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# METABOLIC CHANGES INDUCED IN CHICKENS BY THE ADMINISTRATION OF TREMETOL

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**Abstract**—Tremetol is a toxic extract of the White Snakeroot plant (*Eupatorium urticaefolium* Reichard). The milk of cows grazing on Eupatorium-infested pastures causes "milk sickness", characterized in part by ketoacidosis and changes in blood glucose levels. In this paper the metabolic effects of chronic administration of tremetol in the chick were studied. Blood glucose and blood free fatty acid levels respond with a biphasic pattern to tremetol administration, first rising above, then decreasing below the control values. In contrast, the blood levels of β-hydroxybutyrate show a sustained and major increase during the treatment period. Tremetol had no demonstrable effect on hepatic glycogen content in the treated group. Studies on hepatic enzyme levels revealed an almost total suppression of citrate synthase activity in the experimental group without a parallel decrease in succinate dehydrogenase activity. A selective toxic action on mitochondria resulting in an inability to effectively metabolize acetyl CoA is put forward as the possible cause of the ketoacidosis.

TREMETOL is the active extract obtained from the White Snakeroot plant (*Eupatorium urticaefolium* Reichard). Ingestion of milk from cows who have grazed on this weed produces serious and sometimes fatal intoxications. The syndrome, called milk sickness, is associated with a metabolic disturbance characterized by severe ketoacidosis. Prior to recognition of the causative factor, outbreaks of milk sickness caused many early American communities to disband as the settlers sought healthier areas. The most renowned victim was Abraham Lincoln's mother who succumbed to the disease in 1818. An occasional episode is still encountered.[1]

From the extensive literature accumulated over more than a century and a half dealing with this condition, several major reviews should be mentioned: Couch;[2,3] Wolf *et al.*[4,5] Stenn;[6] Marsh;[7] Moseley;[8] Snively;[9] Christensen;[10] Schuchardt[11] and Drake.[12] A bibliography covering the period from 1811 to 1966 was prepared by Furbee and Snively.[13]

Tremetol has at least six components, three of which have known structures and are related methyl ketone derivatives of benzofuran,[14–16] i.e. tremetone, dehydrotremetone and hydroxytremetone. It has not been established whether any of the known components alone or in combination are responsible for the action of tremetol or whether as yet unidentified compounds may be involved. In view of this we choose the crude but effective extract as our experimental substrate. The choice of the chicken for our studies was based on the detailed description by Butler[11] of tremetol intoxication in this species.

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## METHODS

White Leghorn baby chicks were maintained in a heated coop and supplied with water, Purina chicken feed and crushed oyster shells. The animals were randomly divided into control and test groups. Tremetol was administered daily in 1 mg/g dosage i.m. The material was dissolved in sterile corn oil or propylene glycol in 250 mg/ml concentration. Control animals were injected with the solvent. Glucose was determined on whole blood with glucose oxidase (Glucostat reagent, Worthington Biochemical Corp., N.J.), after deproteinization, according to Somogyi.[18]

Tremetol was prepared by the method of Couch[19] from the plant *E. urticaefolium*, kindly supplied by Dr. James E. Robbers of the School of Pharmacy and Pharmacal Sciences, Purdue University. A voucher specimen is in the Herbarium of the Fairchild Tropical Gardens at Miami, Fl.

The product was characterized by demonstrating the presence of one of tremetol's characteristic components, tremetone. This was accomplished by chromatography on thin-layer plates: Silica gel G (E. Merch A.G., Darmstadt, Germany), layer thickness 250$\mu$m, chloroform as solvent. The spots were visualized either under u.v. light or by using an ethanolic solution of 2,4-dinitrophenylhydrazine hydrochloride. A reference sample of (—)-tremetone was kindly supplied by Dr. W. A. Bonner, Department of Chemistry, Stanford University. One component of our tremetol matched that of the tremetone reference $R_f$ 0·51. The identity of this compound with tremetone was further corroborated by the infra-red spectrum of the eluate, which matches the spectrum of tremetone reported by DeGraw.[16]

The free fatty acid analyses were carried out on whole blood according to the method of Itaya and Ui.[20]

D (—)-$\beta$-Hydroxybutyrate was determined enzymatically by the method of Williamson *et al.*[21] as modified by Young and Renold.[22] The D (—)-$\beta$-hydroxybutyric dehydrogenase (*Rhodopseudomonas spheroides*) was obtained from C. F. Boehringer u. Soehne GmbH, Mannheim, Germany, NAD from P–L Biochemicals, Inc., Wis., and sodium DL-$\beta$-hydroxybutyrate (A grade) from CalBiochem, Calif. The assays were carried out in a Gilford model 2000 spectrophotometer, at 25°.

