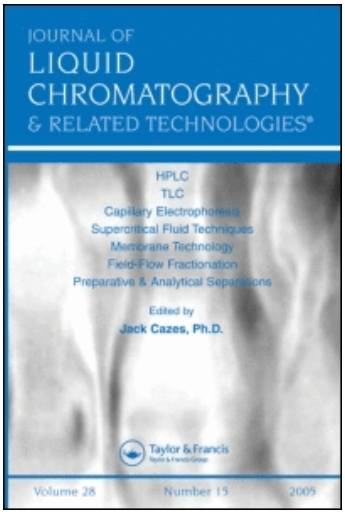

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

A Simple Liquid Chromatographic Method for Analysis of Insulin and Its Derivatives

Y. Pocker[a]; Subhasis B. Biswas[a]
[a] Department of Chemistry, BG-10, University of Washington, Seattle, Washington

To cite this Article Pocker, Y. and Biswas, Subhasis B.(1982) 'A Simple Liquid Chromatographic Method for Analysis of Insulin and Its Derivatives', Journal of Liquid Chromatography & Related Technologies, 5: 1, 1 — 14
To link to this Article: DOI: 10.1080/01483918208068814
URL: http://dx.doi.org/10.1080/01483918208068814

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(1), 1-14 (1982)

A SIMPLE LIQUID CHROMATOGRAPHIC METHOD FOR ANALYSIS OF INSULIN AND ITS DERIVATIVES

Y. Pocker and Subhasis B. Biswas
Department of Chemistry, BG-10
University of Washington
Seattle, Washington 98195

ABSTRACT

This communication reports on our recent application of size-exclusion high performance liquid chromatography for the rapid analysis of insulin and monitoring the progress of its enzymatic hydrolysis. Insulin, a protein hormone, exhibits a complex association behavior in solution. Consequently, analysis of insulin and its derivatives by conventional methods is difficult. We are able to analyze insulin and some of its derivatives, and also to study the trypsin-proteolysis of insulin by using size-exclusion high performance liquid chromatography under the denaturing condition of 7 M urea. This analytical method is rapid, reproducible, selective and simple.

INTRODUCTION

High performance liquid chromatography (HPLC) is a versatile and convenient tool for the analysis and purification of organic and biological compounds (1). Recently HPLC techniques have been extended to biological macromolecules (2-4). An important development in the field of protein analysis is the design of columns which separate proteins and polypeptides as a function of their relative size. Although the general principles of operation of such columns are well known, there

is an urgent need for their detailed and extensive characterization in order to make them valuable tools for protein analysis. Studies in this laboratory have been recently directed towards developing different elution conditions for the analysis of various native and semisynthetic proteins.

This report is based on our recent studies of insulin and its derivatives. Insulin is a protein hormone of molecular weight 5734 (bovine). It exhibits a complex association behavior in solution, where it exists as an equilibrium mixture of monomers, dimers, tetramers and hexamers (5-11). As a result, under native conditions, size-exclusion HPLC (SEHPLC) analyses result in broad chromatograms. To eliminate this problem we have used the denaturing conditions of 7 M urea. Urea is an inexpensive, easily available denaturing agent which can be removed with ease from the column by washing it with distilled, deionized, degassed water. Therefore, urea is a choice denaturing agent to be used with columns where reproducible resolution and selectivity over long periods are desired.

Several groups have reported separation of polypeptides and small proteins by reversed phase high performance liquid chromatography (RPHPLC) (2,3). By comparison, the SEHPLC is more advantageous because the separation is based mainly on size and hence the chromatogram is more predictable; furthermore, molecular weight and subunit structure determinations are also possible. The SEHPLC technique will also provide simpler means of isolating mono component proteins from natural and recombinant DNA sources especially with the development of preparative SEHPLC column.

EXPERIMENTAL

Instrumentation

The HPLC system used in this study is a Waters High Performance Liquid Chromatograph, consisting of an M600A solvent de-

livery system and a U6K universal liquid chromatographic injector, coupled to a Waters Model 440 Ultraviolet absorbance detector. The output of the absorbance detector is attached to a Honeywell Electronik-19' Recorder. The columns used are a μBondapak C-18 reversed phase column and a Waters I-125 protein separation column. Sample injections are performed with a Hamilton microlitre syringe. Solvents and buffer solutions are filtered in a millipore filter just prior to use. Values of pH are determined on a Beckman Research pH meter using a Cole Parmer combination glass electrode.

