10 divided doses over a 5-day period. Hence it may be presumed that either the TSH-inhibitor was not long-acting or the endogenously secreted TSH was not neutralized. Thus far the urinary preparations have been utilized successfully to repress only exogenous TSH effect on the thyroid.

Summary. A technic for detection of TSH-inhibitor in human urinary preparations is described. For an effective partial block of TSH response, mg amounts of a urinary preparation were injected one or more hours before an intraperitoneal stimulatory dose of TSH. TSH response was then measured using I^{131} blood levels which were significantly decreased following administration of urinary TSH-inhibitor.

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Utilization of Serum Lipids by Cultured Mammalian Cells.*† (24745)

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The nutritional significance of the lipids present in the serum medium commonly used for growing mammalian cells in tissue culture is little understood. The effect of serum lipids on growth has been studied by Baker and Carrel(1) and a similar study on lipids from embryonic tissues was made by Giudetti(2). Davidson *et al.*(3) measured the changing lipid composition of chick heart explants during growth, and Simms *et al.*(4) studied factors affecting fat deposition *in vitro*. Verne (5) has examined the effect of various compounds of biological origin on lipid metabolism in tissue cultures. Cholesterol and fatty acids were included in the chemically defined medium of Evans *et al.*(6), but it was later reported that there was no noticeable effect on growth when these were omitted(6a). Sato, Fisher and Puck(7) claim that addition of small amounts of cholesterol to a medium containing exhaustively dialysed serum, increased the plating efficiency of HeLa cells many fold. Rutstein *et al.*(20) have studied cholesterol uptake by primary explants from human aorta, and cholesterol synthesis from labelled acetate has been demonstrated for the L strain of mouse fibroblasts(8) and for a strain of human uterine fibroblasts(9). Azarnoff(10) has noted differences in the ability to synthesize cholesterol between cells from different species. We have studied the differential uptake of the various components of the serum lipids using principally the MB III strain of mouse lymphoblasts(11) as our model system. We have also examined a number of other cell lines, but in less detail. The pattern of lipid uptake for each was similar to that observed with the MB III cell, so that it may be assumed that the results obtained by the more detailed examination of the MB III system are fairly typical.

Materials and methods. Human placental cord serum, ox embryo extract and balanced

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salt solution used for routine maintenance of the cells were prepared according to the methods of Gey(12). Stock cell cultures were maintained in 16 x 150 mm roller tubes(13) in a medium consisting of human placental cord serum (5 parts), ox embryo extract (1 part) and Gey's balanced salt solution (4 parts). The medium also contained penicillin (100 units/ml) and streptomycin (50 μg/ml) and sterile culture conditions were maintained throughout the experiments. Incubation was at 37° in a roller drum turning at 12 r.p.h. Tubes were inoculated with approximately one to 2 million cells in 0.5 ml of medium, and were treated every 4 days by removing approximately two-thirds of the old fluid plus cells and adding fresh medium. The MB III strain used in most of the quantitative studies, grew rapidly under these culture conditions and the cells were only loosely attached to the walls of the glass roller tube. Satisfactory cell suspensions were readily obtainable for cell growth enumeration, without use of trypsin, merely by shaking the tube to agitate the fluid over them. For studies of lipid uptake, the cells were harvested by centrifugation and resuspended in the test medium consisting in most cases of human placental cord serum diluted with one part of the balanced saline solution. Duplicate roller tubes were inoculated with 1 ml of the suspension containing approximately one to 2 million cells. Control tubes in which the medium was incubated alone were prepared at the same time. In prolonged incubations to obtain greater depletion of the lipids, the medium was fortified with an additional 0.2% glucose. Under these conditions the cultures attained a final population density of from 10 to 12 million cells/ml. This population density was two to three times higher than that attainable with most other cell lines, and resulted in measurable depletions of some of the more abundant and less rapidly utilized components such as cholesterol, which otherwise would have been difficult to determine. At the end of the desired incubation period the cell population was determined, the cells were harvested by centrifugation at 1000/g and were washed with 2 successive 1 ml portions of balanced saline to remove the last traces of medium. The washed cells were extracted by refluxing with two successive 5 ml portions of 1:1 ethanol/diethyl ether, for 10 minutes and the extract was evaporated to dryness in a stream of nitrogen to prevent oxidation. The dried extract was then extracted twice with 2 ml portions of boiling petroleum ether (B.Pt. 30-60°), and the final solution containing the total cell lipids was diluted to 5 ml with petroleum ether. The depleted medium and the control medium were acidified to pH 5 with hydrochloric acid, and extracted under reflux for 20 minutes with 2 x 40 volumes of 1:1 ethanol/ether. The extract was evaporated and redissolved in petroleum ether as described above. The following determinations were performed upon aliquot portions of the extracts: 1. Total lipid was determined by the dichromate method of Bloor(14), as modified by Bragdon(15). Since some lactic acid was also extracted, this was determined separately by the method of Barker and Summerson(22), and the values for total lipid were corrected accordingly. 2. Total cholesterol was determined by the method of Pearson *et al.*(16), or where necessary by the more sensitive method of Brown (17). Free and esterified cholesterol were separated by chromatography on silicic acid as described by Wykoff(18), and determined by the method of Brown. 3. Phospholipids were ashed by combustion with perchloric acid-hydrogen peroxide mixture, and inorganic phosphate was determined on the residue by the method of Allen(19). 4. Triglycerides were hydrolyzed with 1 ml 0.4 N NaOH at 100° for 30 minutes and the glycerol liberated was determined by the method of Bailey (21). Since the procedure also brought about hydrolysis of the phospholipids, triglycerides were determined by difference. 5. Unsaturated lipids were determined by a modification of the iodine-bromide method of Wizs and Hanus(23). Purified lipids used in the studies on uptake from defined media free from serum protein were obtained from The California Foundation for Biochemical Research, Los Angeles. Cholesterol suspensions were made by dispersing an alcoholic tincture

