# THE INFLUENCE OF CHOLINE, CYSTINE, AND OF α-TOCOPHEROL UPON THE OCCURRENCE OF CEROID PIGMENT IN DIETARY CIRRHOSIS OF RATS

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Since the original publication of Lillie, Daft, and Sebrell in 1941 (1), the yellow fluorescent, acid-fast "ceroid" pigment, so abundantly present in the nutritional cirrhosis of rats, has been described and studied by a number of investigators (2–8). Regarded at first as an essential and distinctive feature of the lesions, it has now been shown that dietary cirrhosis unaccompanied by pigmentary deposits can be produced in rats (9, 10).

The precise factors which condition the appearance of the pigment are not known. The fact that cod liver oil may be transformed by oxidation *in vitro* into a fluorescent, acid-fast material (11) and that subcutaneously injected cod liver oil acquires similar properties (12, 11) suggested that this component of the experimental diets may have provided a source for the pigment. However, Wachstein (10) has recently shown that ceroid may be formed even when cod liver oil is omitted from the diet.

It is well known that vitamin E deficiency in female rats produces a brown coloration of the uterine muscle and it has been demonstrated in our laboratory, and by Mason and Emmel (19) that this pigment, like ceroid, resists decolorization with acids after staining with carbolfuchsin. Its fluoresence has been described by Moore and Wang (20). Since this pigmentation is preventable by administration of vitamin E, the question arises whether lack of this vitamin may not be a factor in the deposition of the liver ceroid pigment as well. Bearing on this possibility are the recent observations of Dam and Mason (21), that a diet containing 20 per cent cod liver oil brings about the deposit of acid-fast pigment globules in the adipose tissue of young rats, and that the pigmentary changes are prevented by the administration of α-tocopherol.

In view of this suggestive relationship, it seemed of interest to pursue the matter further and to determine experimentally to what degree the presence of ceroid in nutritional cirrhosis could be influenced by the administration of vitamin E. A careful scrutiny of the various diets which have been used in the production of nutritional cirrhosis discloses that most of them were deficient in vitamin E. Those that did contain adequate amounts of vitamin E, as that used by Blumberg and McCollum (3) in which the rats received

3 to 5 cc. of wheat germ oil daily, led to the formation of relatively small amounts of ceroid. In the production of cirrhosis without ceroid, as has been reported by Endicott, Daft, and Sebrell (9), $\alpha$-tocopherol was included in the experimental diet for the first time.

Certain other observations have led us to study the possible modifying effect of choline upon this pigmentation. The fact that choline prevents fat accumulation in the liver cells and that most of the diets leading to nutritional cirrhosis with ceroid pigmentation also brought about the formation of large quantities of fat, raises the point as to whether the fatty change is a necessary precursor to the ceroid.

Since it has been found that excess dietary *l*-cystine produces cirrhosis unaccompanied by fat deposition (22, 23), this offers another means of investigating the fat-pigment relationship, and the possible modifying influence of vitamin E.

The following experiments bear upon these points:—

### *Ceroid Production in Cystine Cirrhosis: Comparison of Effects of Stock Diet and Low Protein Diet*

Two groups, one of 15, the other of 14 young male rats weighing 50 to 80 gm. were fed respectively 5 per cent cystine in McCollum's stock diet (24), and 5 per cent cystine added to a low protein diet of the following composition (22):—

| | *per cent* |
|---|---|
| Commercial casein | 5 |
| Lard | 20 |
| Sucrose | 56 |
| Cod liver oil | 5 |
| Salt (28) | 4 |
| *l*-Cystine | 5 |
| Yeast | 5 |

McCollum stock diet contained in parts per cent: bran 67.5, whole milk powder 15, commercial casein 10, butter 5, NaCl 1.5, and $Na_2CO_3$ 1.0.

The animals were sacrificed at intervals between 3 and 43 days in order to determine the time of first appearance of the ceroid. Sections were stained with Ziehl-Neelsen, and unstained sections studied with the fluorescent microscope.

