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Received June 26, 1961. P.S.E.B.M., 1961, v108.

## Sterol Synthesis by Cells Cultured on Serum from Heat-Stressed Chickens.* (26915)

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Stress has frequently been linked to increased serum lipid levels as an etiologic factor(1,2). Experiments with hypophysectomized and adrenalectomized animals have indirectly incriminated stress hormones in hyperlipemia(3). Adrenaline and adrenocortical hormones have been shown not only to produce hyperlipemia *in vivo*(4) but also *in vitro* in cultures of rat adipose tissue(5,6,7,8). Similarly, corticotropin has been found to increase unesterified fatty acid levels *in vivo* (9). These reports are largely concerned with stress-hormone-induced fatty acid mobilization, however, mirroring the present increased interest in the role of unesterified fatty acids (UFA) in over-all animal lipid metabolism.

Bailey, Gey and Gey(10) found that MBIII lymphoblasts *in vitro* remove cholesterol esters from the nutrient medium, presumably by pinocytosis, and expel unesterified cholesterol into the medium. Azarnoff (11) has observed that organ cultures of the coronary artery and aorta of chickens, guinea pigs and rabbits convert $C^{14}$ acetate to cholesterol, whereas this is not a characteristic of humans, dogs, cats and rats, although the latter do synthesize some type of digitonin precipitable steroid. Simms *et al.*(12) obtained a so-called "B-factor" or "lipfanogen" from human and animal sera (including chicken), which evidently causes lipid aggregation, resembling spontaneous atherosclerosis, in organ cultures of chicken and human arteries. In none of these instances, however, have these phenomena been linked to stress.

Recently(13) studies were conducted by us on the use of tissue culture techniques in biological tests for ascertaining stress in animals. Mouse fibroblasts cultured on a medium containing "stressed" serum (hereafter referred to as S-serum) produce a large number of small lipid droplets. This phenomenon is not elicited by "non-stressed" serum (hereafter referred to as NS-serum). It is demonstrable in rapidly growing, healthy cultures and is not a manifestation of degeneration.

The present report consists of results of tests made in an attempt to determine the nature of the lipid found in these cells after exposure to serum from stressed animals.

*Materials and methods.* The "Low line" strain of mouse fibroblasts (NCTC #2445) (14) was used for these tests. They were cultivated by inoculating 300 to 500 thousand cells per ml into either T-15 or T-60 flasks containing 2 or 10 ml, respectively, of a 10% horse serum 109 medium. After growth was well established (average cell numbers 1.5 million cells per ml), this medium was replaced, in half of the cultures, with a balanced salt solution (B.S.S.) containing up to 20% serum from stressed chickens and in the other half with serum from non-stressed chickens. Young White Leghorn chickens (age: 3-4 months) were subjected to heat stress for 24 hours at 102°F as described in a previous report(13). Total cell numbers in both control and test cultures were essentially identical at the end of the test period

* Supported by a grant from University of Nebraska Research Council.

TABLE I. Sterol Content of Mouse Fibroblasts (NCTC #2445) Cultured on Sera from Stressed and Non-stressed Chickens.

| Conc. of serum in medium | Time of exposure to serum, days | Cholesterol content in mg/$10^7$ cells | |
|---|---|---|---|
| | | Non-stressed | Stressed |
| 12% | 2* | .420 | .571 |
| 20% | 2† | .428 | .555 |
| 10% | 6* | .104 | .150 |

* 3 exp. † 4 exp.

(approximately 0.9 million cells per ml at 48 hours; 3.4 million per ml at 6 days).

