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Oxidation of Unsaturated Fatty Acids in Embryonic and Adult Tissues of Golden Hamster.* (22735)

B. THEODORE COLE† (Introduced by F. G. Hall)

Department of Physiology and Pharmacology, School of Medicine, Duke University, Durham, N. C.

Kohn and Liversedge(1) gave evidence that rat tissue homogenates as well as slices incubated aerobically produced a compound which condensed with either p-amino benzoic acid (PAB) or thiobarbituric acid (TBA) and gave a pink color. Bernheim and co-workers showed that the color obtained by Kohn and Liversedge upon addition of TBA to incubated tissues was due to a product of the oxidation of certain unsaturated fatty acids (2). Crystalline hemoglobin and ascorbic acid were found to catalyze the reaction involved. Analysis of the TBA compound showed that a 3-carbon fragment, probably a peroxide, had combined with TBA. Such a reaction, namely, the oxidation of a double bond and the breaking of a fragment containing 3-carbon atoms from the end of the chain could occur in linolenic acid. Wilbur's group suggested the use of TBA as a test for oxidation of highly unsaturated fatty acids, principally linolenic(3). Donnan, working with rat tissues, noted that the results obtained in using the TBA test to estimate the amount of oxidized highly unsaturated fatty acid present in tissue preparations paralleled those obtained by the usual procedure for fat estimation(4). Holman, Lundberg and Malkin(5) reemphasized the TBA test as a means of qualitative and indirect measure of the oxidative products of highly unsaturated fatty acid (*i.e.*, linolenic, linoleic and oleic) content of tissues. Increased concentration of highly unsaturated fatty acids has been associated with increased rate of cell division(6). The following investigation was carried out to compare the oxidation of highly unsaturated fatty acids (HUFA) in terms of their oxidative products (HUFA-p) in brain, testes and liver, tissues of the embryo and young animal, with those of the adult. Experimental procedures were designed also to give evidence for *in vivo* oxidation of highly unsaturated fatty acids. Correlation between HUFA content and development of the tissues, as well as evidence of *in vivo* HUFA-p formation are presented.

Materials and methods. All pregnant females and weaned (15 days) animals derived HUFA's from a diet of Purina rabbit chow checkers supplemented with lettuce twice each week. The analysis of this diet, supplied by the Ralston Purina Co., is shown in Table I. About 50% of the 2.5% fat is made up of unsaturated fatty acids. Entire organs (with exception of adult livers, where only the left lobe was used) were removed immediately from decapitated animals, weighed wet, homogenized at 0-4°C and diluted with phosphate buffer, pH 6.0 to final concentration of 50 mg/ml. One 0.5 ml aliquot of this stock homogenate was added to 1.5 ml of buffer or to a mixture of 1 ml of buffer and 1.5 ml of ascorbic acid (concentration 0.2 mg/ml.) These mixtures contained in 50 ml Erlenmeyer flasks were either incubated in a con-

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† Department of Zoology, Physiology and Entomology, Louisiana State University, Baton Rouge.

TABLE I. Purina Rabbit Chow Checkers

Protein, %	15.00
Fat, %	2.50
Carbohydrate, %	55.00
Fiber, %	16.21
Ash, %	8.04
Calcium, %	1.12
Phosphorus, %	.65
Magnesium, %	.19
Iron, parts/million	161.00
Manganese, " "	55.00
Copper, " "	7.40
Cobalt, " "	.13
Iodine, " "	5.20
Potassium, %	1.03
Carotene (vit. A activity), parts/million	23.00
Thiamin, parts/million	3.70
Riboflavin, " "	7.40
Niacin, " "	38.80
Vit. D, U.S.P. units/g	2.21
Pantothenic acid, parts/million	6.62
Choline, " "	711.00

