Di-aniline Salt of Arsenic Acid.—This was prepared by dissolving sirupy arsenic acid in alcohol, adding to it an excess of aniline and warming until the di-aniline salt was in solution. On cooling, it crystallized in pearly leaflets; m. p., 143°.

Analyses. Calc. for $C_{12}H_{17}O_4N_2As$: N, 8.53; As, 22.86. Found: N, 8.11, 8.10; As, 23.23, 23.32.

Summary.

1. The reaction between aniline and arsenious chloride has been studied and a new method for the preparation of secondary arsonic acid derivatives devised.

2. Tri-aniline-arsine hydrochloride has been prepared in a pure state and its properties described.

3. Several new compounds, phenarsazine oxide, phenazine-arsonic acid, dinitro-phenazine-arsonic acid, and disodium dinitro-phenazine-arsonic acid have been prepared and their methods of preparation and properties described.

4. A new vacuum sublimation apparatus has been described.

5. Directions for the preparation of mono-aniline and di-aniline salts of arsenic acid have been given.

The writer wishes to express his gratitude to Professor Richard Fischer, under whose direction this work has been carried out, and also to Professors Loevenhart and Kremers, for their interest in this research.

MADISON, WISCONSIN.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF COLUMBIA UNIVERSITY, No. 374.]

EFFECT OF CERTAIN ANTISEPTICS UPON THE ACTIVITY OF AMYLASES.

BY H. C. SHERMAN AND MARGUERITE WAYMAN.

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In experimental studies of enzyme action, antiseptics are frequently used to prevent or suppress any activities of microörganisms. It is obviously important to know as definitely as possible whether antiseptics can be thus used without influence upon the activity of the enzyme which it is desired to study; and furthermore we may hope that a systematic determination of the behavior of enzymes toward antiseptics of different types may ultimately throw light upon the chemical nature of the enzymes themselves and the discussion of enzyme dispersions in terms of colloid chemistry.

The experiments here recorded have to do with the influence of toluene, formaldehyde, and copper sulfate upon amylases of both animal and vegetable origin and when tested in both natural and purified form. The literature affords many statements of a general nature regarding the relation of antiseptics to enzyme action, usually giving the impression that

those of the type of toluene or chloroform have little if any influence upon enzymes while such antiseptics as formaldehyde and copper sulfate are more likely to prove injurious. Precise statements accompanied by experimental evidence appear to be few.

Chittenden and Painter[1] in the course of a study of the effects of numerous salts upon saliva, found copper sulfate to be very injurious to its starch-digesting action.

Grützner and Wachsmann[2] reported that ether, chloroform and thymol retard the action of pancreatic amylase, chloroform being the most destructive of the three.

Kopaczewski[3] could observe no effect of chloroform or toluene in a concentration of 0.1% upon the action of takadiastase, which action, however, was prevented by 1.0% formaldehyde.

Myers and Scott[4] found salivary amylase to be relatively stable in the presence of chloroform, toluene or thymol, toluene appearing to preserve the activity of the enzyme the best of these three antiseptics.

Bokorny[5] reported that maltase was injured by 0.1%, and destroyed by 1% of formaldehyde.

Materials and Methods.—The amylase preparations employed in this investigation consisted of: (1) commercial pancreatin, Lab. No. 8; (2) purified pancreatic amylase, preparations T-8-B, T-18-A, T-18-B made as described in a previous paper[6] and having amylolytic powers of about 3000 according to the method and scale in use in this laboratory since 1910;[7] (3) human saliva as secreted; (4) malt extract; (5) purified malt amylase, Lab. No. 151, amylolytic power[7] 1274; (6) takadiastase, commercial; (7) the purified amylase of *Aspergillus oryzae* prepared from takadiastase and having about 30 times its amylolytic power. We are indebted to Parke, Davis and Company for the pancreatin and takadiastase used.

The starch, water, and other materials employed were purified as described in previous papers from this laboratory.

The purest obtainable chloroform, toluene, copper sulfate, and formaldehyde were employed. The percentage strength of the last substance was determined by the peroxide method.

