of muscle. This may prehaps be explained by low levels of some flavoprotein moiety in muscle which is shunted in the assay for AGPD.

It has been reported(7) that female rat livers contain 4 times as much soluble AGPD as male rat livers, with both groups demonstrating extremely low activity. However, in a preliminary study in this laboratory with 3 female and 5 male rats (Sprague-Dawley), essentially similar mean values for soluble AGPD were found. These data [5.5 (5.2-5.9) in female and 5.4 (3.9-7.5) in male animals] are more consistent with usual findings of similarity of enzyme levels in the two sexes of the same species. The routine use of high levels of iodoacetate in the spectrophotometric method may account for the low levels of measurable AGPD activity described earlier.

*Summary.* AGP oxidation has been determined and characterized in kidney, liver, brain and muscle of the mouse. The assay technic utilized is simple, results are reproducible and only readily available laboratory equipment is required. In addition, enzyme inhibitors which might interfere with the accuracy of the determination are not used. It is felt that the present method may serve to broaden the limited lines of clinical investigation dealing with the significance of AGPD in regulation of metabolic processes.

1. Estabrook, R. W., Sacktor, B., *J. Biol. Chem.*, 1958, v233, 1014.

2. Lee, Y., Takemori, A. E., Lardy, H., *ibid.*, 1959, v234, 3051.

3. Boxer, G. E., Shonk, C. E., *Cancer Research,* 1960, v20, 85.

4. Shelton, E., Rice, M. E., *J. Nat. Cancer Inst.*, 1957, v18, 117.

5. Pearson, B., Defendi, V., *Cancer Research,* 1955, v15, 593.

6. Handler, P., Klein, J. R., *J. Biol. Chem.*, 1942, v143, 49.

7. Young, H. L., Pace, N., *Arch. Biochem. Biophys.*, 1958, v76, 112.

Received April 23, 1961. P.S.E.B.M., 1961, v107.

## Lipid Formation by Fibroblasts Cultured on Serum from Stressed Animals.* (26676)

KENNETH D. ROSE, SAMUEL I. FUENNING AND RICHARD MACA
(Introduced by D. M. Pace)
*Institute for Cellular Research, University of Nebraska, Lincoln*

Tissue cells cultivated *in vitro* are particularly sensitive to altered environment(1). An example is the tendency toward fat accumulation characteristic of most tissue culture cells, particularly those grown in presence of certain hormones(2). Preliminary investigations into possible physiological and morphological changes in tissue culture cells cultured on media containing serum from stressed chickens revealed an apparent alteration in cell physiology. Mouse fibroblasts were seen to accumulate lipid as intracytoplasmic droplets when cultured in the presence of serum from stressed chickens. This was not true in the presence of serum from non-stressed chickens or horses. The present paper constitutes a report on several experiments undertaken to establish and elaborate on the earlier preliminary observation.

*Materials and methods.* Young Leghorn cockerels were chosen as the test animals because of their availability, their notoriously labile emotional nature, and because of the ease with which their blood could be drawn. Early experiments revealed that hens could not be employed, evidently because of the toxicity of high serum calcium levels. Control animals were maintained in an isolated, enclosed, temperature-controlled ($24 \pm 2°C$)

* This research supported by a grant from Univ. of Nebraska Research Council. We wish gratefully to acknowledge the professional advice and assistance given by Drs. Donald Pace, I. William Brill and John L. Adams, Univ. of Nebraska.

incubator with subdued light. Care was taken to provide adequate bird space and proper pen flooring (to allow normal scratching), and to prevent disturbing influences by using indirect feeding and watering systems, in order to reach a basal level of external stress.

Crowding(3) and heat provide 2 excellent forms of controlled stress for chickens, and these methods have been employed in the present studies. Test animals were maintained up to 8 days under (a) crowding conditions of 0.4 square foot to a bird—normal need is one square foot or more per 6 week old bird; and (b) conditions of elevated temperature, the test temperature being 41 ± 1°C.

