

rated. The residue was crystallized from methanol, m. p. 179–180°, yield 1.5 g.

Anal. Calcd. for $C_{23}H_{34}O_4$: C, 73.7; H, 9.2. Found: C, 73.8; H, 9.1.

The above material, 500 mg., was hydrolyzed by boiling with 250 mg. of sodium hydroxide in 50 cc. of ethanol. The neutral fraction melted at 223–225° after crystallization from aqueous methanol.

Anal. Calcd. for $C_{21}H_{32}O_3$: C, 75.8; H, 9.7. Found: C, 75.5; H, 9.6.

The hydrolysis product could be reacylated with hot acetic anhydride to give the original acetate melting at 179–180°.

Treatment of Δ^{16} -allo-Pregnenol-3(β)-one-20 Acetate with Hydrogen Peroxide.—A mixture of 5 g. of Δ^{16} -allo-pregnenol-3(β)-one-20 acetate, 250 cc. of acetic acid, and 50 cc. of 30% hydrogen peroxide was heated to 70° for five hours. The reaction mixture was worked up as described for pregnenolone acetate. The neutral material melted at 185–186° after crystallization from methanol, yield 2.5 g.

Anal. Calcd. for $C_{23}H_{34}O_4$: C, 73.7; H, 9.2. Found: C, 73.8; H, 9.0.

Hydrolysis of this material with alcoholic sodium hydroxide gave a product which melted at 181–182° after crystallization from methanol. This substance gave a large depression in melting point when mixed with the acetate, m. p. 185–186°.

Anal. Calcd. for $C_{21}H_{32}O_3$: C, 75.8; H, 9.7. Found: C, 75.5; H, 9.6.

Reacylation with hot acetic anhydride gave the original acetate melting at 185–186°.

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Crystalline Avidin

BY DERROL PENNINGTON, ESMOND E. SNELL AND ROBERT E. EAKIN

Using essentially the procedures described in a previous communication for obtaining concentrated material,¹ we have obtained the biotin-inactivating protein, avidin, in crystalline form. Highly concentrated material obtained by this means was dissolved (50 mg. per cc.) in one-half saturated ammonium sulfate solution, at room temperature, and saturated ammonium sulfate solution was added to incipient precipitation. After centrifuging out this first turbidity, the clear solution was placed in a refrigerator. Within an hour, the avidin precipitated in the form of fine needle-like crystals. When care was taken that the crystallization proceeded more slowly, larger plates (Fig. 1) were obtained. Both forms were equally active. Such material was recryst-

(1) Eakin, Snell and Williams, *J. Biol. Chem.*, **140**, 535–543 (1941).



Fig. 1.—Avidin crystals magnified 1000 times.

tallized repeatedly by the same method and retained high activity. For assay purposes, salt-free avidin was obtained by dissolving the crystallized material in dilute salt solution, and dialyzing the solution against distilled water until the avidin was completely precipitated.

The crystallization procedure was somewhat destructive to the activity of the avidin. The potency of three times recrystallized material was approximately 4000 units per gram, while the most active amorphous material previously obtained had a potency of approximately 7000 units per gram. Crystals which stood in the refrigerator in contact with the mother liquor lost approximately three-fourths of their activity in three weeks. Dry crystals which stood for three months at summer temperature lost no activity.

Analyses on two independent batches of crystallized avidin gave the following results:

	C	H	N	S	Residue
Sample 1	43.72	7.60	12.10	1.32	2.09
Sample 2	44.26	7.28	12.83	...	0.75

Both samples gave a positive Molisch test for carbohydrate, and the analyses indicate that the substance may be a protein with a large carbohydrate moiety. Further study of the substance will await its production on a large scale.

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An Improved Method for the Preparation of Benzenediazonium Salts

BY WILLIAM SMITH AND CHAS. E. WARING

The usual method of preparing diazo salts is that given by Hantzsch and Jochem.¹ Essen-

(1) Hantzsch and Jochem, *Ber.*, **34**, 3337 (1901).

tially, this procedure involves the slow addition of isoamyl nitrite to the appropriate aniline salt suspended in cold glacial acetic acid. In order to precipitate the benzenediazonium salt in a crystalline form, dry ether must be added dropwise to the solution over a period of three to four hours.

