3. Values for four apparent dissociation constants have been determined and the magnitude of another calculated.

4. Preliminary experiments have been performed on the inhibition of *n*-hexanamide hydrolysis by carboxylate ions other than *n*-hexanoate.

5. The effect of temperature on the hydrolysis has been studied: the apparent energy of activation is 10.5 kcal. between 8.1 and 37.2°.

6. The optimum pH of the reaction is about 8.7 at 37.2°: at higher pH values irreversible inactivation takes place.

7. The results are discussed in terms of the relative affinities of two groups on the catalytic site of the enzyme for substrate/or inhibitor.

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Interference by Amino Acids in the Estimation of Sugars by Reductometric Methods

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It is well known that amino acids interfere with the determination of reducing sugars by alkaline copper and ferricyanide reagents. The interference is negligible in blood-sugar analysis owing to the low amino acid content of blood, but in such materials as protein hydrolysates or bacteriological culture media the interference may be very large. It was indeed the unreliability of reductometric methods for the estimation of carbohydrate groups in proteins that led Sørensen & Haugaard (1933) to apply the orcinol method to this problem, and similar techniques have since been developed using carbazole (Gurin & Hood, 1939), anthrone (Morris, 1948), etc. While these colorimetric methods are more specific than reductometric methods, they are less precise, and the concentrated sulphuric acid employed in most of them produces charring with amino acids such as tryptophan. Many analysts would prefer to use reductometric methods if means could be found for overcoming amino acid interference, and this has been the object of the present investigation.

As a preliminary, the effects of pure amino acids and protein hydrolysates on alkaline copper and ferricyanide reagents were investigated. Some observations along these lines have been reported by Holden (1926), Hewitt (1938) and Munday & Seibert (1933). Marked differences were found in the reducing effects of different amino acids and between different sugar reagents.

To remove interfering amino acids, a number of the deproteinizing reagents used in blood-sugar analysis was tried, since many of them are claimed to remove other interfering substances. None of them was found effective, however, when applied to materials such as protein hydrolysates.

Synthetic ion-exchange resins have been used by a number of workers to remove various interfering substances prior to sugar analysis. Serbiá (1947) used them to remove breakdown products of sugars ('glucose') which had reducing power and were produced by heat treatment of molasses. Partridge & Westall (1948) treated extracts of biological materials with resins as a preliminary to the chro-

matographic analysis of sugars. Williams, Potter, Bevenue & Scurzi (1949) used resins for clarifying extracts of potatoes before reducing-sugar analysis, and Williams, Bevenue & Washauer (1950) extended this work with a comprehensive, comparative study of the use of various resins in the analysis of fresh and dehydrated vegetables.

We describe the use of ion-exchange resins in the present paper, and it is hoped that the results obtained in estimating sugar in the presence of relatively large amounts of amino acids will be of use to other workers.

MATERIALS AND METHODS

Sugars. 'AnalaR' D-glucose and Laboratory Reagent Grade galactose, fructose, mannose, ribose from British Drug Houses Ltd., dried over P_2O_5 .

Amino acids. B.D.H. Laboratory Reagent Grade.

Casein hydrolysate. 'Difco Bacto Casamino Acids' supplied by Baird and Tatlock (London), Ltd. This is a purified acid hydrolysate and the powdered material was used as supplied.

Synthetic resins. IR-120 (H), IR-4B (OH) and IRA-400 (OH) from B.D.H. Ltd. were used as supplied after air drying. Zeo-Karb 225 from the Permutit Co. Ltd. was converted into the H form by treatment with 10% HCl and thorough washing. Details of these resins and regeneration procedures are given in 'Notes on the Laboratory Use of Ion-Exchange Resins' from B.D.H. Ltd.

