phenyliodonium sulfide decomposes at room temperature in the presence of tellurium and antimony to give diphenyltellurium and triphenylstibinic sulfide.

2. To account for these reactions, it is suggested that at least a part of the iodonium salt decomposes by way of a non-ionic mechanism.

EDMONTON, ALBERTA, CANADA RECEIVED JULY 3, 1939

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

Tyrosinase from the Wild Mushroom, *Lactarius Piperatus*

BY H. R. DALTON AND J. M. NELSON

Kubowitz[1,2] has shown that potato oxidase (tyrosinase) contains copper and that the activity of the oxidase toward catechol as the substrate is proportional to the metal content. After considerable purification a product was obtained which in the presence of acetone, at 0°, occurred in a globular form and contained 0.20% copper. Keilin and Mann[3] have also prepared a highly purified polyphenolase (*i. e.*, tyrosinase) from the common mushroom, *Psalliota campestris*, which had a copper content of 0.30% and an activity toward catechol proportional to the copper content.

The tyrosinase described in the present communication was prepared from the wild mushroom, *Lactarius piperatus*. Highly purified preparations from this source were found to contain 0.23% copper. The activity of these preparations toward catechol was also proportional to the copper content (see Fig. 1). The copper was

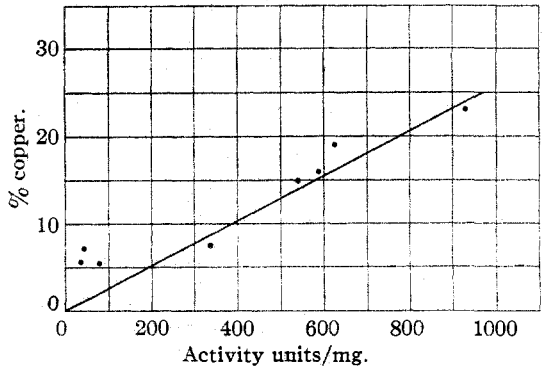

Fig. 1.

easily removed from the enzyme preparation by dilute acids just as Kubowitz noted in the case of the oxidase from potatoes. Contrary to the claims by Yakushiji[4] that the polyphenolase from *Lactarius piperatus* contains a hematin residue; the data in the table clearly show that the iron can be removed completely by one adsorption to kaolin. Only in the case of the two crude preparations, nos. 1 and 2, was iron found to be present and then only in very small amounts. It therefore appears that iron was present only as an impurity in the crude preparations.

Keilin and Mann[3] found no relationship between the activities of their oxidase preparations, from *Psalliota campestris*, toward the two substrates, *p*-cresol and catechol, and were inclined to attribute the activity toward *p*-cresol as involving the presence of some other factor in the reaction mixture. Likewise Adams and Nelson[8] were able to vary, in the case of the oxidase from *Psalliota campestris*, the ratio between the activities toward these two substrates. On the other hand, the last-mentioned investigators were unable by similar procedures, such as the use of various adsorbing materials, to alter the ratio of these two activities in the case of the tyrosinase from *Lactarius piperatus*. Further attempts in the present study to vary this ratio by treating the enzyme solutions with lead acetate and with silver acetate also were unsuccessful. All the preparations examined showed about 10 cresolase units to 1 catecholase unit.

The quantity of copper corresponding to one unit of cresolase is given in the table for several preparations. The rather crude preparations 1, 2 and 3 contain an appreciable quantity of undialyzable copper, which was also noticed by Keilin and Mann[3] in their crude preparations from *Psalliota campestris*, and hence show a high copper value per cresolase unit. Preparations 4 to 8 have lost this extraneous copper and the copper per cresolase unit reaches a constant value of approximately 0.0027 γ. These preparations represent enzyme in which all of the copper seems to be in an active form.

