ruses(7). On the other hand, filtration studies showed that HA component of adenovirus passed more readily through Seitz pads than CF antigen. Many factors might underline this discrepancy, *e.g.*, the difference in electrical charge on the particles or concentration of the different components.

HA activity was found earlier than CF antigen in fluids of MK cells infected with adenoviruses while the opposite usually obtains with viruses(8). This difference may reflect sensitivity of the tests or formation of each component at different times.

The increase in numbers of the adenovirus family has necessitated a search for a simple serologic test for identification. The value of CF test is rather limited, since it measures only a common soluble antigen, although modifications of this test by Pereira(5) and by Binn, *et al.*(6) have increased its usefulness. The specificity of the HI test with ADV-3, 4, and 7 Type 3 definitely indicates its value in diagnostic work. The HI test can be completed in less than 3 hours, thus it is relatively simpler than the CF test. Further, the reagents used in the HI are more stable than those used in the CF test.

Summary. Hemagglutinins of adenovirus Type 3, 4 and 7 were found to be separable from soluble CF antigen and infectious virus. Inhibition of HA activity with rabbit serum antibodies appeared to be type specific. The HA component was relatively stable to heat, formalin and storage at 4°C. It was more readily filterable than CF antigen and was produced earlier than the CF in infected MK cells.

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Received January 11, 1961. P.S.E.B.M., 1961, v107.

Lipid Metabolism in Cultured Cells. I. Factors Affecting Cholesterol Uptake.* (26524)

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The contribution of serum lipids to the nutrition of mammalian cells in culture has been described by Bailey *et al.*(1). It was shown that cells utilize triglycerides most rapidly, but also take up considerable quantities of cholesterol.

Rutstein *et al.*(2) reported that explants of human aorta accumulate cholesterol from a serum growth medium. It was claimed that cholesterol uptake was inhibited by addition of polyunsaturated fatty acids. A number of cell strains synthesize cholesterol *de novo* from simpler constituents in the medium(3). With an adequate supply of serum lipids, however, most of the cell cholesterol is derived from the serum(1). In view of the implication of cholesterol desposition in the etiology of atherosclerosis, it was of interest to determine the factors affecting cellular cholesterol uptake in an *in vitro* system.

Materials and methods. Preparation of growth media and propagation of the MB III strain of cells of mouse lymphoblasts has been described(1). Growth medium used for propagation of stock cultures was human

* This work was supported in part by a research grant from Nat. Heart Inst., Nat. Inst. Health, U.S.P.H.S.

placental cord serum diluted with one part of Gey's balanced salt solution (BSS). Adult human serum was prepared from the blood of normal individuals drawn after 12 hours of fasting. Rabbit serum was prepared from normal rabbit blood obtained by heart puncture from 12-hour fasted animals. In comparative studies on cholesterol uptake all sera were 'deactivated' by heating at 58° for 30 minutes to destroy a toxic factor found in most freshly prepared samples of human and rabbit serum. The heating did not change the cholesterol content of cells grown on the serum. Crystalline bovine albumin was obtained from Nutritional Biochemicals Inc., and highly purified samples of linoleic acid and stearic acid were supplied by the California Foundation for Biochemical Research. Cholesterol-4-C^{14} was obtained from Nuclear Chicago Inc. Radioactive samples were assayed using a halogen-quenched end-window Geiger-Muller tube in conjunction with the usual amplifier and scaler. Cholesterol was determined by the method of Pearson *et al.* (4) or where necessary by the more sensitive method of Zlatkis *et al.*(5). Determinations of cell population were made by counting suspensions in 0.2 × 0.625 Spiers-Levy hemocytometer chambers, and measurements of the dry weight of the cells were made by the colorimetric method of Bailey and Nejad(6). Free and esterified cholesterol were separated on small silicic acid columns as described by Wykoff(7).