stant temperature bath for 2 hours at 37° or placed in cold centrifuge tubes and kept in ice bath for not longer than 30 minutes. The latter solutions so refrigerated were considered to represent a measure of the amount of oxidative product of the highly unsaturated fatty acid present in tissue *in vivo* at the time of removal. Values obtained from tissues incubated without ascorbic acid represent levels of HUFA oxidation as influenced by *in vivo* levels of vit. C, hemoglobin and perhaps other unknown protoplasmic catalysts. Total HUFA content of tissues is represented by TBA values for tissues incubated with ascorbic acid added *in vitro*. At end of incubation, or immediately, in the case of unincubated tissues, 1 ml of 20% trichloroacetic acid (TCA) was added and the precipitated protein was removed by centrifugation. To extract the chromogen precursor, at this step bound to the protein in the pellet, 2 ml of 5% TCA was added to the pellet. To the supernatant, and to tubes containing protein and 5% TCA, was added 2 ml of 0.75% thiobarbituric acid. These mixtures were placed in boiling water bath for 15 minutes. All tubes were subsequently returned to total volume of 5 ml with distilled water and the color read and recorded at wave length 525 on Coleman Junior Spectrophotometer. The optical density of supernatant was expressed in terms of Q values read directly from Spectrophotometer. These values were interpreted as a qualitative expression of the fraction of HUFA-p unbound, and values representing color extracted from the pellet were interpreted as a measure of the HUFA-p bound to protein. All values were read against a blank of 2 ml of 20% TCA, 2 ml of 0.75% TBA and 1 ml distilled water. All studies were carried out in duplicate. Values shown in Tables and Figures are in terms of color density produced by 25 mg of tissue and represent total color produced (bound plus unbound fractions).

Results are presented in Figs. 1, 2, and 3. Data on tissues from embryos 14-15 days old are plotted above abbreviation "Emb" and those for tissues from animals within 8 hours *post partum* above the letters "JB". Incubated tissues at every age gave considerably higher values than unincubated tissues. It is also evident that the catalytic action of ascorbic acid is manifest only in incubated tissues. Statistical analysis of unincubated liver and testes tissue data shows no significant difference between values obtained for different aged animals. In brain tissue, however, the values are significantly higher

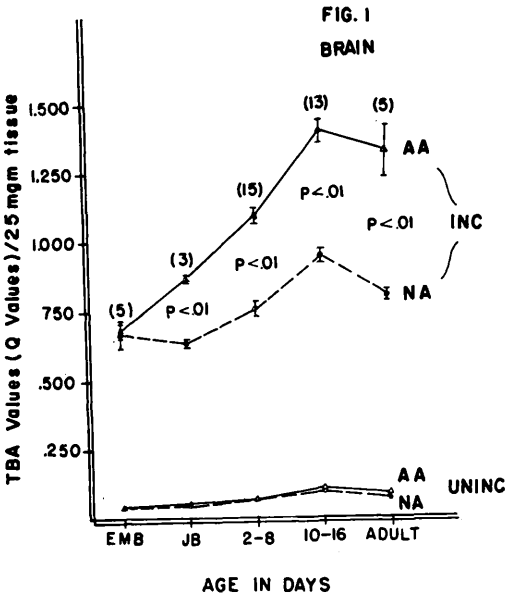

FIG. 1. Mean TBA values which reflect amount of "HUFA-p" available under experimental conditions in brain tissue of different aged hamsters are shown. AA: ascorbic acid added. NA: no ascorbic acid added.

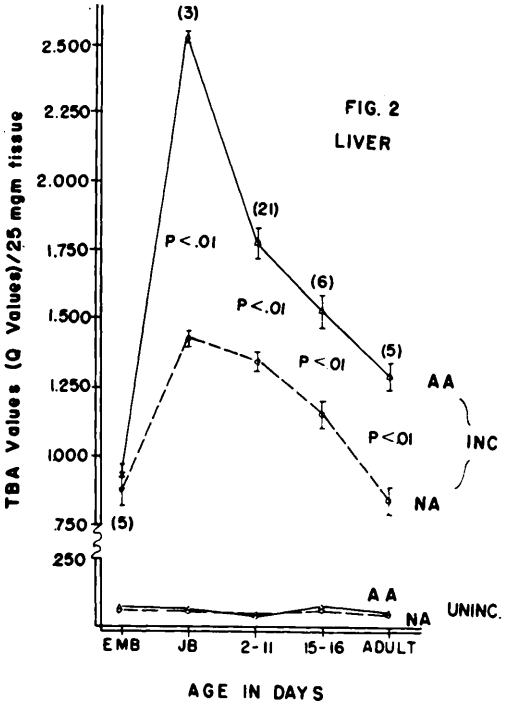

FIG. 2. Mean TBA values which reflect amount of "HUFA-p" available under experimental conditions in liver tissue of different aged hamsters are shown. AA: ascorbic acid added. NA: no ascorbic acid added.

($P < .01$) in animals 10 days of age and older, than in embryos or in tissues of animals at birth. Brain values of animals above 10 days old are higher than either liver or testes values, indicating the presence of greater quantities of HUFA oxidized *in vivo* in this tissue.