The plan of experimentation was to allow a suitable amount of an amylase solution to act upon 100 cc. of 2 % starch paste with and without the antiseptic to be tested in the presence of suitable "activating" salts for 30 minutes at 40° in the same manner as for the determination of diastatic power by the gravimetric method of Sherman, Kendall and Clark,[7] the reducing

[1] Chittenden and Painter, "Studies from the Yale Laboratory of Physiological Chemistry," 1–3, p. 55 (1884–8).

[2] Grützner and Wachsmann, Pflueger's Archiv, **91**, 195–207 (1902); *J. Chem. Soc.*, **82**, II, 614 (1902).

[3] Kopaczewski, *Biochem. Z.*, **44**, 351 (1912).

[4] Myers and Scott, THIS JOURNAL, **40**, 1713 (1918).

[5] Bokorny, *Biochem. Z.*, **94**, 71 (1919).

[6] Sherman and Neun, THIS JOURNAL, **41**, 1855 (1919).

[7] Sherman, Kendall and Clark, *ibid.*, **32**, 1082 (1910).

sugar formed by the action of the amylase upon the starch being estimated by heating with an excess of Fehling solution and the result expressed in terms of mg. of cuprous oxide reduced, allowance being made for the reduction resulting from the starch alone or the starch plus antiseptics as determined by blank tests.

Experiments with Toluene.

Of the antiseptics of the "lipoid solvent" type, the ones most commonly used in enzyme work are chloroform and toluene. Preliminary experiments indicated that chloroform at a concentration of 0.0000124 *M* measurably diminished the action of purified pancreatic or malt amylase but not of a simple malt extract. Chloroform, however, did not lend itself readily to experimentation by the method here used because of its reducing effect upon Fehling solution. Since chloroform and toluene are so largely used interchangeably in work with enzymes and toluene does not appreciably influence the reduction of Fehling solution, it was chosen in preference to chloroform for the following series of experiments. In testing its influence upon the enzyme, 5 cc. of toluene was shaken with the 100 cc. of digestion mixture and the insoluble surplus allowed to remain upon the surface of the substrate.

The results of parallel determinations with and without toluene are shown in Table I.

TABLE I.

EFFECT OF TOLUENE UPON VARIOUS AMYLASES.

Enzyme. Kind.	Amount. Mg.	Reduction of Cu_2O. No C_7H_8. Mg.	5 cc. C_7H_8. Mg.	Loss of Activity. %.
Commercial pancreatin, No. 8....	0.96	333.6	331.8	?
Purified pancreatic amylase, No. T 18A	0.05	200.7	191.0	5
Saliva.....	0.06 cc.	201.7	194.5	4
Malt extract.....	0.035 cc.	238.4	216.0	9
Purified malt amylase, No. 151..	0.15	329.6	304.7	8
Commercial takadiastase, No. 7.	5	308.6	304.4	1
Aspergillus amylase, No. 22b ...	0.25	293.4	285.9	3

From these results it appears that commercial pancreatin and takadiastase were influenced to only a negligible extent if at all by the saturation of the digestion mixture with toluene, while saliva, malt extract and the purified amylase preparations were measurably inhibited in their action, but not inactivated to any very serious extent. This is in accordance with the results of a previous series of experiments carried out by Miss Jennie A. Walker and one of us, in which the action of purified malt amylase was repeatedly found to be slightly diminished by the presence of toluene, but to an extent little if any greater than the variations to be expected between duplicate experiments with such enzyme preparations.

Experiments with Formaldehyde.

One cc. of pure formalin (35% formaldehyde) was diluted to 100 cc., and of this dilute solution 5, 10 and 15 cc. respectively were added to digestion mixtures whose total volumes were always adjusted to 100 cc. The actual weights of formaldehyde used were therefore 0.000175 g., 0.00035 g., and 0.000525 g., and the final concentrations of formaldehyde were 0.000058 *M*, 0.000116 *M*, and 0.000174 *M*, or expressed as ratios, 1:59,000, 1:28,000, and 1:20,000. Doubtless because of combination of formaldehyde with starch, the reducing action of the starch paste to which formaldehyde had been added was found to be less than the sum of the reducing effects of the starch and formaldehyde when tested separately.

Notwithstanding the fact that in making the experiments the formaldehyde was mixed with the starch paste before being brought into contact with the enzyme, and that a liberal excess of starch was always present, the formaldehyde in the small amounts here added distinctly depressed the activity of each of the amylases tested. The results are shown in Table II.