(1) F. Kubowitz, *Biochem. Z.*, **292**, 221 (1937).
(2) F. Kubowitz, *ibid.*, **299**, 443 (1938).
(3) D. Keilin and T. Mann, *Proc. Roy. Soc.* (London), **125B**, 187 (1938).
(4) E. Yakushiji, *Acta Phytochim. Tokyo*, **10**, 63 (1937).

TABLE I

Prepn.	Units/mg. *p*-Cresol	Units/mg. Catechol	Units/cc. *p*-Cresol	Units/cc. Catechol	Dry weight, mg./cc.	Dry weight mg./1000 cresolase units	Cu, mg./cc.	γ Cu per cresolase unit	% Cu (based on dry weight)	Fe, mg./cc.
1	33.8	3.4	65.2	6.5	1.93	28.8	1.1×10^{-3}	0.017	0.057	4×10^{-5}
2	41.8	4.2	46.0	4.6	1.10	23.9	8.0×10^{-4}	.017	.073	2×10^{-5}
3	79.0	7.6	65.5	6.3	0.83	12.7	5.0×10^{-4}	.0077	.062	$>1 \times 10^{-6}$
4	336	33.4	222	22	.66	3.0	5.0×10^{-4}	.0023	.076	[a]
5	540	54	540	54	1.0	1.9	1.5×10^{-3}	.0028	.15	[a]
6	590	65	1000	110	1.7	1.7	2.7×10^{-3}	.0027	.16	[a]
7	625	72	4000	460	6.4	1.6	1.2×10^{-2}	.0030	.19	[a]
8	930	98	4000	420	4.3	1.1	1.0×10^{-2}	.0025	.23	[a]

[a] Indicates that no iron could be detected when as much as 0.5 mg. of enzyme had been used. γ = 0.001 mg.

PREPARATION OF MATERIALS AND PROCEDURE (TABLE I)

Showing enzyme activity, dry weight per enzyme unit and copper content. All tyrosinase preparations obtained from *Lactarius piperatus*.