With the birds in a fasting state,† serum was obtained by cardiac puncture at the end of each test period. This technic does not appear to disturb the chicken. A chemically defined medium was prepared containing 200 units penicillin, 0.2 mg streptomycin and 10% of this serum(4). After the filter sterilization, the medium was stored in pyrex glass tubes at approximately 5°C and used during the experiment.

Because of its apparently great sensitivity to environmental changes, the "Low-line" mouse fibroblast strain was used in most of the studies reported here(5). Other cell lines were used where noted. The experiments were carried out in Carrel flasks (10 flasks for each condition), using replicate culture technics(6). The flasks were inoculated with 1.5 million cells in 1 ml of the chemically defined medium plus 10% horse serum and incubated at 37°C in 5% $CO_2$-air to allow the cells to become established. After 2 days, this medium was replaced by 1 ml of medium containing 10% chicken serum, either control or test. Replacement was repeated every 2 days for the duration of experiment. Observations were made daily for growth and morphological changes, and the cells enumerated by means of the Coulter counter(7). From time to time, the cells were photographed.

Samples of each serum specimen were tested for total cholesterol by the method of Pearson, *et al.*(8) and for total fats by the Lipitest method(9). Sera were analyzed for ionic fatty acids (FFA, NEFA, UFA) by the method of Trout, *et al.*(10) further modified by the use of 0.02N KOH as titrant, and 0.1% thymolphthalein as the indicator.‡

*Results.* Serum from chickens subjected to stress by heating or crowding stimulates intracytoplasmic lipid droplet formation by "Low-line" mouse fibroblasts cultured on media containing that serum. Sera from non-stressed chickens do not elicit this lipid accumulation (Fig. 1). Growth curves of control *vs* "stressed" serum cultures (24, 48 and 96 hours stress) reveal that there is no appreciable variation in total cell counts (Fig. 2). Individual counts suggest a slight stimulation of growth by "stressed" sera at 24 hours which is not apparent in later counts. Droplets become apparent in 8 hours after addition of the "stressed" serum and while the cells are in the stage of active proliferations.§ They reach a maximum concentration in about 24 hours and maintain this maximum so long as the cells are in the presence of "stressed" serum. When "non-stressed" chicken or horse serum medium is substituted, the droplets slowly disappear and are gone by 6 days.

The phenomenon can be demonstrated easily in physiologically immature chickens but becomes difficult to demonstrate in adult chickens. Droplet formation is readily demonstrable in a 10% serum medium from a young cockerel, but a 30% serum medium is required to demonstrate comparable droplet formation in a rooster of 6 months or older. The phenomenon appears to be limited to mesenchymal cells of a non-malignant nature. Six cell lines in addition to the "Low-line" were studied in one experiment in which all were grown on the same pooled sera from 2 birds subjected to 24 hours of heat stress. The "Low-line" control again produced lipid

† Ten to 12 hours after last feeding.

‡ These modifications were recommended by Dr. Herbert Davis, Dept. of Biochemistry, Univ. of Nebraska Coll. of Med.

§ Time-lapse cinematomicrography, not reported in detail here, reveals cellular division proceeding in presence of lipid droplets.