TABLE II.

EFFECT OF FORMALDEHYDE UPON VARIOUS AMYLASES.

Enzyme. Kind.	Amount.	Cu_2O (corrected). No HCHO.	5 cc. HCHO (0.000058 *M*).	10 cc. HCHO (0.000116 *M*).	15 cc. HCHO (0.000174 *M*).	Loss of Activity. 5 cc. HCHO 0.000058 *M*).	10 cc. HCHO (0.000116 *M*).	15 cc. HCHO (0.000174 *M*).
	Mg.	Mg.	Mg.	Mg.	Mg.	%	%	%
Comm. pancreatin, No. 8.....	0.96	277.6	247.2	226.9	194.9	11	18	30
Pur. pancreatic amylase, No. J18A	0.06	223.0	189.3	172.4	144.0	15	22	36
Saliva.....	0.06 cc.	250.4	221.2	203.7	179.8	12	19	28
Malt extract.....	0.035 "	266.4	238.3	218.1	190.5	11	18	29
Pur. malt amylase, No. 151	0.15	313.1	275.8	249.1	221.0	12	21	30
Comm. takadiastase, No. 7.....	5.00	296.9	270.3	246.7	230.0	9	17	23
Aspergillus amylase, No. 22b.....	0.25	292.0	265.2	237.6	221.5	9	19	24

The percentage loss of activity is seen to be very nearly alike for commercial pancreatin, saliva, malt extract and purified malt amylase, slightly higher for purified pancreatic amylase and slightly lower for commercial takadiastase and aspergillus amylase. It would seem from these results that takadiastase is the most resistant enzyme and purified pancreatic amylase the least resistant to small amounts of formaldehyde. Since the amount of destruction is greater in the case of purified pancreatic amylase than in that of commercial pancreatin it appears that impurities in the commercial pancreatin exerted a protective action.[8]

Further experiments showed that the use of even so little as 0.000035 g. of formaldehyde or a concentration of about 0.00001 *M* resulted in a measur-

[8] Compare Rosenthaler, *Biochem. Z.*, **26**, 9–13 (1910).

able loss of activity of commercial pancreatin and that the loss increased regularly with the concentration of the formaldehyde. Because of the reduction of Fehling's solution by the formaldehyde itself, it was not feasible to carry these experiments to a higher concentration than approximately 0.00017 *M*.

In order to see whether the concentration of the enzyme solution made any difference in the amount of inhibition caused by 10 cc. of formaldehyde solution, 0.4 cc., 0.6 cc., and 0.8 cc. of the enzyme solution were used. The results are given in Table III.

TABLE III.

EFFECT OF FORMALDEHYDE UPON VARIOUS CONCENTRATIONS OF PANCREATIN.

Pancreatin.	Reduction of Cu_2O.		Loss of Activity.
Cc.	Mg.	No HCHO. Mg.	10 cc. (0.000116 *M*). Mg.	%
0.4	0.48	132.3	110.0	17
0.6	0.72	204.4	166.8	18
0.8	0.96	269.4	218.5	19

According to these results, it made practically no difference in the percentage of retardation whether 0.48 mg., 0.72 mg., or 0.96 mg. was used with 2% starch paste in the presence of 10 cc. of formaldehyde solution.

To see whether the concentration of starch affected the result, 1%, 2%, and 4% starch pastes were used with 0.96 mg. of commercial pancreatin in the presence and absence of formaldehyde. The results are shown in Table IV.

TABLE IV.

EFFECT OF FORMALDEHYDE UPON THE ACTION OF PANCREATIN ON DIFFERENT CONCENTRATIONS OF STARCH.

Conc. of Starch.	Reduction of Cu_2O.		Loss of Activity.
%	No HCHO. Mg.	10 cc. (0.000116 *M*). Mg.	%
1	222.8	173.9	22
2	259.1	208.5	20
4	277.5	228.2	18

The percentage of destruction did not vary much no matter whether 1%, 2% or 4% starch was used.

From Tables II to IV it can be seen that the percentage loss of activity of commercial pancreatin depends not upon the concentration of the enzyme or the concentration of the substrate, but only upon the concentration of the antiseptic.

Experiments with Copper Sulfate.

