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The poisons of Amanita phalloides and Amanita solitaria

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The Poisons of Amanita Phalloides and Amanita Solitaria

by

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I. Introduction

1 Nature of the problem

The chemical nature of antigens is an interesting point of immunological investigation. It is generally believed that all antigens are proteins. At various times, claims have been made concerning the antigenic properties of different non-protein substances, such as (1) products of protein hydrolysis, (2) protein-free ricin, (3) lipoids, (4) substances occurring in the poison ivy plant (Rhus toxicodendron) and in the mushroom, Amanita phalloides. Most of these experiments have been disproved.

On this point, Zinsser (42) remarks "Summarizing our present knowledge of the chemical nature of antigens, then, we must conclude that, with the exception of Ford's glucosids, no protein-free antigens have been thus far demonstrated."

Wells (38) states "....it is still not proved that any non-protein substance can function as an antigen." He further comments "These observations of Ford are of so much importance in their relation to the entire question of the nature of antigens that they should be repeated for verification. If accepted as they stand they constitute the strongest evidence yet presented as to the possibility of non-protein antigens. The newer developments in immunological research, moreover, make it seem entirely plausible that a complex glucoside, which can be hydrolyzed by enzymes, can act as an antigen."

With his hemolytic glucoside from the mushroom Amanita phalloides, Ford (17) produced typical antihemolytic sera with a strength of 1-1000, that is, 1 cc of 1-1000 dilution completely neutralized the amount of glucoside that would give solution of 1 cc of a 5 per cent blood suspension in 18 hours.

2 Review and criticism of the literature

Publications regarding the antigenic substances of Amanita phalloides come chiefly from two sources, (1) Dr. W. J. Ford, and his associates, at Johns Hopkins University, and (2) Dr. R. Kobert, and his associates, at the

University of Rostock, Germany. Criticism of the findings has been made by Branham (25). The history of the earlier inconclusive investigations has been well covered by Ford (14)(11). Since 1913, nothing concerning the antigenic nature of the poisons has been published. However, Codina (6), Steinbrinck (34), and Hyman (24) have made observations regarding cases of poisoning by A. phalloides.

(a) The hemolytic substance, Phallin

There are at least two poisons in A. phalloides. One is a hemolysin, to which Kobert gave the name "Phallin". It is highly unstable, easily destroyed by acids and alcohol, and rendered inactive by exposure to a temperature of 70°C. Animals immunized with the aqueous extract were found by Ford (11)(13) to produce a serum antihemolytic and antitoxic in character. Thus far, Ford and Kobert agree. Ford (2) (12) also found that the hemolysin was precipitated by alcohol, endured treatment with basic lead acetate, and was destroyed by pancreatic juice. Preliminary analysis by Abel and Ford (3) yielded a percentage composition of C= 48.93, H = 6.08, N = 10.83, S = 1.94, O = 32.322. From this analysis, and from various tests for pentoses, Ford (2)(3) concluded the hemolysin to be a nitrogenous glucoside of the saponin type. Branham (25) does not believe it possible, on a basis of the percentage composition, for the substance to belong to this class of glucosides. Protein was eliminated by Ford by precipitation with metaphosphoric acid and uranyl acetate. The resulting filtrate, which was used for the immunization of animals, gave no biuret test. The glucoside passed through a collodion membrane, but the hemolytic power of the glucoside was destroyed in its passage. Ford (2) mentions that Kobert's hemolysin would not pass through parchment. Pathologically, Phallin characteristically produced hemoglobinurea and pigmentation of the spleen. There was some increase in fat in the various organs, but this was not present to any considerable extent. Ford decided that the second poison, the Amanita-toxin, was more important in poisoning than the hemolysin.

Kobert and his associates disagreed with some of these findings. According to Ford (11), Kobert postulates three poisons in A. phalloides, (1) the hemolytic principle, Phallin, (2) the alcohol soluble alkaloid not producing fatty degeneration, and (3) a toxalbumin present in the alcoholic precipitate of the aqueous extract, allied chemically

to the substances thujon and pulegon, and capable like them of causing fatty degeneration in the internal organs. It is possible that Kobert's views have been modified by a later publication of his associate Rabe. Rabe (28) found that the hemolysin was precipitated by alcohol and sugar of lead. Although Kobert seems to consider the hemolysin as apart from the toxalbumin-alcohol-precipitated-fatty-degenerative-substance, Rabe states that pathological fat found in the liver and in the heart muscle was caused by the hemolysin as well as by the alkaloid. He leaves undecided the matter of hemoglobinurea, and does not mention examination of the spleen. Kobert and Rabe feel that the hemolysin is intensely poisonous, and that it does not hold a subordinate place in the poisoning of people, compared to the toxin.

As contrasted with Ford's characterization of the hemolysin as a nitrogenous glucoside, Kobert and Rabe are certain that it is a toxalbumin. Rabe found that the Phallin could be precipitated almost completely from the aqueous extract by alcohol and lead sugar, could not be precipitated completely by uranyl acetate, that the precipitated sediment then gave an albumin reaction (Millon's), although negative with the biuret test, and that it became weak on drying. The filtrates also gave a positive protein test. A positive Millon's test in the sediment would, of course, be expected, since the reagents used are protein precipitants. A positive test in the filtrates is also possible, since none of the three reagents is a quantitative protein precipitant. It may be noted that a negative biuret test by Ford in his procedure, was no evidence of the quantitative elimination of proteins. However, it would appear that his method of obtaining a non-protein glucosidal substance was less open to question than Rabe's method of obtaining the toxalbumin in any condition approaching purity and isolation.

The chief criticism of Ford's work on the hemolysin has been indicated above. As Branham (25) has well pointed out, a negative biuret test is not conclusive of quantitative protein elimination. Neither are the reagents, metaphosphoric acid and uranyl acetate, quantitative protein precipitants. It would be well that the immunization be attempted with filtrates obtained after the precipitation of protein by more effective reagents. Some evidence on these points is presented later in this paper.

The possibility of a protein-glucoside-hemolysin

complex is strengthened by the outcome of Abel and Ford's (2) dialysis experiments. It is well to remember, however, that dialysis, as mentioned by Bigelow and Gemberling (4) (5), is no certification of the absence of protein in the diffusate. Abel and Ford (2) dialyzed their glucoside.

Branham (25) remarks on this point "Abel and Ford have shown that when their hemolytic antigen was dialyzed in collodion bags, part of the glucoside passed through the dialyzing membrane. Although the glucosides recovered both from within and without the dialyzing sac appeared to be identical, that which had passed through the membrane was not hemolytic. Unfortunately, no test for antigenicity of this non-hemolytic fraction was made. Hence it is difficult to accept the conclusions of these investigators that the active hemolytic principle of Amanita phalloides is a nitrogenous glucoside capable of producing antibodies."

It is possible that the glucoside in the diffusate might be restored to hemolytic activity. Bigelow and Gemberling noted that "Bierry and Giaja made the interesting observation that pancreatic secretion having passed through a colloidal membrane, no longer acted on starch or maltase, but that adding an electrolyte, preferably one containing a chlorine or bromine ion to this inactive material restored its lost activity and ability to act on starch."

(b) The Amanita-toxin

The second poison in A. phalloides has been provisionally named "Amanita-toxin" by Ford (12). Schlesinger and Ford (31) and Ford and Prouty (18) isolated the toxin in a state of considerable purity, by a rigorous analytical separation, later described in this paper. They found that the toxin was thermostable and resistant to acids. Ford (12) determined that the toxin was not affected by pepsin and pancreatin. It gave no alkaloidal reactions, except that it was precipitated, in the purification process, by phosphotungstic acid. Combustion analysis by Ford and Bronson (15) gave the following ash-free percentage composition:

	I	II	III
C	42.89	43.46	
H	6.79	7.29	
N			14.16
S		2.91	

Schlesinger and Ford concluded that the toxin can not be a protein, a glucoside, or an alkaloid, and that "although not necessarily an indol derivative, (it) is at least an aromatic phenol so combined with an amine group that it readily forms an indol or pyrrol ring." The pathological changes produced by the alcoholic extract, observed by Ford (11), were gastric and intestinal ulcers, hemorrhages, necrosis, and especially fatty degeneration. The same pathological changes were produced by the toxin as purified by Schlesinger and Ford (31).

Kobert has considered the amanita-toxin to be an alcohol-soluble alkaloid, now producing fatty degeneration, according to Ford (11). Habe (28) also considered the amanita-toxin an alkaloid, and obtained certain positive alkaloidal tests -- phosphotungstic acid, phosphomolybdic acid, Esbach, Mayer, picric acid, Dragendorff, Kraut, Kiliani, and other tests being used. His method of purifying the toxin was to extract with alcohol, evaporate off the alcohol, and dissolve out impurities with ether and chloroform. The toxin was therefore alcohol-soluble and ether and chloroform-insoluble. This process, however, leaves with the toxin a number of substances which might give an alkaloid reaction. This is evident from experiments reported later in this paper, for no alkaloidal reactions could be obtained with the toxin prepared by the method of Schlesinger and Ford (31). Kobert's views in regard to the question of the production of fatty degeneration (which he thought was caused by a toxalbumin and not by the alkaloid or toxin) may have changed after the experiments of Habe. Habe (28) reported that more fatty degeneration in the liver was produced by his alkaloid-toxin than by the hemolytic substance.