TBA values for brain tissue incubated with ascorbic acid show significant increases from embryonic tissues through 10 days of age (Fig. 1). Values for tissues of embryo and those of animals through 8 days of age, incubated without ascorbic acid, are significantly lower than values for brain tissue of animals beyond 10 days of age. Catalytic action of ascorbic acid on oxidation of HUFA in embryonic tissue is not apparent.

TBA values for liver tissue incubated with ascorbic acid show a pattern in reverse to that of the brain (Fig. 3 and 4). Although oxidation of HUFA in embryonic liver tissues as in brain, does not reflect the catalytic action of vit. C, values for tissues incubated with ascorbic acid are extremely high at birth and thereafter show a gradual and significant decline to adult levels. The latter are substantially elevated above values for embryonic tissue. Tissues incubated without ascorbic acid indicate a similar trend except that levels in the adult are not significantly different from those in the embryo.

Values for testes tissue incubated with ascorbic acid show an increase from a mean level of .701 in animals 15-30 days of age to a peak of 1.331 in animals 55-65 days of age (Fig. 3). Tissues incubated without ascorbic acid reflect a similar trend with peak in group 35-65 days old.

Discussion. Consideration of TBA values for unincubated liver, brain and testes tissues give evidence of a measurable amount of HUFA oxidized *in vivo* with oxidative products present in tissue at time of sacrifice. These conclusions are based upon the fact that measurable quantities of HUFA-p were found in tissues maintained at low temperature and in consideration of the fact that if oxidation occurred *in vitro* it appears to be a valid assumption that it would be catalyzed by the ascorbic acid added. Bernheim, Wilbur and Kenaston(7) have suggested *in vivo* formation of oxidized fatty acid products may act as normal regulators of some aspects of

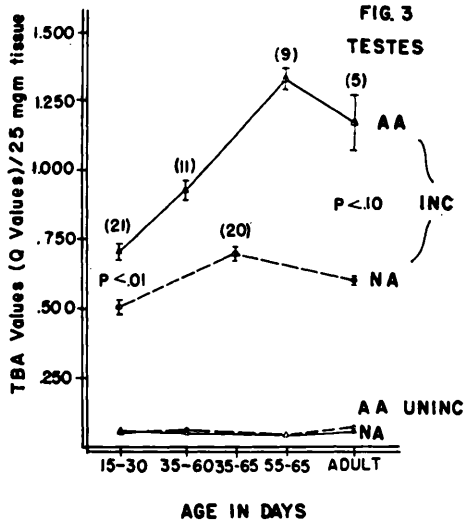

FIG. 3. Mean TBA values which reflect amount of "HUFA-p" available under experimental conditions in testes tissue of different aged hamsters are shown. AA: ascorbic acid added. NA: no ascorbic acid added.

metabolism. Wilbur and co-workers(8) suggest a role of HUFA-p in inhibition of cell division. The oxidative product may well be labile *in vivo* and the values here defined represent those "stabilized" by death of animal and its constituent protoplasm. Except in brain tissue of animals above 10 days old, there is no significant correlation between change of *in vivo* levels of HUFA-p and age. Variation in TBA values of tissues incubated without ascorbic acid reflect *in vitro* oxidation of HUFA in presence of unknown concentration of ascorbic acid which is a constituent part of the tissue under investigation. Therefore, variation in these TBA values may be interpreted as evidence for variation in ascorbic acid content of tissues of animals at various ages, rather than a quantitative expression of HUFA present. Values for tissues incubated with ascorbic acid, under these experimental conditions, probably give a qualitative estimate of total highly unsaturated fatty acid content of the tissue studied.

In the case of brain tissue the content of HUFA gradually rises to that of adult levels in the 10-day-old animal. Since neither the adult nor the embryo is known to synthesize highly unsaturated fatty acids, and in light of the work of Bickenback and Rupp(9) it can be assumed that the only source of HUFA to the embryo is via maternal circulation(10, 11,12). It is significant to note that TBA values in the animals here studied are established at adult levels when the animal is weaned and begins to eat "hard" food, 10-16 days *post partum*. Values for tissues incubated without ascorbic acid do not rise appreciably in the adult, which fact eliminates the probability of an inhibitory action of ascorbic acid added *in vitro*. The abrupt rise in HUFA content of liver tissues correlates well with alteration in liver function known to occur at birth. The findings may be exaggerated by catalytic action of hematopoietic components in the liver during this interval. The gradual rise in total HUFA content of testes tissue from 15 days to a peak in the 55-56 age bracket suggests a significant relationship between fatty acid content, development, and function of the organ. The peak in HUFA content correlates well with age of sexual maturity in the hamster. The gradual, increased difference in TBA values correlated with age, between tissues incubated with and without ascorbic acid suggests a gradual alteration in the *in vivo* content of ascorbic acid.

