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# GRADIENT ELUTION PAPER CHROMATOGRAPHY OF CRYSTALLINE INSULIN

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Paper strip chromatography of commercial samples of insulin using Whatman No. 1. filter paper and ten serial dilutions of 3 M sodium chloride in veronal buffer pH 8.6 as eluent has been presented.

There is much evidence in literature about the inhomogeneity of commercial insulin samples. Numerous chromatographic methods have been proposed either for the separation of insulin from crude pancreas extracts or for determining impurities associated with insulin in therapeutically serviceable preparations.

*Porter* (1) achieved to isolate insulin from a crude protein mixture using a silane treated silica gel column. *Porath and Li* (2) used, for the same purpose, a charcoal column successfully.

However, many workers have tried to separate various protein mixtures containing insulin by paper chromatography. *Robinson and Fehr* (3) were able to make a quantitative estimate of insulin in mixtures with protamine. *Grodsky and Tarver* (4) isolated insulin from the liver proteins in the similar way, and *Light and Simpson* (5) described a paper chromatographic technique for detecting insulin in pancreas extracts.

In several communications (6, 7, 8, 9) we reported that the protein components of a mixture could be chromatographically separated by the application of consecutive elution with salt solutions of increasing ionic strength.

In this communication we wish to demonstrate some general features of this procedure when applied to the separation of insulin from biological inactive impurities as found in commercial samples of insulin.

## EXPERIMENTAL

*Materials.* Several insulin preparations were used for experiments.

1. International standard for insulin, 24 IU/mg; National Institute for Medical Research, Mill Hill, London; 50 per cent porcine and 50 per cent beef pancreas insulin.
2. Beef pancreas insulin, ten times recrystallized from citrate buffer pH 6.1; 24 IU/mg; Pliva, Zagreb.
3. Crystalline beef pancreas insulin, Organon; 0.4% zinc, 2.6% $H_2O$; dry material 25.6 IU/mg.
4. Crystalline insulin, Hoechst; a ten years old sample.
5. Crystalline insulin, Pliva; porcine, four times recrystallized, 22.7 IU/mg.

*Paper chromatography.* For many of the chromatograms Whatman No. 1. filter paper strips 25×300 mm were used. Schleicher-Schüll papers No. 2024a and 2042 showed no significant differences.

The original elution procedure for paper strip ascending chromatography has been already published elsewhere (8). All chromatograms were developed by ten serial dilutions of sodium chloride, so that the total dilution range was 1 : 1000. Dilution factor $f = \left[\frac{C_{10}}{C_1}\right]^{\frac{1}{9}} = 2.15$; 3 M of sodium chloride in a veronal – veronal sodium buffer pH 8.6 (1.84 g veronal – 10.30 g veronal sodium in 1000 ml $H_2O$) were used as a stock solution. Other dilutions were prepared with the same veronal buffer.

The optimal concentration of insulin was 17 mg/ml of a N/100 HCl, and was found by experience.

The chromatograms were stained with 0.1 per cent bromophenolblue in methanol for 30 minutes and washed in 5 per cent aqueous acetic acid.

Bioassays of insulin fractions for hormone activity were performed at Pliva laboratories by the mouse convulsion test (with 24 mice per assay). The insulin was eluted from the chromatograms with 10 per cent sodium chloride in N/1000 hydrochloric acid. The control group of mice was tested with the eluant obtained from the correspondent portion of a developed filter paper without insulin.

Paper electrophoresis was performed by the apparatus and technique described by *Cremer and Tiselius* (10). Veronal – sodium veronal buffer pH 8.6; $\mu = 0.05$; 2.2 mA; 160 V; 16 h; or 33 per cent acetic acid pH 1.7, 0.6 mA 110 V, 8 h were used.

## RESULTS AND DISCUSSION

The zone electrophoresis of porcine insulin (22.7 IU/mg) in veronal – veronal sodium buffer (pH 8.6) or acetic acid (33 per cent, pH 1.7) always revealed two bands. The insulin samples of high hormone activity

(24–27 IU/mg) showed, under the same experimental conditions, one component only.

The chromatography (Fig. 1.) of crystalline insulin (100 mg in 10 ml N/100 HCl) on the column (3×10 cm) prepared from shreded filter paper revealed two peaks indicating two components produced by acid catalysis. The third component could not be eluted with one per cent sodium chloride and remained on the top of the column.