Prepn. 1.—Mushrooms dried by being spread out in the laboratory for several days. Broken up and water added—pressed in hydraulic press and the press juice dialyzed for forty-eight hours against water in a rocking dialyzer which kept the liquid in the cellophane sausage casing, continuously in motion.[5] *Prepn. 2.*—Prepn. 1 precipitated with 0.4 satd. ammonium sulfate, precipitate dissolved in water and dialyzed on rocker for forty-eight hours. *Prepn. 3.*—Prepn. 2 adsorbed to kaolin (*p*H 4.5), eluted with disodium phosphate (*p*H 8.4), treated with 0.3 satd. ammonium sulfate and precipitate discarded, made 0.6 satd. with ammonium sulfate and precipitate formed dissolved in water and dialyzed 48 hours on rocker. *Prepn. 4.*—Dried mushrooms treated with water, pressed and liquid made 0.6 satd. with ammonium sulfate, precipitate dissolved in water and enzyme material adsorbed to kaolin (*p*H 4.5), eluted with disodium phosphate (*p*H 8.4), reprecipitated with 0.6 satd. ammonium sulfate, precipitate dissolved in water, dialyzed fourteen hours on rocker followed by dialysis for twenty-four hours in a large bottle of glass distilled water in the icebox. *Prepn. 5.*—Dried mushrooms water pressed, precipitated with 0.6 satd. ammonium sulfate, dissolved in water, reprecipitated with 0.6 satd. ammonium sulfate, dissolved in water, adsorbed to kaolin (*p*H 4.5), eluted with disodium hydrogen phosphate (*p*H 8.4), reprecipitated with 0.6 satd. ammonium sulfate, dissolved in water, reprecipitated with 0.6 satd. ammonium sulfate, dissolved in water, dialyzed for forty-eight hours on rocker. *Prepn. 6.*—Enzyme material in Prepn. 5 adsorbed to kaolin (*p*H 4.8), eluted with disodium hydrogen phosphate (*p*H 8.4), precipitated with 0.4 satd. ammonium sulfate and precipitate formed discarded, solution remaining was made 0.6 satd. with ammonium sulfate and precipitate formed dissolved in water and dialyzed forty-eight hours on rocker. *Prepn. 7.*—Dried mushrooms, water added, pressed, precipitated with 0.6 satd. ammonium sulfate, dissolved precipitate in water, made 0.3 satd. with ammonium sulfate and precipitate discarded, remaining liquid made 0.6 satd. with ammonium sulfate and precipitate formed dissolved in water, adsorption to kaolin (*p*H 4.5), eluted with disodium hydrogen phosphate at *p*H 8.4, reprecipitated with 0.6 satd. ammonium sulfate, precipitate dissolved in water and enzyme material adsorbed to alumina (*p*H 6.5), eluted with disodium hydrogen phosphate (*p*H 8.4), dialyzed fourteen hours on rocker. This solution had only a slight brown color. *Prepn. 8.*—Dried mushrooms, water added, pressed, precipitated with 0.6 satd. ammonium sulfate, dissolved in water, precipitated with 0.2 satd. ammonium sulfate and precipitate discarded, solution made 0.5 satd. with ammonium sulfate, precipitate dissolved in water, adsorbed to bentonite (*p*H 4.5), eluted with disodium hydrogen phosphate (*p*H 8.4), precipitated with 0.6 satd. ammonium sulfate, dissolved in water, treated with alumina (*p*H 6.5) and portion not adsorbed set aside and marked A. Material adsorbed to the alumina was eluted with disodium hydrogen phosphate (*p*H 8.4) and precipitated with 0.6 satd. ammonium sulfate, dissolved in water and treated with alumina (*p*H 6.5) and solution containing portion of enzyme material which failed to adsorb added to A. Process repeated until 90% of the original enzyme material was recovered as material from portions not adsorbed to alumina. The combined solutions were made 0.6 satd. with ammonium sulfate, precipitate dissolved in small volume of water and treated with norite charcoal (*p*H 6.5), filtered, reprecipitated with 0.6 satd. ammonium sulfate, precipitate dissolved in 13 cc. of water (*p*H 6.7), dialyzed four days in large bottle (3 liters) of glass distilled water placed in refrigerator. Water was changed two times each day. The solution thus obtained was nearly colorless.

Enzyme activity was determined by means of the Warburg respirometer,[6] temp. 25°. Graubard and Nelson's[7] method as modified by Adams and Nelson[8] was used for determining units of enzymic activity toward both *p*-cresol and catechol as substrates. In each case one unit represents the amount of enzyme, measured under prescribed conditions, necessary to bring about an oxygen uptake of 10 cu. mm. per minute. Since the oxidations of the two substrates are distinctly different reactions[9] the two kinds of units must not be confused. For this reason the two enzymic actions have been termed for convenience, cresolase and catecholase activities.

Iron was determined by the *o*-phenanthroline method of Saywell and Cunningham.[10] No interference was observed by them from copper, aluminum and magnesium in a solution containing 1×10^{-5} mg. of iron to which had been added 2×10^{-5}, 1×10^{-4} and 1×10^{-4} mg. of copper, aluminum and magnesium, respectively. Copper was determined by the method of Warburg[11] as modified by Warburg and Krebs.[12] Each determination was made with enzyme containing sufficient copper to give a rate of oxygen uptake between 20 and 30 cu. mm. per ten minutes.

The purest enzyme preparation obtained contained 0.23% copper, possessed an activity of 1000 cresolase units per mg. dry weight and 0.0027 γ copper per cresolase unit.

