sugars dissolved in water. Then the columns were eluted with aqueous ethanol, the concentration of the ethanol being continuously increased from 1 to 30%.[10] The effluent from the columns was collected in 100-ml. portions. Each portion was analyzed by paper chromatography. Similar fractions were combined and concentrated to dryness. The results of the separation are summarized in Table I.

Identification of D-Fructose and D-Glucose.—The fraction no. II (Table I) was treated on a 4.5 × 50 cm. charcoal column using the original Whistler and Durso technique.[9] The sugars were separated in two fractions: monosaccharides and sucrose. D-Fructose and D-glucose were identified using authentic specimen by paper chromatography. Monosaccharide fraction was fermented with baker's yeast[18] for 2 days. D-Fructose and D-glucose were fermented completely. Only some sugar alcohols and pentoses were detected in the residue.

Identification of Sucrose.—Fraction no. IV (Table I) crystallized in large crystals. They were the characteristic sucrose crystals. Paper chromatography showed only sucrose. **A. Hydrolysis.**—Sucrose (*ca.* 0.1 g.) was heated with sulfuric acid (4%; 2 ml.) in a test-tube in a boiling-water bath for 2 hr. After neutralization with barium carbonate and filtration, the filtrate was evaporated to dryness and the sugars were estimated by paper chromatography; two equally large spots of D-fructose and D-glucose appeared. The hydrolysis by invertase[19] gave the same result. **B. Fermentation.**—A portion of sucrose was fermented with baker's yeast.[18] Chromatograms showed that sucrose was completely fermented, and only some unidentified substances appeared in traces. **C. Diazouracil Reaction (Raybin Test).**[20]—Twenty mg. of sucrose was dissolved in 2 ml. of 0.05 *N* NaOH and shaken with 5 mg. of diazouracil at 8°; it gave a deep yellow-green solution. After adding some drops of 0.1 *M* $MgCl_2$, this gave a blue precipitate, which was identical with the authentic specimen of sucrose. **D. Properties.**—M.p. 164°; $[\alpha]^{20}D$ +66.5° (H_2O; *c* 4).

Isolation of Raffinose and Stachyose.—Fraction no. VII (Table I) was treated on a 4.5 × 50 cm. charcoal column using aqueous ethanol, the concentration of the ethanol being continuously increased from 5 to 20%.[10] The effluent was collected in 100-ml. portions. The results are summarized in Table II. Portions 32–67, containing only raffinose and stachyose, were combined and the sugars separated on a 2.5 × 30 cm. charcoal column using the original Whistler and Durso technique.[9]

TABLE II

SUGAR COMPOSITION OF ELUATES FROM A CHARCOAL COLUMN OF FRACTION NO. VII (TABLE I)

Eluate in 100-ml. portions	Sugars present
1–9	Nil
10–12	D-Fructose, D-glucose, sucrose, several unidentified substances
13–19	Sucrose, melibiose
20–24	Manninotriose, several unidentified substances
25–31	Manninotriose, raffinose
32–67	Raffinose, stachyose

Identification of Raffinose. A. Hydrolysis.—Raffinose (*ca.* 10 mg.) was hydrolyzed in the case of sucrose; a paper chromatogram showed three equally large spots of D-fructose, D-glucose and D-galactose. The hydrolysis by invertase[19] gave D-fructose and melibiose. **B. Fermentation.**—The portion of raffinose was fermented with bakers' yeast.[18] After fermentation, the chromatogram showed melibiose. **C. Diazouracil Reaction.**[20]—This reaction gave a definite bluish precipitate, which was identical with the authentic specimen. **D. Properties.**—After several reprecipitations from 90% ethanol, the m.p. was 79°; $[\alpha]^{20}D$ +105.2° (H_2O; *c* 2).

Identification of Stachyose. A. Hydrolysis.—Stachyose was hydrolyzed with sulfuric acid as in the case of sucrose. Paper chromatograms showed the presence of D-fructose, D-glucose and D-galactose. The spot of D-galactose was approximately twice as large as the spots of D-glucose or D-fructose. Hydrolysis by invertase[19] gave D-fructose and manninotriose. **B. Fermentation.**—The portion of stachyose was fermented with baker's yeast.[18] After fermentation, the chromatogram showed manninotriose. **C. Diazouracil Reaction.**[20]—A definite bluish precipitate was identical with the authentic specimen from stachyose. **D. Properties.**—The m.p. was 167°; $[\alpha]^{20}D$ +148° (H_2O; *c* 2).