Ford (17) was unable to produce antibodies for the Amanita-toxin. He summarizes this point as follows: "....it is important to determine how far animals can be immunized to the pure toxin, free from admixture with the blood-taking material. Thus far no success has been met with in this attempt. Animals will withstand the introduction of low multiples of a fatal dose and apparently have a heightened resistance to the action of the poison but in no instance has a definite artificial immunity been established. It is evident that it was the presence of this powerful toxin in our original extracts which was the source of the discrepancy already referred to, namely that a powerful antihemolytic serum may show but a low degree of antitoxin action, and that immune animals succumb to the introduction of high

multiples of a fatal dose even when their serum is endowed with neutralizing substances for the hemolytic glucoside. A curative serum for this variety of fungus intoxication can thus be prepared to the same degree that the hemolysin acts as an etiological agent, unless further investigation shall prove that active immunity towards the Amanita-toxin can be brought about by some other methods."

3 Object of the present study

The original object of the present study was to repeat the isolation of the glucoside by the method of Ford (2), using an improved technique in protein precipitation and in tests for the presence of proteins after precipitation. Then immunization was to be attempted with the presumably non-protein solution. With this plan in mind, during 1928-29, a study was made of protein precipitating agents and protein tests, as presented herein.

In the fall of 1929, the first mushrooms were obtained. It soon became evident, by experiments included in this paper, that the hemolysin was absent, so the course of the study had to be directed to the Amanita-toxin.

In two attempts, the Amanita-toxin failed of isolation at the last step in the procedure. This used up a considerable portion of the mushrooms. The remaining mushrooms were extracted, carried through the process of purification to the final operation, and the procedure there stopped. With this extract, which at every step had been tested for toxicity, tests were made for proteins, alkaloids, and glucosides. An immunological experiment was tried. Permanent mounts were made of representative tissues from rabbits dying from subcutaneous injection with the toxin in its various stages of preparation, and the tissues were examined for pathological change.

II The delicacy of various color and precipitation tests for the presence of proteins, glucosides, and amino acids

1. Historical

Reports on the exact delicacy of color and precipita-

tion tests for the presence of proteins are not extensive. The biuret test is sensitive to one part of protein in 10,000 parts of water, according to Brannam (25). Rakuzin, Braudo, and Pekarskaja (29) found that Millon's test was more sensitive than the biuret, Liebermann's, Adamkiewicz's, the xanthoproteic, Molish's, Pettenkofer's, or Ostromisslenski's, and detected one part of protein per 15,560 of solution. Abernalden and Schmidt (1) report that the ninhydrin reagent shows one part of glycine in 65,000 and about one part in 15,000 to one part in 25,000 of the other amino acids. Folin and Denis, as reported by Hawk (22) can detect with their reagent one part of tyrosine in a million. Matthews (27) gives the delicacy of Spiegler's test as one part of protein in 250,000. Various references on protein tests are given in the supplementary bibliography of this paper.

2 Experimental

It seemed wise to become acquainted, at first hand, with the delicacy of the protein tests, since technique and keenness of vision vary with the operator.

Reagents were prepared and the tests were made in the manner described in the text references given in table 1.

(a) Preparation of the test solutions

Unless otherwise specified, all solutions were in distilled water at room temperature. Each solution was used on the same day as it was prepared, to minimize bacterial decomposition (except the thymol solution).

Edestin did not dissolve in distilled water. Ten per cent sodium chloride allowed a temporary suspension which was well shaken before dilutions were made or a few cubic centimeters drawn off for a test.

Egg albumin, well pulverized, dissolved almost completely, but was shaken before using to insure uniformity.

Gelatin in a 1-200 solution was heated to 60°C; 1-5000 solution was heated to 96°C; both were allowed to cool somewhat before using; solution was complete.

Peptone was treated as was egg albumin.

Asparagin in 1-200 solution was heated to 80°C; 1-5000 solution was heated to 65°C; both were allowed to cool somewhat before using; solution was complete.

Cystine in 1-1000 solution would not entirely dissolve on boiling, but the small residue formed a good suspension on shaking. The 1-5000 solution at 96°C dissolved completely in 15 minutes with trituration. It was allowed to cool to 42°C before using.

Phenylalanine in 1-200 solution heated to 56°C completely dissolved. A 1-5000 solution heated to 80°C completely dissolved. Both were allowed to cool before using.

Tyrosine in 1-500 solution heated to 60°C completely dissolved. A 1-5000 solution heated to 55°C completely dissolved. Both were allowed to cool before using.

Digitalin in 1-250 solution heated to boiling completely dissolved. A 1-5000 dilution heated to 86°C completely dissolved. Both solutions were allowed to cool before using.

Salicin in 1-200 and 1-5000 solutions dissolved immediately at room temperature.

Thymol was allowed to stand several days until completely dissolved.

(b) Procedure in the tests

The shades of color were too delicate to permit satisfactory work by artifical light. By artifical light, it was particularly difficult to distinguish in high dilutions the milky or bluish tinge indicative of a positive test with acetic acid and potassium ferrocyanide, Folin and Denis, Millon, Morner, ninhydrin, Tanret, and xanthoproteic reagents. A set of controls was run on each test, but only on Ehrlich's diazo and Folin and Denis's tests was it necessary to run controls every time a solution was tested.

Acetic acid and potassium ferrocyanide: A modification in technique improved the delicacy of this test. The potassium ferrocyanide was added drop by drop and allowed to run down the inclined test tube so as to form two indistinct layers of solutions. A milky or bluish tinge slowly developing in the upper solution is a positive test.

Ehrlich's diazo-benzene-sulphonic acid: Since distilled water gave an orange solution, a control was always used.

Folin and Denis: Since distilled water gave a bluish color, a control was always used. It was easier to differentiate shades of color (also in Ehrlich's diazo) by looking into the two test tubes (control and test) side by side, than by looking at them at right angles to their vertical position.

Millon: A reddish color appeared only in low dilutions; a positive test in high dilutions was indicated by a milky or bluish tinge.

Xanthoproteic: The yellow color did not always appear; a positive test was sometimes indicated by a milky or bluish tinge.

Table 1, following, with its accompanying notes, gives the results of all the tests.

(c) Notes on table 1

(1) Not an amino acid itself, but the monamide of the amino acid aspartic acid. Asparagin occurs widely in the vegetable world.

(2) e.g.-H168 refers to page 168 of Hawk and Bergeim, "Practical Physiological Chemistry", P. Blakiston's Son & Co., Philadelphia, 1927. M982 refers to page 982 of Mathews "Physiological Chemistry", William Wood & Co., New York, 1927.

(3) The test solution would not color, but in low dilutions, visible particles of edestin would color yellow, and adhere to the sides of the test tube.

(4) This test was impracticable, since the light blue color of the edestin solution confused the blue color which would indicate a positive result in this test.

(5) After the test had stood for one hour.

(6) A light brown color appears, possibly due to the charring of the organic particles by the sulphuric acid.

(7) A second solution of phenylalanine and a second solution of cystine were prepared and tested, to make certain of the variation on this reaction between the two amino acids.

(8) A faint green ring appears.

(9) There is no reaction with para-dimethyl-amino-benzaldehyde, but before or after boiling with hydrochloric acid, a milky or flocculent precipitate appears.

(10) No green appears, but a light milky bluish color.

3 Summary

Gram equivalents of the dilutions are only approximate, since about two cubic centimeters of the tested solution was used in making each test. Wells (38) indicates that the minimum sensitizing dose of egg albumin is not far from 0.000,000,05 gram. Branham (25) emphasizes the fact that "antibody response has been secured with amounts of protein as small as 0.000,001 gram." The most sensitive reaction shown in table 1 is 1-80,000 or about 0.000,025 gram. It is evident that although a solution may give no color or precipitation test for the presence of protein, nevertheless, there may be sufficient protein in solution to allow antibody formation. The negative biuret test of Abel and Ford (2) seems, therefore, to be little certification of the absence of protein in their hemolytic glucoside.

III The precipitation of proteins

I. Historical

Trichloroacetic acid: Seibert (33) showed that when pure proteins are used, trichloroacetic acid precipitates the protein quantitatively. It does not precipitate proteose or residual nitrogen (monamino acids and undetermined nitrogen). Hiller and Van Slyke (23) state "It appears, therefore, that trichloroacetic acid is especially fitted for use with solutions of partially digested proteins when it is desired to remove the proteins, and to regain in their filtrates not only the amino acids, but also a mixiмum proportion of the intermediate products such as 'albumoses' and 'peptoses'." Wasteneys and Borsook (37) and Shonle and Waldo (32) have also shown that trichloroacetic acid fails to precipitate proteose and the other intermediate products. Wasteneys and Borsook (37) with an albumin solution analyzing 94.5 per cent protein, 4.4 per cent proteose, and 1.1 per cent peptone, used trichloroacetic acid in a final concentration not exceeding 2 per cent, and precipitated 96 per cent of the protein nitrogen. They decide, therefore, that trichloroacetic acid completely precipitates the protein. Similarly, using nitrogen determinations as a criterion, they found that meta-protein was completely precipitated.

Greenwald (19) used trichloroacetic acid followed by kaolin treatment. He gives his results as follows: "The

filtrate from the kaolin is absolutely clear and colorless. The liquid remains clear after the addition of picric acid or of potassium-mercuric-iodide, even upon standing for twenty four hours, and gives only the slightest turbidity with phosphotungstic acid. The precipitates obtained upon the addition of phosphotungstic acid to filtrates....were used for the determination of nitrogen. The amounts obtained were no larger than in the blanks."

M. Richter-Quittner (30), Paul Cristol (7), A. Grigaut and P. Zizine (21), P. Cristol and M. Simonnet (8), and H. Wunschendorff (40), have also reported on the use of trichloroacetic acid.

It appears that trichloroacetic acid precipitates pure protein, but does not precipitate the intermediate products (proteoses, peptones, peptides, and amino acids). When followed by kaolin, precipitation of all protein and non-protein nitrogen is complete.

