probably on the outside of the protein molecule, and the glycyl α -amino groups are at the ends of the shorter 'A' chains (Sanger, 1948) and perhaps therefore less accessible, while the least reactive ϵ -amino groups of the lysyl side chains are now known to be located, one in each 'B' chain, adjacent to the alanine residue which provides the terminal carboxyl group of these chains (Sanger & Tuppy, 1951).

It is hoped to extend these investigations to other proteins and peptides with the object of obtaining some insight into the factors influencing the reactivity of amino groups towards reducing sugars.

SUMMARY

1. The reaction at 37° between insulin and glucose freeze-dried together from solution at pH 7 has been investigated.

2. The rate of reaction has been shown to increase with increasing activity of water to a maximum at a water content in equilibrium with a relative humidity of approximately 70 %, and thereafter to

decrease again with further increase in water content.

3. The reactivities towards glucose of the α -amino groups attached to the terminal phenylalanyl and glycyl residues, and of the ϵ -amino groups attached to the lysyl residues of insulin have been determined by the fluorodinitrobenzene method. After 4 days at 65 % relative humidity, 70 % of the phenylalanyl amino groups, 37 % of the glycyl amino groups and only 21 % of the lysyl amino groups had reacted. After 16 days the corresponding figures were 80, 66 and 55 %.

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A Micromethod for the Serial Determination of Organically Bound Halogen

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The main methods for the determination of organically bound halogen are: (1) combustion with nitric acid in the presence of silver nitrate and gravimetric determination of silver halide, (2) dry combustion yielding the free halogen which may be determined gravimetrically or volumetrically, (3) fusion with potassium and volumetric determination of the ionized halide. The authors were faced with the problem of having to follow at frequent time intervals the metabolic fate of

halogen-containing compounds in micro-organisms. For this purpose methods (1) and (2) were found to be too long and cumbersome, whilst in the potassium-fusion method difficulty was experienced in bringing all of the organic substance into intimate contact with the potassium. The following alternative method was therefore devised allowing the rapid determination of small amounts of organically bound halogen (1.5–15 μ mol.) in series.

PRINCIPLE

The halogen-containing compound is subjected to combustion by fuming nitric acid in the presence of silver nitrate.

The precipitate is collected by centrifugation in the combustion tube, dissolved in water by adding a complex-forming substance (aqueous ammonia in the case of chloride and bromide, and potassium cyanide in the case of iodide) and the silver halogen complex reduced (by hydrazine hydrate in the case of chloride and bromide, and zinc powder in the case of iodide) to metallic silver and ionized halogen.

Fig. 1. Evaporation device. *A*, air inlet tube; *B*, support with set screw; *C*, manifold; *D*, cork; *E*, glass tube; *F*, rubber tubing; *G*, hypodermic needle; *H*, combustion tube containing solution to be evaporated. All dimensions in mm.

The metallic silver precipitates and the ionized halogen remains in aqueous solution. (When this work was completed a paper by Iwantscheff (1950) came to the notice of the authors in which a similar principle had been applied to the determination of traces of chloride in selenium.)

The amount of halogen present can be determined by volumetric estimation of the precipitated metallic silver after dissolving in nitric acid or by direct titrimetric determination of the ionized halogen. For the determination of silver and ionized halogen any of the conventional titrimetric methods can be used. The authors have preferred the potentiometric method for the determination of silver, and the diffusion methods of Conway (1947) for the determination of ionized halogen.

EXPERIMENTAL

*Reagents (all of analytical grade)
for preparation of solutions for titration*

Silver nitrate; fuming nitric acid; ammonia, *d*, 0.880; potassium cyanide, 2% (w/v) aqueous solution; hydrazine hydrate, 45% (w/v) aqueous solution; zinc powder; sodium laurylsulphonate, aqueous solution, saturated at room temperature; sodium sulphite; potassium chloride, 0.003 *N* aqueous solution.

Fig. 2. Micro-Carius furnace. *A*, support for thermometer tube; *B*, tinplate shield; *C*, wire support. All dimensions in mm.