Glycogen determinations were carried out by the method of Hassid and Abraham.[23] Citrate synthase was assayed by the method described by Shepherd and Garland.[24] The assay was carried out on a 100,000 *g* 30-min supernatant of chicken liver homogenate. Homogenization was carried out for 5 min in 0·1 M Tris buffer, pH 7·2, in a Potter–Elvehjem homogenizer with Teflon pestle at a 20 per cent tissue concentration. The homogenate was subjected to sonication at 75 W/cm$^2$ and 20 kHz for 1 min. (Heat Systems, Ultrasonics, Inc., Plainview, N.Y., model W1850). Under assay conditions, the supernatant was employed in a 10-fold dilution with buffer. Correction was made for CoA deacylase activity from oxaloacetate-free rates.

Succinate dehydrogenase was determined in acetone powders by the method described by Veeger *et al.*[25] The preparation of acetone powder and subsequent extraction were performed as follows.

Chilled, scissor-minced liver was quickly weighed, added to 20 vol. of acetone at −10°, blended for 2 min and rapidly filtered on a Büchner funnel. The residue was washed with acetone and peroxide-free ether. The acetone powder was quickly weighed suspended in 10 vol. of 0·06 M Tris HCl buffer, pH 8·9, homogenized for 45 sec in a Sorvall Omni-mixer at 0°, and stirred for 30 min in the cold. The homogenate was

centrifuged at 4000 *g* for 45 min in a refrigerated centrifuge. The slightly opalescent yellow supernatant was assayed for succinic dehydrogenase. Blank rates for each liver sample were determined separately and used for correction in calculation of the enzyme activity.

## RESULTS

The experimental animals showed anorexia and subsequent weakness 6–13 days after initiation of the injections. This was followed by trembling and ataxia. Growth retardation was noticeable around day 3 and became more pronounced with time.

The blood glucose values are shown in Table 1. A marked increase is apparent after 1 week of treatment followed by a hypoglycemic state preceding death.

The free fatty acid levels shown for the same period are given in Table 2 and show a parallel pattern observed with glucose.

TABLE 1. DETERMINATION OF BLOOD GLUCOSE*

| | Days after beginning treatment | Concentration of blood glucose (mg/100 ml ± S. D.) | | P |
|---|---|---|---|---|
| | | Control | Treated | |
| Exp. 1 | 0 | 122·0 ± 23·3 (5) | 108·4 ± 17·6 (5) | 0·4 |
| | 8 | 112·2 ± 14·0 (5) | 178·8 ± 15·3 (5) | 0·001 |
| | 14 | 130·8 ± 16·5 (5) | 79·6 ± 21·5 (5) | 0·005 |
| Exp. 2 | 0 | 120·2 ± 18·7 (5) | 127·6 ± 20·1 (16) | 0·5 |
| | 7 | 118·0 ± 14·0 (5) | 174·6 ± 19·1 (16) | 0·001 |
| | 13 | 124·3 ± 6·7 (5) | 68·9 ± 22·5 (16) | 0·001 |

* All chicks were fasted for 8 hr prior to sampling.
Number of animals in the group is given in parentheses. In Exp. 1 the treated chicks received tremetol in propylene glycol at 1 mg/g daily dose for 10 consecutive days. In Exp. 2, it was given in corn oil at the same dose for 9 days.

TABLE 2. DETERMINATION OF BLOOD FREE FATTY ACIDS*

| | Days after beginning treatment | Concn of blood free fatty acids (μequiv./l. ± S. D.) | | P |
|---|---|---|---|---|
| | | Control | Treated | |
| Exp. 1 | 0 | 560 ± 143 (5) | 512 ± 58 (5) | 0·6 |
| | 8 | 470 ± 35 (5) | 602 ± 146 (5) | 0·1 |
| | 14 | 408 ± 34 (5) | 338 ± 51 (5) | 0·05 |
| Exp. 2 | 0 | 388 ± 64 (5) | 406 ± 64 (5) | 0·7 |
| | 7 | 374 ± 23 (5) | 530 ± 59 (5) | 0·001 |
| | 13 | 448 ± 38 (5) | 350 ± 68 (5) | 0·025 |

* For conditions of treatment, see Table 1. Number of animalsi n the group is given in parentheses.