This, and analogous, methods not only have the disadvantage of being tedious and time-consuming, but also it not infrequently happens that an oil, instead of a crystalline precipitate, is produced. Further, the yields obtained by these methods are rather poor.

The following method has been found to be rapid (fifteen to thirty minutes), to always produce good crystalline precipitates, and to give yields which approach the theoretical.

Preparation.—Five grams of aniline hydrochloride is suspended in a mixture of 30 ml. of glacial acetic acid and 30 ml. of anhydrous, peroxide-free dioxane. The mixture is cooled in an ice-salt-bath and ethyl nitrite bubbled in until all the solid material is dissolved. It has been previously established that the complete diazotization of the hydrochloride coincides with the disappearance of the solid phase and the first appearance of an excess of ethyl nitrite. Hence, the instantaneous coloration of starch iodide paper produced by a drop of the solution gives evidence that diazotization is complete. 150 ml. of dry dioxane at room temperature is then added in one portion to the completely diazotized solution, and benzenediazonium chloride immediately precipitates in a fine, white crystalline form. The solid is filtered on a Büchner funnel and washed twice with two 25-cc. portions of dry dioxane to remove any last traces of acetic acid.

The yield of dry benzenediazonium chloride from seven different preparations was, in all cases, calculated to be better than 95% of the theoretical.

It was previously found² that benzenediazonium chloride could be stored under anhydrous ether in an icebox for several weeks without deterioration. We now find that the salt may be kept in good condition for at least a month by merely placing it in a stoppered bottle, at room temperature, under dry dioxane. During the course of time the supernatant liquid gradually changed color, going from water-white to amber. The salt, however, retained its original white, crystalline properties. It appears, then, that any decomposition products which are formed under these conditions are readily soluble in the dioxane and hence their contamination of the solid material is prevented to a large extent.

The rates of decomposition, in water, of freshly prepared benzenediazonium chloride, and of that which had been stored for one month, were compared. The rates were identical ($k_{30} = 1.04 \times 10^{-4}$ sec.⁻¹) within the limits of experimental error. In both cases, the nitrogen evolved at 30° was 98% of the theoretical.

(2) Waring and Abrams, *THIS JOURNAL*, **63**, 2757 (1941).

The method described here has been found equally successful in the preparation of benzenediazonium sulfate. It is reasonable to assume that this method should give equally good yields of high purity for diazo salts from all acids.

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The Use of Phenylhydrazine to Characterize Organic Acids¹

BY G. H. STEMPEL, JR., AND GERSON S. SCHAFFEL

Most of the common methods for the preparation of derivatives for the identification of organic acids require the use either of the acid chloride or of a solution of the sodium salt of the acid. A chance observation of the ease with which acetic acid reacts with phenylhydrazine in benzene solution suggested the possible use of phenylhydrazine as a reagent for characterizing organic acids. A search of the literature revealed the application of this reagent by Latimer and Bost² to the identification of aliphatic sulfonic acids. We have investigated the reaction of phenylhydrazine with a number of carboxylic and sulfonic acids and have found that in most cases a white, crystalline material suitable as a derivative is formed readily. The procedure developed utilizes a reagent which is readily available and requires less time than most methods commonly used to identify organic acids. We have also worked out a simple method for obtaining derivatives of monobasic aliphatic acids formed in the saponification of esters.

The general method used in preparing these derivatives was to boil a solution of the acid in phenylhydrazine for thirty minutes. When the solution was cooled, the solid product either separated in good yield or was precipitated out by the addition of benzene. It was then recrystallized from benzene or from an alcohol-water mixture. In a few cases precipitation occurred immediately upon mixing the acid and phenylhydrazine in the cold. By this procedure weaker acids, in general, form phenylhydrazides, while the stronger acids, such as chloroacetic and various sulfonic acids, form salts. The dibasic acids investigated yield a bis-phenylhydrazide when

(1) Presented before the Division of Organic Chemistry of the American Chemical Society at its 102nd meeting at Atlantic City, New Jersey, in September, 1941.

(2) P. H. Latimer and R. W. Bost, *THIS JOURNAL*, **59**, 2500 (1937).