*Results.*—On the stock diet, containing 5 per cent cystine, 2 of 15 livers had small amounts of ceroid in a few histiocytes within the periportal scars. These livers were from rats which had been on the diet for 39 days. Five of the series of 15 had slight cirrhosis—2 examined on the 6th day, 1 on the 7th day, and 2 on the 39th day (Table I).

In contrast, 11 of 14 rats on the 5 per cent *l*-cystine low protein diet developed ceroid within histiocytes and liver cells. It was first noted on the 6th day, and was present in all after the 15th day.

The food intake of each group was the same, averaging 6.1 gm. daily.

*Influence of 0.2 Per Cent α-Tocopherol Added to l-Cystine Low Protein Diet*

A. In this experiment, 6 young rats weighing 65 to 80 gm., which had previously been on stock diet, were placed on the 5 per cent *l*-cystine low protein diet in which 0.2 per cent α-tocopherol had been incorporated. The average daily food intake of this group was 8.5 gm., so that each rat received approximately 1.7 mg. of α-tocopherol daily. Six control rats of similar age, weight, and stock were given the 5 per cent *l*-cystine low protein diet without α-tocopherol. The average daily food intake was 7.1 gm.

Since the previous experiment had shown that all animals on the *l*-cystine low protein diet had ceroid after the 15th day, the experiment was terminated on the 28th day and the animals were sacrificed.

TABLE I

*Ceroid on Diets Containing 5 Per Cent l-Cystine*

*Comparison of Stock, Low Protein, and Low Protein Plus Choline Diets*

| Stock diet | | | | Low protein diet | | | | Low protein plus 1 per cent choline | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | Days on diet | Ceroid | Cirrhosis | No. | Days on diet | Ceroid | Cirrhosis | No. | Days on diet | Ceroid | Cirrhosis |
| 7-60 | 3 | − | − | 5-25 | 3 | − | − | 7-61 | 3 | − | − |
| 7-62 | 6 | − | ± | 5-29 | 5 | − | − | 7-65 | 6 | − | ± |
| 7-63 | 6 | − | ± | 7-64 | 6 | + | − | 7-73 | 7 | − | − |
| 7-70 | 7 | − | + | 5-34 | 8 | − | − | 7-72 | 7 | − | − |
| 7-19 | 9 | − | − | 5-44 | 15 | + | − | 7-71 | 7 | − | ± |
| 7-22 | 16 | − | − | 5-50 | 20 | ++ | − | 7-83 | 14 | − | − |
| 7-38 | 21 | − | − | 7-88 | 21 | + | − | 7-91 | 21 | − | − |
| 7-39 | 21 | − | − | 7-89 | 21 | + | − | 7-92 | 21 | − | − |
| 7-47 | 24 | − | − | 7-90 | 21 | + | − | 7-93 | 21 | − | − |
| 7-45 | 24 | − | − | 5-59 | 28 | + | − | 7-94 | 24 | − | − |
| 7-46 | 24 | − | − | 8-01 | 34 | +++ | + | 7-95 | 24 | ± | + |
| 7-48 | 24 | − | − | 8-02 | 34 | +++ | + | 7-96 | 24 | − | − |
| 7-49 | 24 | − | − | 5-78 | 40 | + | + | 7-97 | 24 | − | − |
| 8-06 | 39 | ± | + | 5-85 | 43 | ++ | − | 8-04 | 38 | ± | + |
| 8-07 | 39 | ± | ± | | | | | 8-05 | 38 | ± | + |

B. This experiment was a repetition of A, using two groups of rats which were the offspring of females maintained for eight generations on vitamin E-deficient diet, except for the single dose necessary to ensure the delivery of living young. The results are shown in Table II. The average daily food intake for the α-tocopherol-treated group was 7.3 gm. and for the control it was 6.7 gm.

*Comment.*—Several deductions emerge from this experiment.

1. α-Tocopherol does not prevent, but strikingly inhibits the accumulation of ceroid pigment in the liver under the conditions described. Whereas in the α-tocopherol-treated rats, it was present in very small amount and confined to a few histiocytes and liver cells, it was abundantly present in the untreated controls.

2. The α-tocopherol had no influence on the onset of the cirrhotic changes produced by the 5 per cent *l*-cystine low protein diet.