Tungstic acid and picric acid: Hiller and Van Slyke (23) state: "From the results with Witte's peptone it appears that tungstic acid and picric acid are distinguished by the relative completeness with which they precipitate protein intermediate products without precipitating amino acids." However, the intermediate products are not completely precipitated, and from their results with blood, it appears that tungstic acid, picric acid, metaphosphoric acid, trichloroacetic acid, and colloidal iron, all precipitated pure protein quantitatively, but did not completely precipitate amino acids or peptides. H. Wunschendorff (40) reports that tungstic acid gives complete precipitation with less adsorption (of non-protein material than trichloroacetic acid), but filtration is so slow that there may be appreciable hydrolysis of the protein. P. Cristol and M. Simonnet (8) state "....whilst trichloroacetic acid and tungstic acid are excellent precipitants for albumin in the estimation of the total non-protein nitrogen of the serum, and allow neither lipoid nor protein-nitrogen to pass into the filtrate...." R. C. Lewis and S. R. Benedict (26) and M. Richter-Quittner (30) have also reported on the use of these acids, but their original articles were not available.

It seems that picric and tungstic acids precipitate pure protein quantitatively. They precipitate most, but not all,

of the peptides, and none of the amino acids. Nothing definite could be gathered in regard to proteoses, but since the peptides are almost completely removed, it would seem that proteose precipitation would be complete.

Metaphosphoric acid, colloidal iron, and uranyl acetate: Hiller and Van Slyke (23) state "metaphosphoric acid, colloidal iron, and mercuric chloride are intermediate between trichloroacetic acid and tungstic acid in the completeness with which they precipitate the intermediate products of Witte's peptone." They believe the acid (metaphosphoric) to precipitate blood proteins completely. But Munschendorff (40) says that metaphosphoric acid fails to precipitate proteins completely. P. Cristol and M. Simonnet (8) report also that metaphosphoric acid does not completely precipitate proteins. H. Munschendorff (41) describes a modification of the Michaelis method of precipitating proteins with colloidal ferric hydroxide which is stated to give filtrates entirely free from protein. Van Slyke, Vinograd-Villchur, and Losee (36) report that colloidal iron completely precipitates native proteins, but does not precipitate amino-acids or any intermediate products up to the albumoses, and none of those except some of complexity but little below that of the original proteins. Abel and Ford (2) affirm "that Kowalewsky has shown that uranyl acetate will completely remove from various albuminous fluids every trace of proteid giving a biuret reaction, and that Jacoby and others have used this reagent for the removal of proteids from faintly alkaline solutions." Sara E. Branham (25) in a criticism of the results of Ford and Abel, writes as follows: "neither of these reagents (metaphosphoric acid and uranyl acetate) are quantitative precipitants for proteins." Also see A. Grigaut and P. Zizine (21), P. Cristol (7), and M. Richter-Quittner (30), all of which original articles were not available.

From these sources it appears that metaphosphoric acid probably does not completely precipitate even pure proteins, and certainly not the intermediate products. Colloidal iron precipitates pure protein quantitatively, but probably does not precipitate proteoses, peptides, and amino acids.

Phosphotungstic acid, and acetic acid-kaolin: Seibert (33) used phosphotungstic acid as the equivalent of tungstic acid in the precipitation of proteins, and shows that phosphotungstic acid precipitates all proteose and diamino acids (cf. Van Slyke (35)) leaving only monamino acids and undetermined nitrogen in the filtrate. Tungstic acid does not

precipitate diamino acids. Greenwald (20) describes a method with acetic acid and kaolin which removes all protein and protein intermediate products as follows: "Determinations of the N in such filtrates agree with those obtained by the trichloroacetic acid-kaolin method. Both methods are inaccurate, however, through the removal by the kaolin of the N substance other than protein. This is most marked with substances of a basic nature, including diamino acids and NH_3 (present as NH_4Cl) and is absolutely quantitative with creatinine...."

2 Summary

It would appear that for the purposes of the experiment with the glucoside of Amanita phalloides (1) metaphosphoric acid is unsuitable, (2) the use of uranyl acetate is doubtful, (3) trichloroacetic acid tungstic acid, picric acid, and phosphotungstic acid are preferable.

3 Experimental

Short experiments with egg albumin solutions and uranyl acetate, metaphosphoric acid, trichloroacetic acid, phosphotungstic acid, tungstic acid, and picric acid, bore out the summary above. However, some difficulty was experienced with trichloroacetic acid, but finally a filtrate was obtained which would give none of the tests in table 1. Uranyl acetate was effective in eliminating all substances capable of giving positive tests with the more delicate reagents in table 1.

IV The properties of Amanita solitaria

1. The hemolytic and agglutinative power of aqueous extracts of Amanita solitaria

(a) Historical

Ford (9) found that "aqueous or saline extracts of Amanita solitaria are hemolytic, but not to such a degree as are other Amanitas. The lysis is preceded by a typical agglutination of the corpuscles, which sink to the bottom of the tube in a densely adherent mass. The agglutination

is slow, requiring one to three hours, and after this time a slow solution of the corpuscles takes place, requiring four to five hours. ...if extracts be evaporated to a small bulk in vacuo at 35°C, and then precipitated with ethyl alcohol, the precipitate contains the agglutino-hemolysin."

(b) Experimental

(1) Preparation of material

Professor J. H. Miller of the University of Georgia was so kind as to collect, in September 1929, in the vicinity of Athens, Georgia, the A. Solitaria and A. phalloides used in all the following experiments. The A. solitaria were large and almost pure white. The A. phalloides were smaller, and a pale brown. Ford (14) in 1906 definitely used only the white forms of A. phalloides, but in his later experiments he does not specify the color of the fungi. The specimens were dried by Professor Miller on a slow electric drier, and shipped packed in cotton. They were allowed to dry further at room temperature in the biology laboratory at the State University of Montana for several weeks before being used.

To 100 grams of A. solitaria ground in a cereal mill was added 800 cc distilled water. The mixture was triturated in a porcelain mortar, and then set on ice for 48 hours. The mixture was filtered through linen, two thicknesses of Denver Fire Clay #2043 filter paper, and through two thicknesses of #42 Whatman filter paper under suction. Number 42 Whatman paper broke under suction, and it was found that #50 was better. The 620 cc of extract was neutralized to litmus with a small amount of sodium bicarbonate, and concentrated to 60 cc under reduced pressure at a temperature not exceeding 35°C. This concentrated solution was designated Solitaria extract IA.

The remaining plant mash was placed on ice with 500 cc distilled water and extracted for 24 hours. It was then filtered and neutralized as above. The filtrate was concentrated under reduced pressure at a temperature not exceeding 37°C, and reduced from 514 cc to 76 cc. It was designated Solitaria extract IB. This method of extraction follows that of Ford (2)(9)(10)(16).

(2) Solitaria extracts 1A and 1B

Table 2

Hemolytic and agglutinative power of Solitaria extract 1A, for cat cells
(all tests were in duplicate, and in a water bath at 37°C)

Extract 1A	10 per cent sodium chloride	5 per cent by volume washed cat cells in saline	Hemolysis and agglutination readings		
			1 hour	11 hours	18 hours
0.9 cc	0.1 cc	1 cc	negative	negative	slight
1.8 cc	0.2 cc	1 cc	negative	negative	slight
1 cc saline (control)		1cc	negative	negative	slight
1cc saline and excess $NaHCO_3$ (control)		1cc	negative	negative	slight

Solitaria extract 1B gave similar results. Both extracts gave similar negative results with 2.5 per cent washed human red corpuscles.

(3) Solitaria extract II

The raw unconcentrated extract also was negative. Amanita solitaria extract II was prepared from 50 grams dried A. solitaria and 500 cc distilled water. It was further treated as was extract 1A, and was also filtered through a Mandler filter. It was not concentrated. The final volume was 250 cc. It was not neutralized with sodium becarbonate.

Table 3

Hemolytic and agglutinative power of Solitaria extract II, for human cells
(all tests in triplicate, and at 37°C)

Extract II	10 per cent sodium chloride	5 per cent washed human cells in saline	Hemolysis and agglutination Readings			
			1 hour	5 hours	12 hours	22 hours
0.9 cc	0.1 cc	1 cc	all readings were negative			
1 cc saline (control)		1 cc	all readings were negative			

Chncentrated extracts of A. solitaria, prepared for the chemical experimentation following, were also negative.

(c) Summary

All aqueous extracts, concentrated and unconcentrated, from specimens of dried Amanita solitaria, were non-hemolytic and non-agglutinative for human and cat cells. These findings are not in accordance with those of Ford, since his extracts contained a hemolysin and an agglutinin. It is suggested that specimens of Amanita solitaria may vary in their hemolytic and agglutinative properties according to their geographical location. Ford (9) seems to have secured his specimens of A. solitaria in the vicinity of Woods Hole, Massachusetts.

2 The toxicity of aqueous extracts of A. solitaria

Ford (9) heated extracts of Amanita solitaria to 70°C for 30 minutes, and found them innocuous to rabbits and guinea pigs.

In verification of this observation, the following experiment was performed. A portion of A. solitaria extract Ia, which had been kept with a small piece of thymol, in a refrigerator for 20 days, was neutralized with a small amount of sodium carbonate. The extract was heated for a few minutes until there was considerable protein coagulum, filtered, and then heated at 65°-70°C for 30 minutes. Three cubic centimeters injected subcutaneously in the belly of rabbit number 2, weighing 2660 grams, and 3 cc injected in rabbit 4, weighing 2100 grams, were non-toxic, and caused no edema at the point of innoculation. The rabbits were alive several months later.