Redfield[13] and Dhéré and Burdel[14] have found a similarity in the visible region of the spectrum

(5) M. Kunitz and H. S. Simms, *J. Gen. Physiol.*, **11**, 641 (1927).
(6) M. Dixon, "Manometric Methods," University Press, Cambridge, 1934.
(7) M. Graubard and J. M. Nelson, *J. Biol. Chem.*, **112**, 135 (1935).
(8) M. H. Adams and J. M. Nelson, THIS JOURNAL, **60**, 2472, 2474 (1938).
(9) C. A. Bordner and J. M. Nelson, *ibid.*, **61**, 1507 (1939).
(10) L. G. Saywell and B. B. Cunningham, *Ind. Eng. Chem., Anal. Ed.*, **9**, 67 (1937).
(11) O. Warburg, *Biochem. Z.*, **187**, 255 (1927).
(12) O. Warburg and H. A. Krebs, *ibid.*, **190**, 143 (1927).
(13) A. C. Redfield, *Biol. Bull.*, **58**, 150 (1930).
(14) C. Dhéré and A. Burdel, *J. physiol. path. gen.*, **18**, 685 (1919).

among a large number of bloods and solutions containing oxyhemocyanin. In general a rather strong band occurs in the region of 570 mμ. Since the hemocyanins are copper proteins like tyrosinase, purified preparations of the latter, from *Lactarius piperatus*, were examined by means of a Zeiss microspectroscopic ocular attached to a microscope, but no similar absorption could be detected in the visible region.

The preparations were also examined in a Hilger quartz spectrophotometer. The quartz cells used for holding the tyrosinase preparations were 1 cm. in depth. Figure 2 represents a curve plotted from data obtained. Dhéré[15] and Kubowitz[2] investigating the ultraviolet absorption spectrum of oxyhemocyanin obtained strong absorption bands in the region of 278 and 350 mμ which are quite similar to the bands given by the *Lactarius piperatus* tyrosinase. The band at 278 mμ is due to protein.[15]

Ley and Hegge[16] in a study of copper amino acid showed that the band at about 385 mμ is due to copper. The band at about 330 mμ given by the tyrosinase preparations and the band at about 350 mμ for oxyhemocyanin are probably due to the copper in these complexes, their positions being shifted to some extent as a result of the influence of modifying groups. The failure of Ito[17] to find any indication of an absorption in this region for potato oxidase might be due to this absorption being much weaker than the protein band and likely to be hidden by the general absorption in this region.

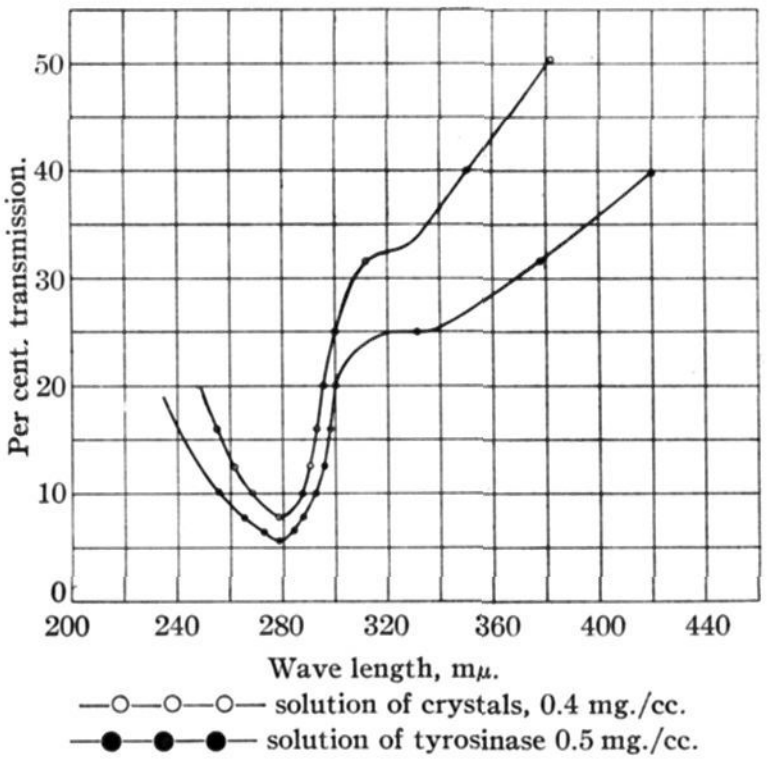

Fig. 2.—Shows the similarity between the absorption curves for a purified tyrosinase preparation and for a solution of the crystals. A weak absorption in the case of the enzyme solution exists in the region from 320 to 340 mμ. The absorption in this region in the case of the solution of the crystals is less pronounced, but when the low concentration of the crystalline material is taken into account, it will be found that the intensity is relatively about the same.