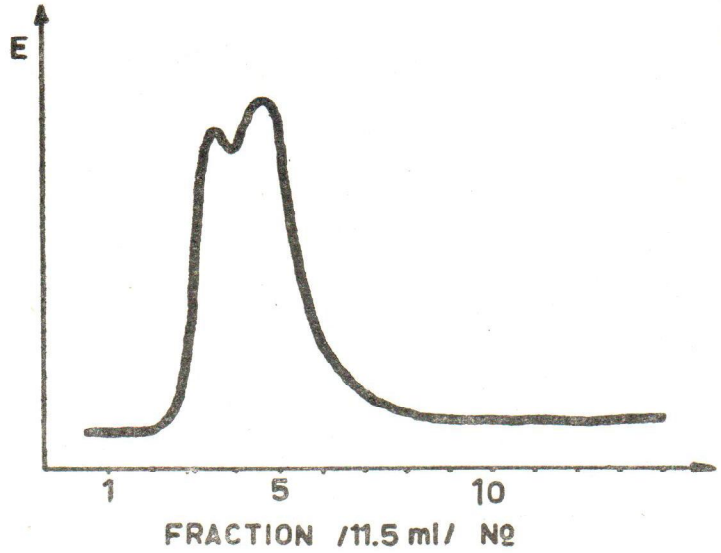


Fig. 1. Chromatographic separation of 100 mg porcine insulin (22.7 IU/mg) on column (shreded Whatman No. 1. filter paper). The column was developed by 1 per cent aqueous sodium chloride: LKB Uvicord absorptiometer, nickel sulphate liquid filter 2537 Å.

The paper chromatography using the upper phase of system n-butanol-acetic acid – 0.1% sodium chloride (3 : 1 : 3), or complex solvents comprising a single phase n-butanol – water – acetic acid (6 : 3 : 1.2 or 6 : 4 : 1.52), according to the proposition of *Grodsky and Tarver* (4), revealed one insulin band only when a fresh prepared insulin solution was used. The lower potency insulin samples showed one additional biologically inactive component on the application spot. After a few days all acidic insulin solutions (HCl N/10 or N/100) were practically transformed and showed two biologically active insulin spots with considerable tailing.

The elution chromatography was performed with ten serial dilutions of sodium chloride (3 M) in veronal – veronal sodium buffer pH 8.6 (1.84 gr veronal – 10.30 gr veronal sodium in 1000 ml $H_2O$) in the total

range of concentrations 1 : 1000. Each centimeter of the chromatogram was developed with increasingly concentrated salt solutions. All insulin samples of high hormone activity showed only one band correspondent to one insulin fraction biologically active. The porcine insulin of lower potency showed another biologically inactive band on the application spot (Fig. 2.)

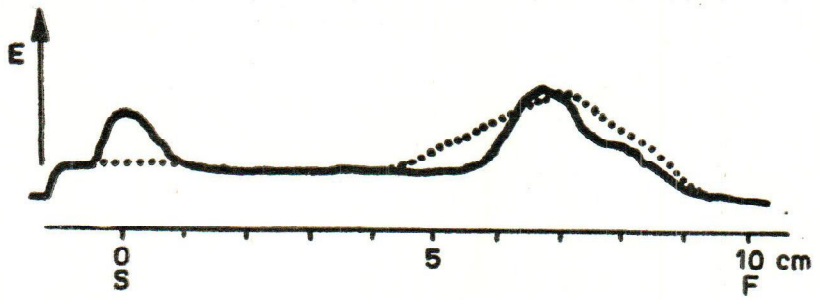


Fig. 2. Photometric evaluation of paper chromatogram developed with ten serial dilutions of sodium chloride gradient $3 \times 10^{-3}$ to 3 M in veronal veronal sodium buffer pH 8,6. ———— porcine insulin 22,7 IU/mg. ..... International standard for insulin, National Institute for Medical Research, London, 24 IU/mg. Spinco Analytrol model RB; interference filter 500 mμ; bromophenolblue.

Preliminary quantitative determinations of protein impurities in insulin by the photometric evaluation of bromophenol – stained chromatograms, using Spinco Amalytrol model RB and 500 mμ interference filter were satisfactory.

*Chrambach and Carpenter* (11) found that commercial preparations of crystalline insulin could be chromatographically resolved into several biologically active components. These insulin transformation derivatives were produced by treating homogeneous insulin with hydrochloric acid for a long time as it occurs during a preparation procedure or chromatography with acid solvents at room temperature. All isolated insulin fractions showed hormone activity. The transformation reaction was accompanied by the liberation of ammonia.

The paper chromatography procedure which we used for the separation of insulin from its contaminants, was the application of Whatman No. 1. filter paper and the elution of protein components with a high number of successively increasing salt concentrations, avoiding organic or acidic solvents which may cause denaturation of insulin.

The results of our experiments confirm that some commercial samples of insulin could be chromatographically resolved into hormone active and inactive components. Highly purified speciments of insulin showed one biologically active component only.

No denaturation of insulin was observed during chromatographic procedure using salt solutions for elution of adsorbed protein from the paper fibers.

Since the hormone activity of commercial insulin varies from 22–27 IU/mg it is reasonable to assume that some inactive protein contaminants must be present. The separation of bovine insulin from the mixture with porcine was not achieved either by salt concentration gradient chromatography or by the complex butanol – acetic acid solvent.

Because of the high cost of the biological assay of insulin, our further investigations will be adapting the described chromatographic method for the quantitative determination of impurities in commercial insulin samples.

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*Sadržaj*

## KROMATOGRAFIJA INSULINA NA PAPIRU

Prikazana je kromatografija na papiru kupovnih uzoraka kristaliničnog inzulina, upotrebom filtrir-papira Whatman br. 1. i razvijanjem kromatograma nizom otopina natrijeva klorida u rastućem gradijentu koncentracija (0,003 – 3 M) u veronalskom puferu pH 8,6.

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