*Preparation of the sample to be analysed
for combustion*

The halogen-containing organic substance to be analysed can be present in aqueous solution or in an organic solvent. The amount to be analysed should be of the order of 1.5–15 μmol . of halogen. A suitable volume of solvent containing the desired amount of halogen was evaporated to dryness in portions of about 1 ml. in combustion tubes which had been carefully cleaned with chromic acid solution. The combustion tubes, length 90 mm., internal diameter 7–8 mm., were made from bomb tubing. These tubes were tapered for about 20 mm. at the bottom to facilitate the collecting of the silver halide precipitates. For the evaporation of the solvent the special arrangement illustrated in Fig. 1 was used. It

consisted of a manifold to which a number of hypodermic needles was fixed in such a way that a fine stream of air could be blown through them. The combustion tubes containing the solution to be evaporated were held in a water bath by means of metal clips and into each tube a hypodermic needle was inserted so that its end was just above the surface of the liquid. The water bath was heated to a temperature just below the boiling point of the solvent and a current of air, as small as possible to avoid splashing, was passed through the needles over the surface of the solution. Under these conditions evaporation was complete in a few minutes.

Combustion and collection of the silver halide precipitates

Solid AgNO_3 , judged to be about twice the calculated amount required, was added to each tube followed by 0.1 ml. of fuming HNO_3 (in the analysis of iodoacetate, conc. HNO_3 was used because fuming HNO_3 caused rapid decomposition immediately). As much as possible of the substance which adhered to the wall of the tube was washed down with the acid by tilting the tube. The tubes were then sealed with the blowpipe and heated for 6 hr. at $270\text{--}300^\circ$ in a furnace placed in the fume cupboard (for precautions in sealing and opening the tubes see Gattermann & Wieland 1946). This heating time may be considerably reduced. For instance, Niederl, Baum, McCoy & Kuck (1940) find that 300° for 0.5 hr. is sufficient for most halogen compounds. The furnace illustrated in Fig. 2 was made from an Al block which was heated by a Bunsen burner. An adequate safety device was found to be a disused tinplate container which was held above the block by a bucket-handle type suspension. The breakage of the tubes under these conditions was found to be very small.

Collection and reduction of silver halides

Silver chloride and bromide. After cooling and releasing the pressure the tubes were opened just below the shoulder by making a scratch and touching it with a hot glass rod. The HNO_3 was carefully diluted with water (1 ml.), stirred (using a thin drawn-out glass rod) and the tubes were heated on a water bath for 5–10 min. to decompose the silver halide- AgNO_3 complex. After the heat treatment, the silver halide precipitate, which should be in a loose powdery form, was centrifuged into the tapered part of the combustion tube, first adding to the contents of the tube a drop of sodium laurylsulphonate solution which serves to prevent particles of the precipitate being held on the surface of the liquid. The supernatant was removed with a Pasteur pipette and the precipitate washed twice on the centrifuge in the same way with water acidified with HNO_3 , stirring each time and adding a drop of sodium laurylsulphonate. This technique of collecting precipitates by centrifuging with laurylsulphonate was used throughout this work.

The halide was dissolved by stirring with conc. NH_3 (0.3 ml.). To the solution at room temperature a solution of $\text{N}_2\text{H}_4 \cdot \text{H}_2\text{O}$ (0.05 ml.) was added. (It is important to carry out the reduction at about 20° ; if the solution is warm the Ag is not all precipitated but tends, in part, to form a colloidal solution.) The solution was mixed by rotating the tube between the palms of the hands and after 2 min. standing, water (about 0.5 ml.) and a drop of sodium laurylsulphonate solution were added. The precipitated metallic Ag was centri-

fuged down. The supernatant was carefully removed and collected in a dwarf tube with a Pasteur pipette. The Ag precipitate was washed once with water (1 ml.), without stirring, collected by centrifuging and the washing added to the first supernatant.

Silver iodide. The AgI precipitate and HNO_3 were diluted with water (1 ml.). To decompose the AgI-AgNO_3 complex which is much more stable than that of Cl^- and Br^- , a double-heat treatment was required. The tube was heated on a boiling-water bath until the precipitate was soft enough to be crushed by a thin glass rod with a ball end; the powdered precipitate was then heated for a further 15 min. The tubes were removed from the water bath and to the hot solution was added a little Na_2SO_3 (about 15 mg.) to reduce to I^- , any IO_3^- which might have been formed. The precipitate was collected by centrifuging with sodium laurylsulphonate and the supernatant removed. Water acidified with HNO_3 was added and, after stirring, the precipitate was heated for a further period of 15 min. on the boiling-water bath. The AgI was collected, washed once more with the dilute HNO_3 and finally with water to remove traces of acid.