3 The chemical nature of aqueous extracts of Amanita solitaria

(a) Historical

Ford (9) evaporated an aqueous extract of A. solitaria

to a small bulk in vacuo at 35°C, precipitated the agglutino-hemolysin with ethyl alcohol, removed protein from the precipitate with uranyl acetate, and neutralized with sodium bicarbonate. The resulting solution reduced Fehling's solution before hydrolysis with hydrochloric acid, and gave no precipitate with phosphotungstic acid. A precipitate was given with basic lead acetate. Tests for pentoses with a-maphthol, orcin, and phloroglucin were negative. From this he concluded that there was in the extract a glucoside different in nature from that in Amanita phalloides.

(b) Experimental

(1) Solitaria extract II

The Solitaria extract II remaining from the hemolytic experiments was divided into two portions. One portion, extract IIA, of 110 cc, was concentrated under reduced pressure at a temperature not exceeding 38°C, to 35 cc. The other portion, extract IIB, was concentrated at not over 42° C from 130 cc to 30 cc. Since the work was briefly interrupted at this point, the solutions were preserved by freezing for two days. They were then thawed out.

Absolute alcohol was added to extract IIA. A small precipitate formed. The precipitate was not dried, but was redissolved, while wet, in water, and a few cubic centimeters of 10 per cent phosphotungstic acid in 5 per cent sulphuric acid was added to precipitate the protein. The filtrate was neutralized with sodium bicarbonate, and filtered. The resulting filtrate reduced Benedict's solution but gave no test for pentoses with Tollen's phloroglucinol-hydrochloric acid reagent, or with Bial's orcinol-hydrochloric acid reagent.

To the 30 cc of extract IIB was added 3 cc of absolute alcohol. No precipitate formed and the solution appeared to be uniform. The volume was reduced to 20 cc at a temperature not exceeding 38°C. To 15 cc was added 2 cc of 10 per cent phosphotungstic acid in 5 per cent sulphuric acid. The filtrate was neutralized to litmus with sodium bicarbonate. There was no precipitate. The solution gave the same reactions as extract IIA. With Barfoed's monosaccharide reagent a green precipitate formed, which Welker (22) accredits to interfering chlorides.

(2) Solitaria extract IA

Solitaria extract IA, used in table 2, was tested five months after its preparation. In the meantime, it had been kept in the ice box with a small piece of thymol.

Absolute alcohol was added to 20 cc of this extract. The small precipitate on filter paper was dried and redissolved in distilled water. Solution was not complete. Boiling with Benedict's solution gave a brilliant green, and on cooling some brown flecks appeared. Barfoed's gave a green flocculent precipitate with a few brown flecks. Picric acid, boiled a minute with sodium carbonate and 1 cc of the extract, was reduced to picramic acid. Tollen's and Bial's for pentoses, Seliwanoff's resorcinol and hydrochloric acid for ketoses, Molish's a-naphthol for carbohydrates, auric chloride for glucosides and alkaloids, and the guaiac test according to Mitthaus (39) for hydrocyanic acid (since hydrocyanic acid easily unites with carbohydrates according to Matthews (27)), all were negative.

Uranyl acetate was added to the remainder of the solution from extract IA, which had given the tests above, in order to precipitate the protein. The filtrate gave a negative Millon's test. Benedict's solution was reduced upon boiling. Barfoed's was unaltered, so it was concluded that some substance precipitated by the uranyl acetate had caused the previous change to green. Picric acid was reduced as before. There was a precipitate with basic lead acetate. Bial's, Tollen's, Seliwanoff's, Molish's, the guaiac test, phosphomolybdic acid, and phosphotungstic acid, all were negative.

(c) Summary

Chemically, then, in Amanita solitaria extracts IA and IB, it would appear from the results with Benedict's solution and with picric acid, that a reducing substance was present. From the absence of any positive results in the other tests, however, the presence of a glucoside would seem unlikely. This does not necessarily contradict the observations of Ford, for his glucosidal extracts were hemolytic and agglutinative, while these were not. The agglutino-hemolysin and the glucoside are probably closely associated.

V. The properties of Amanita phalloides

1 The hemolytic and agglutinative power of aqueous extracts of Amanita phalloides

(a) Historical

Robert, as reported by Ford (14), obtained a dried extract of Amanita phalloides which dissolved ox blood in a dilution of 1-125,000. Ford's (14) Extract V, prepared from 50 grams of A. phalloides and 200 cc of distilled water, not concentrated, had the following hemolytic strength for 1 cc of the respective 5 per cent blood cells:

guinea pig	0.002 cc
rabbit	0.003 cc
beef	0.5 cc
sheep	1.0 cc

From 8 grams of dried A. phalloides and 100 cc distilled water, Ford (14) obtained an unconcentrated extract with a strength of 0.006 cc for 1 cc of 5 per cent guinea pig blood.

With 15 grams of powdered A. phalloides and 200 cc distilled water, Ford (1) secured an extract of the strength shown for 1 cc of 5 per cent blood cells:

guinea pig	0.001 cc
rabbit	0.002 cc
goat	0.02 cc
swine	negative (some discoloration)
beef	negative (some discoloration)
sheep	negative (some discoloration)

Rabe's (28) extracts in a dilution of 1-20,000 dissolved 5 per cent blood cells.

It may be seen that the strength of the extracts bears little relation to the amount of the mushroom employed. The hemolysin is therefore present in variable amounts, or is partially destroyed in the process of extraction.

Entire lack of hemolytic power has been reported. Rabe (28) refers to this fact. Ford (11)(31)(2) mentions it. Apparently the hemolysin is present in larger amounts in the freshly gathered fungi, according to Ford (2). Ford (12) states that A. phalloides does not agglutinate blood corpuscles in vitro.

(b) Experimental

(1) Phalloides extract I

Amanita phalloides extract I was prepared from a dried plant weighing 2 grams and 20 cc distilled water, macerated, and set on ice for 24 hours. The extract was expressed through linen, and filtered through a coarse filter paper and through #42 Whatman filter paper under suction. The filtrate obtained was 4.8 cc. It was not neutralized.

One cubic centimeter of 5 per cent washed cat cells, plus 0.9 cc of Phalloides extract I, plus 0.1 cc of 10 per cent sodium chloride solution, gave an agglutination of the blood cells in 10 hours, in a water bath at 37°C. There was no hemolysis in 19 hours. When the test tubes were shaken, the clot of cells rose to the surface of the liquid as one densely adherent disk. Saline controls were negative.

A similar extract of A. solitaria made at the same time, gave no agglutination or hemolysis, when tested in the same manner.

(2) Phalloides extract II

To determine whether or not the agglutinin was organic, a second extract was made. Phalloides extract II was prepared from three dried plants weighing 8 grams. These were triturated in 100 cc distilled water, set in the ice box for 30 hours, expressed through linen, and filtered as was extract I. The solution was not neutralized. A few cubic centimeters of Phalloides extract II was heated to 84°C for 30 minutes. Protein coagulated,, but redissolved upon cooling.

One cubic centimeter of 5 per cent washed human red corpuscles, plus 0.9 cc of heated Phalloides extract II, plus 0.1 cc of 10 per cent sodium chloride solution, gave no hemolysis or agglutination in 18 hours, in a water bath at 37°C.

Cat cells were similarly negative with heated extract II.

Unheated Phalloides extract II, using 0.9 cc and also 1.8 cc of the extract, was negative with both human and cat cells.

Amanita solitaria extract IA was retested at this point, and was negative, heated and unheated, with human and cat cells.

Controls showed slight hemolysis in 18 hours, as did some of the two extracts. The controls used were:

1. One cc saline plus 1 cc washed human cells
2. One cc saline plus 1 cc washed cat cells
3. One cc Phalloides extract II (no sodium chloride), plus 1 cc human cells
4. One cc Phalloides extract II (no sodium chloride), plus 1 cc cat cells
5. One cc distilled water, plus 1 cc human cells, showed more hemolysis than the other controls
6. One cc distilled water, plus 1 cc cat cells, showed complete hemolysis

This total lack of hemolysis and agglutination in Phalloides extract II can not be reconciled with the agglutination in Phalloides extract I.

It seemed possible that the protein in Phalloides extract II had interfered with the hemolysis or agglutination. To test this theory, part of Phalloides extract II was divided into four portions, and the protein precipitated by four reagents--saturated aqueous picric acid, 10 per cent phosphotungstic acid in 5 per cent sulphuric acid, 10 per cent trichloroacetic acid, and saturated aqueous uranyl acetate. No portion of extract was in acid solution for more than fifteen minutes before being filtered and the filtrate neutralized with sodium bicarbonate.

One cubic centimeter of washed 5 per cent human red corpuscles, plus 0.9 cc of the picric acid extract filtrate, plus 0.1 cc of 10 per cent sodium chloride solution, gave no hemolysis or agglutination in 18 hours, in a water bath at 37°C. The other three filtrates gave similar negative results. Repetition of this experiment with washed 5 per cent cat cells was likewise negative. Secondary changes in some cases occurred because of the protein precipitant present.

It was concluded that Phalloides extract II was in no degree hemolytic or agglutinative for human or cat cells.

(3) Phalloides extract III

To establish definitely the non-hemolytic power of the specimens, a larger amount of the dried fungi were used and the extract concentrated to a small volume.

The small, or spring form of A. phalloides, is known as A. verna, and is mentioned by Ford (14). Twenty grams of A. verna were triturated with 200 cc distilled water and extracted by the process previously described. This Phalloides extract III was concentrated from 116 cc to 36 cc at a temperature not exceeding 33°C, and neutralized with sodium bicarbonate.