TABLE I. Utilization of Serum Triglycerides by Mammalian Cells Growing *In Vitro*.

Cell line	Approx. final population (millions/ml)	Serum triglycerides (mg %) Before	After	% utilization
HeLa	1.0	45	12	73
T333	.5	98	23	77
14 Pf	1.5	98	42	57
ARF	1.5	98	41	58
MB III	6.0	51	0	100

For key to cell nomenclature see Table IV.

Cells were grown 4 days in medium consisting of human placental cord serum diluted with one part of saline. Total lipid was extracted as described in Methods section, and determinations of triglyceride, phospholipid and cholesterol were made. For first cell strains total decrease in the latter 2 components was less than 5%. For MB III (with the greater cell population) these components decreased by 15% and 9% respectively.

rapidly into a salt-free solution containing 50 mg % 'Tween' 80 (Atlas Powder Co.). A double strength saline was then added in equal volume. In this way stable emulsions of cholesterol microcrystals with sizes approximating one micron, were obtained.

Results. Preliminary experiments were made with 5 cell lines All brought about a rapid removal of the triglycerides (Table I) but the population densities were low, so that for the first 4 cell lines the depletion of phospholipids and cholesterol was less than 5% of the total present. The MB III cultures however contained significantly more cells which resulted in the uptake of all the triglyceride (510 μg), 80 μg (15%) of the phospholipid and 70 μg (9%) of the cholesterol. The harvested cells contained 650 μg of total lipid of which 70 μg (11%) was cholesterol, and 360 μg (55%) was phospholipid. No triglyceride was found in the cells in this 4-day experiment, although in older cultures some increase in the cellular triglycerides at the expense of phospholipids has been seen. The iodine number of the original serum lipid did not differ significantly from that of the lipid utilized or from the cell lipid. This indicates that saturated and unsaturated lipids were utilized equally readily.

The time-course of utilization of the various lipid components by the MB III strain after 4, 7 and 10 days of *in vitro* growth can be followed from the data given in Table II. The 4 and 10-day experiments were carried out in the same pooled human placental cord serum. In the 10-day experiment, initial glucose concentration was doubled to 0.4%. Total lipid in the serum decreased progressively and most of the decrease was accounted for by lipid recovered in the cells. At 4 days the lipid found in the cells represented 125% of the decrease in the medium, at 7 days 116% and at 10 days only 89% of that removed from the medium. This may be interpreted to mean that under normal growth conditions the cell lipid is almost exclusively derived from the medium but that some net synthesis (25%) of lipid does take place in the initial culture period when glucose is present in excess. Under prolonged culture conditions in order to complete exhaustion of the medium (10 days), there may be some net degradation of lipid by the cells (11%) to satisfy energy requirements.

The rapid uptake of triglycerides is apparently followed by conversion to phospholipids

TABLE II. Progressive Depletion of Serum Lipids by MB III Strain after 4 Days, 7 Days and 10 Days of *In Vitro* Growth.

