Time, temperature and *p*H when greater than 7.5, had little or no effect on the course of the reaction.

The concentration of sodium borohydride greatly influenced the completeness of reaction up to a point of maximum reduction. One equivalent of sodium borohydride only partially reduced an equivalent of streptomycin. With increasing quantities of sodium borohydride, the reduction of streptomycin rapidly approached completion until a maximum value (as determined by assay for residual streptomycin by the maltol method[6]) was reached. Beyond this point a very great excess of borohydride reagent only slightly lowered the residual streptomycin value.

The amount of borohydride needed for maximum reduction varied with different samples of streptomycin and appeared to be associated to some degree with the purity or previous processing of the material. Samples of pure commercial grade streptomycin sulfate were readily reduced to a satisfactory level (less than 1% residual streptomycin by maltol test) with only a small excess of the sodium borohydride reagent. Partially purified materials and ion-exchange resin eluates, however, often gave unsatisfactory reduction with even very large excesses of sodium borohydride. This apparent resistance to complete reduction of some streptomycin samples has also been observed in the catalytic hydrogenation procedure.

One sample of dihydrostreptomycin sulfate which was treated with a large excess of sodium borohydride still contained 20% of residual streptomycin on the basis of the maltol test. When this material was submitted to paper strip chromatographic analysis according to the procedure of Winsten,[7] only a single dihydrostreptomycin zone was observed; no streptomycin zone was present. Thus, it would appear that sodium borohydride completely reduced the streptomycin component of the sample.

The presence of substances in streptomycin broth which are not active against microörganisms but which hydrolyze to give an absorption at 322 mμ like that of maltol has been postulated.[8] The data obtained in our experiments indicate that there may be associated with crude streptomycin some biologically inactive substance which gives a positive maltol test similar to that of streptomycin. This entity may be carried along in streptomycin processing and continue to give a positive color test after treatment with reducing agents, thus causing an apparent incomplete reduction of streptomycin by sodium borohydride or by catalytic hydrogenation.

Experimental

Preparation of Dihydrostreptomycin Sulfate with Sodium Borohydride.—Five grams of streptomycin sulfate assaying 719 μg. base/mg. was dissolved in 35 ml. of water at room temperature to give a solution containing approximately 100,000 μg/ml. The *p*H was adjusted to 8.0 with triethylamine and 0.15 g. of sodium borohydride[9] in 5 ml. of water was added with stirring. A slight elevation in temperature and mild gas evolution occurred. After thirty minutes, 6 *N* H_2SO_4 was added slowly to *p*H 1.5. Some gas was evolved. The acidified solution was kept at room temperature for 10 minutes and then added to 175 ml. (5 volumes) of methanol with vigorous stirring. The resulting precipitate was collected, washed with methanol, and dried *in vacuo* over P_2O_5 to give 5.1 g. of boron-free dihydrostreptomycin sulfate with a biological potency of 700 μg./mg. and a residual maltol assay of 4.8 μg. streptomycin/mg. The amorphous dihydrostreptomycin sulfate obtained from the above process was readily crystallized from water–methanol solution.

Effect of Time on Completeness of Reduction.—Thirty milliliters of a partially purified streptomycin sulfate solution at a concentration of 100,000 μg./ml. and at *p*H 7.0 was mixed with 1.2 ml. of a solution containing 192 mg. of sodium borohydride. At these concentrations, three equivalents of sodium borohydride were present for each equivalent of streptomycin aldehyde. The determination of residual streptomycin by maltol determination was made after 7 minutes, 30 minutes, and 18 hours. Values obtained for unreduced streptomycin were 3.1, 2.9 and 3.2%, respectively. Similarly, no significant difference in degree of reduction was observed when the streptomycin solution was allowed to stand in the presence of 8 equivalents of sodium borohydride for 7 minutes or for 18 hours.

Effect of Temperature on Reduction with Sodium Borohydride.—Two 20-ml. portions of a partially purified streptomycin sulfate solution of 100,000 μg./ml. concentration were treated with 4 equivalents of sodium borohydride. One sample was held at 25° overnight, the other at 45°. After acidification with sulfuric acid, maltol assays indicated 3.5 and 3.3% residual streptomycin, respectively.

