aldehyde. Vitamin A₁ aldehyde was the predominant form in all except the freshwater perch, pike, and trout and the anadromous salmon, which contained predominantly vitamin A₂ aldehyde.

3. Values are given for the content of total vitamin A₁ and A₂ esters and alcohols, the percentage of the A₂ form and the carotenoid content of the eggs or ovaries.

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The Assay of Insulin Solutions by Paper Chromatography

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The quantitative determination of insulin by paper chromatography has been the subject of numerous publications in recent years. Using the system butanol-acetic acid-water, in varying proportions, Light & Simpson (1956*a, b*) and Grodsky & Tarver (1956) separated insulin from crude pancreatic and liver proteins, Robinson & Fehr (1952) developed a method for the estimation of insulin and protamine in protamine zinc-insulin preparations, and Bouman & Homan (1958) determined insulin potency by radial chromatography. Fenton (1959) described a method for the chromatographic assay of crystalline insulin by using the solvent system butan-2-ol-1% acetic acid (1:1, v/v). This method has since been used in these and other Laboratories, and the procedure has been extended to the estimation of insulin in solutions of composition similar to insulin injection, B.P., for which the present method describes a four-point slope ratio assay. For reasons given in the Discussion it is unlikely that these methods *in vitro* will ever be used in place of the orthodox biological assays of insulin, but attention has once again been drawn

(Rückert & Schöne, 1960) to their value for production control and experimental purposes. These authors reported a simplified chromatographic procedure by which they obtained results in close agreement with those of biological assay, but they were unable quantitatively to assay residual insulin in solutions during thermal-decay tests.

EXPERIMENTAL

Materials and methods

Protein precipitant. This was a saturated aqueous solution of picric acid.

Acidified acetone reagent. Prepared by diluting 2 ml. of conc. HCl to 1 l. with acetone B.P.C. (Thos. Morson and Son Ltd.).

Developing solvent, eluent, volumetric vessels, and protein stain. These were used as described by Fenton (1959), but the following procedure must be adopted in preparing the protein stain. Bromocresol green (0.425 g.) (Hopkin and Williams Ltd.) was dissolved in 125 ml. of 0.1 N-NaOH by sprinkling the dye on the surface of the alkali and stirring. The solution was then added to 2 l. of 0.1 N-acetic acid and the mixture filtered. The reaction should be pH 3.6; the

staining solution may be used for a long time if it is filtered at intervals.

Insulin standards and samples were applied to chromatography papers with an Agla micrometer syringe (Burroughs Wellcome and Co. Ltd.), which was cleaned by washing with water, then dried with acetone when changing from one solution to the next. All precipitates were collected by centrifuging for 10–15 min. at 2000 rev./min., on an M.S.E. medium-sized centrifuge.

Standards. A stock of four-times-recrystallized zinc insulin was distributed in approx. 20 mg. amounts in hard-glass tubes and dried *in vacuo* over P_2O_5 to a moisture content of approx. 1.6%. The tubes were flame-sealed and stored at 2°. The potency of the standard was determined chromatographically (Fenton, 1959) in terms of the Fourth International Standard for insulin.

Solutions of working standard of potency approx. 40 i.u./ml. were prepared in duplicate and in sufficient quantity to last for one week's assays by dissolving accurately weighed amounts of the crystalline insulin in 0.05N-HCl containing 0.3% *o*-cresol and 0.9% NaCl. The precise potencies were calculated to four significant figures. A four-place analytical balance and grade A volumetric glassware were used throughout; the standard solutions were stored at 2°. Subsequently it was convenient and economical to prepare standard solution in bulk, determine the potency with reference to the crystalline standard and store the solution frozen at -30° for daily use. The justification for this procedure is dealt with in the presentation of results.

Procedure

Desalting and concentration of samples. Two 5 ml. portions of standard solution (40 i.u./ml.) and two known volumes of test sample were pipetted into 100 ml. centrifuge tubes. The precise volumes of test solution were chosen from the expected potency of the sample so that approx. 150–250 units were taken. Zinc-insulin suspensions and protamine zinc insulins were dissolved by stirring rapidly with a glass rod wetted with a trace of 5N-HCl and kept for 30 min. before sampling. Protein precipitant (2 vol.) was added with good mixing and the tubes kept for 30 min. The protein picrate precipitates were collected on the centrifuge and the clear supernatants discarded. The picrate precipitates were dissolved in 1–1.5 ml. of acidified acetone reagent, then the protein hydrochloride was reprecipitated from the solution by the rapid addition of an excess (60–70 ml.) of acidified acetone reagent.