3. The deposition of liver ceroid in rats fed 5 per cent *l*-cystine low protein diet is not modified by the previous feeding of a diet adequate in vitamin E.

4. The inhibitory influence of the α-tocopherol was obvious, despite the high content (5 per cent) of the diet in cod liver oil.

5. Ceroid may be present in large amount in liver showing no cirrhosis (rats A1, A3, A6, B5, B7).

TABLE II

*Inhibition of Ceroid Deposition in Liver by α-Tocopherol on l-Cystine, Low Protein Diet*

| 0.2 per cent α-tocopherol | | | Controls | | |
|---|---|---|---|---|---|
| No. | Ceroid | Cirrhosis | No. | Ceroid | Cirrhosis |
| A7 | − | ± | A1 | ++ | − |
| A8 | ± | − | A2 | +++ | ± |
| A9 | ± | − | A3 | + | − |
| A10 | ± | − | A4 | +++ | ± |
| A11 | ± | − | A5 | +++ | + |
| A12 | ± | − | A6 | +++ | − |
| B44 | ± | + | B4 | ++ | ± |
| B45 | ± | + | B5 | +++ | − |
| B46 | ± | ± | B6 | ++++ | ± |
| B47 | ± | ± | B7 | +++ | − |
| B48 | ± | − | B8 | +++ | + |
| B49 | ± | − | | | |

### *Inhibitory Influence of Choline upon Ceroid Deposition*

Fifteen male rats of the same age, stock, and race as those used in the first experiment, were fed 1 per cent choline hydrochloride in a 5 per cent *l*-cystine low protein diet, choline replacing an equivalent amount of sucrose. The rats were sacrificed at intervals between 3 and 38 days. Only 3 of the 15 had ceroid within histiocytes of periportal scars, which were present in one liver at 24 days, and two at 38 days. These three were the only ones with cirrhosis. The average daily food intake of this group was 6.8 gm.

*Comment.*—The addition of choline to the 5 per cent *l*-cystine low protein diet obviously retarded and inhibited, though incompletely, the deposition of the ceroid pigment. Table I does not bring out the fact noted in the sections, that the pigment on the stock and choline diets is restricted to the histiocytes in the portal spaces, whereas in the unsupplemented *l*-cystine low protein diet, it is abundant within the liver cells as well.

Confirming well established observations, the liver of the choline-treated group contained little fat as determined by staining and chemical analysis (22, 23) as compared with those of the untreated group. Whether the in-

hibitory effect of the choline upon ceroid deposition is due to a direct influence upon its production, or whether it acts indirectly by preventing the accumulation of some lipid precursor, cannot be decided with the evidence at hand. It has been pointed out previously (23) that the choline does not prevent the onset of the cirrhosis on the 5 per cent *l*-cystine low protein diet.

### *Influence of α-Tocopherol upon Ceroid Production in Livers of Rats on a Low Protein Diet*

Twenty-four rats belonging to three litters were used in these experiments. The mothers were 6th generation on vitamin E–deficient diet. There were 8 males and 16 females. Five of the rats were started at the age of 49 days, the remainder immediately after weaning. The older animals weighed 125 to 152 gm., the younger 28 to 44 gm. The diet consisted of:

| | *per cent* |
|---|---|
| Commercial casein | 6 |
| Sucrose | 15 |
| Cornstarch | 50 |
| Lard | 23 |
| Cod liver oil | 2 |
| Salts (28) | 4 |

This was supplemented by thiamin 10 mg., riboflavin 20 mg., pyridoxine 10 mg., calcium pantothenate 20 mg., niacin 20 mg., per kilo of diet. This diet is similar to diet L111 of György and Goldblatt (2). For a period of 4 weeks, because of their poor initial growth, 5 per cent yeast was given in place of vitamin B components.

The rats were separated into two groups, in one of which each animal received 2.5 mg. of α-tocopherol in sesame oil by mouth twice weekly, the other group serving as controls. Biopsy specimens of the liver were taken on the days indicated in Table III. At autopsy, sections were taken from all tissues. This analysis, however, will be restricted to the findings in the liver.

