TEXAS AGRICULTURAL EXPERIMENT STATION
A. B. CONNER, Director
College Station, Texas

BULLETIN NO. 650 JUNE 1944

STUDIES ON TOXIC SUBSTANCES OF LOCOWEEDS, ASTRAGALUS EARLEI AND OTHERS

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D-19-744-1500

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The concentrated toxic preparation of the loco weed contains several closely related toxic substances. The compounds precipitated by silicotungstic acid are toxic but do not produce all the symptoms of locoism. The spectrum absorption curve of the purified compound so precipitated resembles closely the spectrum absorption curve produced from the compounds not precipitated. When treated with benzoyl chloride, both the benzoylated and non-benzoylated products contained toxic compounds. Adsorption of the picrates on aluminum oxide separated two or more compounds. Silver nitrate precipitated a compound having the characteristic spectrum absorption curve of the loco compounds. Extraction with iso-amyl alcohol and purification with flavianic acid removed impurities. Every active fraction showed the same general type of spectrum absorption curve, though there were differences in details. Evidence of chemical changes in the toxic substance separated occurred in the solutions after storage even in cold storage, as well as decided losses in the process of separation. The different compounds may be present in the loco weed or some may have been formed during the process of separation.

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STUDIES ON TOXIC SUBSTANCES OF LOCOWEEDS, ASTRAGALUS EARLEI AND OTHERS

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The ingestion of locoweeds by grazing livestock has continued to result in losses among the animals. Many investigations have been undertaken both in the field and in the laboratory to learn more about the disease, about the weed, and about the toxic substances present in the weed.

In a previous bulletin of this Station, No. 537, Fraps and Carlyle (4) described the isolation of a highly toxic concentrate, called 'locoine" from a locoweed, Astragalus Earlei. The work was continued by A. W. Walde, Joseph Semb, and S. H. Wender. The present bulletin reports further progress made in the isolation of the active constituent or constituents of locoweed. It also reports the results of additional studies made in fractionating the locoine concentrate and in investigating the new fractions thus obtained. Three of these new fractions have been found to be toxic to cats; two of these three produced symptoms of locoism.

The most concentrated extract previously reported as capable of producing locoism in cats has now been separated into at least four main fractions, and further separations and investigations have been made on these new substances. The fractions described in this bulletin are more nearly pure and are more potent than those previously reported. The presence in locoweed extracts of several physiologically active substances, instead of only one, has again been experimentally demonstrated to be probable.

Spectroscopic studies in the ultra-violet have been undertaken on the various substances found, and of especial value have been the absorption spectrum curves obtained for these new fractions. Since every toxic fraction thus far studied has a characteristic absorption in the ultra-violet range, the spectrograph method of studying these substances has proved to be a great aid in following the desired compound after experimental separations. Chromatographic adsorption methods and the use of solvent extraction and selective precipitation methods also have been of much value in making the new fractionations.

REVIEW OF LITERATURE

Much of the literature on locoweed investigations has been reviewed in the publications of Fraps and Carlyle (4), of Pease and Elderfield (11), and in the earlier work by Crawford (3). The results of other investigations, as in the field of veterinary science, are found in government publications (17), and in a thorough study of locoism in animals by Mathews (19).

The first printed records of locoweed disease apparently were in the report of the Commissioner of Agriculture, U. S. Dept. of Agriculture, for October, 1873. A description of the symptoms of locoed animals was

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given in detail in this and subsequent reports. The very early work was done by Prescott in 1878 (13) and by Power and Cambier (12) in 1891. Crawford (3) found barium in locoweed plants. He reported that barium compounds gave symptoms of poisoning similar to loco poisoning but this was not confirmed by Marsh (7) or Alsberg and Black (1).

Attempts to isolate the toxic principle of the locoweed have encountered many difficulties. Couch (12) isolated a toxic fraction from the locoweed, Oxytropis Lambertii, but not in crystalline form. The solution prepared from the precipitate with silicotungstic acid was non-toxic to cats. The filtrate after treatment with basic lead acetate and charcoal yielded a light yellow, amorphous mass, of honeycomb structure, very soluble in water, extremely hygroscopic, and insoluble in ether, in chloroform, and in the hydrocarbon solvents. Couch believed his compound not to be of ester, ether, or of glucosidal nature. It was not basic, but nitrogenous and highly hydroxylated. It did not affect a ray of polarized light. Couch was unable to find any precipitant for the substance.

Fraps and Carlyle (4) isolated a toxic principle from the locoweed, Astragalus earlei, in highly concentrated and toxic form, but not in a definitely pure state. This toxic principle, called "locoine" was shown to be a strong base, very soluble in water and in alcohol, but only slightly soluble in ether, petroleum ether, and in chloroform. This "locoine" was found to be precipitated by phosphotungstic acid and recovered from the precipitate by treatment with barium hydroxide. The base was acetylated, forming an acetylacetate. This "locoine" was found to be stable towards reagents and apparently not affected by boiling with dilute acids or alkalis. The most concentrated preparation required a feeding of only one gram over a period of fifty days to loco a cat. The toxic solution was found to give reactions with many of the reagents used to test for alkaloids.

Pease and Elderfield (11) have isolated two non-toxic substances from the locoweed, Astragalus earlei, which they call alpha-earleine and beta-earleine. They were obtained after a separation involving chromatographing a solution of their picrates on aluminum oxide. Stempel and Elderfield (14) report that alpha-earleine is identical with betaine, and beta-earleine with choline. Pease, Reider, and Elderfield (10) report the isolation of the non-toxic sugar, d-pinite (monomethyl ether of inosite) from Astragalus earlei and from Oxytropis lamberiti. Stempel and Elderfield (15) isolated from Astragalus earlei a non-toxic dihydroxyvalerolactone, or an isomer thereof, along with glycerine. They found that Reinecke salt precipitates a highly active fraction, from which a crystalline substance was isolated. The solution obtained on decomposition of the Reineckate produced a typical locoism in cats, but they were unable to demonstrate whether this compound was responsible for such symptoms, or whether a still un-isolated substance carried down in the precipitate was the active material.