Cholesterol emulsions for addition to tissue cultures were prepared by 2 methods as follows: (1) An alcoholic solution of cholesterol was dispensed rapidly with stirring into a sterile solution of distilled water containing 50 mg% Tween 80 (Atlas Powder Co.). Sterile balanced salt solution of double concentration was then added in equal volume. In this way stable emulsions of cholesterol microcrystals were obtained having particle sizes of from 0.5 to 1.5 μ when examined by dark-field microscopy. Clumping of the microcrystals resulted if the suspensions were autoclaved, or if the alcoholic solution was run directly into a Tween 80 solution in saline. Unstable emulsions also were obtained if cholesterol concentration exceeded 100 mg% in the final solution. (2) Finely powdered cholesterol was shaken overnight in a sterile solution of crystalline albumin (2%) in isotonic saline. Excess cholesterol was then removed by high speed centrifugation. Use of these solutions was not practicable in those experiments involving alterations in serum protein:cholesterol ratios.

For studies on cholesterol uptake, cells from 3- or 4-day-old stock cultures were harvested by centrifugation, washed in balanced saline solution and resuspended in saline. Portions of the suspensions containing approximately one million cells were dispensed into roller tube cultures and the test medium was added in a final volume of 1 ml. After 4 days growth, cells were harvested by centrifugation and washed with 1 ml of saline to remove traces of medium. Cholesterol was extracted by heating at 45° for 1 hour with 3 ml of 1:1 ethanol-ether mixture. Extracted cells were centrifuged off, the extract was decanted and cells were air dried for 1 hour in an oven at 110°. Dry weight was determined colorimetrically as described previously. The ethanol-ether extract was evaporated to dryness and the residue was extracted twice with boiling petroleum ether. Where necessary, an aliquot of the petroleum ether was used for measurement of radioactivity, and the remainder was then evaporated to dryness for determination of cholesterol. Cholesterol content of serum samples used in the growth medium was determined by using a similar double solvent extraction method.

Results. Variation of serum type in medium. MB III cells were grown on adult human serum (11 samples), human placental cord serum (8 samples) and adult rabbit serum (6 samples). Each serum was used at a concentration of 50% in the medium and was diluted with an equal volume of balanced saline. Cells were harvested after 4 days. Growth was more rapid in cord serum (mean population increase 5.7 fold) than in adult serum (mean increase 3.5 fold). Cells grown on placental cord serum had a mean cholesterol content of only 2.25% and mean serum cholesterol was 97 mg% (Table I). Cells

TABLE I. Cholesterol Content of MB III Cells Grown on Different Serum Samples.

Type of serum	No. of samples	Serum cholesterol (mg %)	Cell cholesterol (%)
Human adult	11	218.7 ± 49.6*	3.74 ± .62
" cord	8	97.4 ± 35.3	2.25 ± .23
Adult rabbit	6	85.8 ± 29.5	7.80 ± 2.29

* Mean and stand. dev.

Cells were grown for 4 days on the different serum samples diluted with one part of balanced saline. Cells were harvested and cholesterol content was determined as described in *Methods* section.

grown on human adult serum had a higher cholesterol content of 3.74%, and mean serum cholesterol was 219 mg%. Cells grown on rabbit serum had the highest cholesterol content, mean 7.8% although rabbit serum itself had the lowest cholesterol level (mean 86 mg%).

Variation of concentration of serum in medium. Concentration of cord serum in the medium was varied from 20% to 100%. The cholesterol level was thus varied between 16 and 80 mg% without altering the ratio of cholesterol to other serum components. Cells grew more slowly at the lower serum concentrations. There was no significant change in cholesterol content of cells despite the 5-fold range of cholesterol concentrations in which they were grown (Table II).

Addition of free cholesterol to medium. The medium, consisting of 50% human placental cord serum and balanced salt solution, was supplemented with relatively small amounts of free cholesterol in the form of an emulsion with Tween 80. The medium contained initially 40 mg% cholesterol provided by the serum, and the emulsion was added to the cell cultures in the concentrations shown (Fig. 1). The highest concentration (56 mg%) represented an additional 16 mg % over that normally provided by the serum (Fig. 1). At the end of experiment cells were harvested by low speed centrifugation to prevent sedimentation or flotation of cholesterol. Sedimented cells were resuspended twice in balanced saline and recentrifuged to remove any particulate matter adhering to the exterior of the cell membrane. Microscopic examination of the cells using phase optics, and also staining with Sudan black, confirmed the presence of small cholesterol microcrystals within the cell. The cells were analyzed for cholesterol in the usual manner. Addition of relatively small amounts of excess cholesterol to the medium brought about a striking increase in cholesterol content of cells (Fig. 1). The stability of the single microcrystals in the emulsion was good up to levels of 10 mg%, but above this level, clumping of the microcrystals was brought about following a few hours incubation in the serum medium.