Nine tenths cubic centimeter of the neutralized concentrated Phalloides extract III, plus 0.1 cc of 10 per cent sodium chloride solution, plus 1 cc of washed 5 per cent human corpuscles, gave no hemolysis or agglutination in 18 hours, in a water bath at 37°C.

Amanita phalloides extract III, before neutralization, was also negative.

Precipitation of protein by the method and four reagents used with Phalloides extract II gave filtrates which were negative, except for secondary changes.

Controls giving no hemolysis were:

1. One cubic centimeter saline, plus 1 cc 5 per cent human cells.
2. One cubic centimeter extract (no sodium chloride), plus 1 cc human cells.

(c) Summary

No hemolysin or agglutinin was present in the aqueous extracts from dried specimens of Amanita phalloides and Amanita verna, regardless of whether the extracts were concentrated or unconcentrated, protein-free or not protein-free. The agglutination in Phalloides extract I was due to an unusual individual fungus. These experiments bear out the previous findings of Robert, Ford, and Kabe, that hemolytic power is a variable quality of Amanita phalloides.

2. The Amanita-toxin

(a) Purification of the Amanita-toxin

(1) Historical

Since the hemolysin was absent in the specimens of Amanita phalloides, an attempt was made to isolate the thermostable second poisonous principle of the fungus, designated by Ford as Amanita-toxin, with a view to repeating his experiments on its toxicity and chemical nature.

Schlesinger and Ford (31) isolated the Amanita-toxin by a process which they describe as follows:

"One hundred grams of fungi, previously dried over sulphuric acid, were finely ground and the powdered material throughly triturated with 300 cc of 65 per cent ethyl alcohol. The residue was twice treated in the same way and finally mixed with 100 cc of alcohol of the same strength and allowed to stand over night. The liquid of this fourth extraction was united with the first three fractions, making a total of 1000 cc. After careful neutralization with sodium carbonate the extract was evaporated under diminished pressure until all the alcohol had been distilled away and the volume of

the remaining fluid had become reduced to 150-200 cc. After filtering from deposited fatty acids, etc., the solution was made very slightly alkaline with sodium carbonate and treated with a 15 per cent solution of silver nitrate. A voluminous precipitate was formed. This, being non-toxic, was discarded. The filtrate was freed from the slight excess of silver by means of sodium chloride and, now neutral, was treated with a solution of basic lead acetate prepared in the usual way. Another non-toxic precipitate was formed and was also discarded. The filtrate (sometimes a considerable amount of toxic material was included in the first lead precipitate. This should, therefore, be treated with a saturated sodium sulphate solution and filtered. The filtrate is again precipitated with basic lead acetate. The precipitate may now be discarded and the new filtrate treated as described) from basic lead acetate was treated with an excess of a saturated sodium sulphate solution for the removal of lead and to this filtrate phosphotungstic acid (10 per cent phosphotungstic acid in 5 per cent sulphuric acid) was added in slight excess. The phosphotungstic precipitate was decomposed with barium hydrate and the filtrate from the barium compounds was filtered off and the resulting fluid found to contain the poisonous substance. On subcutaneous innoculation of both rabbits and guinea pigs this fluid was highly toxic, 1 cc containing 0.0004 gram of organic and no inorganic material, killing the animals acutely in from 24-48 hours and producing the pathological changes characteristic of poisoning by Amanita-toxin."

(2) Experimental

A. Phalloides extracts IVA and IVB

1. Preparation of Phalloides extracts IVA and IVB

Phalloides extracts IVA and IVB were prepared from 102 grams of dried Amanita phalloides. The exact procedure, quoting Schlesinger and Ford where possible, is here given in detail.

The mushrooms were ground and triturated in a porcelain mortar with 300 cc of 65 per cent ethyl alcohol, and the extract expressed through linen. The residue was twice treated

in the same way and finally mixed with 100 cc of 65 per cent ethyl alcohol and allowed to stand over night. The liquid of this fourth extraction was united with the first three fractions, and filtered through coarse filter paper. The volume was then 700 cc. (At this point Schlesinger and Ford neutralized the extract with sodium carbonate. Although this neutralization was overlooked in this experiment, the toxin was not destroyed at this stage, for the extract was toxic after concentration.) The 700 cc was divided into two portions. Portion IVA was concentrated from 466 cc to 140 cc under reduced pressure at a temperature not exceeding 40°C. Portion IVB was concentrated from 234 cc to 60 cc at a temperature not exceeding 40°C. The temperature most of the time was between 25°C and 30°C. The concentrated extracts were then neutralized to litmus with sodium carbonate.

Ford (14) used thymol to preserve his extracts. Thymol gives all of the protein tests that depend upon the benzene ring. It was planned to use the extracts in later experiments that would necessitate testing for the presence of protein. Thymol was therefore unsuitable as a preservative. The effect of other preservatives upon the Amanitatoxin has not been studied. For these reasons, the extracts were kept frozen in a shelter outside the building, and were allowed to melt slowly at room temperature whenever needed for use.

2 Toxicity of concentrated crude Phalloides extract IVA

Five cubic centimeters of extract IVA was heated to 65°C for 30 minutes, cooled, made isotonic, and injected subcutaneously in the belly of rabbit number 3, weighing 2100 grams. The rabbit died in 36 hours. There was a swollen flabby mass at the site of innoculation, and a subcutaneous gelatinous edema.

3 Failure to isolate the toxic principle in Phalloides extract IVA

One hundred twenty cubic centimeters of extract IVA was filtered through #50 Whatman filter paper under suction. To this extract was added 20 cc of 15 per cent aqueous silver nitrate, and filtered. To the filtrate was added 30 cc of the silver nitrate and filtered. The filtrate gave no further precipitate with more silver nitrate. Saturated aqueous sodium chloride was added until small filtered portions of the solution no longer gave a precipitate. To the filtrate was added basic lead acetate (prepared according to Hawk

Schlesinger and Ford, but the resulting filtrate was not utilized.) To the basic lead acetate filtrate was added saturated aqueous sodium sulphate until there was no further precipitate. Ten per cent phosphotungstic acid in 5 per cent sulphuric acid was added. A very small precipitate formed. Upon filtration, the precipitate was a light pink in color, and both the precipitate and filtrate gave off a sweet aromatic odor. The precipitate was allowed to dry, and was scraped into a small volume of saturated aqueous barium hydroxide, and diluted with distilled water. The pink color disappeared, and also the aromatic odor. There appeared to be no reaction, and the precipitate did not seem to dissolve. The solution was filtered, and gave 70 cc of a clear very light yellow liquid with a sour smell, slowly alkaline to litmus. It was made isotonic, and a small piece of thymol was added.

Four cubic centimeters of this final extract heated to 65°-70°C for 30 minutes was injected subcutaneously in the belly of rabbit number 6, weighing 1900 grams. There was no pathological change, and after 7 days, there was no evidence of the site of injection. The rabbit was used later for other toxicity tests, and was alive 3 months later. This same rabbit had received 0.50 cc of the same solution, 5 days previously, in an attempted minimum lethal dose determination, with three other rabbits, numbers 4, 5, and 7, that received 2 cc, 1 cc, and 0.25 cc respectively. All of them lived, with no evidence of ill effect of the injections. This showed that either (1) no toxin was present, or (2) that it was destroyed by the heating to 65°-70°C for 30 minutes.

That the second possibility did not exist was evidenced by the subcutaneous injection, three months after its preparation, of 3 cc of the unheated solution in rabbit 23, weighing 1000 grams. The rabbit lived.

It was therefore concluded that a poisonous substance was entirely lacking in the final solution.

4 Failure to find the toxic principle in aqueous suspensions of the precipitates obtained in the process of purification of Phalloides extract IVA

It was thought that possibly the toxic principle in extract IVA had been carried down by one of the precipitates in the process of purification. With this in mind, each of the dry precipitates was placed in 50 cc distilled water, well shaken at intervals, allowed to stand over night, and then heated to 65°-70°C for 30 minutes. The supernatant fluids of all were non-toxic. Details are as follows:

The first silver nitrate precipitate suspension was neutral to litmus, and was made up to 1 per cent sodium chloride. Four cubic centimeters injected subcutaneously in rabbit 11 weighing 2500 grams left only a small red swelling after 4 days.

The second silver nitrate precipitate suspension was neutral to litmus. Sodium chloride solution produced a precipitate, so none was added. Injection of 4 cc in rabbit 8 weighing 2650 grams caused a pouchy blood-stained sac at the point of innoculation, probably due to free silver nitrate. The rabbit did not appear ill, and since the sore healed very slowly, he was killed 17 days later.

The sodium chloride precipitate suspension was neutral to litmus and was made up to 1 per cent sodium chloride. Rabbit 9, weighing 2410 grams, injected with 4 cc, appeared 4 days later as if he had never been innoculated.

The sodium sulphate precipitate suspension was neutral to litmus, and was made up to 1 per cent sodium chloride. Four cubic centimeters injected in rabbit 10 weighing 2500 grams left him in four days appearing as if he had never received the injection.

The phosphotungstic filtrate was decidedly acid in reaction, and required 2 cc of saturated aqueous potassium hydroxide to make it neutral to litmus. The peculiar sweetish odor remained, but the color turned slightly greenish-blue. The solution was made up to 1 per cent sodium chloride. Four cc in rabbit 12, weighing 2050 grams, produced no ill effects.

The final barium hydroxide precipitate suspension was neutral in reaction. Sodium chloride was added as before.

Four cubic centimeters did not affect rabbit 13, weighing 1960 grams.