Knowles and Elderfield (6) from Astragalus wootoni have isolated pinite, betaine, choline, and trigonelline, but have not yet isolated the active constituent or constituents. In tests on cats, no appreciable difference in symptoms was found in locoism caused by Astragalus earlei and in locoism caused by Astragalus wootoni. This conclusion is in agreement with the earlier work on these weeds by Fraps and Carlyle (4).

Although there are many seleniferous "locoweeds" (16), it has been pointed out by Fraps and Carlyle (4) and by Pease and Elderfield (11) that in the locoweeds they studied, the concentrations of selenium are so low that selenium is definitely not the toxic material.

PREPARATION OF CONCENTRATED LOCOINE EXTRACTS WITH PHOSPHOTUNGSTIC ACID (METHOD A)

The method of preparation of the concentrated locoine extract is based on the one used by Fraps and Carlyle (1) with some additions and modifications.

One-half kilogram of the dry, finely-ground locoweed, Astragalus earlei, was placed in a large evaporating dish, 1500 ml. of 95% ethyl alcohol added, and the alcohol and weed mixed thoroughly. After standing for at least 30 minutes, with thorough mixing in the meantime, the liquid was pressed out by means of a screw tincture press. The residue was returned to the dish, and this time extracted with 750 ml. alcohol. The residue was discarded, but the combined alcohol extracts were filtered, and then concentrated under reduced pressure to about 150 ml. This concentrated liquid was set aside, until the alcohol extracts from at least five kilograms of weed were on hand.

Extractions of the weed itself were carried out in units of one-half kilogram. After combination and concentration of these extracts, however, solutions representing five kilograms of locoweed were used as units. To the combined alcohol concentrates from 5 kg. of locoweed, an equal amount of water was added, and the solution concentrated under reduced pressure. After cooling, the remaining solution, about 1.5 liters, was decanted from the water-insoluble part, and treated with a slight excess of basic lead acetate. The resulting precipitate was filtered off and discarded. Excess lead was then removed from the filtrate with hydrogen sulfide, and the lead sulfide discarded. The lead-free filtrate was concentrated in vacuo to a thick syrup, and the residue was extracted with 500 ml. of commercial absolute alcohol, while thoroughly stirring and warming the solution. The solution was allowed to cool slowly at first, then to remain over-night in the refrigerator. The supernatant alcohol liquid extract was decanted, and the residue was again extracted, this time with 250 ml. of absolute alcohol. After cooling, the two absolute alcohol supernatant liquid extracts were combined, and concentrated in vacuo to about 250 ml. This concentrated solution was allowed to remain for several days in the refrigerator. Water was added to the filtrate, and the alcohol distilled off under reduced pressure. More water was added, and the solution concentrated to about 150 ml.

The concentrated, aqueous solution was cooled, and in an ice bath, a 24% solution of phosphotungstic acid ($P_2O_5.24WO_3.42H_2O$) in 4% sulfuric acid was added until precipitation was complete. The phosphotungstic acid precipitate was transferred to a centrifuge bottle and extracted with 200 ml. of absolute alcohol. After centrifuging, the supernatant liquid, which had been shown to contain the toxic material, was decanted, and an equal amount of water added. Then the alcohol-soluble precipitate was decomposed in the cold with saturated barium hydroxide solution. A slight ex-

cess of barium hydroxide solution was used. The insoluble material was centrifuged off, and the excess of barium in the residual solution was removed by precipitation with dilute sulfuric acid. After removing the precipitated barium sulfate, the filtrate was concentrated in vacuo to about 150 ml. The spectrum absorption curves of one of these preparations before and after purification with iso-amyl alcohol are shown in Figure 1.

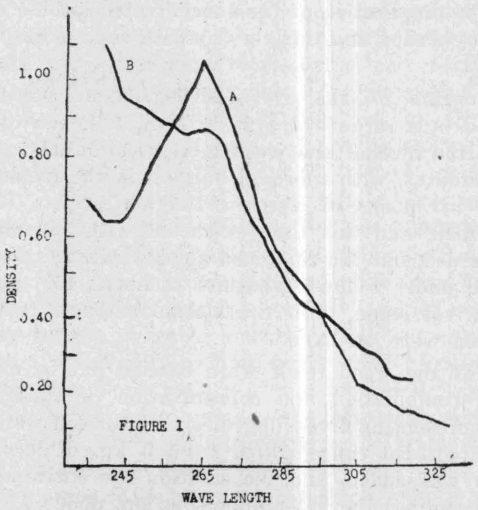

Figure 1. Spectrum curve of locoine concentrate prepared by precipitation with phosphotungstic acid and solution of the phosphotungstate in alcohol, Curve A before, and after, B, purification with iso-amyl alcohol.

PURIFICATION OF LOCOINE PHOSPHOTUNGSTATE WITH ALCOHOL

Fraps and Semb (5) made many experiments to obtain crystalline phosphotungstates instead of the gummy and resinous precipitate usually produced. No definite method was found to produce clean, crystalline precipitates. Fairly good results seemed to be obtained after the crude "locoine" solutions had been first considerably purified. Attempts at recrystallizations of "locoine phosphotungstate" from its alcohol or acetone solutions by addition of a number of solvents did not give favorable results. The use of barium acetate to decompose the phosphotungstates instead of barium hydroxide was found to be unsatisfactory. Carrying out the precipitation with phosphotungstic acid solution in the cold was a preferable way to a precipitation at room temperature. In every method used for the preparation of the "phosphotungstate," losses were found to be fairly large.

Stempel and Elderfield (15) have also recently had difficulties with the phosphotungstic acid precipitation, and believe that the precipitation of the toxic constituent with phosphotungstic acid may possibly be explained by adsorption of the poison on the rather bulky precipitate.

Absolute alcohol was found to be a suitable solvent for extracting the "locoine phosphotungstate" from the other phosphotungstates formed on precipitation. Part of the remaining alcohol-insoluble phosphotungstates was soluble in acetone. When the alcohol-soluble "phosphotungstate" was dried thoroughly, it became difficultly soluble in absolute alcohol, but dissolved easily on addition of acetone.

The solution obtained by Fraps and Semb after decomposition of the alcohol-soluble phosphotungstate when fed to cats produced definite signs of locoism in six cats after one or two months of feeding. Two cats were fed preparations from the phosphotungstate which was insoluble in alcohol but soluble in acetone for more than 3 months but failed to show definite signs of being locoed.