A 0.00056 *M* copper sulfate solution was prepared, and 5 cc., 10 cc. and 15 cc. of this solution used in the experiments. The results are given in Table V.

TABLE V.

EFFECT OF COPPER SULFATE UPON VARIOUS AMYLASES.

Enzyme.		Cu2O.				Loss of Activity.		
Kind.	Amount.	No $CuSO_4$.	5 cc. $CuSO_4$ (0.000028 *M*).	10 cc. $CuSO_4$ (0.000056 *M*).	15 cc. $CuSO_4$ (0.000084 *M*).	5 cc. $CuSO_4$ (0.000028 *M*).	10 cc. $CuSO_4$ (0.000056 *M*).	15 cc. $CuSO_4$ (0.000084 *M*).
	Mg.	Mg.	Mg.	Mg.	Mg.	%.	%.	%.
Comm. pancreatin, No. 8.....	0.96	278.7	82.7	71.2	62.8	70	74	77
Pur. pancreatic amylase, No. T 18B.....	0.07	206.6	51.4	48.0	43.0	75	77	79
Saliva.....	0.06 cc.	267.1	170.3	167.3	166.8	36	37	38
Malt extract.....	0.035 cc.	245.9	215.6	189.1	166.5	12	23	32
Pur. malt amylase, No. 151	0.15	342.6	322.2	306.8	296.2	6	10	14
Comm. takadiastase, No. 7.....	5.00	303.8	291.5	277.6	266.3	4	9	12
Aspergillus amylase, No. 22b...	0.25	279.0	257.9	241.9	232.0	8	13	17

The results in Table V show that all of the amylases studied were affected by from 0.000028 *M* to 0.000084 *M* copper sulfate. The pancreatic and salivary amylases were affected considerably more than malt amylase and takadiastase.

Assuming that the iso-electric points of the amylase proteins are in the range of other proteins previously reported by Loeb and others, namely in the vicinity of $C_H{}^+ 10^{-5}$, and since the activity of the pancreatic amylase was measured in the presence of electrolytes at a reaction of about $C_H{}^+ 10^{-7}$ while the reaction of the substrate solutions in the cases of malt amylase and takadiastase was about $C_H{}^+ 10^{-4.5}$, it would appear that the greater destruction of pancreatic amylase may be due to the formation of insoluble copper-proteinate[9] which would form at the hydrogen-ion concentration of the substrate solution as prepared for the pancreatic, but not as prepared for the malt and aspergillus amylases. We hope soon to study the iso-electric points of some of these amylase preparations.

Saliva was affected only about half as much as was the pancreatic amylase. This may be due to the presence in the saliva of a relatively large amount of other protein which would tend to protect the amylase by removing copper in the manner suggested above.

The results of a second series of experiments with a new solution of copper sulfate, used in such amounts as to give a wider range of final concentrations than in those above described, are shown in Table VI.

It may be seen from Table VI that in the presence of exceedingly minute amounts of copper sulfate its inhibitory effect is much more pronounced in the case of the pancreatic than of the malt amylase. In all cases the percentage loss of activity increases with increasing concentration of copper. Purified pancreatic amylase was tested with somewhat larger amounts

[9] Loeb, *J. Gen. Physiol.* **1918–19**, *passim.*

TABLE VI.

EFFECT OF COPPER SULFATE UPON CERTAIN AMYLASES.

Enzyme. Kind.	Amount.	Cu_2O. No $CuSO_4$.	1 cc. $CuSO_4$ (0.000006 *M*).	10 cc. $CuSO_4$ (0.00006 *M*).	50 cc. $CuSO_4$ (0.0003 *M*).	Loss of Activity. 1 cc. $CuSO_4$ (0.000006 *M*).	10 cc. $CuSO_4$ (0.00006 *M*).	50 cc. $CuSO_4$ (0.0003 *M*).
	Mg.	Mg.	Mg.	Mg.	Mg.	%.	%.	%.
Comm. pancreatin, No. 8.....	0.96	264.1	135.5	66.0	43.7	49	75	83
Pur. pancreatic amylase, No. T 18B.....	0.07	298.3	122.4	71.7	41.6	59	76	86
Saliva.....	0.06 cc.	183.1	131.4	116.1	105.6	28	37	42
Malt extract.....	0.035 cc.	263.1	257.4	197.9	92.1	2	25	65
Pur. malt amylase, No. 151.....	0.15	308.4	308.2	279.9	199.5	..	9	35

than in the above experiments with the result that copper sulfate at 0.00042 *M* was found to deprive it of 89% of its activity; and at 0.00054 *M*, 92% of the activity was lost.