*Comment.*—From the data presented in the table, it seems fair to draw the following inferences:—

1. α-Tocopherol, in the dosage employed, for a time virtually inhibits the appearance of the ceroid. Thus the pigment was absent in all but one of the 13 biopsy specimens obtained from the 27th to the 57th day, whereas it was found in 8 of 11 untreated controls. This effect was still apparent on the 85th to the 100th day; only one of the 5 rats examined during this period had ceroid, whereas all the untreated controls had ceroid. Later, the protective effect of the tocopherol is much less evident, although the amount of the pigment in the tocopherol group is slightly less than in the untreated.

Since fatty changes and cirrhosis were similar in both groups, it is evident that the development of these lesions is not influenced by the tocopherol. However, one might surmise that the more severely damaged livers are less responsive to the action of the tocopherol in inhibiting the appearance of the pigment.

2. Another significant effect of the tocopherol was evident in the general appearance and growth of the treated animals. The weight curves of the

two groups were similar for 4 months; from this time on, the untreated rats declined in weight, so that at the time of death (163 to 174 days), the mean weight of the survivors was only 113 gm. as compared with 177 gm. in those which had received tocopherol. Although a flattening of the weight curve in vitamin E–deficient rats on an otherwise adequate diet has been described by Evans and his coworkers (25, 26), marked loss of weight does not occur in uncomplicated vitamin E deficiency in rats, and has never been observed by

TABLE III

*Inhibitory Influence of α-Tocopherol on Accumulation of Ceroid Pigment in Livers of Rats on Low Protein Diet*

| α-Tocopherol, 2.5 mg. semiweekly | | | | | | Controls | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | Biopsy | | Autopsy | | | No. | Biopsy | | Autopsy | | |
| | Days | Ceroid | Days | Ceroid | Body weight | | Days | Ceroid | Days | Ceroid | Body weight |
| | | | | | gm. | | | | | | gm. |
| A4-1 | 56 | – | Died after operation | | | A20-2 | 57 | ± | 85 | ++ | 148–169 |
| A27-2 | 56 | – | 94 | – | 40–112 | A27-9 | 56 | + | 94 | +++ | 32–110 |
| A4-5 | 56 | – | Died after operation | | | A27-10 | 56 | + | 94 | ± | 35–149 |
| A27-3 | 56 | – | 94 | – | 40–164 | A27-6 | 27 | ± | 100 | + | 38–120 |
| A20-4 | 57 | – | 85 | ++ | 125–150 | A27-7 | 56 | – | 100 | + | 44–148 |
| A27-1 | 27 | – | 100 | – | 40–184 | | | | | | |
| A27-5 | 48 | – | 100 | – | 40–150 | | | | | | |
| A20-3 | 57 | ± | 167 | ++++ | 152–221 | A4-8 | 56 | + | 163 | +++ | 30–80 |
| A20-5* | 57 | – | 167 | ++++ | 146–208 | A4-9 | 56 | – | 163 | ++++ | 34–101 |
| A4-2 | 27 | – | 163 | ++ | 28–134 | A20-1 | 57 | – | 167 | ++++ | 145–165 |
| A4-3 | 56 | – | 163 | +++ | 36–165 | A27-8‡ | 48 | + | 171 | ++++ | 38–97 |
| A27-4 | 56 | – | 171 | + | 42–157 | A4-6 | 56 | + | 174 | +++ | 36–150 |
| A4-4 | 56 | – | 171 | ++ | 34–174 | A4-7 | 27 | ± | 174 | ++++ | 34–84 |

* Jaundiced.

‡ Thrombosis of portal vein and ascites.

us in many hundreds of rats maintained for long periods on a vitamin E–deficient diet.

Associated with this weight loss, there was noted a marked reduction of food consumption, calculated as food intake per 100 gm. of body weight. The average daily intake of the tocopherol-treated rats was 7.2 gm. per 100 gm. of body weight, as contrasted with 5.2 gm. for the untreated animals—a difference of 40 per cent.