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(18) Fleischmann's yeast (Standard Brands, Inc.) was purchased from a local distributor.

(19) Difco Laboratories, Inc., Detroit, Michigan.

(20) D. French, G. U. Wild, B. Young and W. S. James, THIS JOURNAL, **75**, 709 (1953).

Synthesis of β-Cyclocitral

BY J. D. CHANLEY, E. CHOW AND H. SOBOTKA

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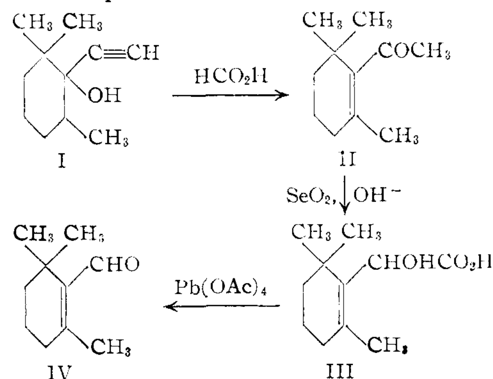
We have investigated the action of formic acid on various cyclohexanol acetylenes and have reported[1] the cases of 1-ethynylcyclohexanol and its 2,2-dimethyl homolog. At that time, we had also prepared 1-acetyl-2,2,6-trimethylcyclohexene (II) from 1-ethynyl-2,2,6-trimethylcyclohexanol (I), but were puzzled by its low molecular extinction coefficient ϵ_{max} 1300 at λ 242 mμ, which suggested that the reaction product did not consist exclusively of an α,β unsaturated ketone; in fact, it did not yield any keto derivatives. Recently, Henbest and Woods[2] have independently prepared this compound by the same method and also by hydration of 1-ethynyl-2,2,6-trimethylcyclohexene, employing a mercuric oxide–boron trifluoride catalyst. They also observed a low extinction coefficient at 243 mμ and likewise failed to obtain keto derivatives. Nevertheless, they deduced that the compound is an α,β-unsaturated ketone.

We have now obtained conclusive proof for the postulated structure of this compound. We converted it by the following sequence of reactions into β-cyclocitral: Oxidation of the compound with selenium dioxide yielded trimethylcyclohexenyl glyoxal, which on treatment with sodium hydroxide rearranged to the glycolic acid of m.p. 173° (III). This was oxidized with $Pb(OAc)_4$ in acetic acid to β-cyclocitral (IV). The latter compound and its semicarbazone were found to be identical with authentic samples.



1-Acetyl-2,2,6-trimethylcyclohexene (II).—One hundred grams of 1-ethynyl-2,2,6-trimethylcyclohexanol[3] was refluxed with 530 ml. of 90% formic acid for one hour. After

(1) J. D. Chanley, THIS JOURNAL, **70**, 244 (1948).

(2) H. B. Henbest and G. Woods, *J. Chem. Soc.*, 1150 (1952).

(3) H. Sobotka and J. D. Chanley, THIS JOURNAL, **71**, 4136 (1949).

removal of formic acid, the residue was carefully fractionated; 61 g. of product, b.p. 88° (13 mm.), was collected, $n^{20}D$ 1.4780, λ_{max} 242 mμ, ϵ_{max} 1330; reported[3] 88–90° (16 mm.), $n^{17}D$ 1.4778, λ_{max} 243 mμ, ϵ_{max} 1400.

Anal. Calcd. for $C_{11}H_{20}O$: C, 79.47; H, 10.91. Found: C, 79.46; H, 10.93.

2,2,6-Trimethylcyclohexene-1-glycolic Acid.—Five grams of the above compound and 4 g. of seleinous acid in 20 ml. of dioxane were refluxed for 3 hours. The ether extract of the residue, after removal of dioxane, was treated with sodium carbonate and then with 10% sodium hydroxide. Acidification of the hydroxide extract yielded the acid which after recrystallization from alcohol–water melted at 173° (with gas evolution).

Anal. Calcd. for $C_{11}H_{18}O_3$: C, 66.63; H, 9.14; neut. equiv., 198. Found: C, 66.48; H, 9.22; neut. equiv., 194.