PURIFICATION BY TWO PRECIPITANTS WITH PHOSPHOTUNGSTIC ACID AND FULLER'S EARTH

A crude locoweed fraction was prepared by two precipitations, as follows: After the first precipitation of the "locoine" solution with phosphotungstic acid, the absolute alcohol soluble part of the precipitate was decomposed with barium hydroxide, treated with basic lead acetate, filtered, and the excess lead removed, as in the method of Fraps and Carlyle (4). Then the filtrate was reprecipitated with phosphotungstic acid solution. This second precipitate was extracted with absolute alcohol, water added, and the alcohol-soluble portion of the phosphotungstic acid precipitate decomposed with barium hydroxide solution. The neutralized, concentrated, aqueous solution resulting from the filtrate was called 2PP (twice precipitated by phosphotungstic acid). Trichloracetic acid was added and the aqueous solution extracted with iso-amyl alcohol, then with ether, which removed most of the color. The purified aqueous portion had in its absorption spectrum in the ultra-violet an extremely sharp maximum at 265 mu. and a minimum at 245 mu, see Figure 4. This is one of the sharpest maxima obtained on any locoweed extract.

Measured portions of the fraction 2PP, after having been adjusted with dilute sulfuric acid or barium hydroxide to pH values of 1.5, 3.8, 7.5, and 9.8, were each treated separately with weighed portions of fuller's earth previously washed with distilled water. More of the greenish-yellow color remained in the solution which had been adjusted to pH 9.8 than in the ones which had been adjusted to the other pH values.

The supernatant liquids were neutralized, and the absorption spectra determined. The curves of the solutions at pH 1.5 and pH 3.8 gave a maximum at 265 mu and a minimum at 245 mu. The curve of the solution at pH 7.5 gradually rose to a slight maximum at 255 mu and fell to a minimum at 240-245 mu. The one at pH 9.8 failed to exhibit a maximum or minimum.

Elution of fuller's earth with dilute barium hydroxide after its treatment with a solution of 2PP at pH 1.5 produced a solution which had a maximum at 365 mu and a minimum at 245 mu.

Toxicity Tests of Fraction 2PP on Cats

Five cats fed on various solutions prepared from material adsorbed on fuller's earth were locoed after about two to two and one-half months of

feeding. Four cats fed on solutions prepared from fractions not adsorbed on fuller's earth gave varying results. One showed definite symptoms of locoism; another appeared to be locoed; but two failed to exhibit definite signs of locoism.

Diagram 1—Separation of locoine concentrate preparations.

P-1 Alcohol-soluble phosphotungstate, locoed cats—Fig. 1

P-2 Alcohol-insoluble phosphotungstate, soluble in acetone, did not loco cats

P Precipitated with silicotungstic acid, from alcohol-soluble phosphotungstate—Fig. 2, SP

Slightly physiologically active but not locoine—Fig. 3

SF. Not precipitated by silicotungstic acid—Curve A

A. Extracted by phenol

B. Not extracted by phenol

Chromatographed on aluminum oxide with picric acid

A. Colorless percolate

B. Yellow percolate

C. Adsorbed, soluble in water

D. Adsorbed, not soluble in water

NB. Not benzoylated with benzoyl chloride, see diagram 2. Produced symptoms of locoism. Fig. 2, NB- A

E. Benzoylated, ether soluble

BP. Separated from solution on partial evaporation (BP), and purified (BPS), not locoine.

BF. Slightly soluble in ether.

BFRS. Ether soluble saponified and base extracted BFRS.

BFRS. Chromatographed in alcohol solution
BFRS-W-Adsorbed—Fig. 3
BFRS-A-Not adsorbed—Fig. 4

BFRS. Chromatographed with picric acid.
A. Adsorbed
B. Not adsorbed

Diagram 2—Separation of unbenzoylated Fraction NB.

NB. Chromatographed through aluminum oxide

NBW. Adsorbed—eluated with water

NBA. Not adsorbed—Symptoms of locoine when fed to cats. Fig. 2

Treatment of NBA with silver nitrate.

Filtrate—no maximum or minimum in absorption spectrum curve.

Precipitate—locoine spectrum curve.

Treatment of NBA with flavianic acid.

Precipitate—no maxima or minima in absorption spectrum curve.

Filtrate—chromatographed through aluminum oxide.

Adsorbed—purified—absorption spectrum maximum 625—minimum 243

Not adsorbed—purified—absorption spectrum maximum 270, minimum 243

Treatment of NBA with picric acid and chromatographed

Not adsorbed

Adsorbed, yellow zone

Purification With Iso-Amyl Alcohol

When the locoine solution was made slightly acid and extracted with iso-amyl alcohol and then with ether, substances were removed which were not locoine. The purified solution had given a sharper spectrum curve (See Fig. 1). This method of purification was used on a number of preparations.

PRECIPITATE WITH SILICOTUNGSTIC ACID

When the concentrated locoine solution secured in Method A was treated with silicotungstic acid, a portion was precipitated but most of the organic matter remained in solution. The fraction precipitated by this reagent was toxic but did not produce all the symptoms of loco disease. The fraction not precipitated was toxic and produced the usual symptoms.

The concentrated, aqueous solution was separated into two fractions by precipitation with a 25% solution of silicotungstic acid (Merck $SiO_2.12WO_3.26H_2O$) in 4% sulfuric acid. A grayish, curdy precipitate was formed, rapidly becoming resinous-like and dark. This precipitate was centrifuged off and washed with cold water. Additional water was added to the washed precipitate, then saturated barium hydroxide solution was added in slight excess and the mixture shaken thoroughly. Mechanical breaking up of the precipitate was sometimes necessary. The excess of barium was removed with dilute sulfuric acid and the neutralized aqueous solution was concentrated in vacuo. This solution was treated again with silicotungstic acid solution. This time, a much smaller amount of sticky material accompanied the precipitate. After decomposition with barium hydroxide in the usual manner, after neutralization and concentration, a third precipitation was made with silicotungstic acid solution. This precipitate, when kept cold, did not become resinous-like, nor much darker. This precipitate was washed with cold water, then decomposed with barium hydroxide. The resulting solution was neutralized and concentrated. This solution was called SP (silicotungstic acid precipitate). Some was used for toxicity tests on cats, and proved to be slightly physiologically active,

