FRACTIONS OF TREMETOL AND THEIR TOXICITIES

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HISTORICAL INTRODUCTION

According to Couch[3], tremetol is an oily, high molecular weight alcohol, present as an ester in rayless goldenrod, Aplopappus heterophyllus (Gray) Blake, and white snakeroot, Eupatorium urticaefolium Reichard. Tremetol is the poisonous principle responsible for trembles in sheep and horses, and milksickness in either calves or man, when poisoned by the milk of cows grazing on pastures contaminated by either of these two plants, or by Aplopappus fruticosus (Rose and Stanley) Blake[4,7]. Rayless goldenrod is found in Arizona, New Mexico, Texas, Oklahoma, and Colorado, U.S.A., and in Sonora and Chihuahua, Mexico[7]. White snakeroot is found in the eastern United States, and in Minnesota, Nebraska, Oklahoma, Texas, and Louisiana[3,7]; an apparently closely related species is found in eastern China[8].

Lathrop[5], following Couch's procedure, isolated a small quantity of tremetol from A. heterophyllus. Further experiments by Lathrop showed that larger amounts of the toxic principle could be obtained by slightly modifying Couch's procedure. He also found that the color test proposed by Couch is not specific for tremetol, but is given by the non-toxic essential oil. Lathrop fractionally distilled tremetol at 80-100°C and 1 mm Hg, in a Hickman still, and isolated a trace of a crystalline unsaturated ketone m.p. 84°. Cleverdon[7], using guinea pigs, attempted to assay the toxicity of the original tremetol, the two fractions, and the residue obtained by Lathrop. The liquid materials were given by intraperitoneal injections. The solid residue and second fraction were dissolved in sterile olive oil before injection. Because of the lack of material, the small

number of injections at infrequent intervals, and the small number of experimental animals, no significant results were obtained.

The present work was undertaken in the hope of finding a convenient susceptible experimental animal, and obtaining by fractionation the pure toxic principle of tremetol for further study.

EXPERIMENTAL

PART I : ISOLATION OF TREMETOL

Rayless goldenrod was mowed while in bloom, sun-dried, and baled by Lathrop in New Mexico. Seven bales (500 pounds) were sent to Abbott Laboratories, Chicago, Illinois, for extraction, according to Lathrop's[5] modification of Couch's procedure. Approximately fourteen pounds of tarry extract were recovered from the rayless goldenrod. Several one hundred-gram quantities of this material were saponified and extracted according to the procedure recommended by Lathrop. In this manner it was experimentally determined how to recover the maximum amount of poisonous ether extract. The fractional precipitation of impurities from diluted alcohol proved superfluous and was abandoned.

The remainder of tarry material was divided into six 1000 g portions. Each was dissolved in 2,000 ml of 95% C_2H_5OH and saponified by adding 150 g KOH and refluxing for nine hours. The strongly alkaline reaction mixture was then extracted in an all-glass continous ether-extraction unit*, using isopropyl ether vaporized by a water bath. The ether solution was dried over CaO, evaporated to 500 ml on a water bath under reduced pressure to remove most of the isopropyl ether, and then cooled to -5° and filtered to remove wax. The filtrate was extracted at 45° with 3 liter portions of 5% HCl until the HCl did not become colored nor contain any material insoluble in 5% NaOH. The ether layer was neutralized by shaking with 5% Na_2CO_3 solution, dried over CaO, and concentrated under reduced pressure on a water bath to 100-150 ml of a thick orange-red oil.

* This extraction unit was designed by the author and built by Dr. H. M. Trimble, of this department.

PART II : EXPERIMENTAL ANIMALS

Preliminary susceptibility tests, using the crude tarry extract in capsules at the dosage of 0.5g per kg body weight, were made on cats, dogs, guinea pigs, rabbits, rats, chickens, and ducks. Chickens appeared to be the most sensitive and cats and dogs the least. One rabbit died with typical symptoms; another developed a tolerance for the extract, and lived for over one month with the daily dosage being doubled every week.