In contrast with these two metabolic parameters which rise and fall in parallel, β-hydroxybutyrate levels showed a further 3-fold increase during the second period, as shown in Table 3.

Table 3. Determination of blood β-hydroxybutyrate*

| | Days after beginning treatment | β-Hydroxybutyrate concn (μmole/ml ± S. D.) | | P |
|---|---|---|---|---|
| | | Control | Treated | |
| Exp. 1 | 0 | 0·137 ± 0·013 (5) | 0·132 ± 0·006 (5) | 0·5 |
| | 8 | 0·131 ± 0·009 (5) | 0·565 ± 0·080 (5) | 0·001 |
| | 14 | 0·131 ± 0·011 (5) | 1·868 ± 0·147 (5) | 0·001 |
| Exp. 2 | 0 | 0·131 ± 0 011 (5) | 0·135 ± 0·017 (5) | 0·7 |
| | 7 | 0·136 ± 0·007 (5) | 0·637 ± 0·319 (5) | 0·01 |
| | 13 | 0·136 ± 0·019 (5) | 1·824 ± 0·308 (5) | 0·001 |

* For conditions of treatment, see Table 1. Number of animals in the group is given in parentheses.

The biphasic behavior of glucose levels posed the question of correlating these changes, at least in the hypoglycemic terminal phase with the levels of glycogen in the liver. The results of these determinations are shown in Table 4.

Table 4. Determination of liver glycogen*

| | Glycogen content (mg/g) | |
|---|---|---|
| | Control | Treated |
| Exp. 1 | 2·62 (2) | 2·71 (2) |
| Exp. 2 | 2·73 (2) | 2·97 (8) |

* Treated chicks received tremetol at a daily dose of 1 mg/g in corn oil (250 mg/ml) for 7 days and were sacrificed for glycogen determination when moribund. Both control and treated animals were fasted for about 9 hr prior to sacrifice. The livers were treated with 30% KOH immediately after removal. Number of animals in the group is given in parentheses.

Coincidental with the drastic changes observed in the carbohydrate and lipid metabolism of the animals is a fatty metamorphosis of the liver, which is not accompanied by necrosis.

In order to account for the precipitating causes of these observations, especially for the characteristic severe ketosis, further experiments were carried out to assess the functional state of the hepatic tricarboxylic acid cycle in these animals.

The first enzymatic step investigated was the formation of citrate by determining the levels of citrate synthase of treated and control animals. A dramatic drop in the

activity of this enzyme to approximately 5 per cent of the controls was demonstrated as shown in Table 5.

TABLE 5. CITRATE SYNTHASE ACTIVITY*

| | Citrate synthase activity (Units/g ± S. D.) | |
|---|---|---|
| | Control | Treated |
| Exp. 1 | 6·59 (2) | 0·43 (2) |
| Exp. 2 | 4·27 ± 1·93 (6) | 0·204 ± 0·180 (13) |

* Treated chicks were given tremetol at a daily dose of 1 mg/g in corn oil (250 mg/ml) for 8 days and were sacrificed for citrate synthase assay when moribund. Number of animals in the group is given in parentheses.

The question whether the drop of citrate synthase level is simply a reflection of a general drop of mitochondrial enzyme activity accompanying the liver damage seen in the histological preparations was tested by measuring succinate dehydrogenase levels in control and treated animals. The results of these experiments are shown in Table 6. The data show no significant difference between the experimental and control groups.

TABLE 6. DETERMINATION OF SUCCINIC DEHYDROGENASE*

| Group | Serial no. | Succinic dehydrogenase activity (Units/ml supernatant) | (Units/mg protein) | (Units/g liver) |
|---|---|---|---|---|
| Control | 424 | 2·00 | 2·82 | 72·7 |
| | 425 | 1·52 | 3·96 | 51·2 |
| Treated | 426 | 2·86 | 2·85 | 71·8 |
| | 428 | 2·00 | 4·69 | 41·7 |
| | 432 | 2·00 | 5·16 | 42·0 |
| | 433 | 2·48 | 5·62 | 44·0 |
| | 434 | 3·52 | 2·92 | 98·0 |
| | 436 | ND† | ND | ND |
| | 437 | 5·14 | 2·89 | 76·9 |

* Conditions for succinate oxidation: 0·15 m-mole of phosphate, pH 7·6, 1·0 ml of the supernatant to be assayed for enzyme activity, 3 mg of bovine serum albumin, 2 mg of *N*-methylphenazonium methosulfate, 0·015 μmole of 2,6-dichlorophenol-indophenol and 3 μmoles of cyanide in 2 ml total volume at 38°. One unit is defined as 1 ml of succinate oxidized per min. For conditions of treatment of animals, see Table 5.