Perhaps the most significant finding is lack of evidence for catalytic activity of vit. C on *in vitro* oxidation of HUFA constituents of embryonic tissues. This could be due to inhibitory effects of high concentration of ascorbic acid (resulting from high *in vivo* levels), or could be the result of an inhibitory substance peculiar to embryonic tissues and not found in tissues *post partum*. The possible role of variable tissue concentrations of tocopherols and other metabolic antioxidants should be mentioned. Although no bio-assay of vit. E was made, the diet used is sufficient in every respect to sustain our colonies of hamsters indefinitely without showing symptoms of vitamin deficiency. The possibility exists that where TBA values for tissues incubated without ascorbic acid are low, tissues themselves may contain high concentrations of antioxidants not readily identified. Where no difference can be demonstrated between levels of HUFA-p in tissues incubated with and without ascorbic acid (*i.e.*: embryonic tissues) a reaction between some unidentified antioxidant may be manifest. Ability of specific tissues of various aged animals to concentrate tocopherol remains a possibility. Current investigation of this problem is being undertaken.

Summary. The Thiobarbituric Acid Test for oxidative products of essential fatty acids (HUFA), primarily linolenic, present in tissues *in vivo* and after aerobic oxidation *in vitro,* with and without ascorbic acid, was applied to brain, liver and testes tissues of hamsters of various ages. Minute measurable quantities of oxidative products (probably peroxides) are formed *in vivo*. The quantity of HUFA present in brain tissue increased gradually with *post partum* development of the brain, with constant levels apparent after 10-16 days of age. The quantity of HUFA present in testes tissue correlates well with development and function of that organ

with constant levels established, following a peak during age of sexual maturity. There is a significant rise in total HUFA content of liver tissue, associated with emergency of birth. Lack of catalytic activity of ascorbic acid upon *in vitro* oxidative reaction involved in embryonic tissue has been demonstrated.

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Influence on Circulating 17-Hydroxycorticosteroid Concentrations of Compounds Structurally Related to Salicylate.* (22736)

ALAN K. DONE,† ROBERT S. ELY, AND VINCENT C. KELLEY

Department of Pediatrics, University of Utah College of Medicine, Salt Lake City

Earlier publications(1,2) reported the finding of marked elevations in circulating 17-hydroxycorticosteroid (17-OHCS) concentrations in guinea pigs following intraperitoneal or oral administration of large doses of sodium salicylate. Hypophysectomy, as well as adrenalectomy, abolished this effect(2). In the present investigation, various other derivatives of benzoic acid were studied with regard to their effects on circulating 17-OHCS concentrations in guinea pigs in an attempt to ascertain whether these effects are related to the structural configuration of the administered compound.

Methods. Male guinea pigs weighing 450 to 750 g were used in these experiments. Sodium salts‡ of the compounds listed in Table I were prepared by dissolving the acids in an equivalent amount of 1 N NaOH; the pH of each solution then was adjusted to neutrality using the Beckman glass electrode. Solutions were diluted with distilled water to contain the equivalent of 100 mg of the acid per ml. Compounds tested were injected intraperitoneally in doses equivalent (on the basis of molecular weight) to 200 mg salicylic acid per kilo body weight. Four hours later the animals were rapidly anesthetized in an ethyl ether atmosphere and bled from the abdominal aorta into heparinized syringes. Additional groups of animals treated with the monohydroxy derivatives of benzoic acid were sacrificed at 1 and 2 hours following injection. Plasmas were separated by centrifugation immediately and stored in deep-freeze until used for analyses. Plasma concentrations of 17-OHCS were determined by the method of Nelson and Samuels(3) as modified by Eik-Nes, Nelson and Samuels(4).

Observations. Data concerning the influence on plasma 17-OHCS concentrations of the various compounds tested are shown in Table I. Benzoic acid had no significant effect. For purposes of statistical analysis the

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‡ For convenience, compounds will be referred to as acids.