Total lipid (days)	mg % Before	mg % After	% decrease	Found in cells (mg)*
4	222	170	23	65
7	245	180	27	76
10	222	120	46	87
Cholesterol				
4	86	79	9	7
7	93	79	18	14
10	86	67	22	13
Phospholipid				
4	54	46	15	36
7	39	24	38	17
10	51	9	82	37
Triglyceride				
4	51	0	100	0
7	45	3	94	21
10	47	10†	79	8
Iodine number				
7	72	73	0	79

The 4- and 10-day experiments were carried out in same pooled human placental cord serum, diluted with one part balanced salt solution. In the 10-day experiment glucose concentration was doubled to 0.4%. In the 7-day experiments, cord serum (a different sample) was diluted with one part NCTC 109 synthetic medium(6).

* Calculated on basis of cells grown on 100 ml serum.

† Apparent increase in triglyceride after 10 days growth was caused by release of lipid droplets from degenerated cells.

TABLE III. Average Metabolic Quotients for Uptake of Serum Lipids from Serum and Synthetic Media by MB III Strain Cells.

Compound	Emulsifier	Q (μg/million cells/hr)
Total lipid	50% serum medium	.42
Cholesterol	*Idem*	.08
Phospholipid	"	.18
Triglyceride	"	1.8
Synthetic medium		
Triolein	Lecithin	3.2
"	"Tween 80"	3.7
Lecithin	None	1.4
"	Plus Triolein	.75
Cholesterol	"Tween 80"	.6
"	Albumin	.07
Lactic acid		2.2
Glycerol		2.3
Glucose		21

The synthetic medium consisted of NCTC 109 (6) with glucose concentration reduced to 0.05% to prevent excessive pH changes. Lipid uptake was measured over the first 48 hr only. Purified lipids were added in concentrations similar to those found in cord serum, *i.e.* 50-100 mg %. Rates given for uptake from serum medium in upper part of Table are approximate values only. They are calculated on basis of linear population increases between times at which population determinations were made (0, 4, 7 and 10 days). Over the first few days, however, growth is more approximately logarithmic. Values for lactic acid, glycerol and glucose are given for comparative purposes.

in the cell. When all the triglyceride has been utilized, the phospholipids in the medium are taken up more rapidly. The apparent subsequent increase in the medium triglyceride after 10 days (Table II) is probably due to release of fat droplets which was observed in the older cultures when some cells were beginning to show signs of degeneration. The rapid disappearance of triglyceride represented true uptake rather than extracellular hydrolysis since in experiments where triglycerides were added to synthetic medium (Table III) it was shown that the total lipid in the medium (which would have included any such hydrolysis products) decreased at the same rate as triglyceride The rate of uptake of some pure lipids added to a chemically defined medium paralleled the rate observed in serum (Table III). The rate of uptake of triolein was less when it was emulsified with lecithin and a similar sparing effect on rate of uptake of lecithin was noted when triolein also was present. The free acids and the methyl esters were toxic when used at similar concentrations, but from microscopic observations of the cells and the medium it was concluded that this toxicity probably resulted from the binding of calcium or other ions to form insoluble precipitates rather than to any specific toxicity due to structure.

Cholesterol uptake could not be measured directly using other cell lines, for the reasons already stated. Cholesterol content of a number of strains was however determined directly. The results (Table IV) show that appreciable amounts are present in all cells, ranging from a low of 0.6% of the dry weight for a strain of rat adrenal cells to as high as 2.8% for the FL strain(28) of human amnion cells. Cholesterol content seems to be fairly characteristic of the type of serum in which the cell is grown. For example the cholesterol content of the MB III strain grown on a large number of human placental cord serum samples has ranged only between 1.9 to 2.5% of the dry weight. For adult serum medium the cholesterol content of the cells may be almost doubled; for cord serum which was supplemented with only small additional amounts of free cholesterol or for serum samples from certain coronary patients, MB III cells containing as high as 10% of the dry weight as cholesterol have been grown. Total cholesterol extracted from the medium and from the MB III cells was separated into free cholesterol

TABLE IV. Cholesterol Content of Mammalian Cell Strains Growing *In Vitro*.