Fig. 3. Chlorine determinations. $\times$, *o*-Chlorobenzoic acid (argentometric method); $\circ$, *o*-Chlorobenzoic acid (Conway method). The line drawn corresponds to that for the theoretical values.

The precipitate was dissolved in the aqueous solution of KCN (0.2 ml.). The reduction of the AgCN complex was carried out by addition of Zn dust (about 30 mg., the large excess being necessary to provide a large surface for the reaction) and stirring for about 1 min. After the reduction, water (1 ml.) and a drop of sodium laurylsulphonate solution were added and the solution centrifuged. The I^- -containing supernatant was separated by means of a Pasteur pipette, the precipitate containing metallic Ag and excess Zn was washed once by stirring with water (1 ml.) and after centrifuging, the wash-water was added to the first supernatant.

Titrimetric

Metallic silver. The Ag precipitates were dissolved in 50% (w/v) HNO_3 by warming (0.2 ml. for the Ag precipitated by reduction of AgCl and AgBr ; 0.25 ml. for the precipitate containing Ag and excess Zn, arising from the reduction of AgI). The Ag solutions were then titrated potentiometrically with the standardized KCl solution, using a glass reference

electrode and a continuous-reading electronic voltmeter (see Lykken & Tuemmler, 1942).

Chloride ions. The supernatant solutions from the Ag precipitates and their washings were evaporated to dryness by blowing an air stream over their surfaces as described above. The Cl^- -containing residues were transferred to the outer rim of the Conway units in two portions of 0.5 ml. of water and the halogen was determined in the manner described by Conway (1947). A silicone fixative was used as small losses of halogen occurred with the normally used paraffin fixative.

Bromide ions. To the solution containing the Br^- was added 0.05 ml. 10% (w/v) KOH. The solution was evaporated to dryness and the residues were then heated in the oven at 100–110°, for 1 hr. to remove all traces of excess

0.5 ml. portions of water and transferred to the outer chamber of the Conway unit. In order to liberate the I_2 , 0.1 ml. 4.2 N- H_2SO_4 was added, and the determination then carried out as described by Conway (1947), except that a silicone fixative was used. A control experiment showed that under these conditions Br_2 was not liberated from a solution containing Br^- .

RESULTS

The results are given in Figs. 3–5. Slightly lower results were obtained by the Conway method, but this method has the advantage of providing a means of estimating separately chloride, bromide and iodide in mixtures of all three.

Fig. 4. Bromine determinations. ●, Bromoterephthalic acid (argentometric method); ×, potassium *p*-bromophenylacetate (argentometric method); ○, potassium *p*-bromophenylacetate (Conway method). The line drawn corresponds to that for the theoretical values.

$\text{N}_2\text{H}_4 \cdot \text{H}_2\text{O}$ and then transferred to the Conway units in two portions of 0.5 ml. of water. If the heating was omitted the presence of excess $\text{N}_2\text{H}_4 \cdot \text{H}_2\text{O}$ in the solution led to N_2 production and the formation of a foam on the surface of the $\text{K}_2\text{Cr}_2\text{O}_7$ solution. This interfered with the diffusion of Br_2 from the outer rim into the inner chamber of the Conway unit. The KOH was added to convert NH_4Br to KBr , since it was found that heating the former salt alone caused a significant loss of Br^- , presumably due to volatilization of the salt. The Conway determinations were carried out in the prescribed manner (Conway, 1947), but using a silicone fixative.

Iodide ions. The tubes containing the I^- solutions were clipped in a water bath placed in the fume cupboard, 0.8 ml. 0.066 M-phosphate buffer (pH 7) was added and the solution was evaporated to dryness by the air-stream method. Thus the CN^- was removed by volatilizing it as HCN . The residues were extracted with two successive

Fig. 5. Iodine determinations. ×, Potassium iodoacetate (argentometric method); ○, potassium iodoacetate (Conway method). The line drawn represents the theoretical values.

SUMMARY

1. A titrimetric micromethod for serial determinations of chlorine, bromine and iodine in organic compounds is described.

2. The organically bound halogens are transformed into silver halides by combustion with fuming nitric acid, the silver halides are dissolved by a complex-forming agent (ammonia in the case of chlorides or bromides, and potassium cyanide in the case of iodides) and reduced to metallic silver, which precipitates, and halogen ions, which remain in the supernatant solution.

3. The halogen present can be estimated by determination of the precipitated metallic silver, or by direct titration of the halogen ions in the supernatant solutions using the diffusion methods of Conway.

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