Materials

Insulin. Bovine Zn-insulin was purchased from Sigma Chemical Co. (Lot No. 47C - 0264) and was also obtained as a gift of Dr. Ronald E. Chance of Lilly Research Laboratories, Indianapolis, Indiana. Zn-free insulins were prepared by the method of Goldman and Carpenter (8). TPCK treated trypsin was purchased from Worthington Biochemical Corporation.

Highly purified insulins were prepared by chromatography of Zn-free insulin on a Sephadex G-50 (superfine) column (2.5 cm x 90 cm) using 10% acetic acid solution as eluant.

Urea was purchased from Bio-Rad Laboratories (Electrophoresis purity reagent grade). All urea solutions were prepared with distilled, deionized, degassed water and analytical reagent grade tris (recrystallized) and HCl.

The present communication also demonstrates a unique exploitation of this technique for the study of proteolysis. Thus we were able to monitor the specific hydrolytic cleavage of insulin by trypsin using SEHPLC and to determine, not only the optimum conditions for maximum proteolysis, but also ways of avoiding unnecessary precipitation during the course of this reaction.

Procedure

(i) Column: A Waters associates column I-125 for the separation of proteins having a molecular weight in the range of between 2000 - 80000 was used for this study. The column was stored in methanol. The change from methanol to urea buffers was achieved by washing in the sequence methanol, methanol water, water, Tris-HCl buffer, urea buffer. After analysis, the column was stored in methanol by changing the solvents in the reverse order. Caution: Since the column is highly pH sensitive the solution must be kept below pH 8.0 and at the end of the analysis an extensive wash (2 ml/min for 60 min) of the column with deionized water is absolutely necessary in order to maintain a smooth and reproducible performance. The column should be stored in methanol or methanol water (1:1).

(ii) Preparation of Mobile Phase and Sample: The mobile phase used for these analyses was 7 M urea in 0.01 M Tris-HCl/0.05 M NaCl buffer of pH 7.0 at 25°C. The buffer was prepared by adding 0.01 moles of Tris and 0.05 moles of NaCl per liter of 7 M urea solution, which was prepared with deionized water and further deionized by passing through an anion exchange column. The final pH was adjusted with HCl. The buffer solution was filtered and degassed. Methanol was filtered and degassed before use. The solutions of the samples were prepared in the mobile phase buffer.

(iii) Trypsin Modification Studies: Trypsin (TPCK-treated) cleaves insulin at B_{22}-Arg and B_{29}-Lys to form desoctapeptide-(B_{23-30})-insulin, a heptapeptide-(B_{23-29}) and B_{30}Ala. Zn-free bovine insulin was dissolved in 0.0025 M $CaCl_2$ at pH 9.5 and 37°C. TPCK-trypsin was added to the insulin solution at an enzyme-substrate ratio 1:20. The solution was incubated at 37°C using a thermostat, and the pH was maintained at 9.5 ± 0.02 by employing a PDP/8E computer controlled pH-state and titrating with 0.1 N NaOH. Aliquots of 200 µl were taken at different time

intervals and frozen immediately in dry ice-acetone bath. The aliquots were lyophilized and, just prior to HPLC analysis, were dissolved in 200 µl urea buffer. Aliquots of 20 µl, containing ca. 20 µg of total protein, were then injected in the column for analysis. The peaks were monitored at 254 nm.

RESULTS

The SEHPLC chromatogram of a bovine insulin preparation obtained from Eli Lilly Co. is shown in Figure 1a and compared with the SDS-PAGE patterns shown in Figure 1b. The SEHPLC chromatogram indicates several minor impurities, whereas, electrophoresis pattern shows only one minor impurity. To purify this insulin preparation, it was dissolved in 0.25 N HCl and precipitated with 18 volume acetone. The precipitate of insulin hydrochloride was collected and washed with acetone. The purified insulin perparation was dried overnight under vacuum and was analyzed by SEHPLC. Figure 2 shows the chromatogram of purified insulin. This preparation contains only two very minor impurities. These very minor impurities, however, are not detectable by electrophoresis. These SEHPLC and SDS-PAGE analyses indicate that the SEHPLC technique is a powerful tool for protein analysis. The HPLC analysis has higher resolution and takes a few minutes in contrast to electrophoresis which takes several days. The retention time of insulin under the conditions used was 8.5 minutes. Retention times, peak areas and peak shapes were highly reproducible under the conditions used.