Influence of Nature of the Sample upon Completeness of Reduction with Sodium Borohydride.—Aliquots of a solution of crude streptomycin sulfate containing 50,000 μg./ml. were treated at *p*H 8.5 with 2, 4 and 8 equivalents of sodium borohydride solution. After 30 minutes the solutions were acidified to destroy excess borohydride and assayed for residual streptomycin, giving values of 4.2, 5.0 and 4.2%, respectively.

A partially purified streptomycin was treated similarly. After reduction with 2, 4, 8 and 16 equivalents of sodium borohydride, residual streptomycin values of 2.0, 3.6, 2.7 and 2.2% were obtained.

Solutions of pure commercial streptomycin sulfate were treated in the same manner with 1 and 2 equivalents of sodium borohydride. Unreduced streptomycin values following such treatment were 2.5 and 0.9%.

Catalytic Reduction *vs.* Borohydride Reduction.—A sample of partially purified streptomycin sulfate at a concentration of 200,000 μg./ml. was placed in a Parr hydrogenator. Activated platinum catalyst (2.5 g./100 g. streptomycin sulfate) was added and the mixture shaken continuously for 15 hours at 25° under 50 lb. pressure of hydrogen. The solution was added to 5 volumes of methanol to give solids assaying 5.7 μg. streptomycin/mg. To an aliquot of the above starting solution before reduction was added 6 equivalents of sodium borohydride. Fifteen minutes after mixing, the solution was acidified to *p*H 1.5 with 6 *N* H_2SO_4 and added to 5 volumes of methanol to give solids with an apparent streptomycin content of 9.7 μg./mg.

In a repeat run comparing catalytic *vs.* sodium borohydride reduction in which pure streptomycin sulfate was used as starting material, it was found that by both procedures the unreduced streptomycin was less than one-half per cent.

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(6) *Federal Register*, **15**, 9460 (1950).

(7) W. A. Winsten and E. Eigen, THIS JOURNAL, **70**, 3333 (1948).

(8) Hazel M. Doery, E. C. Mason and D. E. Weiss, *Anal. Chem.*, **22**, 1038 (1950).

(9) Metal Hydrides, Inc., Beverly, Mass.

The Deuteration of Aniline in the Presence of Raney Alloy

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Methods for the introduction of deuterium into aniline have been investigated since deuterated anilines in which deuterium was in a specific position were required for a separate study. Accordingly,

a method which involved replacement of bromine with deuterium in the case of brominated anilines by means of Raney nickel–aluminum alloy in the presence of deuterium oxide and sodium deuteroxide was studied. *o*-Bromoaniline yielded aniline which was found to contain an amount of deuterium corresponding to the presence of 0.79 atom of deuterium for each aniline molecule. Furthermore, bromination of this sample of aniline gave rise to 2,4,6-tribromoaniline which contained only 0.03 atom of deuterium for each aromatic nucleus, and conversion of the deuterated aniline to *p*-bromoacetanilide yielded a product which showed a deuterium content equivalent to 0.80 atom for each aromatic nucleus. Thus, practically all of the deuterium was in the *ortho* position of the aniline, and the conversion of the bromoanilines to the corresponding deuteroanilines in the presence of Raney alloy appeared to be feasible. However, when this procedure was applied to *m*-bromoaniline and *p*-bromoaniline anomalous results were obtained. Instead of *m*-deuteroaniline and *p*-deuteroaniline, mixtures resulted and the compositions of these mixtures indicated a pronounced tendency for the formation of *o*-deuteroaniline. In order to obtain further information it was decided to ascertain whether aniline undergoes protium–deuterium exchange in the presence of deuterium oxide and sodium deuteroxide. Accordingly, ordinary aniline was refluxed for two hours with heavy water (*ca.* 99% D_2O) and sodium deuteroxide. After separation, the aniline was treated with about 20 times its own volume of methanol in order to transform N–D to N–H bonds. The methanol was removed by evaporation and the aniline was analyzed for deuterium. The mole fraction of deuterium [D/(D + H)] was found to be 0.0067. The mole fraction of deuterium in monodeuteroaniline is $^1/_7$ or 0.143. Therefore little or no protium–deuterium exchange occurs in the presence of base alone. Facile deuteration of aniline takes place, however, if Raney alloy is added. Aniline, after having been heated under reflux for 3.25 hours with deuterium oxide (*ca.* 95%), sodium deuteroxide and Raney alloy was separated, treated with methanol, as previously described, and distilled. Analysis[1] of this sample of aniline for deuterium was performed after 5.11-fold dilution with ordinary aniline. This dilution was made in order to avoid having to take into account the mass 4 peak on the mass spectrometer. In order to determine the distribution of the deuterium in the aromatic nucleus, the derivatives listed in Table I were also prepared and analyzed. In the case of sample II, the conditions were the same except that the aniline was heated under reflux for 24 hours.