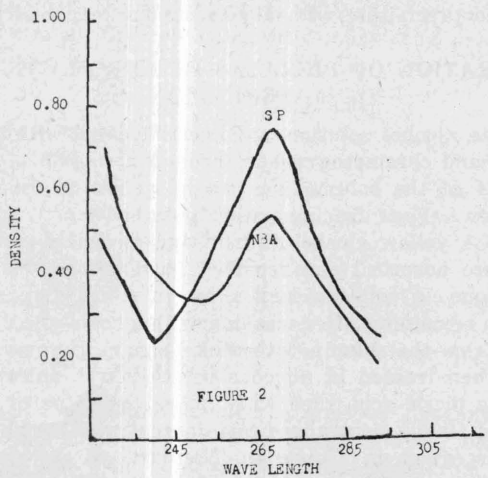

FIGURE 2

Figure 2. Spectrum curve, SP, of locoine preparation which was precipitated with silicotungstic acid, purified, toxic to cats, did not produce all symptoms of locoism. NBA—Not precipitated with silicotungstic acid, not benzoylated, not adsorbed with aluminum oxide, locoed cats.

though not producing many of the typical symptoms of locoism. The absorption spectrum is shown in Figure 2 and is similar to that for the previous preparation (Figure 1).

Not Precipitated With Silicotungstic Acid

The filtrate containing the material not precipitated by the silicotungstic acid solution, and still containing an active principle, was treated with a saturated barium hydroxide solution until no more precipitation occurred, even on long standing. The precipitate was filtered off and discarded, and the excess of barium removed with dilute sulfuric acid. The aqueous solution was concentrated to about 100 ml. under reduced pressure. A drop of this solution was tested to see if any precipitate was still formed with the silicotungstic acid solution. If so, the silicotungstic acid precipitation was repeated. When the concentrated aqueous solution no longer gave any precipitation with silicotungstic acid solution, it was used for further separations. This solution was called SF (silicotungstic acid filtrate).

Alkaloid Tests on the Fraction SF. Alkaloid tests on the preparation SF were made according to the methods of the A. O. A. C. Silicotungstic acid solution, ferric chloride solution, Wagner's reagent, Mayer's reagent, Kraut's reagent, Marme's reagent, mercuric chloride solution, potassium iodide solution, picric acid solution, and potassium ferrocyanide solution all failed to give any precipitate. Phosphoric acid solution produced a heavy amorphous precipitate. Reinecke's solution was decolorized, as was also a 2% potassium permanganate solution.

An alcoholic solution of SF yielded a trace of precipitate with alcoholic mercuric chloride solution, and a precipitate with alcoholic tannic acid solution, but no precipitate with alcoholic picrolonic acid solution.

SEPARATION OF PICRATES BY CHROMATOGRAPHIC ADSORPTION

An absolute alcohol solution of SF was treated with picric acid in absolute alcohol, and chromatographed through a column of aluminum oxide. The upper half of the column was bright yellow in color, and the lower half, pale yellow. Four fractions were obtained. (A) A colorless alcohol filtrate. (AA) A yellow alcohol filtrate and alcohol washings. (B) A pale yellow substance adsorbed and removed from the column by elution with water. The aqueous solution had a brownish-red tingle. (C) A bright yellow fraction remaining after washing with alcohol and elution with water was removed from the column with dilute barium hyroxide solution. Each solution was then treated in aqueous solution with sulfuric acid and benzene to remove picric acid, then with 150 ml. portions of iso-amyl alcohol, then ether, and finally neutralized and concentrated. The absorption spectrum curves are given in Figure 4. The curve of the alcohol filtrate had a sharp maximum at 264 mu and no minimum. The curve of the aqueous filtrate had a sharp maximum at 262 mu and a sharp minimum at 240 mu; while that of the barium hydroxide eluate had an extremely sharp maximum at 256 mu.

Another alcoholic solution of SF without picric acid was first passed through aluminum oxide and divided into the alcohol filtrate A and the

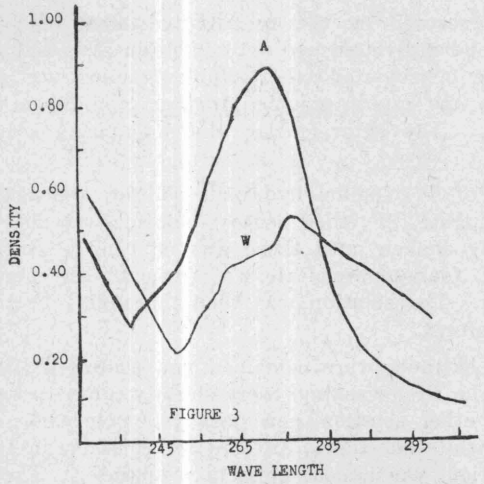

Figure 3. Spectrum curves of compounds not precipitated by silicotungstic acid, benzoylated, not adsorbed by aluminum oxide, A. The same but adsorbed by aluminum oxide, W.

water eluate W. Each of these solutions, transferred to absolute alcohol, was separately treated with alcoholic picric acid solution and separately chromatographed. Both fractions from the chromatographed "picrates" of A showed a maximum at 268-270 mu. The alcohol filtrate A had a maximum of 264 mu, and the water eluate W had a maximum at 268-270 mu and a minimum at 237 mu. Evidently more than one compound was present.

Separation With Phenol

Most of the solution SF was removed on extraction with liquified phenol. The remaining aqueous phase, after removal of traces of phenol and purification with iso-amyl alcohol, showed on its absorption spectrum curve in the ultra-violet a broad maximum at 269-283 mu and a major minimum at 245 mu. Also present were a very weak maximum at 297-306 mu and a weak minimum at 286 mu. Of all the newer fractions studied and tested, this solution was the only one to show the extra maximum and minimum. This extra maximum and minimum, however, has been found in some crude locoweed extracts before they had been separated into the newer fractions.

The phenol extract, after transfer to aqueous solution, gave a sharp maximum at 268-270 mu. Thus at least one substance, maximum 268-270 mu, was soluble in the phenol and had been separated from the solution by means of the extraction.