We applied this HPLC technique to monitor the trypsin cleavage of bovine insulin. When insulin is treated with trypsin, two major fragments are produced: desoctapeptide-(B_{23-30})-insulin (DOPI) and a heptapeptide (Scheme I). Insulin with a molecular weight ∿ 5750 and DOPI with a molecular weight ∿ 4900 are indistinguishable on our short column by HPLC analysis. However, the heptapeptide + octapeptide fragments which elute as a single peak,

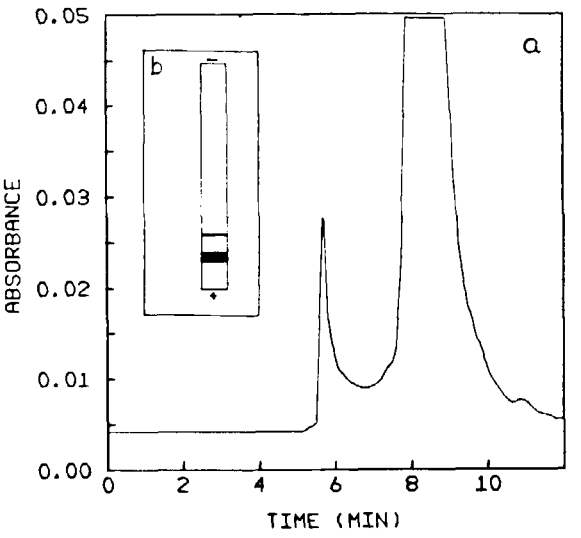

Figure 1. (a) The size exclusion high performance liquid chromatogram of insulin (a preparation obtained from Eli Lilly Co.). The conditions used were: buffer, 0.01 M Tris-HCl/0.05 M NaCl in 7 M urea; pH 7.0 at 25°C; protein quantity 150 µg; flow rate 1 ml/min. Protein was initially dissolved in mobile phase buffer and the peaks were monitored at 254 nm.

(b) The SDS-PAGE pattern of the same insulin preparation in 7.5% polyacrylamide gel stained with coomasie blue and destained in 7% acetic acid by diffusion.

Both Figure 1a and 1b indicate the major impurity but several minor impurities can be observed only in Figure 1a.

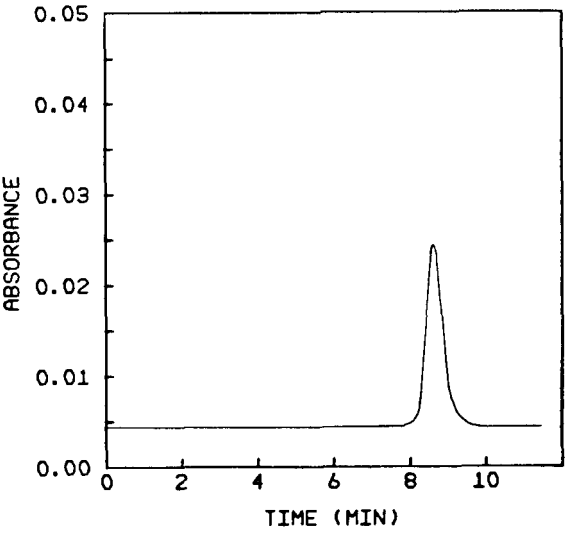

Figure 2. The size exclusion high performance liquid chromatogram of highly purified insulin under conditions described in Figure 1a and the protein was ∿ 20 μg.

Scheme 1

Bovine Insulin

↓ Trypsin (TPCK treated), pH = 9.5, 37°C

Desoctapeptide-(B_{23-30})-Insulin

+

Octapeptide-(B_{23}-B_{30}) + heptapeptide-(B_{23}-B_{29}) + Ala-(B_{30})

can be observed. Therefore, we used the peptide peak (hepta - peptide plus octa peptide) to monitor the trypsin digestion of insulin.