The sodium sulphate-basic lead acetate precipitate of the retreatment process was neutral in water. Addition of sodium chloride caused a precipitate, so none was added. Four cubic centimeters did not affect rabbit 14 weighing 1900 grams.

This experiment shows that the reagents employed were not toxic in themselves, and also that the toxic principle of the fungus was not carried down mechanically, or if so, in such an altered state, that it would not redissolve or retain its toxicity.

5 Determination of the particular stage in the process of purification, at which stage the Amanita-toxin was destroyed

The concentrated crude Phalloides extract IVB was used to determine the stage in the purification process, at which the toxin was destroyed.

Two and one half cubic centimeters of this extract IVB heated to 65°-70°C for 30 minutes, injected subcutaneously in the belly, killed rabbit 7, weighing 2520 grams, in 36 hours. The pathological changes are described later in this paper.

To 53 cc of extract IVB was added silver nitrate and sodium chloride solutions, in the manner described in the purification of extract IVA. The filtrate, after the sodium chloride, was made neutral to litmus with sodium carbonate. Three cubic centimeters of this filtrate, heated to 65°-70°C for 30 minutes, was injected in rabbit 15, weighing 3050 grams. This rabbit lived, although the site of innoculation was red and blood-shot. Three cubic centimeters, heated as usual, injected in rabbit 9 weighing 2410 grams, caused death in 20 hours. Three cubic centimeters, heated as usual, injected in rabbit 10, weighing 2020 grams, caused death in 56 hours. Evidently a toxic substance was present.

To the remaining 35 cc of the silver nitrate-sodium chloride filtrate was added basic lead acetate and sodium sulphate in the manner before described. Four cubic centimeters of this filtrate, heated as usual, killed rabbit 12, weighing 2050 grams, in 36 hours, and killed rabbit 13, weighing 1960 grams, in 60 hours. Rabbit 5, weighing 2180 grams, died in 44 hours.

Schlesinger and Ford (31) mention that a considerable amount of the toxic material was sometimes included in the first lead precipitate. To test this, the first lead precipitate from extract IVB was treated in the manner of Schlesinger and Ford. The process gave 17 cc of solution. Four cubic centimeters of this liquid, heated as usual, and injected subcutaneously in the belly of rabbit 4, weighing 2100 grams, produced no ill effects.

To the remaining 20 cc of the basic lead acetate-sodium sulphate filtrate (which had killed rabbits 5,12,13) was added phosphotungstic acid and barium hydroxide in the manner before described. This time, however, the barium hydroxide mixture was heated to 60°C, but there was no change. The cold filtrate took one drop of concentrated sulphuric acid to induce a neutral reaction to litmus. Three cubic centimeters of the filtrate, heated as usual, did not affect rabbit 11, weighing 2500 grams, nor rabbit 15, weighing 3050 grams.

The phosphotungstic filtrate from the process just above was neutralized with sodium carbonate. Three cubic centimeters, heated as usual, did not affect rabbit 6, weighing 1900 grams. Two cubic centimeters, heated, did not affect rabbit 14, weighing 1900 grams.

6 Summary

The toxic principle was present (1) in the crude extract, (2) in the silver nitrate-sodium chloride filtrate, and (3) in the basic lead acetate-sodium sulphate filtrate.

The toxic principle was absent (1) in the filtrate from the retreatment process, (2) in the phosphotungstic acid reagent filtrate, (3) in the final barium hydroxide filtrate,

and (4) in the supernatant fluid of all precipitates immersed in water.

It is evident that: (1) If the toxin was present in the phosphotungstic filtrate, then its toxicity was destroyed. (2) If the toxin was present in the final barium hydroxide filtrate, then its toxicity was destroyed. (3) If the toxin was present in the barium hydroxide precipitate (which included all of the phosphotungstic precipitate that did not go either into the phosphotungstic filtrate or into the barium hydroxide filtrate), then (a) The toxicity was destroyed by either the phosphotungstic acid reagent, or by the barium hydroxide, or (b) The toxin was so altered that the barium hydroxide precipitate would not yield a toxic fluid upon being placed in water.

Certain chemical indications later given in this paper, show that a portion of the toxin, in an altered condition, was found in the phosphotungstic filtrate.

B Phalloides extract V

At this time, there remained of the specimens of Amanita phalloides only 32 grams of Amanita verna. It was therefore necessary to bring the investigation to as complete a close as the limited amount of the fungus permitted. With this in mind, the specimens were extracted, and the purification process carried down to, but not through, the phosphotungstic acid reagent stage. The toxicity of the extract was tested at each step. Immunization of a rabbit was attempted, and a chemical investigation was made concerning the nature of the toxin, with the basic lead acetate-sodium sulphate filtrate.

1 Preparation of Phalloides extract V

Since the hemolysin had been shown to be absent in the specimens, it seemed feasible to extract with distilled water, instead of 65 per cent ethyl alcohol. The 32 grams of A. verna were extracted with 320 cc distilled water in the usual manner. The dried remains from the preparation of Phalloides extracts IVA and IVB, amounting to 62 grams, were extracted with 500 cc distilled water, and added to

the A. verna solution. The combined extracts, amounting to 675 cc after filtration, were neutralized to litmus with sodium carbonate, and concentrated to 150 cc under reduced pressure, at a temperature not exceeding 30°C. No thymol was added at this time.

2 Toxicity of Phalloides extract V

Three cubic centimeters of concentrated extract V, heated to 65°-70°C for 30 minutes, and injected subcutaneously in the belly, killed rabbit 16, weighing 1150 grams, in 20 hours, and killed rabbit 17, weighing 1150 grams, in 20 hours.

3 Toxicity of the silver nitrate-sodium chloride filtrate

Extract V was treated with silver nitrate and sodium chloride in the manner previously described. A 3 cc dose, heated and injected as usual, killed rabbit 18, weighing 1250 grams, in 40 hours, and killed rabbit 19, weighing 1000 grams, in 48 hours.

4 Toxicity of the basic lead acetate-sodium sulphate filtrate

The silver nitrate-sodium chloride filtrate of extract V was treated with basic lead acetate and sodium sulphate in the usual fashion. The solution was neutral to litmus. A 3 cc dose, heated and injected as before, killed rabbit 20 weighing 1000 grams, in 40 hours, and killed rabbit 21, weighing 900 grams, in 48 hours.

5 Lack of toxicity in the controls

A silver nitrate-sodium chloride control was prepared by adding 2 cc of the 15 per cent aqueous silver nitrate solution to 100 cc of distilled water, and precipitating with 5 cc saturated aqueous sodium chloride. The filtrate gave no further precipitate with sodium chloride solution and was of course neutral to litmus. One and a half cubic

centimeters in rabbit 22 weighing 1000 grams, and 3 cc in rabbit weighing 900 grams, were not toxic in any degree.

To the remainder of the silver nitrate-sodium chloride solution was added basic lead acetate solution until there was no more precipitation. To the filtrate aqueous sodium sulphate was then added until precipitation was complete. The filtrate was neutral to litmus. Three cubic centimeters injected in rabbit 24, weighing 1000 grams, and 3 cc injected in rabbit 25, weighing 1100 grams, were not toxic in any degree.

6 Summary

Phalloides extract V was toxic (1) in the crude extract, (2) in the silver nitrate-sodium chloride filtrate, and (3) in the basic lead acetate-sodium sulphate filtrate. Control solutions prepared in the same manner as extract V were non-toxic.

(b) The chemical nature of the Amanita-toxin

(1) Historical

Rabe's (28) alcohol-soluble, ether and chloroform-insoluble toxic substance gave the positive alkaloidal tests--phosphotungstic acid, phosphomolybdic acid, Esbach, Mayer, picric acid, Dragendorff, Kraut, Kiliani, and others. Ammonical silver nitrate was blackened by warming. Gold chloride was discolored, and Fehling's solution was positive only after long heating with hydrochloric acid. Rabe is uncertain whether the reductor in the Fehling's test belongs to the toxin or not.

Schlesinger and Ford (31) obtained different reactions. They describe their final toxic product as follows: "Amanita-toxin is very soluble in water, less so in 80 per cent alcohol and only very little soluble even in hot absolute alcohol; it is insoluble in the ordinary organic solvents. Its aqueous solution is optically inactive. It is a fairly stable compound for it can be boiled in absolute alcohol and in aqueous solution for some time without suffering serious

loss in toxicity; it is only very slowly affected by acids at room temperature, retaining its toxicity for several days when thus treated. Boiling acids, however, rapidly destroy the poison. It does not reduce Fehling's solution either before or after prolonged boiling with 5 or 10 per cent hydrochloric acid. With the exception of phosphotungstic acid, this toxin reacts with none of the alkaloidal precipitants, nor does it respond to any of the alkaloidal color reagents. (For a list of these, see Kippenberger, Nachweis von Giftstoffen.) It does not give the biuret test or Millon's reaction. We may, therefore, conclude that this poison is neither a glucoside, an alkaloid, nor a proteid in the generally accepted sense of these terms. The following reactions give us a clue to its identity, and we are convinced that these reactions are due to the Amanita-toxin itself because of our rigorous method of purification and because the tests become more pronounced as the process of purification advances. Fusion with metallic potassium and subsequent treatment in the usual fashion shows the presence of nitrogen and sulphur. By boiling a concentrated solution of the purified toxin with hydrochloric acid and subsequently treating it with barium chloride the sulphur was shown to be present as conjugate sulphuric acid. (The solution gave no test for sulphates before boiling.) While making the fusion with potassium a strong odor of fatty amines was observed, and the gas evolved gave white fumes when a drop of hydrochloric acid on a glass rod was brought near. To determine whether the toxin is a substance from which amines may be split off by reagents ordinarily used for this purpose a small portion of the dried material (Boiling with a solution of potassium hydrate gave no noticeable amine odor or alkaline fumes) was mixed in a test tube with powdered potassium hydrate. The amine odor was noticeable at once, but after heating, the persistent and unmistakable odor of indol completely masked that of the amines and a pine splinter moistened with concentrated hydrochloric acid gave the characteristic pyrrol red when held in the mouth of the test tube. The application of the tryptophan test of Hopkins and Cole gave negative results."