TABLE I

Compound	$\frac{D}{D+H}$ for the undiluted sample I	II	No. of deuterium atoms per aromatic nucleus I	II
Aniline-d_x	0.144	0.313	1.02	2.19
Acetanilide-d_x	.117		1.05	
2,4,6-Tribromoaniline-d_x	.0276	.0680	0.11	0.27
p-Bromoacetanilide-d_x	.112	.235	0.89	1.88

(1) W. M. Lauer and W. E. Noland, THIS JOURNAL, **75**, 3689 (1953).

These results indicate the following percentage distribution of deuterium in the two samples.

	I	II
ortho	76.5	73.5
meta	10.8	12.3
para	12.7	14.2

It is evident that under the above-described conditions, exchange in the *ortho* position predominates. These results perhaps can be accounted for by assuming that the aniline molecule is anchored to the catalyst through the electron pair on the nitrogen; the rest of the molecule being less firmly attached. As a consequence, the *ortho* positions are in closest proximity to the catalyst and the probability of exchange in these positions is therefore the highest.

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The Amino Acid Composition of Human Salivary Amylase[1]

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The present study was undertaken in continuation of some work on the interaction of salivary amylase and chloride ions. Evidence for combination of chloride with the amylase, other than the well known activation of the enzyme, has been presented.[2] It seemed possible that some indication as to the nature of the structure responsible for the particular affinity of amylase for this anion might be revealed by amino acid analyses.

Salivary amylase was crystallized according to a previously described procedure.[2] Following hydrolysis of the protein its amino acid content was determined by chromatography on the ion-exchange resin Dowex-50, 8% cross-linked, according to the method of Moore and Stein.[3]

Experimental

Four times crystallized amylase was dissolved by adding 0.2 *N* sodium hydroxide dropwise to reach a *p*H of 10.4. The solution was then adjusted to *p*H 8.0 with 0.1 *N* acetic acid and the amylase was precipitated with three volumes of acetone at −14°. It was washed twice with acetone and dried *in vacuo* over sulfuric acid at room temperature. The amylase was used in this form for the amino acid analyses. Dry weight was determined on an aliquot by drying at 78° *in vacuo* over phosphorus pentoxide. The dry protein contained 0.78% ash and, on the basis of dry, ash-free protein, 17.04% nitrogen and 1.69% sulfur.[4] All the following results are reported on the basis of dry, ash-free protein.

Samples of the amylase, approximately 25 mg. in each portion, were hydrolyzed with 5 ml. of constant boiling hydrochloric acid in sealed tubes at 110° for 24 or 48 hours. The hydrochloric acid was removed *in vacuo*, the sample was made up to 5 ml. with water and 1 ml. containing approximately 5 mg. of amino acid mixture was applied to each column.

The Dowex-50 was a sample kindly provided by Drs.

(1) This work was carried out at the Rockefeller Institute for Medical Research. The generosity of Dr. Gertrude E. Perlmann in making the necessary laboratory space and equipment available is gratefully acknowledged.

(2) J. Muus, *Compt. rend. Lab. Carlsberg, Ser. Chim.*, **28**, 317 (1953).

(3) S. Moore and W. H. Stein, *J. Biol. Chem.*, **192**, 663 (1951).

(4) These determinations were done by Mr. S. Theodore Bella.