In another series of susceptibility tests the stems, leaves, and blooms of Aplopappus heterophyllus were ground to a coarse powder. This was then mixed into a balanced feed in proportions up to 10% and given to six chickens and twelve rats. The animals were weighed daily and the weight of feed and water consumed was also taken daily. At the end of eight weeks all animals had lost about half their original weight and were eating less than one-third of the amount of feed that was taken at the start. The animals were suffering from starvation but would eat less feed if the percent of rayless goldenrod was increased or more if it was decreased. Thus the quantity of rayless goldenrod consumed remained practically constant. It was noted that the animals took normal quantities of feed and water for the first three or four days. Following this, the feed consumption was almost nothing for two days, and there was a sudden drop in water consumption, usually on the second day. This refusal to drink caused a sharp drop in weight due to dehydration. The animals seemed to repeat this cycle of eating and drinking, drinking but not eating, neither eating nor drinking, throughout the experiment.

PART III : THE FRACTIONS COLLECTED AT 100°

A. The Distillation

The residual oil described at the end of Part I, in 25 ml portions, was fractionally distilled in a Hickman molecular still on a water bath at 80-100° and 0.1 to .00001 mm Hg*. To help prevent contamination of the vacuum line with volatile organic substances, a trap was placed next to the Hickman still. This trap consisted of a cold-finger condenser immersed in a pint thermos bottle filled with acetone and dry ice. About 3 ml of a thin colorless oil was ultimately taken from the condenser trap. This oil was similar in physical properties to the essential oil and was probably a component of it.

The results of distillation are given in Table I.

The residue, a hard, red, glassy solid, was removed by dissolving it in warm diethyl ether. At the end of two weeks, white cubical crystals, m.p. 258°, had separated from the ether solution. These gave positive color and solubility tests for a sterol, but were present in too small amount for toxicity tests.

* The mercury diffusion pump and McLeod gauge used in the vacuum line were built by Dr. H. M. Trimble.

DATA ON DISTILLATION OF CRUDE TREMETOL

Fraction	Time interval of collection (hours)	Temperature (°C)	Pressure (mm Hg)	Description of distillate
I	first hour on water bath	80 - 85	1. - .02	thin colorless oil with strong odor of rayless goldenrod. Probably rich in essential oil of the plant
II	2nd - 4th	97 - 99	.02 - .001	clear orange oil, approximately twice the viscosity of I, slight odor same as that of I
III	5th - 8th	97 - 99	.001 - .0001	oil with viscosity, color, and odor approximately that of II
IV	9th - 15th	97 - 99	.0001 - .00001	extremely viscous, clear, dark orange-red oil; odor same as for II
V	16th - 28th	97 - 99	.00001	semi-solid opaque red-amber oil; odor same as that of II, but weaker
A	first hour on glycerin bath	80 - 90	1. - .02	same as I, above
B	2nd - 3rd	90 - 110	.02 - .0001	similar to II and III combined
C	4th - 8th	110 - 130	.0001 - .00001	similar to IV and V combined
D	8th - 20th	130 - 180	.00001	a hard orange-yellow wax

B. The Relative Toxicities

The relative toxicities of whole tremetol, each of the four fractions, the residue, and the original tarry extract were determined as follows:

The several materials were weighed into capsules and given to twenty-five four-week-old Rhode Island Red cockerels that weighed 85 ± 5 g each. This was repeated, using seventy-five White Leghorn cockerels that weighed 90 ± 5 g. The dosages used were 0.05, 0.1, and 0.2 g per day. Fraction I proved non-toxic. Fractions II, III, IV, and V, and residue were increasingly toxic, in that order with the whole tremetol nearly as toxic as V. This indicated that the toxic principle is slightly volatile and can undergo distillation at 99°, but that most of it remained in the residue.

Autopsies were performed* on all cockerels that died during the experiment, and on all survivors at the end.