Sterol determinations were made by the Bloor-Sackett modification(15) of the original Liebermann-Burchard reaction on cell-free media before and after culturing at 37°C for 2 days, 3 days and 6 days respectively. Error in the procedure was found to be ± 1.63 mg % by multiple tests on known cholesterol standards. Cell extractions were prepared by washing all of the cells in a T-60 culture with B.S.S., after which they were extracted 3 times for 20 minutes each with 3:1 alcohol-ether solution. The combined extracts were evaporated to dryness over a water bath, the residue extracted 3 times with chloroform and this extract analyzed for sterol as above. Error in the procedure was found to be ± 0.22 mg %. It is recognized that many sterols give the same, or very nearly the same, color reaction as cholesterol with this technique(16). Other currently used methods(17), employing precipitation of the cholesterol digitonide in 96% alcohol, followed by colorometric analysis, are apparently more specific, but even they are open to question. Azarnoff(11) studying utilization of sodium-acetate-$C^{14}$ by arterial tissue in organ culture, found that human aorta and coronary artery were capable of incorporating this substance into a digitonin-precipitable substance, but that this substance was *not* cholesterol, since "all of the radioactivity disappeared from the cholesterol fraction with preparation of the dibromide." Others have observed the same(18). Because of this lack of specificity, we prefer to use the term sterol concentration rather than cholesterol concentration, but feel that this terminology does not alter the significance of the findings.

*Results and discussion.* Tests were run to ascertain sterol content, and Table I presents the results with cell extractions. There is a definite increase in the amount of sterol in cells cultured for 48 hours on serum from stressed animals. Longer exposure (6 days) does not increase the amount. Although the total amount of sterol was decreased in cells cultured in S- and NS-serum at the end of 6 days, relative difference was of the same order as that in cells exposed for only 2 days. Concentrations of serum in the medium apparently had no significant effect on total sterol in the cells. The data suggest that sterol synthesis is stimulated by "stressed" serum but that the product is not retained within the cell.

Table II presents results of analyses of the media before and after exposure to the cells. The change in sterol content of the NS-serum component after 48 hours culture was negligible, varying from –0.67 to 1.19 mg% (average 0.37 mg %). In S-serum the sterol content increased considerably. The increase varied between 6.66 to 13.88 mg % (average 9.92 mg %). In another experiment, the cells were cultured for 6 days in 10 ml of medium containing 10% S- or NS-serum. Every 48 hours the medium was replaced by 10 ml fresh medium. Thus, at the end of the experiment, there were 30 ml of pooled medium to analyze in each instance. The sterol content of these specimens represented total

TABLE II. Sterol Content in Serum Component of Media in Which Mouse Fibroblasts (NCTC #2445) Have Been Cultivated.

| Sample No. | Conc. of serum, % | Sterol conc. in mg %: Unused medium | Medium after 48 hr culture | Increase |
|---|---|---|---|---|
| | | "Non-stressed" serum | | |
| 1 | 12 | 98.14 | 98.90 | .76 |
| 2 | 20 | 88.25 | 88.84 | .59 |
| 3 | 20 | 88.25 | 89.44 | 1.19 |
| 4 | 20 | 88.25 | 88.25 | .00 |
| 5 | 20 | 88.25 | 87.58 | –.67 |
| | | "Stressed" serum | | |
| 1 | 12 | 113.89 | 127.77 | 13.88 |
| 2 | 12 | 118.05 | 127.77 | 9.72 |
| 3 | 20 | 125.33 | 136.00 | 10.67 |
| 4 | 20 | 136.00 | 142.66 | 6.66 |
| 5 | 20 | 125.33 | 133.33 | 8.00 |
| 6 | 20 | 125.33 | 136.00 | 10.67 |

TABLE III. Characteristics of Lipid Droplets Produced in Media Containing UFA or Serum from Stressed Chickens.

| | Oleate induced | Stressed serum induced |
|---|---|---|
| Size: | Large, up to 15 $\mu$ | Small, about 1-2 $\mu$ |
| Rate of formation: | Appear in 6 hr, fully formed in 24 hr | Appear in about 12 hr, fully formed in 48 hr |
| Rate of utilization: | 48 hr | Not utilized |
| Heat inactivation: | Not inactivated | Inactivated by 89°C for 30 min. |
| Insulin-glucose effect: | Inhibits formation | No effect |

output of these cells over a 6-day period under nearly ideal growing conditions. Total sterol increase in NS-medium was found to be 5.79 mg %; in S-medium it was 26.40 mg % or 4½ times as much.