β-Cyclocitral.—One gram of the glycolic acid in 25 ml. of glacial acetic acid was oxidized at 60–65° with 2.2 g. of lead tetraacetate added in two portions during two hours. To the cooled mixture 7 ml. of 1 *N* sulfuric acid was added, lead sulfate removed, the filtrate diluted with 300 ml. of ether and neutralized with a paste of sodium bicarbonate. The neutral ether layer yielded on evaporation 500 mg. of practically pure β-cyclocitral, $n^{25}D$ 1.4950, λ_{max} 248 mμ, ϵ_{max} 7880.

The semicarbazone was prepared and recrystallized from ethyl acetate; m.p. 165°, λ_{max} 274 mμ, ϵ_{max} 20700.

Anal. Calcd. for $C_{11}H_{19}ON_3$: C, 63.10; H, 9.16; N, 20.10. Found: C, 63.25; H, 8.90; N, 20.21.

All the recorded physical properties of β-cyclocitral and its semicarbazone are in agreement with those in the literature.

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Interactions of Homologs of Carcinogenic Azo Dyes and Bovine Serum Albumin[1-3]

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Many studies have been made on the interactions between proteins and azo dyes, but the majority of these studies have been concerned with interactions involving cationic or anionic dyes.[4] Very little has been reported regarding the interactions of uncharged azo dyes and proteins.[5] This latter type of interaction might be of interest since certain of these uncharged azo dyes are involved in tumor formation.[6]

It has been reported that slight structural modifications in azo dye ions can affect the ability of these ions to complex with proteins.[7-9] This suggests that slight structural changes in uncharged azo dyes might also result in a similar effect. If this were true then we might have another clue to the relationship between carcinogenicity and chemical structure.

Protein–dye interactions involving 4-dimethylaminoazobenzene, 3′-methyl-4-dimethylaminoazobenzene and 4′-methyl-4-dimethylaminoazobenzene might be of value since these three dyes possess widely differing values for their relative carcinogenicities.[6] Studies with these dyes, however, are complicated by the fact that they are extremely insoluble in the aqueous solutions usually used for protein–dye interaction studies. The aminoazobenzene homologs of these compounds, however, are more soluble in water and yet possess the same structural relationships found among the members of the trio of carcinogens. Accordingly, it was thought desirable to study the interactions of 4-aminoazobenzene, 3′-methyl-4-aminoazobenzene and 4′-methyl-4-aminoazobenzene with proteins.

Bovine serum albumin was selected as the protein for this study since much is known concerning the nature of the complexes involving this protein. Hence any results obtained with bovine serum albumin might be more easily interpreted than results obtained with some less well characterized protein. If studies with bovine serum albumin proved to be successful, then the more complex systems involved in tumor formation might also be successfully studied.

Experimental

Synthesis and Properties of Dyes.—4-Aminoazobenzene was prepared in two steps by the preparation and rearrangement of diazoaminobenzene. 3′-Methyl-4-aminoazobenzene and 4′-methyl-4-aminoazobenzene were prepared in a manner similar to that used by Witt[10] in that *p*-nitrosoacetanilide was condensed with the appropriate toluidine in glacial acetic acid to yield the acetyl derivatives of the desired dyes. These were then hydrolyzed to yield the final products. All of the dyes were recrystallized from alcohol–water mixtures to give compounds melting at 122.5–123°, 96–97° and 151–152°, respectively (lit. values 125–126°[11] and 126°[12,13]; 89–91°[13]; 144–146°[13] and 153–154°,[14] respectively.)

The ionization constants for these dyes were determined colorimetrically by use of the Beckman model DU spectrophotometer. The solubilities of these dyes were determined by placing small amounts of the dyes in the phosphate buffer used for interaction studies and these mixtures agitated at 25°. Periodically, samples of each solution were withdrawn, filtered and diluted to concentration ranges wherein Beer's law was known to obtain. The concentrations of these solutions were then determined by use of the Beckman model DU spectrophotometer and from these values and the corresponding dilution factors the solubilities were obtained.

Interactions.—The spectral shift and equilibrium dialysis techniques developed by Klotz and his co-workers were used for these studies.[15,16] All spectral and analytical data were obtained by use of a Beckman model DU spectrophotometer using either 1 or 5 cm. cells. The dialysis studies were performed at 25°. The spectral and analytical data were taken at room temperatures. The concentrations of

(1) Contribution No. 517, Department of Chemistry, Kansas Agricultural Experiment Station, Manhattan, Kansas.

(2) Supported by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

(3) Presented in part before the Biological Section of the 124th National Meeting of the American Chemical Society, Chicago, Illinois, September 6, 1953.

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