Reductometric methods. For details of the techniques the original papers should be consulted. (a) The copper method of Somogyi (1945), as described for the estimation of up to 3 mg. of glucose in the sample, 0.01 N- $Na_2S_2O_3$ was used; (b) the ferricyanide method of Fujita & Iwatake (1931)—(F & I), modified by Herbert (unpublished), for the estimation of up to 2.5 mg. of glucose in the sample, 0.05 N- $Na_2S_2O_3$ was used; (c) the ferricyanide method of Hagedorn & Jensen (1923)—(H & J) as described for the estimation of up to about 0.36 mg. of glucose in the sample, 0.005 N- $Na_2S_2O_3$ was used. The reagents described for deproteinization in each method were used only as stated in the text.

EXPERIMENTAL AND RESULTS

Reduction by solutions of pure amino acids. Nineteen amino acids were examined in eleven groups, similar ones such as glycine, alanine, valine, leucine and isoleucine being included together. Tyrosine, cystine and tryptophan were dissolved in a small volume of HCl, diluted to the required concentration, and samples were adjusted to near neutrality with NaOH before testing. Appreciable reduction was obtained as follows: of all three reagents by cystine, tryptophan and tyrosine; of F & I reagent by proline and hydroxyproline; of H & J reagent by methionine. The following amino acids gave little or no reduction of copper and ferricyanide: glycine, alanine, valine, leucine, isoleucine, arginine, lysine, histidine, serine, threonine, glutamic acid, aspartic acid and phenylalanine.

In general the amount of reduction of copper was less than that of ferricyanide and was usually not proportional to the amount of amino acids present, e.g. the reducing values as glucose for 2 and 4 mg. of DL-tryptophan were 0.41 and

0.49 mg. A more quantitative effect was obtained with L-cystine, 1, 3 and 5 mg. giving reducing values equivalent to 0.65, 1.6 and 2.2 mg. as glucose respectively.

Reducing power of casein hydrolysate. Casein contains little cystine (0.4%), tryptophan is destroyed by acid hydrolysis and the hydrolysate did not significantly reduce alkaline copper; 12.5, 18.75 and 25 mg. gave reducing values with the Somogyi reagent equivalent to 0.08, 0.1 and 0.1 mg. glucose respectively. With the F & I reagent, these amounts of casein hydrolysate gave values of 0.76, 1.16 and 1.6 mg. as glucose respectively.

The reducing powers of mixtures of amino acids and glucose.

The reducing powers of samples containing from 5 to 50 mg. of casein hydrolysate and 2 mg. of glucose together with similar solutions made just alkaline to phenolphthalein were determined by the Somogyi method. Neutralized samples were included in the test in case the buffering power of the larger amounts of amino acids was affecting the results. Results are shown in Table 1, and it may be seen that either low or high recoveries of glucose were obtained according to the amount of casein hydrolysate present. Mixtures of tyrosine and glucose gave less reduction than the same quantity of glucose alone, e.g. 2 mg. of glucose with 0.25, 0.5 and 1.0 mg. of tyrosine gave recoveries of 1.58, 1.85 and 1.87 mg. of glucose. It is evident that interference by amino acids in this method is not necessarily explained by their reducing power, and in fact inhibition of reduction by glucose occurs in certain cases. This complicated interference in the presence of glucose was found to be very dependent on the time the sample and the copper solution were left in the boiling-water bath. Whereas glucose alone gave maximum reduction at or just after 8 min. in the bath, the maximum reducing power of mixtures of glucose and amino acids was not reached until about 12 min. Casein hydrolysate (12.5 mg.) with 2 mg. of glucose gave reducing power values after 8, 10 and 12 min. equivalent to 2.04, 2.26 and 2.28 mg. of glucose respectively. Samples containing 25 mg. of casein hydrolysate and 0.5, 1.0 and 2 mg. of glucose were estimated by the F & I method with recoveries of 1.85, 2.09 and 2.5 mg. glucose respectively.