Strain	Cell type	% cholesterol
FL	Human amnion(28)	2.8
D-1 Re	" chondromyxosarcoma	2.6
ARF	Articular ridges of femur (rat)	2.1
MB III	Tumorous mouse lymphoblast	2.0
319	Walker rat sarcoma	1.9
Maben	Human lung carcinoma (Frisch)	1.7
256	Walker rat carcinoma	1.6
72-624	Tumor from subcut. areolar tissue (rat)	1.6
HeLa	Epidermoid carcinoma of cervix (human)	1.3
3G29	Rat adrenal (*Zona fasciculata*)	.6

Cells were harvested from the growth medium after 4 days. Cholesterol was extracted and determined as described in Methods section. Medium in most cases was human placental cord serum diluted with one part of balanced saline. Cholesterol values are expressed as % of dry wt after extraction of lipid and air drying at 120°. Dry wt was determined by direct weighing or by combustion as total carbon by dichromate method of Bloor(14).

TABLE V. Selective Utilization of Serum Lipids by MB III Cells Growing *In Vitro*.

Component	% in serum lipid	% in lipid utilized	% in cell lipid
Cholesterol	40	20	15
Phospholipids	25	20	50
Triglycerides	20	60	5
% unsaturated (as linoleic)	40	40	45

Values given are approximated from avg values for a number of human placental cord serum samples. Values for lipid utilized are avg over whole growth period. If these values are calculated over the early stages of growth, the disproportionality between triglycerides and cholesterol is even more pronounced than these figures indicate.

and esterified cholesterol by chromatography on silicic acid columns. In this experiment total cholesterol in the original medium was 59 mg % of which 40 mg % was ester and 19 mg % free. Total cholesterol in the medium after the cells had been harvested was 43 mg % of which 19 mg % was ester and 24 mg % was free, and the harvested cells contained 24 mg (corrected for comparative purposes to cells harvested from 100 ml serum) which was all free cholesterol. Although at first sight this may be interpreted to mean that the cells take up the esterified cholesterol exclusively and then hydrolyze it, exchange experiments using labelled cholesterol, and designed to follow more closely the mechanism of lipid uptake (see discussion) have shown that the correct explanation probably is that the cells take up both the free and the ester, hydrolyze the ester and then excrete the excess free cholesterol. In addition, free cholesterol added to chemically defined medium (Table III) was taken up at a high rate. This higher rate of uptake in synthetic medium is probably the result of some phagocytic ingestion of the cholesterol crystallites in the emulsion in addition to the usual method of lipid uptake. The question of ability of the MB III strain to synthesize cholesterol could not be decided in our experiments because of the large excess present in the medium. It can be calculated however that under the culture conditions used here, less than 5% of the total cell cholesterol is the result of net synthesis. There is little if any degradation of the cholesterol or interconversion into other steroid compounds since even after prolonged incubation of 4-C^{14} cholesterol with MB cells, less than 0.1% of the radioactivity could be detected in products other than cholesterol on paper chromatograms.

Discussion. The lipids are relatively large molecules with average molecular weights of the order of 500, and in addition, in serum are bound in lipoprotein complexes with molecular weights of many thousands. These large molecules however enter the cell at rates comparable to those for much smaller molecules (*cf.*, glycerol, glucose and lactic acid Table III) Current theories to explain lipid absorption in the gut follow two main schools of thought. 1) The so-called 'Lipolytic' theory which assumes that triglycerides are first completely hydrolyzed to the fatty acids and then are absorbed as a bile acid complex, (or a variation of this in that they are converted into phospholipid in the cell membrane during active transport) and 2) the 'particulate' theory which assumes that small droplets of fluid can pass directly into the cell. (For review see Deuel(24)). In the consideration of uptake of large molecules by cells in tissue culture it has generally been assumed that some process analogous to the 'particulate' theory above was responsible. Tissue cultured cells observed under moderate magnification on the glass walls of the culture vessel are seen to take up large numbers of droplets of the medium by a process of 'cell drinking' or pinocytosis(25,26). The apparent mechanism of this process has been discussed by Gay(25), and Lewis(26) has estimated that the cell is capable of taking up in this way a volume of fluid equal to the cellular volume in as little as 3 hours or less. In Table IV are collected average values for the composition of the lipid of the medium, for the lipid taken up by the cell and for the cell lipid itself. The composition of the lipid taken up by the cell is very different from that of the medium. It is difficult to reconcile this with the apparently random and non-specific process of pinocytosis where it would be expected that the composition of the droplets enfolded by the cell membrane, (and which are often many microns in diameter), would be the same as that of the outside medium. We have considered two al-