The suspensions were stirred thoroughly with glass rods to ensure complete flocculation, covered with cellophan caps and kept for 30 min. The precipitates were collected by centrifuging, the supernatants rejected, and excess of acetone was evaporated from the precipitates by directing a gentle stream of air from a glass jet into the centrifuge tubes. The moist precipitates were dissolved in 5–10 drops of 0.05N-HCl, and each solution was transferred to a 1 ml. volumetric vessel by means of a capillary transfer pipette. The centrifuge tubes were washed repeatedly with portions (5–10 drops) of 0.05N-HCl, each wash was transferred to its volumetric vessel, 3–5 min. being allowed for the walls of the centrifuge tubes to drain between transfers, and the contents of all volumetric vessels were adjusted to the calibration mark and mixed thoroughly. Tests and standards were treated in the same way.

Chromatography. Four sheets of Whatman no. 1 chroma-

tography paper were cut, folded and marked out to dimensions described by Fenton (1959) and the concentrated samples and standards applied with a micrometer syringe. In this assay 16 spots were distributed between the four papers in the following Latin-square order:

Paper 1	S1	T1	S2	T2
Paper 2	T1	S2	T2	S1
Paper 3	S2	T2	S1	T1
Paper 4	T2	S1	T1	S2

where S1 = standard at low concn. level, S2 = standard at high concn. level, T1 = test at low concn. level, and T2 = test at high concn. level. The precise volumes to be expelled from the micrometer syringe were calculated so that approximately 1 unit of insulin per spot was delivered at each low level of test and standard, and about twice this amount at each high level. Since the diameter of each spot should not exceed 1 cm. (equivalent to about 10 μ l.), volumes in excess of this amount were applied in portion, each portion being allowed to dry at room temperature between applications.

For each complete assay the four papers were placed in one tank (height 15 in., base 11 in. $\times$ 8 in.), fitted with a retractable-rod assembly as described by Fenton (1959). The chromatograms were allowed to equilibrate for not less than 16 hr. (overnight), then developed for 7½–8 hr. during the following day. Subsequent stages were carried out as described by Fenton (1959), up to calculation of results.

Calculations. The following data were collected for each complete assay: potency of standard, P (i.u./ml.); calc. vol. (μ l.) of standard/spot, Z_1 (low level) and Z_2 (high level); calc. vol. (μ l.) of test/spot, Z_3 (low level) and Z_4 (high level). The mean extinctions of four eluates of Z_1 , Z_2 , Z_3 and Z_4 are Y_1 , Y_2 , Y_3 and Y_4 respectively. The calculated volumes were obtained by multiplying the volume applied per spot by V_1/V_2 , where V_1 is the volume of original sample or standard (ml.) and V_2 is the capacity (ml.) of the volumetric vessel.

If the results of an assay are expressed graphically (Fig. 1), the potency of the test sample can be calculated simply from the ratio of the volumes of test/standard which

Fig. 1. Data for chromatographic assay of sample of insulin solution of potency 30.4 i.u./ml., in terms of reference standard solution (stored frozen), of potency 39.87 i.u./ml. ●, Standard, $Z = 87.664 Y + 0.264$; ○, test, $Z = 112.635 Y + 0.7898$; - - - -, values of Y within range of observations. Volume was calculated in terms of original solution.

Table 1. *Data and calculation of relative potency of an insulin solution (see also Fig. 1)*

The use of a calculating machine for computing the data in this table made it convenient to retain several places of decimals throughout; values were rounded off at the end of the calculation.

Standard (frozen solution) of potency $P = 39.67$ i.u./ml.			Test sample		
$V_1 = 5$ ml.	$V_2 = 1.3202$ ml. for Z_1	$Y_1 = 0.213$.	$V_1 = 5$ ml.	$V_2 = 1.2539$ ml. for Z_3	$Y_3 = 0.170$.
	$V_2 = 1.3233$ ml. for Z_2	$Y_2 = 0.428$.		$V_2 = 1.2575$ ml. for Z_4	$Y_4 = 0.346$.
5 μ l. applied per spot for Z_1 .	$\therefore Z_1 = 5 \times \frac{5}{1.3202} = 18.9365$ μ l.		5 μ l. applied per spot for Z_3 .	$\therefore Z_3 = 5 \times \frac{5}{1.2539} = 19.9377$ μ l.	
10 μ l. applied per spot for Z_2 .	$\therefore Z_2 = 10 \times \frac{5}{1.3233} = 37.7843$ μ l.		10 μ l. applied per spot for Z_4 .	$\therefore Z_4 = 10 \times \frac{5}{1.2575} = 39.7614$ μ l.	
Regression coefficient for standard line,			Regression coefficient for test line,		
$b_s = \frac{37.7843 - 18.9365}{0.428 - 0.213} = 87.664$.			$b_t = \frac{39.7614 - 19.9377}{0.346 - 0.170} = 112.635$.		
$C_s = 18.9365 - \frac{0.213 (37.7843 - 18.9365)}{0.428 - 0.213} = 0.264$.			$C_t = 19.9377 - \frac{0.170 (39.7614 - 19.9377)}{0.346 - 0.170} = 0.7898$.		
Range of observation, $E_{95\% \text{ m.u.}}$			$\frac{[(Yb_t) + C_t] \cdot P}{(Yb_s) \times C_t} = \text{potency of sample (i.u./ml.)}$		
	(Y)	$(Yb_s) + C_s$		$(Yb_t) + C_t$	
0.20	0.20	17.797		23.317	
0.25	0.25	22.180		28.948	
0.30	0.30	26.563		34.580	
0.35	0.35	30.946		40.212	
			Geometric mean potency = 30.4 i.u./ml.		