Ford and Prouty (18) later corrected this analysis to the extent of deciding that no conjugate sulphate was present.

(2) Experimental

The basic lead acetate-sodium sulphate filtrate from

extract V (which had been shown to contain a poisonous substance) was examined. Inasmuch as this filtrate contained an excess of sodium sulphate and possibly some lead acetate, or traces of other compounds of the purification process, a control solution was prepared from distilled water, and put through the same process as was the mushroom extract. All tests made upon the toxic solution were also made upon the control solution, and the results compared.

By the comparative procedure above, the following protein tests were found negative: (1) Millon's, (2) Tanret's, (3) Heller's, (4) Spiegler's, (5) acetic acid and potassium ferrocyanide. The aromatic odor, so noticeable in the phosphotungstic filtrates of extracts IVA and IVB, was present in Millon's test, was less strong in Heller's, and was doubtful in Spiegler's and Tanret's. The reagents of these tests are composed largely of strong acids, and it was thought that the odor might be due to the acid decomposition of the toxin. This belief was strengthened by the fact that no such odor was given by the control solution when either phosphotungstic acid (reagent) or any other acid, was added to the control.

Ninhydrin gave a persistent dark violet color, with a tinge of red in direct sunlight. Hawk (22) notes that this test is given by certain amines, so it was thought that this reaction was given by the amine in Schlesinger and Ford's toxin, rather than by an amino acid.

Sulphuric acid, nitric acid, 10 per cent phosphotungstic acid in 5 per cent sulphuric acid, and 10 per cent aqueous phosphomolybdic acid, gave no alkaloid or glucoside reactions upon being added to the toxic solution. All gave the aromatic odor.

Other negative tests were: (1) tannic acid, (2) auric chloride, (3) Mayer's, (4) the prussian blue test with potassium hydroxide, ferrous sulphate, and hydrochloric acid for hydrocyanic acid, (5) the guaiac test for hydrocyanic acid, (6) Guy's or Denzell's, (7) the furfural reaction with cane sugar and sulphuric acid.

In contrast with the possible amine reaction with the ninhydrin, no amine odor or pyrrol red test with a pine

splinter could be obtained upon boiling with powdered potassium hydroxide. Benzenesulfonyl chloride gave no amine reaction, and an attempt to diazotize with hydrochloric acid and sodium nitrite was unsuccessful.

One and a half cubic centimeters of the toxic solution was evaporated to dryness in a small test tube. Elementary analysis by sodium decomposition showed the presence of nitrogen and sulphur. Since a positive sulphur test was also given by the control, the sulphur determination lacked conclusive value.

(3) Summary

The basic lead acetate-sodium sulphate filtrate from extract V gave only four definite reactions: (1) a light pink-colored precipitate, and a sweetish-vinegar-odor, with 10 per cent phosphotungstic acid in 5 per cent sulphuric acid, (2) the characteristic odor, with sulphuric acid, nitric acid, and phosphomolybdic acid, (3) a persistent dark red-violet color with ninhydrin, and (4) nitrogen by elementary analysis. It was concluded that the toxic principle present was essentially that of Schlesinger and Ford, and definitely not alkaloidal in nature.

(4) Attempt to find, by chemical means, the toxin lost in extracts IVA and IVB

With the above chemical summary in mind, an attempt was made to locate, by chemical reaction, the toxin which had disappeared in the process of purification of extracts IVA and IVB. The following solutions were examined: (1) the phosphotungstic acid reagent filtrates (neutralized) from IVA and IVB, (2) the barium hydroxide filtrates (neutralized) from IVA and IVB, (3) the barium hydroxide precipitates (in aqueous suspension) from extracts IVA and IVB.

Phosphotungstic filtrate IVA gave a deep violet with ninhydrin, and no nitrogen test by elementary analysis of 1.5 cc evaporated to dryness. Phosphotungstic filtrate IVB gave a wine red with ninhydrin, changing to tan on cooling, and no nitrogen test.

Barium hydroxide precipitate suspension IVA gave a light blue with ninhydrin, which was also given by a control solution. The other three fluids were negative with ninhydrin. No nitrogen tests were made for them.

Auric chloride gave, on boiling with the phosphotungstic filtrates, a black-brown precipitate that could not be explained by the action of a control solution.

From these reactions, nothing definite could be learned, except that possibly some of the inactivated toxin lay in the phosphotungstic filtrates, in an amount sufficient to give a positive ninhydrin test, but insufficient to give a nitrogen test by elementary analysis of 1.5 cc evaporated to dryness.

(c) Attempted immunization

Immunization of a rabbit was begun, although Ford, as previously stated, was unable to obtain a definite artificial immunity for the Amanita-toxin.

To establish roughly the minimum lethal dose, on February 20, a 1000 gram rabbit was given 2 cc. A 900 gram rabbit was given 1 cc (heated as usual). The 1000 gram rabbit died in 30 hours from the 2 cc injection, while the 900 gram rabbit was still alive two months later. Therefore, the minimum lethal dose for a 1000 gram rabbit, seemed to lie between 1 and 2 cc.

Rabbit 15, weighing 3050 grams, was chosen for the immunization. This rabbit had previously survived a non-toxic dose of 3 cc of the barium hydroxide filtrate from Phalloides extract IVB on February 6. Strangely enough, the rabbit had not succumbed on January 27 to a lethal injection of 3 cc of the silver nitrate-sodium chloride filtrate of Phalloides extract IVB which had killed 2 other rabbits at that time. In fact, rabbit 15 was the only animal presenting an inconsistency in the behavior of the rabbits to lethal doses. The immunization injections were made subcutaneously, and in each case, the fluid was heated to 65°-70°C for 30 minutes.

Table 4

Immunization of Rabbit 15

Date	Dose	
February 24	0.25 cc	
March 3	0.50 cc	
March 10	0.75 cc	
March 17	1.00 cc	
March 24	1.50 cc	(about one lethal dose for 1000 gram rabbit)
March 31	2.00 cc	
April 7	3.00 cc	(about two lethal doses)
April 14	4.00 cc	
April 21	5.00 cc	

Rabbit 15 was to have been bled the week of April 28-May 3. Before doing so, the toxicity of the immunization fluid was redetermined. Table 5 gives the number of the rabbits used in this determination, weight, previous non-toxic injections, and present dose.

Table 5

Minimum lethal dose of immunization fluid as of April 28

Rabbit number	weight in grams	Prior non-toxic injections	Present Dose	Date
2	2660	4.5 cc BaOH control filtrate		Jan. 8
		3.0 cc Solitaria extract IA		Feb. 10
		(heated)	3 cc	Apr. 28
4	2100	2.0 cc BaOH filtrate, Phalloides IVA		Jan. 17
		4.0 cc Retreatment filtrate, IVB		Feb. 3
		3.0 cc Solitaria extract IA		Feb. 10
		(heated)	2 cc	Apr. 28
6	1900	0.5 cc BaOH filtrate, Phalloides IVA		Jan. 17
		4.0 cc BaOH filtrate, Phalloides IVA		Jan. 22
		3.0 cc Phosphotungstic filtrate, IVB		Feb. 6
		(not heated)	4 cc	May 2
11	2500	4.0 cc $AgNO_3$ ppt. suspension, IVA		Jan. 23
		3.0 cc BaOH filtrate, Phalloides IVB		Feb. 6
		(not heated)	4 cc	May 4
26	2250	none (not heated)	3.5cc	May 5

The 3.5 cc given rabbit 26 represented all of the remaining immunization fluid. All of the above rabbits lived, with no apparent ill effects from the injections. From this it is evident that the fluid had entirely lost its toxicity by April 28. However, on February 20, two cubic centimeters killed a 1000 gram rabbit in 30 hours. From February 20 to April 28, the neutralized fluid had been kept in the ice box, preserved by thymol.

(1) Summary

The important point here is that the toxic substance was quite unstable. This bears out the results of our previous attempts to purify the toxin, for the toxic principle was destroyed by the phosphotungstic acid reagent-barium hydroxide treatment. Ford's toxin survived this treatment and apparently remained toxic over a comparatively long period of time. It is suggested that Amanita-toxin, like the hemolytic glucoside, is not of uniform quantity, or nature, in specimens of Amanita phalloides.

(d) Pathological changes produced by Amanita phalloides poisoning

(1) Historical

The differences of opinion between Ford and Kobert in regard to the pathology of Amanita phalloides intoxication have been discussed in the introduction to this paper, and it has been suggested that Kobert's views possibly now agree with Ford's. Present observations, in the main, confirm Ford's statements.

Ford (11) summarizes the changes produced:

"2. The lesions produced by the whole extract of the fungus consist of gastric and intestinal ulcers, hemorrhages, necrosis of cells, fatty degeneration, hemoglobinurea, and pigmentation of the spleen and other organs.

3. The lesions produced by the Amanita-toxin consist of gastric and intestinal ulcers, hemorrhages, necrosis, and fatty degeneration, and to a certain extent they approximate the lesions seen in man.

4. The Amanita-hemolysin acts upon animals by virtue of its blood-laking properties, producing the hemoglobinurea and the pigmentation of the spleen characteristic of hemolytic intoxications."