† ND = not detectable.

## DISCUSSION

In this study we have attempted to examine the effect of tremetol administration on delineated areas of carbohydrate and lipid metabolism most likely to be contributory to the ketosis.

The syndrome induced by tremetol intoxication is characterized by severe ketoacidosis. Reports on blood glucose levels in human cases vary from normal Hartman *et al.*[1], to hypoglycemia, Bulger *et al.*,[26] while Couch[27] noted hyperglycemia in animals. It appears from our findings that the blood glucose level is determined by the degree of the progress of the intoxication and passes through an early hyperglycemic period followed by hypoglycemia toward the terminal phase of the disease. Free fatty acid levels show similar behavior. The changes in the latter, while statistically significant are, however, not particularly striking.

β-Hydroxybutyrate levels are marked by a consistent rise throughout the treatment period and terminally reach a level of approximately 14-fold over the controls. The height of the ketonemia coincides with a somewhat lowered free fatty acid level and it is conceivable that mobilization of depot fat is at least a partial source of the ketone bodies. The ketonemia observed in these animals required a more detailed investigation into its potential causes.

It seems unlikely that glucose could be a significant precursor of acetate at least in the late stages of this condition since, as the data show, hypoglycemia and ketonemia are coexistent. It is somewhat surprising that the hypoglycemic state is not reflected by corresponding low levels of liver glycogen, which are the same in both experimental and control groups. The cause for this finding is unknown. It may indicate that tremetol may have an effect on extra-mitochondrial pathways involved in glucose mobilization. It is necessary to point out (see also note to Table 4) that hepatic glycogen levels were determined on animals deprived of food for 9 hr prior to sacrifice. This is the likely explanation for the uniformly low glycogen levels, $< 300$ mg/100 g for both experimental and control groups.

The lowered blood free fatty acid content would indicate that, at least in the late stages of tremetol intoxication excessive mobilization of depot fat is not a likely cause for ketosis. A possible alternate cause for it would be an impairment of acetate oxidation.

In order to test this assumption, the functionality of the tricarboxylic acid cycle was investigated and comparative assays of citrate synthase were carried out. The results of these experiments show a drastic reduction in the activity of this enzyme to approximately 5 per cent of the controls.

A reduction of enzyme activity of this magnitude could be responsible for the ketonemia observed in this condition. In view of the condition of the liver of the experimental animals showing a high degree of fatty infiltration, it was considered possible that the decrease in this enzyme might simply reflect a general hepatotoxic effect possibly connected with a decrease in the number of functioning mitochondria, but a comparison of succinic dehydrogenase activity in experimental and control animals showed no differences between these groups. The isolated sample yielding no activity is in all likelihood due to experimental error.

Citrate synthase is a member of the enzyme group localized in the mitochondrial matrix, while succinate dehydrogenase is formed in the inner membrane. It is not known at this time whether other matrix enzymes are affected by tremetol treatment or not,

but as may be seen from the succinate dehydrogenase assay, a diminution of activity in mitochondrial enzymes, in general, is not the case. The possibility exists that the citrate synthase levels are an expression of a direct enzyme inhibition due to tremetol (or its metabolites) present in the assay mixture while succinate dehydrogenase preparations would have been free from it after acetone treatment. This assumption is made less likely by the fact that the citrate synthase assay solutions contain only 2 per cent tissue extract, and this would result in a considerable dilution of a potential inhibitor.

The sustained increase of β-hydroxybutyrate in the blood also indicates that the dehydrogenase, in all probability the mitochondrial enzyme, is functioning throughout the experimental period.

These findings point in the direction of a rather selective enzyme inhibitory effect of tremetol as contrasted with a non-specific hepatotoxic effect. It remains to be investigated whether other mitochondrial matrix enzymes besides citrate synthase are affected simultaneously.

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