Aliquots were taken at different time intervals and analyzed. Figure 3 shows the chromatograms of the time-dependent analyses of the reaction mixture. Peak I (retention time = 4.25 min) is

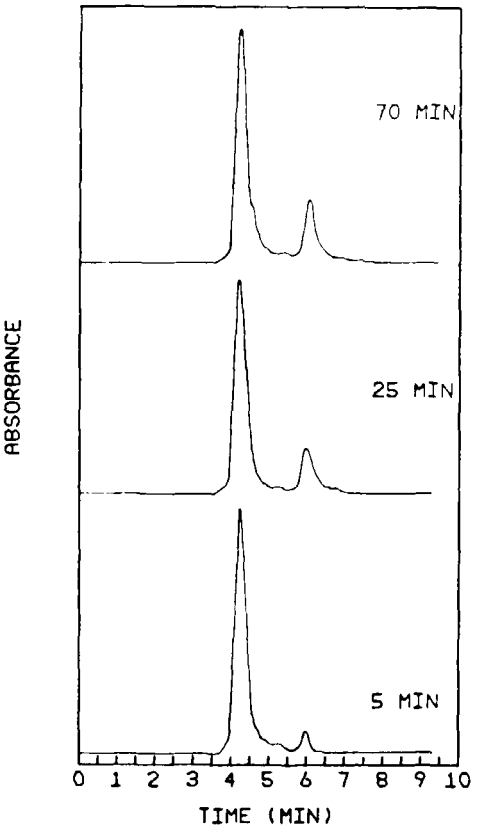

Figure 3. The SEHPLC monitoring of trypsin digestion of insulin at pH 9.5 and 37°C. The enzyme to substrate ratio was 1:20. A ca. 20 µg protein aliquot (taken at various time intervals as indicated) in the mobile phase buffer was injected and the chromatographic conditions were similar to Figure 1a except the flow rate was 2 ml/min. Peak I (with a retention time of 4.25 min) is the insulin + DOPI peak and Peak II (with a retention time of 6 min) is the heptapeptide peak.

made up of a mixture of insulin + DOPI and remains unchanged: however, Peak II (retention time = 6 min) consisting of octapeptide + heptapeptide increases with time. Peak II is a totally included peak and is absent at the start of the reaction but increases steadily with time. After one hour Peak II changes very slightly with time (Figure 4) and reaches a plateau. At 3.0 hours the reaction was terminated and lyophillized. A ca. 1 mg portion was dissolved in 100 µl urea buffer and injected in the column. The second half of Peak I and all of Peak II was collected, partially dialyzed and lyophillized. The samples were analyzed in the Aminoacid analysis laboratory in the Department of

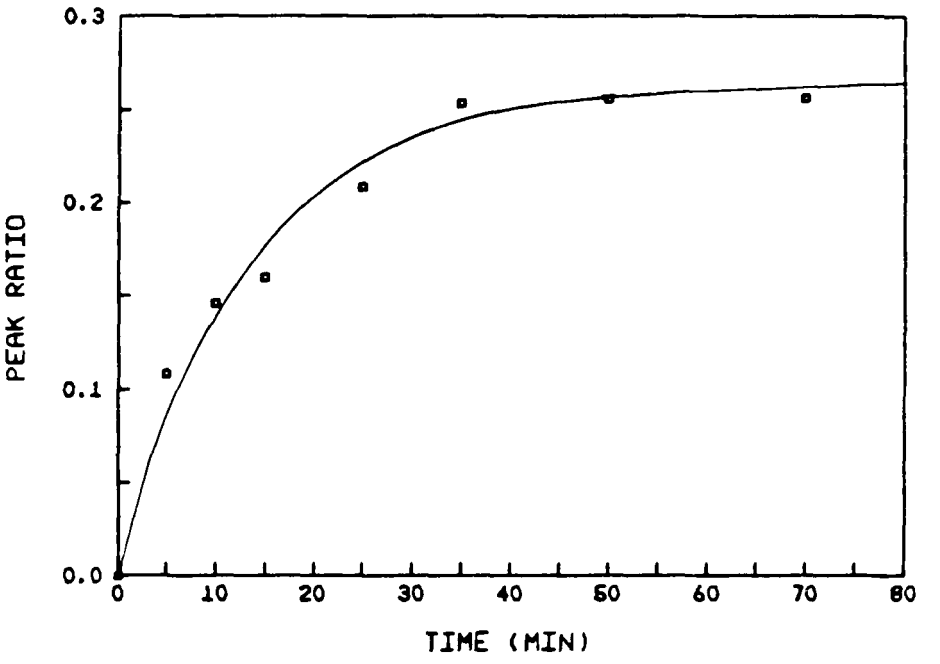

Figure 4. A kinetic profile of the progress of trypsin cleavage of insulin at pH 9.5 and 37°C using an enzyme to substrate ratio of 1:20. The ratio of Peak II/ Peak I (as shown in Figure 3) were plotted against the time at which the aliquot was taken.