BENZOYLATION WITH BENZOYL CHLORIDE

Previous work showed (4) that the locoine base is acetylated with acetyl chloride. The losses in the acetylation and subsequent saponification were high, and for this reason benzoyl chloride was tried. Several

fractions were secured by the benzoyl treatment of fraction SF. One fraction was not benzoylated and not soluble in ether but contained a toxic compound. The benzoylated ether-soluble fraction was divided into two more fractions, one highly soluble in ether, and the other only slightly soluble in ether. The latter fraction did not appear to be poisonous. See Diagram 1.

An excess of 5% sodium hydroxide solution was added to the silicotungstic acid filtrate, SF; then benzoyl chloride was added. The contents were thoroughly shaken until there was no benzoyl chloride present. A greenish-yellow, foamy precipitate was formed. The precipitate was dissolved in ether. The solution was then thoroughly extracted with ether and with chloroform.

The ether extracts were combined and thoroughly washed with distilled water. The first washings were slightly colored; the later ones were colorless. The ether solution, now deep yellow in color, was set aside to evaporate. A white substance, difficult to redissolve in ether, was formed when the solution was almost entirely evaporated. The precipitate was washed free of oily and resinous-like material with cold ether. This white precipitate was called BP ("Benzoate" precipitate). Crystals of BP, before recrystallization, gave a melting point of 151°-4°C. On recrystallization from alcohol, the melting point was 178-80°C. The crystals were long, white, interlacing needles. They were insoluble in water, difficulty soluble in ether and in cold absolute alcohol, but soluble in hot absolute alcohol.

The ether solution from which crystals of BP had separated, was allowed to evaporate still further. An oily and sticky residue was left. It was extracted with cold and then with slightly warm petroleum ether. The petroleum ether extracts yielded another white precipitate, which later studies indicated to be impure benzoic acid. After the petroleum ether extractions a resinous-like material was left which was dark brown in color and soluble in ether. This was called BFR (benzoate filtrate residue).

The crystals of BP and the ether-soluble residue BFR were each separately saponified with sodium hydroxide. BP was found to be extremely difficult to saponify completely. BFR produced an extremely penetrating odor during the treatment. Each saponified solution was cooled, and then acidified with dilute sulfuric acid. Benzoic acid was precipitated in the case of BFR, but none was observed in the case of BP. The latter failed to produce any noticeable precipitate on acidification after its treatment with sodium hydroxide. After thorough extraction of each solution with ether, the aqueous phases were each separately neutralized with barium hydroxide, and the barium sulfate discarded. The neutralized solutions were then evaporated to dryness, and the residues thoroughly extracted with absolute alcohol.

Absorption spectrum studies in the ultra-violet on a neutral aqueous solution obtained after the saponification of BP failed to show the maximum or minimum which indicates locoine poison. An aqueous, neutral solution called BFRS obtained after the saponification of BFR showed a

maximum at 264 mu and a minimum at 243 mu, before purification. Solution BFRS was used for toxicity tests on cats and found to be definitely toxic.

Chromatographic Adsorption Analysis of BFRS. The absolute alcohol solution of BFRS (saponified solution of BFR) was chromatographed through a column of aluminum oxide (c. p. Baker's Analyzed, ignited powder). The absolute alcohol filtrate was pale greenish-yellow in color and was called BFRS-A. The spectrum curve is shown in Figure 3. A

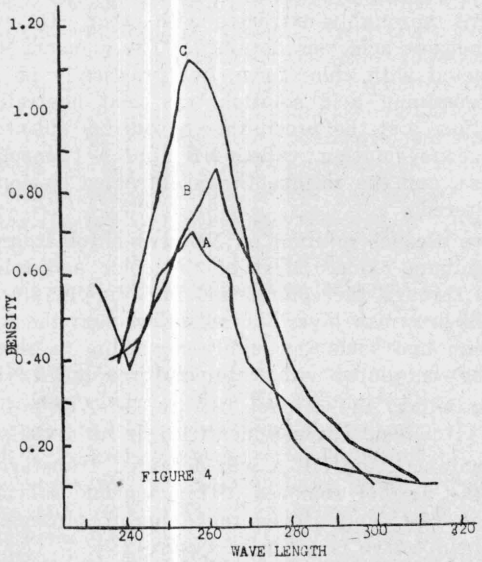

Figure 4. Spectrum curve of fractions separated from preparation SF not treated with benzoyl chloride, by chromatograph of the picrate on aluminum oxide. A, first colorless filtrate. B, eluted with water. C, eluted by barium hydroxide.

brownish ring of material was adsorbed at the top of the column. After thorough washing of the column with absolute alcohol, which failed to remove the adsorbed matter, water was used for the elution. The brown ring now passed into the aqueous filtrate, which was called BFRS-W. The spectrum curve is shown in Figure 3. The two curves are decidedly different and show the presence of two different compounds.

Chromatograph of the Picrate. A solution of BFRS, which had not been chromatographed previously, showed on its absorption spectrum curves in the ultra-violet a maximum at 264 mu and a minimum at 243 mu. The absolute alcohol solution of this was treated with alcoholic picric acid solution and chromatographed through a column of aluminum oxide. A small ring faintly pink was adsorbed at the top of the column. The remainder of the column was yellow in color. The water eluate of the column, after the usual purifications, had a maximum at 268-270 mu. The

column was next eluted with a dilute sulfuric acid solution. The acid removed all noticeable color from the column. The acid eluate, after the usual purifications, showed a maximum at 268-270 mu, and a minimum at about 243 mu.

Portion Not Benzoylated With Benzoyl Chloride

The solution SF, after its treatment with benzoyl chloride, was purified from benzoylated material by extraction with ether, and with chloroform. The alkaline aqueous solution was then acidified with dilute sulfuric acid. The resulting aqueous solution, as well as the benzoic acid precipitate, were thoroughly extracted with ether. On evaporation of the ether, impure benzoic acid was obtained. The aqueous solution, now acid, was also extracted with chloroform, but practically no material was removed. The remaining acid solution was next neutralized with barium hydroxide solution, and the precipitate of barium sulfate discarded. The neutralized aqueous solution, called NB (not a "benzoate") was evaporated to dryness, and the residue thrice extracted by commercial absolute alcohol, and filtered.