TABLE II. Variation in Cell Cholesterol by Varying Serum Cholesterol in the Medium.

Serum cholesterol (mg %)	16	24	32	40	60	80
Cell cholesterol (%)	2.2	2.3	2.5	2.3	2.2	1.9

Cells were grown in a medium consisting of balanced saline supplemented with human placental cord serum having 80 mg % cholesterol. Percentage of serum added was varied to give cholesterol concentrations shown.

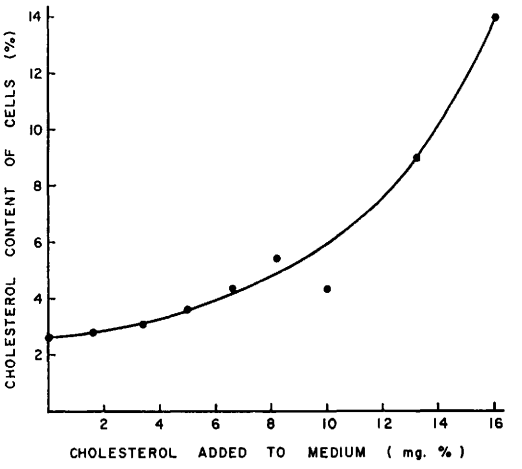

FIG. 1.

It seemed reasonable to assume from the results in Fig. 1 that the added cholesterol was taken up preferentially. To test this, cells were grown in a medium of similar composition, but with an added emulsion of cholesterol 4-C^{14}. By measuring the specific activity of the cellular cholesterol it was possible to calculate the percentage derived from the serum cholesterol and the percentage derived from the added cholesterol. It was found (Table III) that the added cholesterol

TABLE III. Distribution of Serum Cholesterol and Cholesterol-C^{14} in Cells.

C^{14} cholesterol added (mg %)	Total cholesterol in medium (mg %)	Cell cholesterol (%)			
		Total	C^{14}	Unlabelled	Additional unlabelled
0	70	2.0	0	2.0	0
2.5	72.5	2.0	.3	1.7	−.3
5.0	75	4.3	1.3	3.0	1.0
10.0	80	4.6	1.9	2.8	.8
15.0	85	5.9	2.8	3.1	1.1

Cord serum medium was supplemented with increasing amounts of cholesterol-C^{14} emulsion giving final concentrations shown. Specific activity of cholesterol in the harvested cells was measured. In this way the fraction derived from the serum cholesterol (4th and 5th columns) was determined. "Additional unlabelled" cholesterol is calculated as the difference between cholesterol content of cells grown on serum cholesterol alone (2.0%) and the contribution of the serum cholesterol in cells grown on a medium supplemented with labelled C^{14} cholesterol. Addition of unbound C^{14} cholesterol to medium resulted in increased uptake of unlabelled serum cholesterol.

was taken up preferentially compared with its relative concentration in the medium. This, however, did not account completely for the increase in the cells as is shown by the last column in Table III. Addition of free cholesterol also resulted in an increase in uptake of serum cholesterol as compared to uptake from this fraction in control cultures.

Because of the preferential uptake of unbound cholesterol, it was of interest to determine the pattern of uptake when cholesterol was added in a protein-bound form. Cells were grown in 50% cord serum medium at a total cholesterol concentration of 86 mg%. 70 mg% of this was provided by the serum and 16 mg% by adding an equal volume of a solution of cholesterol C^{14} in 2% crystalline bovine albumin (*see* Methods section). After 4 days growth the cells contained 1.3% cholesterol, of which only 14% was derived from labelled albumin-bound cholesterol. Since the albumin-bound fraction provided 18% of the cholesterol in the medium, it was concluded that cholesterol added in combination with protein (in contrast to unbound cholesterol) was taken up at approximately the same rate as serum cholesterol.