TABLE I. Amino Acid Composition of Peak I and Peak II in Figure

	Peak I (DOP-Insulin)		Peak II (heptapeptide)	
Amino Acids	Theoretical	Experimental	Theoretical	Experimental
Asp	3	3.13		
Thr	0	0.12	1	1.03
Ser	3	2.81		
Glu	7	7.19		
Pro	0	0.16	1	1.00
Gly	3	3.05	1	1.00
Ala	2	1.99		
Cys	6	not determined		
Val	5	4.56		
ILeu	1	0.82		
Leu	6	5.50		
Tyr	3	2.83	1	0.6
Phe	1	1.05	2	1.96
His	2	2.00		
Lys	0	0.14	1	1.19
Arg	1	1.08		

Biochemistry at the University of Washington. The results are shown in Table I, which proves that Peak I is DOPI and Peak II is the heptapeptide. The results are in excellent agreement with those of previous workers (12). The pepsin cleavage of insulin at pH = 2.5 and 3°C was monitored in the same fashion (data not shown).

The calibration of the column with respect to molecular weight of proteins was performed using ribonuclease, carbonic anhydrase, horse liver alcohol dehydrogenase, haemoglobin, and bovine serum albumin (BSA). Blue dextran of m.w. 2×10^6 dalton was used to determine the void volume of the column. The plot of log M_w vs (Ve-Vo)/Vo (Ve = elution volume, Vo = void volume) is presented in Figure 5. This plot shows that the use of denaturing conditions of 7 M urea does not alter the linearity of the size exclusion column I-125. It might be possible that the interaction of this column with solute in 7 M urea is hydrophobic in nature. This is not true in the cases we studied. In normal conditions the size of a protein molecule depends on its tertiary structure in addition to molecular weight, consequently the column will not be linear with respect to Mol.wt. alone. However, under denaturing conditions, the tertiary structure will be insignificant as most proteins will acquire a more coiled state, whose size will be a simple function of the molecular weight. As a result the column will be linear with respect to the sizes and Mol.wt. of the proteins.

Discussion

The method described in this paper uses the SEHPLC system and has several advantages. The technique is rapid, exhibits moderately high resolution and provides a simple method for analyzing a complex mixture of proteins. It can be used for the analysis of protein mixtures, and for the isolation, purification and determination of molecular weights of proteins using few micrograms to milligram quantities of proteins.