ternative mechanisms in explanation of these results. First that the lipids are absorbed into the cell by some specific mechanism which may involve chemical transformation and active transport across the cell membrane. Conversion of triglycerides into phospholipids during or immediately following absorption offers some support to this hypothesis). Second, that the lipids ars absorbed into the cell by some non-specific process (micro. or ultramicro. pinocytosis) which is followed by specific utilization of certain components and non-specific excretion of the remainder. Such a sequence of events would of course give a net result which would resemble specific uptake. It is very probable that some process of fluid excretion analogous to pinocytosis is continually going on, otherwise the cell would swell and burst. However in contrast to pinocytosis, it must be taking place at a submicroscopic level(27), since visible droplets are rarely seen leaving the cell. In order to distinguish more certainly between these various possibilities, we have studied the uptake of labelled cholesterol, both in the free state and complexed with crystalline albumin, and its subsequent exchange with the medium when the cells were returned to unlabelled medium. It was found that the cells were continually excreting cholesterol at a rate which was at least 1½ times the *net* rate of cholesterol accumulation within the cell. These results can only be reconciled if some process (*i.e.* uptake followed by excretion) such as that outlined in the second hypothesis above, is taking place. Fillerup *et al.* have studied the uptake of labelled albumin palmitate by ascites tumor cells(29). They found that palmitic acid was readily taken up, but that albumin was not. They concluded therefore that the albumin palmitate complex was probably hydrolyzed at the cell surface and that this was then followed by passage of the palmitic acid into the cell. It would seem however that the operation of some excretion process similar to that described here would also be a valid explanation of these results.

Although the values observed for rate of exchange of labelled cholesterol with the medium are of sufficient magnitude to explain the uptake of both phospholipids and cholesterol by the second mechanism outlined above, rate of uptake observed for triglyceride is still too large to be explained entirely on this basis. Two possibilities are under consideration. First that the labelled cholesterol within the cells is less labile towards exchange than the newly incoming unlabelled cholesterol, or second, that uptake of triglycerides does indeed involve both a specific and a non-specific process.

Summary. 1) A variety of mammalian cells growing in tissue culture took up appreciable amounts of lipid from the serum used in the medium. For the MB III strain of mouse lymphoblasts, triglycerides were most rapidly utilized followed by phospholipids and then cholesterol. This preferential utilization of triglycerides was also shown by 4 other cell strains. 2) The MB III cell utilized saturated and unsaturated lipids equally readily, and there was little net breakdown of lipid to satisfy cell energy requirements. There was however considerable conversion of triglycerides to phospholipid following absorption. 3) Cholesterol uptake appears to be a general phenomenon, and appreciable amounts ranging from 0.6% to 2.8% of the dry weight were found in all strains of cultured cells examined. Cholesterol content of cells was related to the type of serum used in the growth medium. 4) The composition of the lipid taken up by the cells differed markedly from that in the medium. This is discussed from the point of view of: (1). A specific process of uptake or (2). A non-specific uptake by pinocytosis, followed by incorporation and a non-specific excretion. Evidence is presented in favor of this second mechanism.

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Anticoagulant Activity of Phosphatidylserine Free of Lysophosphatidylserine.* (24746)

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In our earlier work(1,2) purified phosphatidylserine (PS) from pork brain was shown to be a potent anticoagulant *in vivo* and *in vitro*. However, these fractions were contaminated with lysophosphatidylserine (LPS). The present paper describes fractions free of LPS, with anticoagulant activity similar to that of the earlier fractions. This substantiates the previously recorded evidence(1,2) that anticoagulant activity is a function of naturally occurring PS.

Methods. Preparation of fractions. In our earlier method(1,2) for purification of PS from the fraction III of Folch(3) the fractions are in prolonged contact with chloroform and methanol. The method was modified by eliminating the *overnight* extraction of each fraction with chloroform and methanol, thus completing the fractionation in one day instead of eight. The extracts derived from the fraction III of Folch are designated as III-1, III-2, III-3, . . . III-8 and the residue as III-R.

Results. Paper chromatography. Fig. 1 illustrates paper chromatography of the pure serine phosphatide fractions on paper impregnated with silicic acid. The methods employed have been described(4,1). To show how purification was followed from the "cephalin" some of the cruder fractions are included. Fractions III-2 to III-8 had only one spot corresponding to PS. This spot was ninhydrin positive and appeared blue-violet under ultra-violet light (UV) in the wet state, after staining with Rhodamine 6G. The yellow center is believed to represent aldehydogenic serine phosphatide. Fraction III-R showed a spot corresponding to PS and a slow-moving component with mobility and staining properties similar to an inositol phosphatide (IP). The IP content of this fraction would explain

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