All controls were normal. All cockerels poisoned with the residue, crude tremetol, or fraction V had enlarged gall bladders and kidneys: in every case, the kidney tubules were distorted and enlarged: the walls of the digestive tract were thin and inflamed; the liver had large yellow spots; and the viscera smelled of acetone in severe or prolonged cases of poisoning. Usually the pelves of the kidneys and the ureters were plugged with ureates. The others, poisoned on fractions II, III, and IV, had inflamed and distorted kidney tubules and ureters plugged with ureates, but were without the other abnormalities characteristic of tremetol poisoning.

* Autopsies by Dr. Lewis H. Moe, D.V.M., Department of Bacteriology Physiology, and Veterinary Science, O.A.M.C.

This condition of the kidneys suggested vitamin A deficiency, although the mash used was fortified with fish liver oil. The experiment with the White Leghorn cockerels was repeated with extra amounts of vitamins A and D concentrate added to the feed. No signigicant differences were noted.

Figure I.
A Chick Dying of
Tremetol Poisoning

The symptoms of typical cases of tremetol poisoning were dizziness, gasping, cyanosis, acetone in the breath, trembling, and spasm. Sometimes the head almost touched either the tail feathers or the toes (see Figure I). In fatal cases, these symptoms were followed by a coma which lasted three to four hours, unless the chick was touched, in which case a brief spasm occured, followed by death within a few minutes. The coma could be temporarily relieved by administering oxygen.

The immediate cause of death from trembles is respiratory failure induced by acidosis. Abnormal functioning of the liver and kidneys is probably responsible for the acidosis. The results of the autopsies and the symptoms are in good agreement with those listed by Couch[3] and Marsh[7], in spite of the differences in experimental animals.

PART IV : FRACTIONS OF TREMETOL OBTAINED AT 180°

A. The Distillation

Because of the toxicity of the residue obtained at 100°, distillation of the ether extract was tried at 180° using the same apparatus with the substitution of glycerin for water in the heating bath. Even at this temperature apparently little of the toxicity was lost, as shown by the feeding experiment reported in Table II.

Fractions were separated on the basis of the rate of distillation, with the pressure, temperature of the bath, and temperature of the condenser water kept constant for each fraction. By this method, if the temperature of the bath and the pressure are low enough to prevent bumping, and the condenser is as cold as possible without solidifying the condensate, the different fractions can be collected separately, with only a drop or so of the previous fraction contaminating the first portion of the succeeding fraction. By mistake, a portion of improperly dried tremetol was placed in the still; it frothed so violently that it was impossible to distill.

White crystals which collected on the condenser were indistinguishable from those previously obtained from the ether solution of the residue after distilling at 97-99°. When distilled at temperatures up to 180°, the four fractions collected (A, B, C, D) corresponded in physical properties to the other fractions (I, II, III, IV, V), as indicated in Table I. The residue was a hard, clear, red, glassy substance, insoluble in diethyl ether, but soluble in hot benzene.

One series of fractions was recombined and redistilled at 180° without leaving any residue or materially affecting the physical characteristics or quantity of any fraction. This indicated that no appreciable decomposition occured at 180°.

All attempts to isolate a poisonous pure compound from the various toxic materials in amounts proportionate to their toxicities, failed. All of the usual derivative reactions for phenols or alcohols either failed or yielded gummy, insoluble, colored products from which no appreciable amount of crystalline compounds could be recovered. Li and Pak[6] report that the toxic principle of Eupatorium chinense L. is coumarin. Since Couch states that poisoning by species of Eupatorium and Aplopappus found in the United States is due to a single compound which he calls tremetol, an attempt was made to isolate coumarin from whole tremetol.