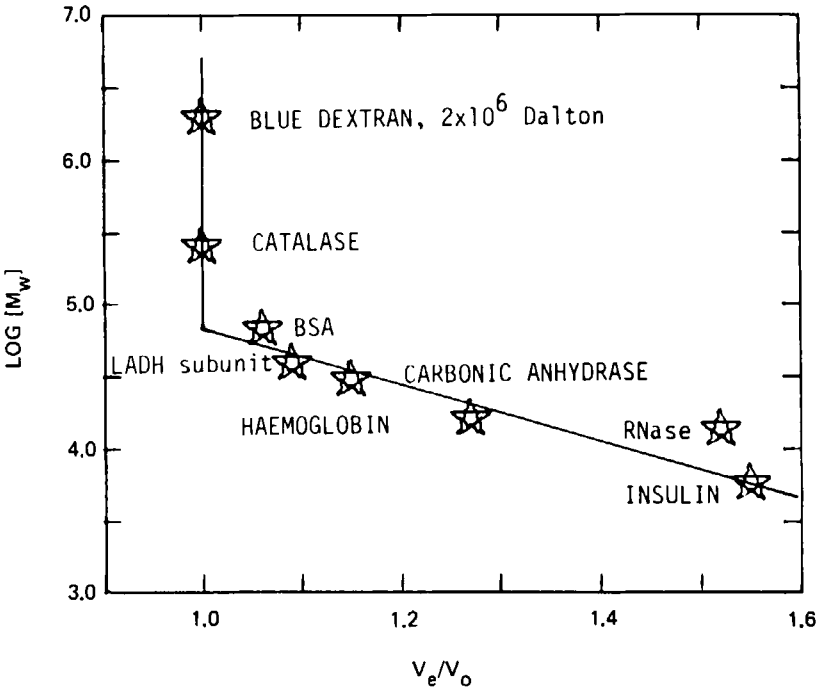

Figure 5. The molecular weight calibration of the I-125 protein-separation column. Chromatographic conditions were as described in Figure 1a and ∿ 20 µg of proteins were injected.

Work in progress attempts to develop a high protein recovery technique suitable for isolation and purification of valuable proteins. Although, at present, only small quantities of protein can be purified by this technique, in the future it will be possible to purify larger quantities of proteins when preparative size-exclusion columns will be available. Large-scale purification of single-component proteins will be extremely valuable for treating hypersensitive insulin-dependent diabetics (13,14).

We have successfully monitored trypsin and pepsin proteolysis of insulin by the SEHPLC technique, and have further demon-

strated that the nomitoring of similar reactions is very fast and convenient and extremely useful in protein-sequencing. This technique will also be useful in monitoring the isolation and purification of proteins by conventional methods. In the light of these preliminary observations this technique seems extremely promising and worthy of further investigations.

ACKNOWLEDGEMENT

We thank Dr. Mark O. Lively of the Department of Biochemistry of the University of Washington for numerous aminoacid analyses.

This work was supported in part by a grant from the National Science Foundation and by a Grant-in-Aid from Sigma Xi, The Scientific Research Society.

ABBREVIATIONS USED

HPLC	High performance liquid chromatography
SEHPLC	Size-exclusion high performance liquid chromatography
RPHLC	Reversed phase high performance liquid chromatography
DOPI	Desoctapeptide-(B_{23-30})-Insulin
PAGE	Polyacrylamide gel electrophoresis
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophorsis
PPCK	L-(tosylamido 2-phenyl)ethyl chloromethyl ketone

NOTE ADDED IN PROOF

After the completion of this manuscript, similar observations have been reported in regard to the SEHPLC of other proteins under denaturing conditions (Imamura, T., Konishi, K., Yokoyama, M. and Konishi, K. (1981). J. Liq. Chromatogr. 4(4), 613).

REFERENCES

1. Snyder, L.R. and Kirkland, J.J. in "Introduction to Modern Liquid Chromatography", Wiley-Interscience, New York, (1974).
2. Rivier, J., Spiess, J., Perrin, M. and Vale, W., in Biological/ Biomedical Applications of Liquid Chromatography II, Ed. Gerald L. Hawk, 12, 223-241, Marcel Dekker, Inc., New York, (1978).
3. Rivier, J.E., (1978) J. Liq. Chromatogr., 1(3), 343
4. Schroeder, R.R., Kudirka, P.J., and Joren, Jr., E.C., (1977) ibid, 134, 83.
5. Fredericq, E., and Neurath, H., (1950) J. Am. Chem. Soc., 72, 2684.
6. Fredericq, E., (1953) Nature (London) 171, 570; (1956) Arch. Biochem. Biophys., 65, 218.
7. Jeffrey, P.D., and Coates, J.H., (1965) Biochim. Biophys. Acta, 109, 551; (1966) Biochemistry, 8, 4108.
8. Goldman, J., and Carpenter, F.H., (1974) Biochemistry, 13, 4567.
9. Biswas, S.B., and Pocker, Y., 34th Northwest Regional Meeting of the American Chemical Society, June 13-15, 1979, Richland, Washington, Abstr. 92, American Chemical Society, Washington, D.C.
10. Pocker, Y., and Biswas, S.B., (1980) Biochemistry, 19, 5043.
11. Pocker, Y., and Biswas, S.B., (1981) Biochemistry, 20, 0000.
12. Bromer, W.W., and Chance, R.E., (1967) Biochim. Biophys. Acta, 133, 219.
13. Jorpes, H.E., (1949) Arch. Int. Med., 83, 363; (1950) Acta Med. Scand. (Suppl. 239), 138, 313.
14. Nichols, M.N., (1955) J. Pediat., 46, 314.