give equivalent extinctions at any value of $E_{525\text{ m}\mu}$ within the range of observations indicated by the dotted lines. It is more precise, however, to tabulate the results as shown in Table 1, and calculate the regression coefficients for standard and test lines in terms of the linear equation $Z = bY + C$, by substitution of the data. Thus for standards:

$$b_s = \frac{Z_s - Z_1}{Y_s - Y_1} = \text{slope constant},$$

$$C_s = Z_1 - \frac{Y_1(Z_s - Z_1)}{Y_s - Y_1} = (\text{intercept}).$$

Coefficients b_t and C_t for the test line were derived from the same equations by substituting the test data and a number of potencies for test calculated as:

$$p (\text{i.u./ml.}) = \frac{[(Yb_t) + C_t]P}{(Yb_t) + C_t},$$

taking Y at different values of extinction within the range of observations. As may be expected, the several estimates of potency were distributed more closely about their geometric mean when the differences between C_s and C_t were small. When in any assay $C_s = C_t = 0$, a single potency estimate is obtained and it is superfluous to take more than one value of Y .

Alternative methods of calculation for a four-point assay may be found in standard works; for example, the method of Finney (1952), when applied to chromatographic assays designed on the Latin square, gave satisfactory evidence of validity and enabled fiducial limits to be estimated. For this purpose the ratios of high to low level, each of test and of standard applied per spot, were kept constant at precisely 2:1, in order to fulfil the conditions required by this method of calculation.

RESULTS

The quantitative efficiency of desalting and concentration was established by assaying dilutions of insulin solution containing salts and preservative and comparing the results with those obtained by direct assay (Fenton, 1959) of comparable salt-free dilutions.

The effects of storing standard solutions in the frozen state were investigated in two separate experiments. Table 3 shows the potencies of the contents of duplicate ampoules assayed in terms of the crystalline reference standard at fixed time intervals during 8 weeks' storage at -30° , with one extra freezing and thawing included in the design.

When the analysis of variance on the results (Table 4) revealed no evidence suggestive of deterioration, we investigated the effects of multiple freezing and thawing in the second experiment in which insulin solution (potency 40 i.u./ml.), contained in 60 sealed glass ampoules, was frozen by immersing in a mixture of industrial methylated spirit and solid carbon dioxide, then allowed to thaw at room temperature. Groups of 20 ampoules were isolated after 5, 15 and 30 complete cycles of

freezing and thawing and their contents assayed against the reference standard (crystalline insulin) as described in the experimental section. Table 5 shows the results. No evidence was found that the number of freezing cycles over the range 5–30 had any significant effect on potency. The overall variance of the 60 pooled results gave a standard

Table 2. *Recovery of insulin by chromatographic assay*

Solution A contained insulin and 0.05N-HCl only, with the required amount of solution applied directly to the papers by repeated addition of 10 μ l. portions; solution B contained insulin solution at pH 3, containing 0.9% NaCl and 0.3% *o*-cresol, chromatographed after desalting and concentration as described in the text. Theoretical potency was calculated from biological assay of the crystalline insulin from which the solutions were prepared.

Theoretical potency (i.u./ml.)	Potency by chromatographic assay (i.u./ml.)	
	A	B
42.0	42.0	43.6
36.5	36.9	36.6
32.0	31.5	32.0
25.5	26.0	24.8
21.0	21.0	22.4
12.5	10.5	11.1

Table 3. *Potency of insulin solutions assayed during storage at -30°*

Compositions of solution: insulin 40 i.u./ml.; NaCl, 0.9%; *o*-cresol, 0.3%; dil. HCl to pH 3. Solutions were stored in 10 ml. sealed glass ampoules.