When the procedure of Li and Pak[6] was followed, coumarin was not obtained from the ether extract or any of the fractions. Oxidation with alkaline $KMnO_4$ gave an acid gum which with p-nitrobenzyl bromide produced white crystals, m.p. 79°, when recrystallized from HOH. This corresponds to the p-nitrobenzyl ether of acetic acid, m.p. 80°. Couch reported obtaining acetic acid by destructive distillation of tremetol.

Crystals similar to those obtained by Lathrop, and found by him to be a ketone, slowly formed in crude tremetol and all liquid fractions. These crystals could not be isolated in amounts sufficient for toxicity studies.

THE EFFECTS OF TREMETOL FRACTIONS AND TREMETOL ON WHITE LEGHORN CHICKS

Group	Number of chicks	Material received	Dose per day in grams	Results in average weight per chick, in grams				Condition
				Start	10 days	20 days	26 days	
One	20	None	None	117	182	274	316	Normal
Two	5	Tremetol	.05	115	186	287	273	Poisoned
Three	5	Tremetol	.1	117	193	220* (234)	223* (312)	Poisoned
Four	5	Fractions B and C combined	.05	115	198	304	398	Normal
Five	5	Fractions B and C combined	.1	117	179	241	250	Poisoned
Six	5	Fraction D	.05	115	194	293	339	Normal
Seven	5	Fraction D	.1	117	186	282	310	Normal
Eight	5	Residue from distillation at 180° and .00001 mm Hg	.05	115	191	291	338	Normal
Nine	5	Mineral oil, medium viscosity	.1	117	190	287	326	Normal

B. The Relative Toxicities

From one hundred White Leghorn cockerels, sixty were selected and sorted into eight groups of five, and one group of twenty, according to weight and appearance, so that each individual of any group of five weighed within one gram of the corresponding chick of each of the other groups. The average weight per chick in four groups was 115g, and in the other five groups, including the group of twenty, 117g. The relative toxicities of crude trometol and its fractions as determined by growth and autopsy of these chicks are given in Table II.

All chicks were kept in the same brooder and were fed and watered from the same troughs. All of Group One appeared nervous and did not gain as rapidly as Groups Two, Three, Five, or Six, probably because of loss handling. All groups except One were caught daily and were given the various substances in capsules. At the end of the first week it was noticed that the controls receiving no capsules or additional material, Group One, quit eating and drinking during the one to two hours required to give the other chicks capsules; whereas the treated chicks, including the controls receiving mineral oil, would continue to eat and drink until caught and would resume as soon as released. This created an unforeseen psychological factor which detracts from the usefulness of Group One as controls for normal gains in weight.

All groups were weighed every other day. At ten days, Group Four was the heaviest and Group Five the lightest.

This order was kept until the end of the experiment, on the twenty-sixth day. All groups that received a 0.05g dosage of any fraction gained more rapidly than either control group. While those receiving a 0.1g dosage of any fraction gained less rapidly than either control group. The only fraction that produced symptoms of tremetol poisoning also induced the most rapid gain.

In order to stop pecking, 3% NaCl was added to the mash on the sixteenth day. To obtain more accurate weights, food was withheld for 18 hours on the twenty-second day. Apparently both the additional NaCl and the withholding of feed had a permanently deleterious effect on the chicks that had previously shown symptoms of tremetol poisoning, and only transient effects on the normal chicks.

DISCUSSION

PART I : ISOLATION OF TREMETOL

By use of the large continous ether extraction unit, a higher recovery of tremetol was obtained than was practicable using separate portions of ether as was done by both Lathrop and Couch. The principal difference in the tremetol obtained by this method is that the amount of the high molecular weight residue is increased in proportion to that of the lighter fractions. Subsequently, this heavy portion proved to be the most toxic.

PART II : EXPERIMENTAL ANIMALS

Different species of animals vary considerably in their susceptibility to tremetol poisoning. Chickens of different ages show different susceptibilities; young, growing chicks are most susceptible.