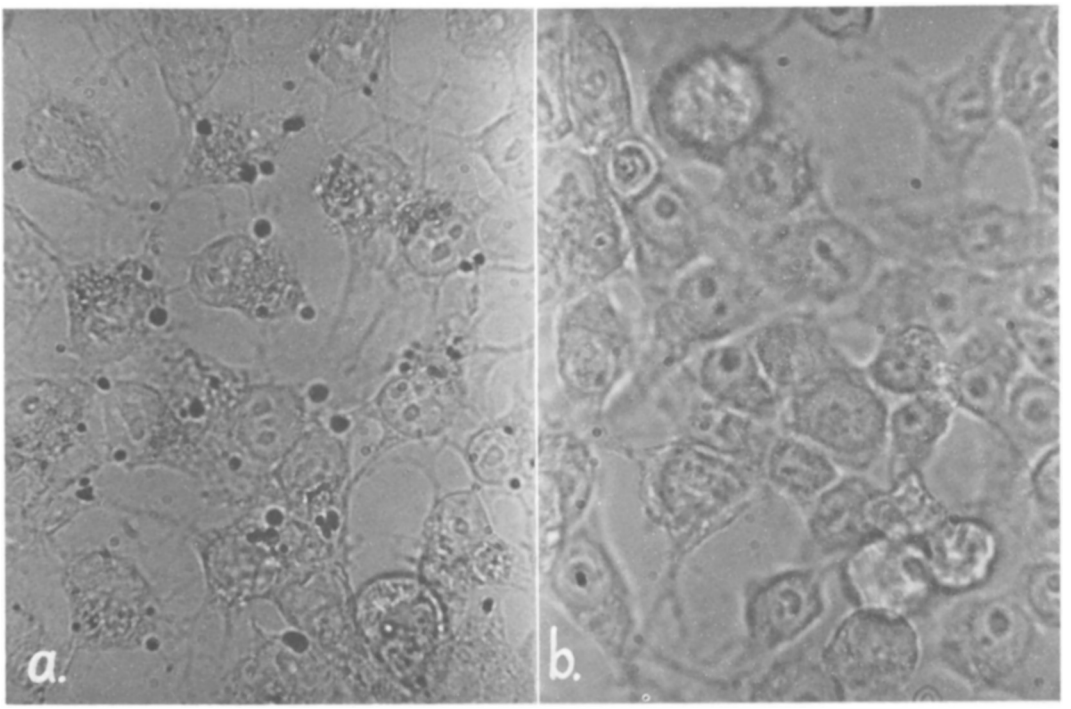


FIG. 1. Sudan III—stained cultures of "low-line" mouse fibroblasts grown on serum from (a) stressed and (b) non-stressed chickens. Approximately 650×.

droplets. Earle's L-strain produced a few. The malignant (High-line) mouse fibroblast did not produce droplets nor did the epithelial or endothelial lines (HeLa, mouse liver, human lung and human skin).

Stress-induced elevation of various serum lipid constituents is well known(11,12), as is the role of pituitary-adrenal hormones in lipogenesis(13,14). It seemed important, therefore, to ascertain whether an increase in available serum lipid might not explain the observed accumulation of lipid. Twenty-eight different sera were analyzed for total lipid and cholesterol (Table I). There appeared to be no relation between the serum lipid levels and development or non-development of intracytoplasmic droplets. As an

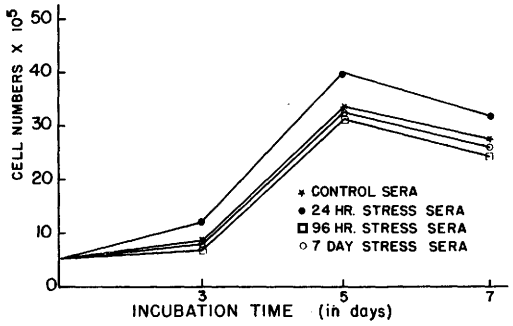


FIG. 2. Growth of mouse fibroblasts on media containing "non-stressed" and "stressed" chicken sera.

TABLE I. Serum Cholesterol and Total Lipid Levels in Stressed and Non-Stressed Chickens.

| | No. | Serum cholesterol (mg %) Range | Avg |
|---|---|---|---|
| Non-stressed controls* | 10 | 108-146 | 126 |
| 24-hr stressed† | 9 | 98-195 | 136 |
| 96-hr " † | 4 | 98-190 | 155 |
| Tranquilized, controls‡ | 3 | 130-132 | 131 |
| " , 24-hr stress‡ | 2 | 130-178 | 154 |
| | | Total lipids (mg %) | |
| Non-stressed controls* | 7 | 227-402 | 290 |
| 24-hr stressed† | 7 | 157-376 | 240 |
| 96-hr " † | 4 | 219-315 | 277 |
| Tranquilized, controls‡ | 3 | 175-310 | 237 |
| " , 24-hr stress‡ | 2 | 157-236 | 196 |

* All control samples produced no droplets in cell culture.

† All test samples produced droplets in cell culture.

