Short Communications

The Accurate Measurement of Insulin Molarity

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In order to study critically some physical characteristics and biological properties of insulin we needed an accurate measurement of the molarity of insulin solutions. Herskovits (1965) has measured the concentration of insulin by nitrogen analysis and published a value for the molar extinction coefficient of 5.96×10^3 at 277 nm. This contrasts with the value of 5.22×10^3 obtained by Praissman & Rupley (1968) from dry-weight determinations corrected for counterion content. It occurred to us that this considerable difference could arise if the wrong value for the nitrogen content of insulin was assumed. The amide nitrogen atoms of insulin are known to be labile (Sundby, 1962) and commercial preparations of insulin have a degree of heterogeneity that has been attributed to random loss of amide nitrogen (Carpenter & Chrambach, 1962; Mirsky & Kawamura, 1966). In an extreme case insulin could lose 6 amide nitrogen atoms/mol., thus decreasing its nitrogen content from 65 to 59 atoms/mol. (Sanger, 1960). Another potential source of error is the small but measurable amount of proinsulin that commercial preparations of insulin are known to contain (Schmidt & Arens, 1968).

We have evidence that the most highly purified samples of insulin available from different commercial sources vary sufficiently in their amide nitrogen content to account for the high extinction coefficient obtained by Herskovits (1965), and we have established that the molar extinction coefficient for insulin is 5.53×10^3 at 277.5 nm. We have also measured the molar extinction coefficient of tyrosine and find it to be 1.38×10^3 at 274.1 nm.

Ten-times-recrystallized bovine and porcine insulin from Novo Industri A.S (Copenhagen, Denmark), two different samples of six-times-recrystallized bovine insulin from Boots Pure Drug Co. (Nottingham) and crystalline bovine insulin from Burroughs Wellcome Ltd. (Beckenham, Kent) were all generously supplied by the Companies concerned. L-Tyrosine was purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.) A.R. $(\text{NH}_4)_2\text{SO}_4$ was dried and stored over P_2O_5 in an evacuated desiccator. Standard solutions of known nitrogen concentrations were made up by accurately weighing and diluting this dried salt.

One sample of Boots bovine insulin was purified by elution from Sephadex G-50 with M-acetic acid and the small peak due to proinsulin was discarded (Schmidt & Arens, 1968). Some samples of insulin were heated on a boiling-water bath for 45 min. with M-NaOH to remove amide nitrogen (Tristram, 1949). To confirm that this did cause the removal of all amide groups we analysed a sample of Wellcome insulin shown by nitrogen analysis to contain 5.7 amide groups/mol. Samples before and after treatment with alkali were hydrolysed with 10M-NaOH at 110° for 16 hr. Previous experience has shown that phenylalanine is quantitatively released under these conditions but that the yield of tyrosine is consistently low. Phenylalanine, tyrosine and NH_3 were separated and determined by using an automatic amino acid analyser (Technicon Instruments Ltd.) and were found to be present in the molar proportions 3.00:3.81:7.08 for the untreated insulin and 3.00:3.84:1.51 for the alkali-treated insulin. The difference between these two values for NH_3 released on hydrolysis agrees within experimental error with the number of amide groups calculated from the nitrogen analysis. All samples of insulin treated with M-NaOH are therefore referred to as 'deshexamido insulin' (Sundby, 1962).

Samples for nitrogen determination were dissolved in 10mM-HCl at concentrations of about 250 $\mu\text{g./ml.}$ Accurately measured volumes of these solutions were transferred to Pyrex glass vials, so that each vial contained between 50 and 100 $\mu\text{g.}$ of N. These samples were then processed by the method of Jacobs (1964), in which nitrogen is first converted into NH_3 by digestion with 18M- H_2SO_4 and the NH_3 is then allowed to react with ninhydrin. The colour produced is measured at 570 nm. and compared with the colour produced from a standard source of nitrogen. Errors can arise in converting the sample nitrogen into NH_3 , and again in sampling and processing the NH_3 solution.