Variation of albumin content of medium. Four samples of serum from normal human adults having low serum cholesterol levels, and 4 samples of serum from atherosclerotic patients were pooled. Cells were grown in both samples of pooled serum at a level of 50% in the medium and cultures were supplemented with increasing concentrations of crystalline bovine albumin (Table IV). Mean cholesterol content of cells grown on the normal serum was 3.2% compared with 4.1% for cells grown on the atherosclerotic serum. No significant change was produced in the values over the range of albumin concentrations studied (Table IV).

Addition of fatty acids. Three groups of cultures were set up in 50% human placental cord serum. Cholesterol emulsion was added, giving concentrations in the final medium of 0, 1, 2, 3, 4, 5 and 6 mg% of added cholesterol. One group of cultures was retained as controls, and the other 2 groups were supplemented with linoleic acid or stearic acid emulsion (0.5 mg%). The cells were harvested after 4 days of growth. There was no significant difference in cholesterol content of the 3 groups (Control 5.4%; linoleic acid supplemented 4.7% and stearic acid supplemented 5.1%). A 3-fold increase in fatty acid concentrations (1.5 mg%) with a different sample of serum gave a similar result

TABLE IV. Effect on Cell Cholesterol of Increasing Concentrations of Albumin in the Medium.

Albumin added (mg %)	0	100	300	1000	2000
Cell cholesterol (%)					
Pooled normal serum	3.2	3.1	3.0	3.0	2.7
" athero. "	4.1	4.2	4.0	4.5	4.5

Four samples of normal human adult serum having low cholesterol levels were pooled. Mean serum cholesterol was 146 mg %. Four serum samples from atherosclerotic patients having a mean serum cholesterol of 233 mg % were also pooled. Cells were grown in samples of both pooled sera diluted with one part of balanced saline solution and supplemented with crystalline bovine albumin at concentrations shown.

(Control 4.2%; linoleic acid supplemented 3.9% and stearic acid supplemented 4.3%). Higher concentrations of free fatty acids than those used here were toxic to the cells.

Relative importance of free and esterified cholesterol. About two-thirds of the cholesterol in serum is normally present in the esterified form. Experiments were performed to determine the relative contributions of these 2 fractions to the cell cholesterol. It was found that the cell cholesterol was almost exclusively (95-100%) in the unesterified form, but that, paradoxically, most of the decrease in the medium occurred in the ester fraction. Supplementing the medium with free cholesterol-C^{14}, however, showed that about half of the cell cholesterol was derived from the free cholesterol of serum. Following incubation for the 4-day growth period, about 20% of the label in the medium was in the ester fraction. The labelled ester cholesterol found in the medium results from esterification exchange catalyzed by the serum. It seems probable also that the cells contain a cholesterol esterase which breaks down the esters to free cholesterol following absorption.

Discussion. It has been shown that uptake of serum cholesterol by cells in culture is related to cell type(1). In a series of cells grown in human placental cord serum, cholesterol content varied from a high of 2.8% for the FL strain of human amnion to as low as 0.6% for a strain of rat adrenal cells. The experiments reported here demonstrate that with a given cell type, cell cholesterol is also dependent upon the type of serum used in the growth medium. It is also apparent that cell cholesterol is determined by factors other than (or in addition to) serum cholesterol level as is seen by comparing cholesterol content of cells grown on rabbit serum with that of cells grown on human placental cord serum (Table I). Cholesterol level in rabbit serum was less than in cord serum. Cells grown on rabbit serum, however, had over 3 times the cholesterol content of those grown on cord serum.