An absolute alcohol solution of NB was chromatographed through a column of aluminum oxide. Most of the color and solid matter of the solution passed through the column and into the filtrate, now to be called NB-A. A small brownish layer was adsorbed near the top of the column. After the column had been thoroughly washed with absolute alcohol, the brownish matter was eluted with water and was called NB-W.

On long standing, the alcohol filtrate NB-A deposited a crystalline substance NB-AP, which was not identified or its toxicity tested.

The supernatant liquid NB-A was decanted, transferred to an aqueous solution, and the alcohol removed. This aqueous solution was used for toxicity tests on cats, and proved to be highly physiologically active on cats. Its spectrum curve is given in Figure 2.

Silver Nitrate Separation

On treatment of a solution of NB-A in absolute alcohol with an absolute alcohol solution of silver nitrate, in subdued light, a white precipitate was formed. The precipitate was washed with absolute alcohol, suspended in water, and decomposed with hydrogen sulfide. After filtering off the lead sulfide, the filtrate was treated with activated charcoal, and the resulting, almost colorless solution showed a flattened maximum at 255-265 mu.

The filtrate from NB-A, after precipitation with silver nitrate, was treated with hydrogen sulfide, the filtrate transferred to aqueous solution, and boiled with activated charcoal. No definite maximum or minimum appeared in its absorption spectrum in the ultra-violet, so it did not belong in the locoine group. Thus alcoholic silver nitrate is one of the very few precipitants found for a toxic fraction of locoweed extract, when the latter is highly purified.

Flavianic Acid Separation

An alcoholic solution of NB-A produced a reddish-orange precipitate on addition of an absolute alcohol solution of flavianic acid (2, 4 di-nitro-

1-naphthol-7-sulfonic acid). This precipitate had a melting point above 250°C. It was somewhat soluble in an excess of the reagent. The precipitate was decomposed with concentrated hydrochloric acid. The solution resulting from the decomposition of the precipitate, after filtration and neutralization, showed no definite maximum or minimum in its absorption spectrum in the ultra-violet. It thus did not contain any loco compound.

The absolute alcohol filtrate, after precipitation with flavianic acid was chromatographed through a column of aluminum oxide. An absolute alcohol filtrate was obtained and another fraction which had been adsorbed on the aluminum oxide but eluted by water. After treatment of both solutions in water with concentrated hydrochloric acid, followed by filtration and neutralization, an absorption spectrum determination in the ultra-violet showed that the alcohol filtrate had a maximum at 270 mu and a minimum at 243 mu. The water eluate had a flattened maximum around 265 mu and a minimum at 243 mu. The two fractions from the chromatographing both had locoine spectrum curves and appear to contain substances definitely different from the one removed by precipitation with the flavianic acid.

Chromatograph Separation of Picrates

Another absolute alcohol solution of NB-A was treated with an absolute alcohol solution of picric acid. No precipitation was observed. The resulting solution was then chromatographed through a column of aluminum oxide. The filtrate was yellow. The column was thoroughly washed with absolute alcohol until no more color passed into the filtrate. The upper one-fifth of the column was pale yellowish green in color, the middle two-fifths was a bright yellow color, and the lower two-fifths was pale yellow. On elution with water, a reddish-brown colored filtrate resulted. After the water elution, only the middle two-fifths of the column remained yellow in color. The top and bottom portions were white. The middle yellow was eluted with dilute barium hydroxide solution. Each filtrate and eluate was transferred to aqueous solution, made acid with dilute sulfuric acid and the picric acid removed with benzene. The resulting aqueous solutions, only very faintly colored now, were extracted with 150 ml. portions of iso-amyl alcohol, then extracted with ether. The ether was removed from the aqueous solution, and each aqueous solution was concentrated. The absorption spectrum of the solution prepared from the absolute alcohol filtrate had a maximum at 268-70 mu and a minimum at about 244 mu. The aqueous eluate had a maximum at 264 mu and a minimum around 240 mu. The results indicated that two or more compounds were present.

STABILITY OF LOCOINE TO ACIDS AND ALKALI

Absorption spectrum determinations in the ultra-violet were carried out on crude locoweed solutions in the earlier studies on the problem to observe the effects of acid and alkaline treatments.

Refluxing a locoweed concentrate with 10% sulfuric acid solution for three hours had no effect on the maximum, which in this solution was 264 mu. A solution of locoine heated at 80°C. for three hours with air bub-

bling through gave practically the same curve as before the treatment. Heating a locoweed solution for three hours with barium hydroxide solution failed to destroy the maximum at 264 mu but made it less sharp than before.

TESTS ON CATS FOR PHYSIOLOGICAL ACTIVITY

The toxicity tests for cats were carried out as in the previous work of Fraps and Carlyle (1). The cats were kept in cages about 3x3x2 feet in dimensions. They were fed suitable canned dog food five times a week and salmon twice a week. A solution of powdered whole milk was also fed daily to those animals which would drink it. The loco extract was mixed with a small amount of the milk or salmon and fed to the cats. After this was eaten, the cats were fed larger amounts of their food. Water was provided all the time. The cages were kept thoroughly clean.

According to Fraps and Carlyle (4), the symptoms of the loco disease in cats vary somewhat with different animals. The head usually shakes up and down, the vision seems distorted in advanced cases, and the animal seems to be unable to judge the distance to its food. The eyes frequently assume a staring expression, and there is often weakness or paralysis in the hind legs, the left leg seeming to be affected more than the right. The usual symptoms are lack of muscular coordination, a weakness of the hind legs, staring eyes, and a shaking head. Other workers have reported the same general symptoms. In an excellent study, Mathews (5) described in detail the symptoms of locoism in domestic animals.

Fraction NB-A was an aqueous solution of a locoweed extract which had not been precipitated when treated with silicotungstic acid solution; had not precipitated nor become ether-soluble when treated with benzoyl chloride; and had not, when in absolute alcohol solution, been adsorbed on passage through a column containing aluminum oxide. Details of its preparation have been described in the experimental procedure. After 30-36 days of feeding of NB-A (1.2-1.4 grams of solid matter) to the cats, each animal began to lose ability to use its left hind leg. For at least ten days prior to the loss of leg muscular control, the animals had become extremely nervous and restless; the fur was quite roughened. The cats seemed to lack energy. On occasions, they moved very slowly, and when the door of the cage was opened and food placed inside the cage, the cat would not locate the food for some time. The cats gradually lost weight, and when finally completely stricken, they appeared quite emaciated. By the time the paralysis of the hind leg set in, the animals had already lost most of their muscular coordination. They could hardly maintain themselves on their feet. While trying to eat food out of a pan, the animal would generally miss the pan or topple over, head-first into the pan. There was some quivering of the head. Within a day or two after the paralysis started, it had spread, and the animal could scarcely move. Within four or six days more, the cats died.