Period of storage at -30° (weeks)	Potency of solution (i.u./ml.)			
	Single freezing and thawing (duplicate ampoules)		Double freezing and thawing (duplicate ampoules)	
0	40.0	—	40.0	—
1	41.5	38.5	39.9	—
2	39.8	41.8	40.9	40.0
3	40.0	40.1	39.7	39.8
5	42.4	42.2	42.5	41.8
8	41.2	40.0	40.6	40.4

Table 4. *Analysis of variance for data in Table 3 after transformation to log potencies (abridged table)*

Source of variance	Degrees of freedom	Mean square
Between times of testing	5	0.00029628
Between frequencies of freezing	1	0.00008932
Interaction, times and frequency	5	0.00000275
Between duplicates	11	0.00009367

deviation of $\pm 4.5\%$ per assay, compared with $\pm 4.3\%$ associated with the estimation of crystalline insulin reported by Fenton (1959).

The results of the chromatographic determination of 27 samples of insulin solution of composition corresponding to insulin injection, B.P., are summarized in Fig. 2. Statistical analysis of the results gave a very significant correlation ($r = 0.98$, D.F. 25, $P < 0.001$) between the results and those of biological assay on the same samples.

Table 5. *Effect of successive cycles of freezing and thawing on insulin solutions*

Composition of solutions: insulin, 40 i.u./ml.; NaCl, 0.9%; o-cresol, 0.3%; dil. HCl to pH 3. Solutions were stored in 10 ml. sealed glass ampoules.

No. of cycles of freezing and thawing	Potency (mean of 20 ampoules) (i.u./ml.)	Limits of error, $P = 0.05$ (i.u./ml.)
0	40.0	39.4-40.6
5	40.0	39.4-40.6
15	39.6	39.1-40.9
30	39.4	39.1-40.9

DISCUSSION

Comparisons between results obtained by chromatographic and biological assays on the same solutions have shown that the best agreement is obtained when, in the chromatographic determination, the amount of insulin present in the developed spots is the same for test and standard. This observation agrees with that of B. G. Overell (personal communication). The present design enables equivalent amounts of test and standard to be compared each at two working levels and fulfils the requirements of a slope ratio assay of the type in which two lines of different slope, intersecting at or close to the blank level, represent the conventional dose-response relationship. If b_s and b_t are estimates of the two regression coefficients, $R = (b_s/b_t)$ is an estimate of relative potency.

The response parameter is a spectrophotometric determination of eluate intensity, but the customary practice of referring experimental points to a fixed standard curve is not permissible because of the inherent errors introduced by positional effects of spots within papers, errors between papers

Fig. 2. Results of the chromatographic assay of 27 samples of insulin solutions compared with the results of biological assay. The regression line is the diagonal, $X = Y$. The co-ordinate values, after transformation to logarithms to base 10, gave $X = 1.08 Y - 0.12$ and $Y = 0.885 X - 0.173$ for the best-fitting lines.

(Fenton, 1959) and temperature fluctuations (Bouman & Homan, 1958). These effects had to be compensated by the presence of standards on every paper in each assay. The Latin-square design balanced out positional and paper effects, since each spot of test and standard occurred once on each paper and occupied all available positions over the four papers.

Several unsuccessful attempts were made to separate insulin from crystalline glucagon (Staub, Sinn & Behrens, 1953, 1955), by the chromatographic method as described and by the radial technique of Bouman & Homan (1958). Although the latter procedure gave somewhat better separation in our hands than the ascending method, there was still some overlapping of the spots. We regard glucagon therefore as a possible source of interference. It has been our experience that on occasions when chromatographic and biological assays disagree on identical samples the higher value is usually the chromatographic one. Although it would be illogical to ascribe this to the specific effect of glucagon, the phenomenon is consistent with our finding that the two substances migrate to almost the same R_f position in the butan-2-ol-acetic acid-water system. There may be other substances of pancreatic origin which behave in a similar way. These findings do not necessarily detract from the value of chromatographic methods for the estimation of insulin but point to the clear distinction to be made between the estimation of insulin in samples of known history and its detection in the presence of foreign substances.

SUMMARY

1. A method for the assay of insulin solutions by ascending paper chromatography is described. The solutions, after desalting and concentration, are applied to papers which are developed in the upper phase of butan-2-ol-1% acetic acid (1:1, v/v), then stained with bromocresol green. The dye is eluted and its concentration measured in a spectrophotometer with respect to standards run

simultaneously. Treatment of the data as a four-point slope ratio assay gave results in good agreement with those obtained on the same solutions by the mouse convulsion method.

2. Solutions of four-times-recrystallized insulin, stored in the frozen state for use as working standards, remained stable for periods up to 8 weeks at -30° , and could be refrozen and thawed up to 30 times in the presence of phenolic preservative without detectable loss of potency.

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