The results (Table II) indicate that the important parameter in determining cell cholesterol levels is the ratio of cholesterol to other components of the serum. This was confirmed in experiments (Fig. 1) where unbound cholesterol was added to the medium in the form of an emulsion. Relatively small increments in unbound cholesterol brought about large increases in cell cholesterol. When the same concentrations of cholesterol were added in the form of a protein complex with crystalline albumin, however, there was no corresponding effect on cell levels. This suggests that one determining factor may be the relative ratio of protein-bound to unbound cholesterol in serum. That this is not the only factor, however, is shown by the fact that increasing the albumin content of the medium up to 80% above normal levels did not alter cholesterol content of the harvested cells (Table IV). Since most of the cholesterol in serum is bound to an alpha-globulin fraction,† it is possible that cholesterol binding by proteins other than albumin is more important. It would be expected that both qualitative as well as quantitative parameters should be considered in evaluating the importance of protein-bound cholesterol. Thus for example, when serum is shaken with free cholesterol-C^{14}, most of the bound radioactivity appears in the alpha-globulins† and relatively little in the albumin fractions. When albumin is shaken alone with cholesterol-C^{14}, however, it binds considerable amounts of radioactivity.

The variation in uptake of cholesterol from different serum samples was not due to differences in free fatty acid content of the serum. Concentration of cellular cholesterol was not affected by addition of linoleic acid or stearic acid to the medium in concentrations of up to 1.5 mg%. We have thus failed to observe in this cell system the effects on cholesterol uptake which were noted by Rustein *et al.*(2) in explants of aorta tissue when similar concentrations of free fatty acids were used.

The question of the relative contribution of free cholesterol and cholesterol esters to the cellular cholesterol is not clear from the re-

† As determined by chromatography on DEAE ion-exchange cellulose columns. (J. M. Bailey and Edward Walsh, unpublished data).

sults. In serum medium alone, only cholesterol esters are depleted, whereas it is apparent from the experiment using labelled cholesterol that both free and ester are taken up. The most probable explanation of the results is that both free and ester cholesterol are taken up by cells. Cholesterol esters are then hydrolyzed by the cells and are deposited as free cholesterol. Any excess is then excreted back into the medium as free cholesterol. We have observed the functioning of this excretion process by using cells grown on C^{14} labelled cholesterol and later transferred to unlabelled medium(8). The role of the serum proteins in this excretion process is being investigated.

Summary. 1. Some of the factors influencing uptake of cholesterol by a strain of mammalian cells growing *in vitro* have been examined. 2. Cholesterol uptake by cells is dependent upon the type of serum used in the medium. Cells grown on rabbit serum had a higher cholesterol content than cells grown on human adult serum or human placental cord serum. Rabbit serum, however, had the lowest serum cholesterol level. 3. Cholesterol content of cells was not influenced by concentration of serum cholesterol in the growth medium provided the relative proportions of cholesterol and serum protein were not changed. 4. When emulsions of free (non-protein bound) cholesterol were added to serum medium, relatively small increases in cholesterol content of the medium resulted in large increases in cholesterol content of the cells. 5. By use of C^{14} labelled cholesterol, it was shown that unbound cholesterol was taken up preferentially as compared with cholesterol in the protein-bound form. 6. Cholesterol content of cells was not influenced by addition of stearic acid or linoleic acid to the growth medium. 7. Cells utilized both free and esterified serum cholesterol. The ester was apparently hydrolyzed intracellularly since the cell cholesterol was almost entirely in the unesterified form. 8. It is concluded that the main factor controlling cellular cholesterol uptake may be the relationship between serum cholesterol and the binding power of the serum proteins, rather than the cholesterol level itself.

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Received January 18, 1961. P.S.E.B.M., 1961, v107.

Interaction of Compound 48/80 and Stilbamidine with Strandin.* (26525)

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Histamine release from mast cells may be effected by a variety of substances, among which are chlorpromazine(1), protamine(2), the thiazine dye, toluidine blue O(2), compound 48/80(2) and stilbamidine(2,3). All of these compounds interact markedly with heparin and chondroitin sulfate. MacIntosh, reviewing the behavior of a number of histamine releasers, has found a parallel between histamine release and heparin combining power(2).

Recent work in this laboratory has indi-

* Supported by a grant from National Tay Sachs' Assn.