Treatment with deproteinizing reagents. As the F & I reagent was the one most affected by amino acids, it was used to investigate the effect of deproteinizing reagents. Both $Cd(OH)_2$ (Fujita & Iwatake, 1931) and $BaSO_4-Zn(OH)_2$ (Somogyi, 1945) removed cystine completely, probably because of the insolubility of this acid under such conditions and barium-zinc precipitation decreased the reducing power of tryptophan.

Table 1. *Reduction of Somogyi's (1945) copper reagent by mixtures of glucose and casein hydrolysate*

All solutions contained 2 mg. of glucose.		
Casein hydrolysate (mg.)	Recovery as glucose (mg.)	
	No treatment	Neutralized (alkaline to phenolphthalein)
5	1.95	1.98
10	2.20	2.28
20	2.24	2.24
30	2.18	2.19
40	1.88	2.08
50	1.61	1.89

The reducing powers of solutions of casein hydrolysate before and after treatment with $\text{Cd}(\text{OH})_2$ were practically identical. Treatment of similar solutions with BaSO_4 - $\text{Zn}(\text{OH})_2$ or by the HgSO_4 method of West, Scharles & Peterson (1929) lowered their reducing powers only slightly.

Treatment with cation-exchange resins. IR-120 (H) and Zeo-Karb 225 (H) are synthetic cation-exchange resins which exchange hydrogen ions for other positively charged ions. To test their efficiency the following experiment was made: 50 mg. of casein hydrolysate was dissolved in 10 ml. of water and about 1 g. of resin was added. The mixture was shaken on a rotary shaker for 15 min. and after the resin had settled, a sample (100 μl .) was applied to paper in parallel with an untreated sample (20 μl .) for examination by paper chromatography (Consden, Gordon & Martin, 1944). After

Table 2. *Shaking times with IR-120 (H) and Zeo-Karb 225 (H) for the removal of amino acids*

Ferricyanide method (Fujita & Iwatake) used. Details are given in the text.

Time of shaking (min.) ...	Reducing power of 25 mg. of casein hydrolysate expressed as glucose (mg.)			
	1	2	3	15
Zeo-Karb 225 (H)	0.34	0.19	—	0.103
IR-120 (H)	0.13	0.12	0.12	0.103
No treatment			1.6	

Table 3. *Reduction of Somogyi's (1945) copper reagent by mixtures of various sugars and casein hydrolysate after treatment with IR-120 (H) and IR-4B (OH)*

Details are given in the text.

Sugar	Recovery as glucose (mg.) in duplicate tests	
	Alone, no treatment	+ 25 mg. casein hydrolysate with resin treatment
Glucose	1.0, 1.0	1.0, 1.0
Galactose	0.815, 0.815	0.815, 0.815
Fructose	0.91, 0.90	0.90, 0.90
Mannose	0.91, 0.93	0.93, 0.93
Ribose	0.67, 0.68	0.66, 0.66

Table 4. *Reduction of Somogyi's (1945) copper solution by barium-zinc-treated solutions of mixtures of glucose, casein hydrolysate and bovine albumen before and after treatment with IR-120 (H) and IR-4B (OH)*

Each sample contained 10 mg. of bovine albumen and 25 mg. of casein hydrolysate. Other details are in the text.

Glucose (mg.)	Recovery as glucose (mg.) in duplicate tests	
	No treatment	Resin treated
0.5	0.66, 0.65	0.51, 0.51
1.0	1.16, 1.19	0.99, 0.99
1.5	1.69, 1.71	1.49, 1.48
2.0	2.14, 2.14	1.98, 1.98

development and spraying, the resin-treated sample showed only the merest trace of ninhydrin-reacting material, in contrast to the untreated sample, which showed a large amount. Neither of these resins removed glucose from solution, but in the presence of large quantities of cations the acid produced was often sufficient to interfere with the subsequent estimation and had to be neutralized. Acid formation could be avoided by including an anion-exchange resin; IR-4B (OH) was found satisfactory, whereas IRA-400 (OH) removed nearly all the glucose present.