That the similarity of the two curves may be due to the enzyme solution containing uncrystallized material, corresponding to the crystals, can hardly be the case. As already stated in the text, not more than 10% of the dry weight in any purified preparation was obtained in the crystalline form. Assuming that Beer's law holds, it can be shown that these two curves are identical, and that it is unlikely that this would be the case if the crystals and enzyme material were not closely related.

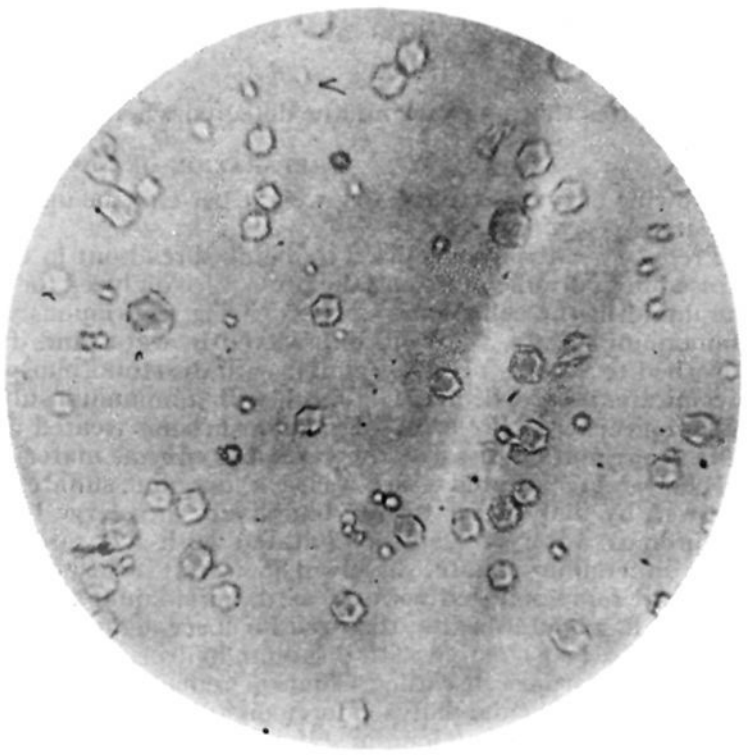

Fig. 3.

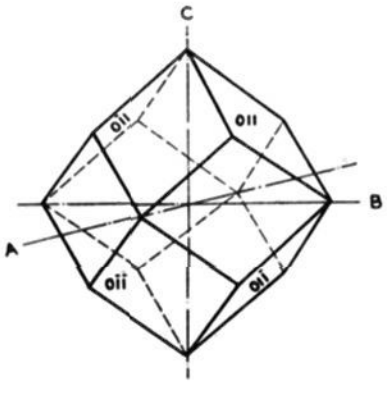

Fig. 4.

As has been stated in a previous communication,[18] a crystalline material was obtained from several preparations of tyrosinase from *Lactarius piperatus*. Rather crude preparations, as well as preparations purified as described in the legend of the table, yielded crystals. In no case, however, was more than 10% of the dry weight of a

(15) C. Dhéré, *J. physiol. path. gén.*, **19**, 1801 (1920).

(16) H. Ley and H. Hegge, *Ber.*, **48**, 70 (1915).

(17) R. Ito, *J. Biochem. Japan*, **24**, 279 (1936).