To secure typical symptoms of chronic tremetol poisoning, a daily dosage of approximately 1g per kg of body weight must be given for seven to twelve days. A lower dosage is likely to induce a tolerance instead of producing a case of fatal poisoning. Chicks that survived as long as twenty days did not succumb at the end of sixty days while continuing to receive tremetol and at the end showed only mild typical symptoms of tremetol poisoning. Exertion would result in attacks of trembling and cyanosis in rabbits and chickens that had been previously given tremetol for six to eight days.

A higher dosage tends to result in a sudden onset of depression followed within a few hours by death. Probably insufficient food accentuates the symptoms of tremetol poisoning, as demonstrated when food was temporarily withheld.

Under natural conditions, animals eat rayless goldenrod only when in poor condition due to a lack of normal forage, as on overgrazed or dry pasture[3]. In this manner animals would eat large quantities of rayless goldenrod only at the time that they were most susceptible to poisoning.

PART III : FRACTIONS OF TREMETOL

Fractionation at 180° differed from that at 100° only by the quantity and toxicity of the residue and in the toxicity of the last fraction collected. At 100° the last fraction was only slightly toxic and the residue was most toxic. At 180° the middle fractions are most toxic and the residue and last fraction are of low toxicity.

Considering the physical properties of the fractions, tremetol is undoubtedly a mixture of at least several substances. Also, to judge from the results of feeding tremetol and its fractions, as shown in Table II, there must be at least two or three components with different physiological activities. The slight apparent loss in toxicity following distillation could be due to the partial separation of these active components. This separation would account for the differences in symptoms and autopsy findings of the various groups that received different fractions, and also for the stimulation of growth by Fractions B and C and the absence of any marked growth stimulation by tremetol itself. This could be due to some vitamin in Fractions B and C whose benefits are obliterated by other substances in tremetol, and whose toxic properties are augmented by substances present in tremetol.

Chicks are sensitive to slight differences in dosage, per gram of body weight, of tremetol or any of its components that are physiologically active. This is easily seen by comparing the action of identical doses of Fractions B and C or of whole tremetol on the heaviest chicks, the next lightest chicks, and the lightest chicks of Groups Two, Three, Four, and Five, in Table III.

TABLE III

WEIGHT INCREASE, AS AFFECTED BY DOSAGE OF TRENETOL OR ITS PHYSIOLOGICALLY ACTIVE COMPONENTS
Groups, fractions, and dosage, as described in Table II

		Start		10 days		20 days		26 days	
Group	Chick	Weight	Percent*	Weight	Percent	Weight	Percent	Weight	Percent
	H	130g	102%	215g	102%	348g	103%	Killed	
Two	NL	110g	100%	171g	96%	241g	90%	204g	65%
	L	105g	100%	168g	97%	Killed			
	H	125g	100%	216g	102%	298g	90%	312g	82%
Three	NL	110g	100%	178g	100%	170g	64%	Died	
	L	105g	100%	173g	100%	Died			
	H	130g	102%	232g	106%	331g	100%	425g	107%
Four	NL	110g	100%	190g	104%	275g	102%	349g	106%
	L	105g	100%	171g	99%	Killed			
	H	125g	100%	203g	97%	306g	92%	Killed	
Five	NL	110g	100%	163g	92%	223g	84%	208g	67%
	L	105g	100%	157g	91%	188g	72%	Died	
	H	125g	100%	209g	100%	331g	100%	375g	100%
Nine	NL	110g	100%	177g	100%	267g	100%	312g	100%
(controls)	L	105g	100%	173g	100%	261g	100%	Killed	

* Percent of corresponding control's weight

H - Heaviest chick
NL - Next to lightest chick in group
L - Lightest chick in group

Four chicks of Group Three and two chicks of Group Five died of tremetol poisoning. None of the other chicks died; none showed symptoms of any disease except tremetol poisoning, as indicated in Table II, in which case all of the chicks of a poisoned group had typical symptoms in various degrees of severity.

The lightest chicks of any poisoned group were always considerably more affected than the heaviest; this is owing to the difference in the ratio of daily dose to body weight.