Fraction BFRS. Fraction BFRS was an aqueous solution of a locoweed extract which had not been precipitated when treated with silicotungstic acid solution; but which had produced an ether-soluble resinous-like substance on treatment with benzoyl chloride. This substance was separated from the ether, saponified, and further purified. Solution BFRS

was fed for 49 days (approximately 1.4 grams of solid matter) to a cat. Paralysis became noticeable at this time. The symptoms were quite similar to those shown by fraction NB-A, in its effect on cats, but the effects were more gradual. Also the fraction seemed to have caused a humping of the cat's back. For about ten days prior to this effect, the animal had refused to eat food mixed with BFRS. Although it did eat other food, its appetite was considerably less than usual. The animal was very easily startled. Sometimes one could approach the cage and the animal without being noticed at all. Then suddenly realizing that some one was nearby, the cat would become startled.

Fraction SP. Fraction SP was precipitated by silicotungstic acid. Solution SP produced a paralysis in two cats in 39-41 days (1.2 grams approximately of solid matter). It did not, however, produce most of the symptoms of locoism. No glassy stare of the eyes, or shaking of the head were observed. For at least two weeks prior to the paralysis, the animals ate very little food, even when SP was omitted from the food. Within four or five days after the beginning of the paralysis, the animals died.

Fraction NB-A and BFRS, especially NB-A, appeared to be definitely physiologically active, producing most of the symptoms of loco poisoning. The fraction SP, though active, did not cause the more usual symptoms of locoism.

LOCO POISON IN SEVERAL VARIETIES OF LOCOWEEDS

Samples of Astragalus mollissimus Torr and Oxytropis saximontana A. Nels were furnished by the Wyoming Experiment Station, with the cooperation of the U. S. Forestry Service, through the courtesy of O. A. Beath and H. F. Eppson. Extracts from these plants fed to cats by A. W. Walde showed that these plants contain the poison which locoed cats. The other two varieties found to contain locoine are Astragalus wootoni and Astragalus earlei.

ABSORPTION SPECTRA

Absorption spectra have been determined in the ultra-violet region for many of the newly isolated fractions as well as for the locoine concentrates of Fraps and Carlyle. From a comparison of these absorption spectra, one finds that some fractions differ from each other chiefly in a shift of the maximum or minimum to longer or shorter wave lengths. Others have the same maxima, but differ in their minima.

Locoweed extracts prepared similarly to the locoine concentrate of Fraps and Carlyle (4), and which have been shown to produce locoism in cats, in their absorption spectrum curves in the ultra-violet exhibited a maximum at 265 mu and generally a minimum of about 240 mu. Practically every locoweed extract used for the new fractionations described in this bulletin showed the same general curve, namely a maximum at 265 mu and a minimum at 240 mu, before the new separations were made. However, after the separations, the new fractions exhibited slight variations. The type of curve would remain the same, but the maximum would appear at a slightly different wave length, such as at 275 mu or at 268 mu or would remain at 265 mu. The minimum also shifted in some frac-

tions to such values as 247 mu or to 237 mu, or sometimes did not appear at all, in some of the fractions. However, the general curve remained essentially of the same type for every fraction proved to be physiologically active on cats.

DISCUSSION

Fractions NB-A which did not form a benzoyl compound and BFRS which formed a benzoyl compound proved to be highly physiologically active in tests on cats, and produced most of the symptoms found in locoed cats. With NB-A, the paralysis of the hind legs and the almost complete loss of muscular coordination came on suddenly. This suddenness is especially striking in comparison with the previous results obtained with the non-fractionated toxic crude locoweed solutions. With such solutions the animal's head would shake, and the eyes would have a glassy stare for many days before the final stages. With NB-A the cats seemed to be acquiring these milder loco symptoms, but in a very short time they would apparently advance to the final stages. Fraction NB-A appeared to be a most active fraction in producing locoism. Solution BFRS also produced symptoms of locoism, but more gradually, and in this respect the preparation was different from NB-A.

Fraction SP precipitated by silicotungstic acid was slightly physiologically active, although it did not produce many of the symptoms of locoism. One cat became dull and depressed, another seemed highly irritable. Both cats had paralysis of the hind leg but did not show the glassy eye stare, or the head shaking usually found in locoed cats.

It cannot as yet be stated definitely whether the active principle in locoweed is a single substance or a combination of several factors. It may be that the fractions obtained by us contain compounds all of which stem from one and the same original toxic substance, but which are changed very slightly one from the other by the various chemical treatments. On the other hand, it is possible that there are several very closely related substances present in the locoweed, each capable of producing some of the symptoms of locoism, but no one alone capable of producing all the effects previously attributed to locoism.

The spectrum curve of the solution prepared from the silicotungstic acid precipitate, SP is similar to that of the solution prepared from fraction NB-A, which was not precipitated by the silicotungstic acid solution, and did not react with the benzoyl chloride. Solution BFRS-A, which was not precipitated by the silicotungstic acid, but had reacted with the benzoyl chloride to produce an ether-soluble substance, also gave a curve of the same pattern as that of NB-A. However, the curves differ in that the maximum of NB-A is about 2 mu toward the higher end of the spectrum and its minimum about 2 mu lower than the others.

It has been found thus far that very physiologically active substances producing symptoms of locoism have the same general type of absorption spectrum curve in the ultra-violet, although in some cases there may be a slight shifting of the maximum or minimum towards the higher or lower wave lengths. The non-toxic fractions have not been found to have this type of curve.

Previous to its separation by treatment with benzoyl chloride and chromatographing through aluminum oxide, practically every crude locoweed solution tested had shown a maximum at 264 mu or 265 mu; whereas, after the further fractionations, variations began to appear, and a larger number of the new locoweed fractions showed maxima around 268-270 mu or higher.

