactation. A second objective was to relate changes in mammary cell numbers and synthetic activity with prolactin and adrenocorticotropic hormone (ACTH) contents in the pituitary, two hormones that have been implicated in regulation of lactation (4).

Materials and Methods. On the third day of lactation thoracic teats of 48 Sprague-Dawley rats were ligated, litter size adjusted to 6 pups, and mother rats assigned to one of four groups to be killed either on day 16, 20, 28, or 36 of lactation. Both 16-day-old original litters and foster litters were replaced every 4 days with 12-day-old foster litters. Cumulative litter weight gains were recorded between days 13 and 16 of age for all litters. Final body weights of lactating rats were recorded at time of killing, and nucleic acid content determined on 6 abdominal-inguinal mammary glands as previously described (5).

Twelve anterior pituitaries were collected from each of 2 additional groups of rats killed on either day 20 or 36 of lactation. Prolactin potency of the 12 pooled pituitaries of each group was estimated by the method of Reece and Turner (6). The ACTH potency of the 2 groups of pituitaries was determined by the method of Saffran and Schally (7). Prolactin

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