(18) H. R. Dalton and J. M. Nelson, THIS JOURNAL, **60**, 3085 (1938).

purified enzyme preparation obtained in a crystalline form. The procedure followed in obtaining the crystals was similar to that usually employed for crystallizing hemocyanin,[19] *i. e.*, by adjusting the *p*H of the enzyme solutions, containing at least 1000 units of cresolase per cc., to about 4.8 by means of sodium acetate buffer and then adding ammonium sulfate until the solution became slightly cloudy. After standing for several hours crystals could be observed. Enzyme preparations which had been treated, in the course of purification, with lead acetate so far have not yielded any crystalline material by the above described procedure.

Since the previous communication it has been possible to obtain perfectly shaped crystals which belong definitely in the isometric system. Their shape is rhombic dodecahedric. They are usually found lying on their 011 faces and when thus viewed through the microscope show hexagonal shapes. Figure 3 is a photomicrograph of these crystals and Fig. 4 is a perspective diagram.

The crystals show thryxotropy, are colorless, stain well with methyl violet, and have an average size of approximately 0.010 mm. Analysis showed 0.25% copper and 13.6% nitrogen. Like solutions of the purified tyrosinase, solutions of the crystals showed no marked absorption in the visible spectrum, but did show an absorption curve similar to that of the enzyme in the ultraviolet (see Fig. 2).

The crystals are insoluble in dilute acids and salt solutions. When freshly prepared they are soluble in dilute alkali (*p*H 8) but become increasingly insoluble on standing. After standing for several weeks they become practically insoluble in 0.1 *M* sodium hydroxide.

The wash water from freshly prepared crystals, after the tenth washing by means of the centrifuge, still showed tyrosinase activity. This activity may possibly be due to enzyme from the concentrated mother liquor still adhering to the crystals. Against this possibility, however, might be mentioned that 3 mg. of crystals dissolved in 20 cc. of secondary sodium phosphate solution and having an activity of 4 cresolase units per cc. showed an increase in activity to 12 units per cc. after standing for three weeks.

Slight increases in activity on standing were also noticed in the case of some solutions of the tyrosinase preparations. The latter observation has been made also by Keilin and Mann[3] and, as they have suggested, may be due to the presence of a proenzyme, which by some unknown process may be activated. Results obtained in this study show that only about 15% of the copper in the crude preparations is in the active form. The remaining copper or at least part of it may be related to this inactive state of the enzyme.

The similarity in composition, spectra and tendency to slight increase in activity on standing, all point to a close relationship between the active enzyme and the crystals. The fact that inactive or only slightly active crystals were obtained from solutions of highly purified preparations in which all the copper appears to be in the active form (γ of copper per cresolase units, see table) suggests the two following possibilities. Either the enzyme becomes, for some unknown reason, inactive when it is crystallized, or an inactive form, such as a proenzyme, accompanies the enzyme as it is purified.

Summary

1. A method is described for preparing highly purified preparations of tyrosinase from *Lactarius piperatus*.

2. The tyrosinase is shown to be a copper protein.

3. The purest tyrosinase preparation contained 0.23% copper and had a dry weight of one mg. per 1000 cresolase units and 0.0027 γ of copper per cresolase unit.

4. The iron present in the crude preparations of the enzyme can be removed by adsorption methods.

5. Purified enzyme preparations showed no definite absorption bands in the visible region of the spectrum, differing in this respect from the hemocyanins.

6. Two absorption bands were observed in the ultraviolet region: a band due to protein at 273 mμ and a band at about 330 mμ probably due to copper.

7. A crystalline material was obtained, from rather crude as well as from purified preparations of the tyrosinase, having a copper content of 0.25% and 13.6% nitrogen. The crystals were colorless and belonged to the isometric system. The average size was about 0.010 mm.

8. Solutions of the crystals were only slightly active toward *p*-cresol and catechol. This slight

(19) C. Dhéré, *J. physiol. path. gén.*, **18**, 503 (1919).

activity showed a tendency to increase when the solutions were permitted to stand for some time. Certain solutions of the freshly prepared tyrosinase also showed this tendency to increase in activity on standing.