‡ Reserpine added to food in concentrations of 5 mg %.

TABLE II. Serum Ionic Fatty Acids in Stressed and Non-Stressed Chickens.

| | No. | Serum ionic fatty acids (meq/l) Range | Avg |
|---|---|---|---|
| Non-stressed controls | 6 | .297-.813 | .484* |
| Stressed (3 to 24 hr) | 9 | .270-.861 | .564* |
| Horse serum | 4 | .559-.608 | .548 |

* Difference between non-stressed and stressed serum averages, calculated as sodium oleate, is 2.4 mg %.

example, some stressed sera contained 98 mg% cholesterol and produced droplets, while some non-stressed sera contained as much as 146 mg% and did not produce droplets.

Simms(15) has reported lipid "granule" formation by fibroblasts cultured on media containing added fatty acid soaps. Similar observations in our laboratory led us to consider the possibility that increased serum fatty acid levels might be the source of the accumulated lipid.‖ However, a series of ionic fatty acid determinations on stressed and non-stressed chicken sera and on horse sera (which does not stimulate droplet formation under the conditions of our experiments) indicate that the level of free fatty acids is not a significant factor in their production (Table II). Most of the sera tested contained less than 20 mg% (0.66 meq l) of free fatty acid when calculated as sodium oleate. Grossfield(2) arrived at a similar conclusion while studying lipid vacuole formation by tissue culture cells, reporting that "The lipid appears predominantly to be produced by the cell and not to come from the medium."

*Discussion.* The results presented here strongly suggest a humoral mechanism for production of a substance eliciting the observed phenomenon of lipid accumulation by fibroblasts cultured on serum from chickens subjected to crowding and heat stress. That the test cell used is a cloned fibroblast and is not, *per se*, a lipocyte, indicates that this is not a usual function of this cell and might, therefore, be considered abnormal. That lipid droplet production occurs during active cell growth and multiplication and not during a period of cultural decay and cell death suggests that the process is not one of degeneration. The apparent limitation of the phenomenon to cells of mesenchymal origin is not without precedent, Kline, *et al.*(16) having reported similar observations with hydrocortisone stimulation of various cell lines.

The relationship between physiological youth and production of this stress factor is interesting in that Weiss(17) has recently reported similar observations, wherein he noted acclimatization to cold was best achieved by the relatively young rat. In this regard also, the frequently observed lipogenic effect of the growth hormone from the anterior pituitary(18) and production of a lipid mobilizer from the anterior pituitary by stress(19) are extremely interesting. Whether all of these widely diverse observations surrounding lipid mobilization by physiologically young animals can be equated with Holman's theory of first stage atherogenesis in humans by "fatty streaking," which occurs between the ages of 0 to 19 years of age(20), remains to be seen, but it appears worthy of consideration.

*Summary.* Certain tissue cells, grown *in vitro* on media containing serum from stressed chickens, produce quantities of lipid deposited in droplets within their cytoplasm. Serum from non-stressed animals does not elicit this phenomenon. Being associated with a period of active proliferation, it cannot be said to be a degenerative process. Evidently it is not related to increased lipid content of the serum. The stress factor, if such there be, is more easily elicitable during physiological youth of the experimental animal. It is suggested that the phenomenon is humoral stimulated and may be a manifestation, *in vitro* of elevated circulating stress hormones *in vivo*.

‖ It was determined that 20 mg% or over of sodium oleate was required to produce droplets similar to those observed on "stressed" serum. A level of 35 mg% or over was found to be cytotoxic.