To assess these errors we measured the colour produced in six replicates from each of six NH_3 solutions obtained by digesting samples from a single solution containing nitrogen. From two such experiments we showed that a greater error arises in sampling from the initial solution than in

Table 1. *Molar extinction coefficients of various insulins and of tyrosine*

The molar extinction coefficients are given as means $\pm$ S.E.M., with the number of N atoms/mol. assumed in calculating the molar extinction coefficient in parentheses. The insulin concentration was obtained from the absorption spectrum by taking ϵ to be 5.53×10^3 , and the number of amide groups/mol. was calculated from the concentration and the total nitrogen content.

Source of insulin	$10^{-3}\epsilon$	$\lambda_{\max.}$ (nm.)	Calc. no. of amide groups/mol.
Boots bovine II ('deshexamido')	5.55 ± 0.04 (59)	277.5	—
Novo bovine ('deshexamido')	5.51 ± 0.04 (59)	277.5	—
Wellcome bovine	5.55 ± 0.03 (65)	277.5	5.7
Novo bovine	5.75 ± 0.03 (65)	277.5	3.5
Boots bovine II	5.84 ± 0.03 (65)	277.5	2.6
Boots bovine II (Sephadex-purified)	5.81 ± 0.03 (65)	277.5	2.8
Novo porcine	5.90 ± 0.03 (65)	277.5	1.9
Boots bovine I	6.06 ± 0.03 (65)	277.5	0.3
Boots bovine II ('deshexamido')	6.12 ± 0.05 (65)	277.5	
Novo bovine ('deshexamido')	6.06 ± 0.05 (65)	277.5	
Tyrosine, pH 2	1.38 ± 0.02 (1)	274.1	
Tyrosine, pH 13	2.40 ± 0.03 (1)	292.1	

sampling and processing the NH_3 solution. Therefore in all nitrogen determinations we took quadruplicate samples from the unknown solution and processed the resulting NH_3 solutions in duplicate. Each determination of nitrogen is thus based on a mean of eight colorimetric measurements.

The u.v.-absorption spectra were measured with a Cary model 14 recording spectrophotometer with cells of 2mm. light-path. Spectra were recorded from 400 to 260nm. so that corrections could be made for Rayleigh light-scattering (Wetlaufer, 1962). Spectra of the insulin solutions used for nitrogen determinations were recorded without further dilutions, and for reasons already described (Garratt & Walson, 1967) no attempt was made to measure the spectrum of insulin in which the

phenolic groups had ionized. Three different dilutions of the tyrosine solutions used for nitrogen determinations were made with either 10mm-HCl or 100mm-NaOH so that the extinction as measured lay between 0.4 and 0.8. The spectra were recorded and the equivalent extinctions of the undiluted samples were calculated. All small volumes were measured with a syringe micro-burette (Micrometric Instrument Co., Cleveland, Ohio, U.S.A.).

The values obtained for the extinction coefficient of the different insulin samples and of tyrosine are shown in Table 1. The purification and removal of proinsulin by Sephadex-gel filtration made no significant difference. The values obtained from the two samples of 'deshexamido insulin' agree within the error of the method. Using the mean of these two values as the true molar extinction coefficient we have calculated the number of amide groups/mol. for each insulin sample. Always this falls within the acceptable range 0-6.

We believe that the value of 5.53×10^3 is the most accurate measure available for the molar extinction coefficient of insulin. It is almost exactly the calculated value obtained by summing the extinctions of the component amino acids. This makes insulin somewhat unusual since in most proteins the $\epsilon_{\text{obs.}}/\epsilon_{\text{calc.}}$ ratio is greater than 1:1 (Wetlaufer, 1962). It is of course possible that lability of amide groups in other proteins as well as insulin has led to errors in determining their molar concentrations and thus to errors in calculating their molar extinction coefficients.

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