Fraction A was identical to Fraction I, which was found to be nontoxic. Fractions B and C combined could apparently either poison or stimulate normal growth, depending on the dosage. Fraction D was only slightly toxic; this indicates that the principal toxic components of tremetol are volatile. The residue was only slightly toxic.

SUMMARY

Chicks are ideal experimental animals for the investigation of rayless goldenrod poisoning. The convenience in handling, economy in first cost and maintenance, and saving in experimental material are important advantages of chicks over cattle. The symptoms of tremetol poisoning in chicks are similar to those found in other animals.

A tolerance to chronic tremetol poisoning was produced in chickens and rabbits.

Tremetol was separated by high-vacuum distillation into several fractions of different physical and physiological properties. Neither tremetol nor any of its toxic fractions so far isolated are individual compounds but are mixtures of at least several oily, resin-forming substances. The symptoms of trembles are probably due to the combined actions of several of these compounds. Some of these active substances were at least partially separated by molecular distillation. Different components were also separated by selective absorption but in quantities too small for experimental use.

An unidentified sterol, m.p. 258, was obtained from tremetol.

Even slight differences in the dosage produced marked differences in the effects of tremetol or any of its toxic fractions. Tremetol and its toxic fractions have a definite threshold dosage which must be passed to induce poisoning. A dosage lower than the threshold amount caused an increased tolerance. Just above the threshold level a slight difference in dosage resulted in symptoms that varied considerably in severity.

All fractions of tremetol either stimulated or retarded the growth

of chicks, depending on the dosage. In the sub-threshold dosages they stimulated growth. At the dosages used the growth stimulation effect varied inversely with the dosage. This indicates the presence in trametol of a toxic compound and a vitamin-like compound.

RECOMMENDATIONS FOR FUTURE WORK

The extraction of pulverized goldenrod should be tried with solvents other than alcohol, possibly ligroin or benzene. Another solvent might extract the toxic principle with less contaminants, such as chlorophyll and essential oil. Successive extractions using different solvents might improve the product.

Other plants such as legumes and grasses could be extracted and examined for the growth stimulating substance present in Fractions B and C. It is a common practice for poultrymen to plant grasses or legumes to serve as a supplement for young growing chicks. Also a smaller dosage of Fractions B and C would be expected to produce even greater increases in growth rate. Qualitative tests for known vitamins should be made of Fractions B and C.

Controls should be given empty capsules so as to receive the same handling as the other experimental chicks. The quanity of toxic material not absorbed from the intestines and the amount excreted by the kidneys should be determined. This would indicate the nature of tolerance development; whether it is due to an increased ability to excrete or to metabolize the toxic material.

Histological studies of the various organs would indicate the extent of injury. This might also explain the symptoms of chronic tremetol poisoning. The different tissues from poisoned chicks should be fed to other chicks, or other experimental animals, to detect retention of tremetol.

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AUTOBIOGRAPHY

I, Stephen O'Neil Butler, was born on a farm east of Walters, Oklahoma, March 7, 1916. All of my elementary and high school education was at the Walters Public School. After receiving my diploma in 1934, I attended Cameron State School of Agriculture, Lawton, Oklahoma, until 1936. The remainder of my schooling has been taken at O.A.M.C., Stillwater, Oklahoma. In 1939, my Bachelor of Science degree was granted, with majors in Chemistry and Biological Science. I took graduate work at Stillwater until September, 1940. From 1938 until 1940, I worked for the Department of Zoology, and the Department of Bacteriology, Physiology, and Veterinary Science. Kay Curran, also a graduate student, and I were married in June, 1940. In September 1940, we moved to Goodwell, Oklahoma, where up to the present, I have taught Biological Science at Panhandle A & M College. We have had three children since coming to Goodwell.

Some graduate work was done at Stillwater during August, 1941. Since then, feeding experiments have been completed in Goodwell.

Typed by:

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