1. Biesele, J. J., Goldhaber, P., *Cancer Res.*, 1955, v15, 767.

2. Grossfield, H., *Experientia,* 1958, v14, 371.

3. Garren, H., *Poultry Sci.*, 1952, v31, 917.

4. McQuilkin, W. T., Evans, V. J., Earle, W. R., *J. Nat. Cancer Inst.*, 1957, v19, 885.

5. Sanford, K. K., Likely, G. D., Earle, W. R., *ibid.*, 1954, v15, 215.

6. Pace, D., Aftonomus, B., *ibid.*, 1957, v19, 1065.

7. Mattern, C. F. T., Brackett, F. S., Olson, B. J., *J. Applied Physics*, 1957, v10, 56.

8. Pearson, S., Stern, S., McGavack, T. H., *Anal. Chem.*, 1953, v25, 813.

9. Merck, & Co., Inc., Rahway, N. J.

10. Trout, D. I., Estes, E. J., Jr., Friedberg, S. J., *J. Lipid Res.*, 1960, v1, 199.

11. Zarafonetis, C. J., Seifter, J., Baeder, D. H., Kalas, J. P., *A. M. A. Arch. Int. Med.*, 1959, v104, 974.

12. Wolf, S., *Mod. Concepts Cardiovasc. Dis.*, 1960, v29, 599.

13. Shafrir, E., Steinberg, D., *J. Clin. Invest.*, 1960, v39, 310.

14. Havel, R. J., Goldfien, A., *J. Lipid Res.*, 1959, v1, 102.

15. Simms, H. S., Parshley, M. S., Pitt, R. B., *J. Geront.*, 1947, v2, 205.

16. Kline, I., Leighton, J., Belkin, M., Orr, H. C., *Cancer Res.*, 1957, v17, 780.

17. Weiss, A. K., *Fed. Proc.*, 1960, v19, 137.

18. Henneman, D. H., Henneman, P. H., *J. Clin. Invest.*, v39, 1239.

19. Seifter, J., Baeder, D. H., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 469.

20. Holman, R. L., McGill, H. C., Jr., Strong, J. P., Geer, J. C., *J. Am. Med. Assn.*, 1959, v170, 416.

Received April 24, 1961. P.S.E.B.M., 1961, v107.

## Modification of Lethal Effect of Bacterial Endotoxin by Substances Altering the Metabolism of 5-Hydroxytryptamine.* (26677)

ROGER M. DES PREZ,† NANCY FALLON AND EDWARD W. HOOK
(Introduced by P. Reznikoff)
*Department of Medicine, The New York Hospital - Cornell Medical Center, New York City*

5-Hydroxytryptamine (5HT), which in free form is a potent stimulator of smooth muscle, normally circulates bound to platelets in an inactive state(1). The occurrence of thrombocytopenia and elevated plasma concentrations of 5HT after intravenous injection of endotoxins from Gram-negative bacteria(2-3) suggests that some of the biologic effects of endotoxins might be related to release of 5 HT from damaged or sequestered platelets. The present studies were undertaken to determine the effect of substances that alter the metabolism of 5HT on the lethal action of bacterial endotoxin in mice.

*Materials and methods.* Three substances that alter 5HT metabolism were investigated: (A) 1-benzyl-2, 5-dimethyl serotonin (BAS),‡ an antimetabolite of 5HT by virtue of similar chemical configuration(4), (B) beta-phenyl isopropyl hydrazine (PIH)§(5), an inhibitor of monamine oxidase which catalyzes the degradation of 5HT to 5-hydroxy indole acetic acid(6), and (C) the metabolic precursor of 5HT, 5 hydroxy tryptophan (5-HTP)‖, administration of which appreciably increases tissue stores and platelet concentrations of 5HT(7). Dosages of BAS, PIH, and 5HTP were based on the reports of Shaw and Wooley(4), Horita(5), and Udenfriend *et al.*(7), and were somewhat larger than necessary for physiologic activity. These substances produced no apparent untoward effects when administered alone to control groups of 10 or more mice.

Eighteen to 25 g male albino mice of the CFW strain (Carworth Farms, New City, N. Y.) were used in all experiments. Groups of 10-20 mice were injected by the intraperitoneal (IP) or subcutaneous (SC) route with the substance to be tested on each of 2 suc-

* This study supported by USPHS grant.

† Edward Livingston Trudeau Fellow of American Thoracic Society.

‡ Merck, Sharp, and Dohme Research Laboratories, Rahway, N. J.

§ Lakeside Laboratories, Milwaukee, Wis.

‖ Nutritional Biochemicals, Cleveland, Ohio.