Experiments with various concentrations of enzyme (pancreatin) and of starch, similar to those described under formaldehyde, were performed with copper sulfate.

The results are shown in Tables VII and VIII.

TABLE VII.

EFFECT OF COPPER SULFATE WITH VARIOUS CONCENTRATIONS OF PANCREATIN.

Pancreatin.		Reduction of Cu_2O. No $CuSO_4$.	10 cc. (0.00006 *M*).	Loss of Activity.
Cc.	Mg.	Mg.	Mg.	%.
0.4	0.48	140.5	36.3	74
0.6	0.72	204.8	55.7	73
0.8	0.96	280.9	74.5	74

TABLE VIII.

EFFECT OF COPPER SULFATE UPON THE ACTION OF PANCREATIN ON STARCH AT DIFFERENT CONCENTRATIONS.

Conc. of Starch.	Reduction of Cu_2O. No $CuSO_4$.	10 cc. (0.00006 *M*).	Loss of Activity.
%.	Mg.	Mg.	%.
1	240.8	71.1	70
2	276.8	79.0	71
4	290.3	88.8	69

From Tables VII and VIII it will be observed that the percentage of loss of activity of pancreatin is practically unaffected by a change in enzyme concentration or by a change in the starch concentration. It is not the ratio of antiseptic to enzyme or of antiseptic to substrate, but the ratio of antiseptic to water which determines the activity of the amylase.

Summary.

Low concentrations of chloroform did not affect commercial pancreatin or malt extract, but did affect the purified preparations of these amylases.

Toluene had very little influence upon the activities of the amylases either in their natural or purified condition.

Commercial pancreatin, purified pancreatic amylase, saliva, malt extract, purified malt amylase, commercial takadiastase, and the purified amylase of *Aspergillus oryzae* were all injured by formaldehyde even in small amounts. Takadiastase was the least, and purified pancreatic amylase the most, affected. The percentage of loss of the enzyme action increased in all cases with increasing concentration of formaldehyde.

A very low concentration of formaldehyde (0.0000116 *M*) gave a 3% destruction of the activity of commercial pancreatin.

All of the enzymes studied were very sensitive to copper sulfate. Pancreatic amylase was much more sensitive than any of the others. Most of the enzymes were injured by as low a concentration of copper sulfate as 0.000006 *M*. Almost complete destruction of the activity of purified pancreatic amylase was caused by 0.00054 *M* copper sulfate. The inhibiting effect of copper sulfate on the activity of amylases increased with increasing concentration of copper sulfate.

The percentage loss of enzyme action due to formaldehyde and to copper sulfate solution did not depend upon the ratio of antiseptic to enzyme or of antiseptic to substrate, but upon the ratio of antiseptic to water, or the concentration of the antiseptic in the system.

The results demonstrate the need of attention to the possible effects of antiseptic upon enzyme in cases in which antiseptics are used to suppress microörganisms in studies of enzyme activity.

The much greater sensitiveness of the amylases to formaldehyde and copper sulfate than to toluene is of further interest in connection with the problem of the protein nature of these enzymes.

We are indebted to the Carnegie Institution of Washington for the use of enzyme preparations which had been purified in connection with work done under the auspices of the Institution.

NEW YORK CITY.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF COLUMBIA UNIVERSITY, No. 375.]

THE INFLUENCE OF CERTAIN AMINO ACIDS UPON THE ENZYMIC HYDROLYSIS OF STARCH.

BY H. C. SHERMAN AND FLORENCE WALKER.

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Our experiments on the influence of amino acids upon the rate of hydrolysis of starch by different enzymes, begun with the study of asparagine and aspartic acid,[1] have been extended to glycine, alanine, tyrosine and phenylalanine. Essentially the same experimental methods have been employed as in our work with asparagine and aspartic acid. In the experiments described below, however, "soluble" starch, prepared by the Lintner

[1] Sherman and Walker, THIS JOURNAL, 41, 1867 (1919).