The above data show that sterol does accumulate in the medium in contrast to intracellular sterol, which does not accumulate (Table I). These observations suggest that intracellular synthesis of sterol is increased under S-serum stimulus, and that the product escapes into the surrounding medium where it accumulates. We have, in fact, observed large accumulations of extracellular lipid in chick embryo heart and artery explants grown in presence of S-serum. Control organ cultures grown on NS-serum medium did not show a similar demonstrable aggregation of lipid. Although past experience indicated the extra cellular cholesterol is not the source of these lipid droplets(13), another experiment was carried out to test this fact further. Cholesterol was added to normal horse serum in concentrations equivalent to an increase of 33 mg % and 67 mg %. The mouse fibroblast cells were grown on this medium for 48 hours without production of lipid droplets. The same experiment was repeated using medium with non-stressed chicken serum with the same results. The evidence here suggests that exogenous cholesterol plays little if any part in the appearance or non-appearance of these lipid droplets.

Simms *et al.*(12) observed that fibroblasts cultured in medium containing fatty acid soaps tend to accumulate lipid droplets within their cytoplasm. This phenomenon was observed in our laboratory also but it was soon realized that the UFA droplets and S-serum droplets were not the same, neither morphologically nor in their physiological relation to the cell's metabolism, since the sterol tends to accumulate and is not utilized as a metabolic substrate by the cells. This is not true of droplets produced in presence of fatty acid as the following experiment demonstrates: Replicate cultures of mouse fibroblasts were grown on 10% S-serum and on a 10% NS-serum medium containing 25 mg % sodium oleate. After 48 hours exposure, at which time an abundance of lipid droplets were present in both cultures, the media were replaced by a 10% horse serum medium. Within 48 hours, the lipid droplets produced in the cultures containing UFA had disappeared. The droplets produced in the S-serum were still present, and remained even after 6 days.

In another experiment, insulin† (0.01 units per ml) and glucose (1 mg %) were added to oleate-containing and S-serum containing media before culture. Lipid droplet formation in the S-serum medium was unimpaired after 48 hours exposure while droplet formation in the oleate medium was prevented.

This latter was not unexpected, since Gordon and Cherkes(8) had previously found that fatty acid aggregation in rat adipose tissue *in vitro* did not occur in presence of insulin-glucose. It is significant, however, that this hormone-substrate combination did not affect lipid droplet formation in S-serum. Furthermore, heating the 2 media for 30 minutes at 89°C prior to inoculation prevents lipid droplet formation in S-serum medium but not in oleate-containing medium. These observations are listed in Table III and further support the belief that this phenomenon is not associated with UFA metabolism.

In a separate experiment, replicate cultures

† Zinc free insulin furnished by Eli Lilly and Co.

containing oleate and S-serum, respectively, were started. After 48 hours, the medium was removed from the cells and used for cultivating fresh cultures. Good growth was obtained in both groups of cultures, but the cells in the oleate medium now no longer produced lipid droplets, whereas lipid droplet formation in the medium containing S-serum was unimpaired. Then the now relatively depleted medium was again removed and used for cultivating additional fresh cells. Although growth was not exuberant, lipid droplets were still formed in the S-serum medium. From these results, it appears as though the active principle causing this phenomenon is not a metabolic substrate but is apparently acting in a catalytic manner.

*Summary.* Young chickens subjected to heat stress at 102°F for 24 hours elaborate a stress factor into their blood stream. When serum from these animals is used as an adjuvant in a serum-balanced salt solution medium, cells grown on this medium accumulate large numbers of small lipid droplets within their cytoplasm. The phenomenon is not related to substrate total fats, cholesterol or unesterified fatty acids. The droplet formation is associated with active cell growth and division and is not a function of degeneration. Evidence is presented to show that these droplets are, at least in part, steroid in nature and possibly cholesterol, and that they are produced intracellularly and escape by some means into the extra-cellular environment where they accumulate. They apparently are not used as metabolic substrate by the cells, but probably represent a by-product of the cell's altered lipid metabolism. It is possible that this type of phenomenon, functioning *in vivo* in animals under stress, may account in part for the hypercholesteremia of stress reported by Shafrir and Steinberg (4) and others. It also may furnish a clue to a source of extrahepatic endogenous cholesterol in the animal body. It is hoped that investigations of this type may renew interest in further use of cell culture techniques for detection of physiological alterations in the animal body.

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Received August 4, 1961. P.S.E.B.M., 1961, v108.