To find the optimum time for treatment with IR-120 (H) and Zeo-Karb 225 (H), samples containing 50 mg. of casein hydrolysate in 10 ml. of water were shaken with approximately 1 g. of resin for various times and after filtering through Whatman no. 42 paper, a 5 ml. portion ($\approx$ 25 mg. casein hydrolysate) was tested for reducing power by the F & I method. The results (Table 2) showed that complete removal of interference was not obtained but the residual effect after 15 min. was not more than 7% of that before treatment and this time was adopted.

Solutions (10 ml. each) containing 50 mg. of casein hydrolysate and 2 mg. of glucose were shaken with approx. 1 g. each of IR-120 (H) and IR-4B (OH) for 15 min. After filtration through Whatman no. 42 paper, 5 ml. samples gave the correct reducing values with the Somogyi reagent. Galactose, fructose, mannose and ribose in the presence of casein hydrolysate also gave correct recoveries after resin treatment (Table 3).

Solutions (10 ml. each) containing 50 mg. casein hydrolysate and 1.0, 2.0 and 4.0 mg. of glucose were treated with IR-120 (H) and after filtration 5 ml. samples gave reducing-power values equivalent to 1.14, 2.13 and 4.2 mg. glucose respectively with the F & I reagent.

Treatment of samples containing protein and amino acids. Duplicate portions (6 ml.) of solutions containing 50 mg. of casein hydrolysate, 20 mg. bovine albumen and 1, 2, 3 and 4 mg. of glucose were treated with 2 ml. 5% $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and 2 ml. 0.3N- $\text{Ba}(\text{OH})_2$. After mixing and centrifuging, the clear supernatants from one series were shaken with 1 g. each of IR-120 (H) and IR-4B (OH) for 15 min. After filtration through Whatman no. 42 paper, 5 ml. samples were tested for reducing power with the Somogyi reagent in parallel with 5 ml. samples of the non-resin-treated supernatants. Correct recoveries were obtained from resin-treated samples (Table 4).

Estimation of similar solutions by the F & I method using the $\text{Cd}(\text{OH})_2$ deproteinizing reagent and IR-120 (H) followed by neutralization of the liberated acid with sodium carbonate gave glucose recoveries close to the theoretical.

DISCUSSION

The results of the present work indicate that sugar cannot be accurately estimated in the presence of relatively large amounts of amino acids by the Somogyi (1945), the Fujita & Iwatake (1931) or the Hagedorn & Jensen (1923) methods, although the copper method is superior for this purpose. Interference in the copper method cannot be explained by the reducing power of amino acids, since certain of these inhibit the reduction by sugar, e.g. tyrosine and glucose gave less reduction than the same quantity of sugar alone. Recovery of glucose from

mixtures of casein hydrolysate and glucose depended on the relative concentrations of each. Amino acids form complexes with alkaline solutions of copper which are easily observed by their strong blue colour and they also form compounds with sugar in alkaline solution. These three factors, i.e. reducing power, copper-complex formation and sugar-amino acid compounds, probably all contribute to the interference found.

The use of ion-exchange resins extends the use of reductometric methods for the estimation of sugars and the Somogyi (1945) method will give correct recoveries with small amounts of sugar and large amounts of amino acids.

SUMMARY

1. Interference by amino acids in the reductometric estimation of glucose was investigated using copper and ferricyanide methods.

2. Copper was appreciably reduced by cystine, tyrosine and tryptophan, but in general was less affected by amino acids than was ferricyanide.

3. Although casein hydrolysate alone had little effect on copper, recoveries of glucose from samples containing it and glucose were incorrect.

4. Precipitation by barium, cadmium or mercury did not substantially lower the reducing power of a solution of acid-hydrolysed casein using the Fujita & Iwatake method.

5. A method using synthetic cation-exchange resins is described which allows the accurate estimation of glucose in the presence of a large excess of amino acids.

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