There was also an obvious difference in the appearance of the two groups. The untreated rats showed a diffuse thinning of the hair; coincident with the weight loss, they became dirty, the tail, face, and ears covered with an orange-brown scurf, which also collected about the outside of the feeding cups. Those

receiving tocopherol looked sleek despite the fact that all had cirrhosis. This effect of tocopherol in conserving growth and well-being on a low protein diet has not, so far as we are aware, been previously emphasized.

3. The usual signs of vitamin E deficiency were found on this standard cirrhosis-producing diet, which was essentially similar to that used by György and Goldblatt (2): in the females, brown pigmentation of the uterus; in the males, complete loss of spermatogenesis with atrophy of the testes; in both sexes, muscular dystrophy of varying degrees. The $\alpha$-tocopherol-treated group showed none of these changes.

## DISCUSSION

The experiments presented offer evidence that vitamin E, in the form of $\alpha$-tocopherol, retards the appearance of ceroid pigment in the livers of rats on low protein diets. The effect is most striking when 5 per cent *l*-cystine, which speeds up the formation and deposition of the pigment, is added to the diet. The inhibitory effect however, with the dosage used, is not a lasting one; after 160 days, the livers of the $\alpha$-tocopherol-treated rats contain almost as much ceroid as the untreated controls.

Another point that has become evident is that the presence of pigment is not dependent either upon fatty changes in the liver cells, or upon the cirrhosis. We have repeatedly seen extremely fatty livers without ceroid, and conversely, ceroid may occur in livers with very little fat accumulation. Although when cirrhosis develops, such pigment as is present tends to become concentrated in the portal scars, cirrhosis may occur in $\alpha$-tocopherol-treated rats without any pigment whatever.

Since pigment having similar staining reactions, morphology, and distribution may occur in human cases of cirrhosis and other nutritional disorders (27), its occurrence in the tissues may perhaps be indicative of an underlying vitamin E deficiency, or when associated with cirrhosis of the liver, may point to an inability of this organ to store or properly metabolize the vitamin E brought to it from the intestine. This is admittedly speculative, but the fact that there are no known specific lesions characterizing vitamin E deficiency in man lends interest to the suggestion. Even though factors other than lack of vitamin E may be concerned, such as lack of choline or excess of *l*-cystine or cod liver oil (10), it may still be a helpful indication of this particular nutritional disturbance, when considered in conjunction with other evidence. This suggestion finds support in the fact that the brown uterine pigment of rats, which by our present tests is indistinguishable from the ceroid pigments in the liver on low protein diets, occurs when the only known dietary deficiency is vitamin E; indeed, it is one of the pathognomonic lesions of this condition (13–19).

Admitting that there is some relationship between ceroid pigment and

deficiency of vitamin E, many questions remain to be investigated. Is the damaged liver unable to handle or store tocopherol brought to it from the intestine? Is the inhibitory effect of the $\alpha$-tocopherol due to its antioxidant properties, or is there some more specific chemical reaction involved? These and other problems cannot be solved until more is learned of the chemistry of the ceroid pigment on the one hand, and of the biochemical action of the tocopherols upon the tissues, on the other.

As for the inhibitory influence of choline, little can be said beyond presenting the experimental data. These do indicate that the appearance of the ceroid does not depend upon a preceding fatty change in the liver cells, since the cirrhosis produced on the *l*-cystine low protein diet in the presence of choline is unaccompanied by fat accumulation in the liver cells; nevertheless, small amounts of ceroid pigment appear after 39 days.

## CONCLUSIONS

1. Five per cent *l*-cystine in a stock or low protein diet produces ceroid deposits in rat liver. This effect of *l*-cystine is much greater in low protein than in stock diets.

2. One per cent choline has an inhibiting effect on deposition of liver ceroid resulting from a low protein diet containing excess cystine.

3. The occurrence of ceroid pigment in the livers of rats on a low protein diet, with or without the addition of excess *l*-cystine, is transiently inhibited by the administration of $\alpha$-tocopherol. Five per cent cod liver oil in the diet did not prevent this effect of $\alpha$-tocopherol.

4. On low protein, vitamin E–deficient diets, there occurs after 4 months, a rapid and progressive weight loss. This does not happen when $\alpha$-tocopherol is added to the diet.

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