9. The ratio of activities of the tyrosinase preparations toward *p*-cresol and catechol could not be altered by various methods of purification, but always remained 10 to 1, respectively. The ratio of the two activities in the case of the solutions of the crystalline material was also 10 to 1.

10. Composition, similarity in absorption spectra, the slight increase in activity when solutions are allowed to stand, all point to a close relationship between the crystals and the highly purified tyrosinase preparations.

NEW YORK, N. Y. RECEIVED AUGUST 9, 1939

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF THE CALCO CHEMICAL COMPANY, INC.]

Sulfanilamide Derivatives. IV. N^1,N^4-Diacylsulfanilamides and N^1-Acylsulfanilamides[1]

BY M. L. CROSSLEY, E. H. NORTHEY AND MARTIN E. HULTQUIST

We have prepared an extended series of N^1-acylsulfanilamide derivatives[2] of the type NH_2—C$_6$H$_4$—SO_2NXCOR, as a continuation of our studies of N^1-substituted sulfanilamides. The scope of the work was extended to include examples where R was alkyl (from one to seventeen carbons in length), alkenyl, aryl, aralkyl, diaralkyl, aralkenyl, cycloalkyl, cycloalkenylalkyl and heterocyclic. Derivatives were also prepared from aliphatic dicarboxylic acids and aryl dicarboxylic acids. A few metanilamide derivatives were prepared, but these appeared to be of little therapeutic interest. X in the above formula was hydrogen, alkyl or a cation.

As intermediates in the preparation of many of the above, N^1,N^4-diacylsulfanilamides of the general formula, R'CONH—C$_6$H$_4$—SO_2NXCOR, were synthesized. In these, R' was usually a methyl group, but several compounds were prepared in which R' was the same as R. This particular series was not extended since the pharmacological results were not encouraging.

Derivatives in which the N^4-nitrogen of the N^1-acylsulfanilamide was substituted by sulfanilyl- or sodium methylenesulfinate groups were also prepared.

Since inception of this work, the first members of both series have been mentioned. A diacetylsulfanilamide, melting at 240–242°, which from the method of preparation was probably impure N^1,N^4-diacetylsulfanilamide, was described by Scudi.[3] N^1-Acetylsulfanilamide has been described by M. Dohrn and P. Diedrich[4] and is being sold abroad under the name "Albucid" for use against gonorrhea.

Several methods of synthesis were employed, but the one most generally useful involved reaction between N^4-acetylsulfanilamide and an acyl halide in the presence of dry pyridine. This reaction proceeded smoothly, giving good yields of the N^1-acyl-N^4-acetylsulfanilamide. The N^4-acetyl group was then removed by boiling with a slight excess of aqueous sodium hydroxide, to give a solution of the sodium salt of the N^1-acylsulfanilamide, from which the free N^1-acylsulfanilamide was obtained by acidification. Other methods successfully used are given in the experimental part.

Attempts to prepare derivatives by reaction of dry N-acetylsulfanilyl chloride with several carbonamides in pyridine failed. The reason for such failure is not understood and is being investigated further.

N^1-Acyl-N^4-sulfanilylsulfanilamides were prepared by condensing an N^1-acylsulfanilamide with N-acetylsulfanilyl chloride in pyridine, followed by hydrolysis of the acetyl group with aqueous sodium hydroxide.

N^4-Sodium formaldehydesulfoxalate derivatives of N^1-acylsulfanilamides were prepared by warming equimolecular amounts of the starting materials in water until solution was complete, then evaporating to dryness *in vacuo*.

(1) Presented in part before the Division of Medicinal Chemistry, Baltimore meeting, American Chemical Society, April, 1939.

(2) For nomenclature, see Crossley, Northey and Hultquist, THIS JOURNAL, **60**, 2217 (1938).

(3) Scudi, *Ind. Eng. Chem., Anal. Ed.*, **10**, 346 (1938).

(4) M. Dohrn and P. Diedrich, *Münch. med. Wochschr.*, **85**, 2017 (1938).