All following references to previous pathological findings refer to the above article by Ford, unless designated as the observation of Rabe.

In discussing the pathology, the rabbits are referred to by number. Table 6 gives the number of the rabbit, the weight, prior non-toxic injections, and the lethal injection, with dates, and hours before death.

Table 6
Lethal experiments with rabbits

Rabbit	Weight in grams	Prior non-toxic injections	Lethal Dose	Date	Hours before death
Lethal injections of crude extracts, heated 65° C, 30 minutes.					
3	2100	none.....	5cc IVA extract	Jan. 14	36
7	2520	0.25 cc IVA BaOH filtrate		Jan. 17	.....
		.....	2.5cc IVB ext.	Jan. 22	36
16	1150	none.....	3cc V extract	Feb. 15	20
17	1150	none.....	3cc V extract	Feb. 15	20
Lethal injections of the silver nitrate-sodium chloride sols.					
9	2410	4cc NaCl ppt.IVA suspension.....		Jan. 23	.....
		.....	3cc IVB $AgNO_3$-NaCl filtrate	Jan. 30	20
10	2020	4cc IVA basic lead acetate-Na_2SO_4 ppt.suspension	.....	Jan. 23	.....
		.....	same as # 9	Jan. 30	56
18	1250	none.....	3cc V $AgNO_3$-NaCl filtrate	Feb. 15	40
*19	1000	none.....	same as # 18	Feb. 15	48
Lethal injections of the basic lead acetate-Na_2SO_4 sols.					
5	2180	1cc IVA BaOH filtrate	.....	Jan. 17	.....
		.....	4cc basic lead acetate-Na_2SO_4 filtrate IVB	Feb. 3	44
12	2050	4cc IVA phosphotungstic	.....	Jan. 23	.....
		.....	same as # 5	Feb. 3	36
13	1960	4cc IVA BaOH ppt.suspension		Jan. 23	.....
			same as # 5	Feb. 3	60
*20	1000	none.....	3cc V basic lead acetate-Na_2SO_4filtrate	Feb. 15	40
21	900	none.....	same as # 20	Feb. 15	48

* not examined for pathology

A. External examination and gross changes

Post-mortem rigidity was a conditon noted in all the animals, but mentioned by Ford as lacking in man. Respiratory distress, evidenced by bloody foam at the nose, was found in rabbits 7, 17, and 18.

The appearance of the site of innoculation varied. Rabbits 7, 16, 10, 5, showed no external injury. Rabbit 3 presented a flabby mass 4 cm. across, typically produced by hemolytic extracts, according to Ford. Since our extracts contained no hemolysin whatever, it appears that this reaction may also be produced by other than hemolytic extracts. A similar, but smaller mass, was found on rabbit 18. Numbers 17, 12, 21, were red and bloodshot. The skin of rabbit 13 was a pale green in a spot 2.5 cm across.

Subcutaneously at the site of innoculation there often was an extensive gelatinous edema extending towards the bladder, particularly noticeable in 3, 17, and 21. The green color noticeable externally in rabbit 13 was evident subcutaneously over almost the entire peritoneum. Also there was present in 13, a mass of connective tissue becoming thicker towards the bladder. Number 10 had a tissue growth 3.5 cm across by 0.75 cm thick, which resembled a piece of commercial dried pressed apple.

Subcutaneous hemorrhage and pigment spots were found in 7, 17, 12, 5, and 21, but were noticeably absent in 9. Hemoglobinurea, emphasized by Ford as prominent in the hemolytic intoxication, but found in only one animal by Rabe, was not sought for in the examination. After the examination of several carcases, it was observed that in most cases the bladder either was well distended with urine, or distended, but empty, showing release of the urine after death.

(1) Summary

From these observations it was concluded that with the exception of post-mortem rigidity, there were no uniform gross changes, although respiratory distress, subcutaneous hemorrhage, and subcutaneous pathological tissue growths were common.

B. Microscopic examination

With the assistance of the histology class of the Winter Quarter 1930, representative tissues were sectioned and mounted permanently in balsam. Table 7 shows the tissues examined.

Table 7

Tissues examined microscopically

Organ	Rabbit and cause of death		
	Crude extract	Silver nitrate-sodium chloride filtrates	Basic lead acetate-sodium sulphate filtrates
spleen	16, 17	9, 18	12, 21
liver	16, 17	9, 18	12, 21
lung	16	9, 18	12
kidney	16	9, 18	12, 21
heart	7	9	12, 13

In the spleen, Ford found, with the hemolysin, considerable extravasated blood and a great increase in blood pigment. The latter was absent in poisoning by the toxin.

The crude extract produced in the spleen of rabbits 16 and 17 more than the normal amount of extravasated blood, and a small, but by no means prominent, amount of blood pigment. This agrees with the fact that the extract was not hemolytic. Rabbits 9, 18, 12, and 21, also showed extravasated blood, but no excess blood pigment.

The liver in all rabbits presented the most striking change. There was a decided fatty infiltration of the liver cells and fatty degeneration. Phknotic nuclei were frequent, and the nuclei of the hepatic cells showed degenerative changes. Many blood corpuscles lay free in the bile capillaries, often completely filling them. This condition of the liver is essentially the same as that found by Ford.

The lungs of 16, 9, and 18 were congested with blood, Rabbit 12 did not present a definite excess of blood. In 18 there was noticeable at times what appeared to be a path-

ological fat content in the tunica adventitia of the small arteries and in the mucosa of the branchioles. Rabbit 12 appeared to have the same excess fat in the adventitia of the small arteries, but not in the large arteries or in the branchioles. Blood pigment was in no case prominent. Ford observed in the lungs many free blood corpuscles and an excess of blood pigment, in poisonings by the whole extract.

Ford marked that, with the whole extract, the kidney showed "a uniform condition of congestion and hemorrhage, the kidney cells are shrunken from the basement membrane and are the seat of hyaline degeneration."

Rabbit 16 died from the injection of the whole extract, but the kidney sections did not show a clear picture of the condition found by Ford. There was a little fat in the tubules, but not sufficient to be definitely abnormal. The kidney cells, in places, did appear to be somewhat shrunken from the basement membrane.

However, the kidneys of rabbits 9 and 18 were decidedly abnormal. Congestion of blood was noted in the enlarged Malpighian corpuscles and in the capillaries adjacent to the tubules, both in the cortex and medulla. Excess fat was found between the basement membrane and the epithelium lining the tubules, also in the lumen of the tubules. Fat was sometimes present in the Malpighian corpuscle, lying between the simple squamous epithelium of Bowman's capsule and the glomerulus. Particularly near a large artery the fat was excessive in the lumen of the collecting tubules and in the ascending limb of Henle's loop. In 18 there seemed to be excess fat in the glomeruli, between the capillaries. Rabe found little change in the kidney, except enlarged Bowman's capsules. He seldom found fat in the rabbit in the collecting ducts and in Henle's loop. The kidney sections of rabbits 12 and 21 were poor, but some fat could be seen in the tubules and in the Malpighian corpuscles.

In the heart, Rabe observed, with both hemolysin and "alkaloid", a rather rich fat content of the heart muscle, and sometimes small capillary bleeding between the muscle fibers. Ford noted with the whole extract and with the toxin, and to a lesser extent with the hemolysin, "hyaline degeneration, nuclear vacuolation, and at times almost complete destruction leading to the appearance of small areas of focal

necrosis." (in the muscle fibers).

No particular pathological lesions of the above nature were found in the heart sections of rabbits 7, 9, 12, and 13. There were a few stray red blood cells, but nothing extensive, and by no means was pathological fat present.

l Summary

Of the various lesions noted, none could be described as peculiar to any one of the three toxic solutions employed. Extravasated blood was noticeable in the lungs. Fatty degeneration in the liver and kidneys confirm the observations of Ford and Rabe. The lack of excess pigmentation in the spleen supports Ford's view that this change is peculiar to the hemolysin. The unchanged condition of the heart muscle is contrary to both Ford's and Rabe's observations, However, sections of both ventricle and auricle were examined under oil immersion, and nothing unusual could be found.

VI Summary

1. The delicacy of various protein tests is given, and it is pointed out that a negative biuret or Millon's test is not conclusive of quantitative protein elimination.

2. Metaphosphoric acid is unsuitable for quantitative protein precipitation, the use of uranyl acetate is questionable, and trichlroacetic acid, tungstic acid, or picric acid would be preferable.

3. Aqueous extracts of dried specimens of Amanita solitaria obtained from the vicinity of Athens, Georgia, were neither hemolytic nor agglutinative for cat or human red corpuscles.

4. Amanita solitaria is non-toxic for rabbits and guinea pigs.

5. The presence of a glucoside in the non-hemolytic dried specimens of Amanita solitaria was found unlikely.

6. Aqueous extracts of dried specimens of Amanita phalloides obtained from the vicinity of Athens, Georgia, were not hemolytic for cat or human red corpuscles. This

confirms the findings of previous investigators that the hemolysin is a variable constituent.

7. The Amanita-toxin is not an alkaloid and probably contains an amine group, but in this investigation, (a) the toxic substance did not survive the phosphotungstic acid reagent-barium hydroxide treatment, and (b) it did not retain its toxicity upon standing for two months. It therefore appears that in specimens of Amanita phalloides obtained from different localities, that the Amanita-toxin is not of uniform nature, and probably varies quantitatively.

8. The Amanita-toxin induced in rabbits post-mortem rigidity, and fatty degeneration, especially in the liver and kidneys. The heart was free from fat. The lack of pigmentation in the spleen confirms the observation of Ford that this condition is caused by the hemolysin.

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Color and precipitation tests for the presence of proteins

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