One preparation of fraction BFRS-W showed a maximum at 275-277 mu and a minimum at 248 mu. This maximum had the highest wave length of any obtained for the isolated fractions.

One preparation of BFRS exhibited a maximum at 264 mu and a minimum at 243 mu, but after the chromatographing, both BFRS-A and BFRS-W had maxima of 270 mu or above. It would appear that in this case, chromatographing under the conditions used somehow produced a shift in the maxima.

The precipitation of the absolute alcohol solution of NB-A by alcoholic silver nitrate offers promise for precipitation of a pure toxic principle. The precipitate contained the substance showing the absorption spectrum curve generally found for the toxic substance in previous experiments.

The use of an alcoholic solution of flavianic acid may also prove of value in further purification of fraction NB-A. A solution made from the flavianic acid precipitate (melting point above 250°), did not show the maximum or minimum in its absorption spectrum in the ultra-violet. The filtrate remaining after separation from the precipitate showed a typical absorption spectrum curve for active loco fractions.

The use of the chromatographic adsorption analysis methods on the picrates of the fractions studied may possibly be of value for further studies. Although no precipitate was formed with the picric acid, some of the fractions combined with the picric acid. In some experiments, several solid picrates were obtained on further concentration. The absolute alcohol filtrate of NB, after the picric acid chromatograph treatment, had a maximum at 268-270 mu, whereas the water eluate had a maximum at 264 mu. Before the chromatographing, the solution NB had a maximum at 264 mu. Here is would seem, as has been found in several other instances, that the alcohol filtrate after the chromatographing, had its maximum at a slightly higher wave length than the water filtrate. Yet both filtrates were in aqueous solution at the time of the determinaion of he absorption spectra, and all alcohol had supposedly been removed.

When another locoweed extract, not treated with benzoyl chloride, was treated with picric acid and chromatographed, all of the maxima in the absorption spectrum curves were this time shifted towards the lower wave lengths. The maximum of the alcohol filtrate was 264 mu; that of the water eluate, 262.5; and that of a barium hydroxide eluate, 256 mu. In this case, there was a descent in the maximum with further elutions.

The chromatographing of these solutions may have caused some change or merely made a separation. Incomplete separation, even with thorough washings, or the adsorption of material not eluted at all by the solvents used are not to be excluded. These reasons, or others not known, may explain some of the variation.

In absorption spectrum studies in the ultra-violet of solutions of the locoine concentrate, the maximum at 265 mu had remained unchanged after strong acid and alkali treatments. Previous workers at this laboratory and elsewhere have stressed the apparent stability of "locoine" towards acid and alkali treatments. In spite of the apparent stability of the locoweed active principle towards acids and alkali, there is evidence of shifts from one compound to closely related compounds, as a result of long standing, of certain chemical treatments, or of evaporation to complete dryness by heat. It is possible that a number of closely related compounds found after fractionations are actually not present as such in the plant, but are a result of the chemical treatment used in the long series of steps in the isolations and separations. In several experiments, after apparently complete removal of all material precipitated by silicotungstic acid from a locoweed solution, a careful test would show no further precipitation with silicotungstic acid but with no further treatment or concentration, but after long standing in the refrigerator, some such solutions were found to produce considerable precipitation on the addition of silicotungstic acid. Some of these solutions when kept in the refrigerator for some time, would become distinctly basic in their reaction to litmus, although they had been neutral when stored. It has been found that after evaporation to dryness of clear absolute alcohol solutions of locoweed extracts, all of the residue could not be redissolved in even larger amounts of absolute alcohol.

Liquified phenol, when used to extract a relatively pure fraction of a locoweed extract, removed a substance which possessed the same general absorption spectrum curve usually obtained for the toxic fractions. This appears to be an excellent means of separation. However, phenol failed to remove the desired material from some less fractionated extracts of locoweed.

As the locoweed extract was carried through additional separations, fewer and fewer alkaloid tests showed positive results with the new fractions. Even with an aqueous solution of SF, which can be fractionated much further, Wagner's, Kraut's, Marme's, and Mayer's reagents and mercuric chloride solution failed to give precipitates. Only silver nitrate and phosphotungstic acid were found to yield precipitates in aqueous solution with SF.

ACKNOWLEDGMENT

Some of the work presented in this bulletin was done by A. W. Walde and Joseph Semb, which is hereby gratefully acknowledged.

SUMMARY

An improved method is given for preparing concentrated solutions of the poisonous constituents of locoweeds.

The poisonous principles are found in Astragalus molissimus Torr, Astragalus Wootoni, Astragalus earlei and Oxytropis saximontana.

The phosphotungstate soluble in absolute alcohol locoed cats; that not soluble in absolute alcohol but soluble in acetone did not loco cats.

The compounds precipitated by silicotungstic acid caused a paralysis of the cats but did not produce all the symptoms of locoism.

Extraction with iso-amyl alcohol often removes impurities which interfere with securing a sharp spectrum absorption curve.

The fraction purified by precipitation with phosphotungstic acid, solution of the phosphotungstate in absolute alcohol, and purified from compounds precipitated by silicotungstic acid, did not give the usual alkaloid tests. This fraction is termed SF.

When chromatographed with picric acid in alcohol solution on aluminum oxide, fraction SF gave a colorless solution not held by the adsorbant, a yellow compound removed by washing with alcohol, a pale yellow layer not removed by alcohol but eluted by water, and a bright yellow layer removed by barium hydroxide. The presence of 4 different compounds was indicated.

Phenol extracted from SF a compound with the characteristic absorption spectrum of the locoines.

Treatment with benzoyl chloride produced benzoylated and non-benzoylated fractions. Both were toxic to cats.

Chromatographic adsorption analysis of the saponified benzoylated fraction on aluminum oxide indicated the presence of two different compounds.

The toxic portion of the non-benzoylated fraction was not adsorbed by aluminum oxide. It was precipitated by silver nitrate but not precipitated by flavianic acid. Chromatographing the filtrate from the flavianic acid on aluminum oxide separated two compounds.

Several closely related but different poisons appear to be present in locoweeds. It is possible that some of these are formed by isomerism in the process of the separations, and are not originally present in the locoweed.

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