tions F3 and F2B run close together but are easily differentiated because of the unique dimer formation of F3 (ref. 11). F3 is the only histone fraction to contain cysteine (1 residue per mole) and partial dimer formation occurs by oxidation under these conditions giving a band of approximately half the mobility of F3. This does not occur with any other histone fraction. F3 and F2B can of course be better separated by extending the length of migration.

Using this method, any histone fraction or mixture of fractions can be identified and characterised, and if quantitative methods are subsequently employed[8] the relative amounts of each fraction can be determined.

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Electrophoretic heterogeneity of crystalline insulin: A comparison of methods

The heterogeneity of crystalline insulin has been demonstrated by a number of methods. On paper[1] or cellulose acetate[2] electrophoresis with high concentrations of urea present to dissociate aggregated proteins, about six fractions may be separated, which apparently correspond to a series of deamidated insulins. MIRSKY AND KAWAMURA[3] studied crystalline insulins from eleven species by polyacrylamide gel electrophoresis, and found marked heterogeneity in each case, all the separated fractions having the biological properties of insulin. The presence of 8 *M* urea, however, had no effect on the electrophoretic pattern, so aggregated insulin molecules may have been absent. Other separation techniques have nevertheless shown the presence of molecules very different in size from insulin: gel filtration of bovine insulin on Sephadex G-50 resulted in the separation of several fractions one of which, with a molecular

weight of *ca.* 9,300, was identified as the single chain insulin precursor, proinsulin[4]. The present work compares the results obtained when crystalline insulin samples were fractionated using electrophoresis on conventional and gelatinised cellulose acetate membranes and polyacrylamide gels. The molecular weights of some of the fractions present have been estimated by introducing sodium dodecyl sulphate and iodoacetamide into the acrylamide gel electrophoresis buffers.

Materials and methods

Three different crystalline samples of bovine insulin (standard grade, *ex* English pancreas; standard grade, *ex* American pancreas; Isophane grade) were kindly donated by Boots Pure Drug Co. Ltd., and others were obtained from Mann Research Laboratories, Calbiochem and Sigma; a sample of porcine insulin was also obtained from Mann Research Laboratories. All samples had activities of *ca.* 25 I.U. per mg.

Cellulose acetate electrophoresis was carried out using a Tris–barbiturate buffer, pH 9.0, $I = 0.025$, using both conventional (Millipore U.K. Ltd. and Shandon Scientific Co. Ltd.) and gelatinised ("Cellogel RS", Arnold R. Horwell Ltd.) membranes. 2–3 μl samples at 2% w/w concentration were used with the conventional strips, and 20 μl samples at 8% w/w concentration with Cellogel RS. Protein fractions were stained with 0.5% w/v Ponceau S (Ed. Gurr Ltd.) in 5% trichloroacetic acid and densitometric traces of the stained membranes were obtained by reflectance using a Joyce-Loebl "Chromoscan". Disc electrophoresis on 7.5% w/w polyacrylamide gels at pH 8.5 were performed according to ORNSTEIN AND DAVIS[5]. Experiments were performed at ambient temperatures of 3° and 22° and the temperature in the gels monitored using a calibrated thermocouple. The gels were stained with Amido Black and scanned by transmission densitometry in the Chromoscan. Molecular weight determinations on 10% w/w polyacrylamide gels were performed using the method of WEBER AND OSBORN[6]. The buffer contained 1% w/v sodium dodecyl sulphate and 0.002 *M* iodoacetamide (British Drug Houses, Ltd.). Bovine serum albumin, pepsin, trypsin, β-lactoglobulin, lysozyme, cytochrome *c* and protamine sulphate (B.D.H. Ltd. and Sigma) were used as molecular weight markers and the protein bands were stained with 0.6% w/v Coomassie Blue in methanol–acetic acid (3:1).

All the insulin samples examined were revealed to be heterogeneous by all the methods used. With each method, however, the electrophoresis patterns given by the different insulins were similar and it was not possible to distinguish individual samples.

Results and discussion

Electrophoresis on conventional cellulose acetate was not a very satisfactory method of analysis; not more than four weakly-stained and poorly-separated bands could be distinguished. The use of Cellogel RS gelatinised cellulose acetate, however, produced superior resolution. A typical densitometer trace is shown in Fig. 1(a). Fig. 1(b) shows the results of the analysis of the same insulin sample by polyacrylamide gel electrophoresis. Although the protein bands are sharper and better resolved in the latter case there is an unmistakable similarity between the two traces, approximately six fractions being separated in each case. Since separations effected by polyacrylamide gel electrophoresis are normally due in part to a molecular sieving effect, which is presumably absent in cellulose acetate membranes[7], these results

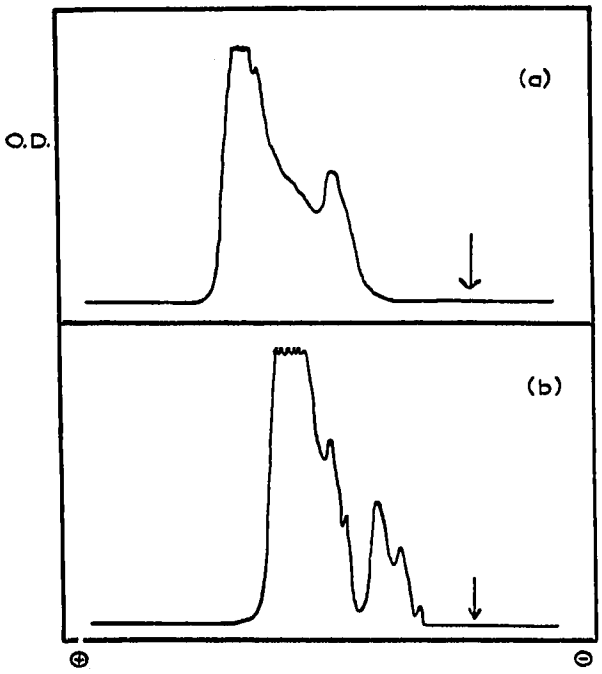

Fig. 1. Electrophoretic separation of crystalline insulin (Boots Pure Drug Co. Ltd., *ex* English pancreas) on (a) Cellogel RS gelatinised cellulose acetate, and (b) 7.5% polyacrylamide gel. Densitometer traces of stained protein bands. Arrows mark the position of sample application.

indicate that most of the separated fractions may have molecular weights similar to that of native insulin, *i.e.* that they are modified insulins. Because of the possibility of different dyebinding capacities in the separated fractions no attempt was made to estimate their relative concentrations.

When gel electrophoresis runs were performed at an ambient temperature of 3° rather than 22°, there was no improvement in the separations obtained. The thermocouple showed that the gel temperature after the first few minutes of the run was not greatly dependent on the ambient temperature. In all experiments, the temperature at first rose sharply but reached a constant value after 40–50 min. With a constant current of 5 mA per gel this final temperature was *ca.* 43° when the ambient temperature was 3°, and *ca.* 46° when the ambient temperature was 22°. Representative data are given in Table I. Similar temperature increases for runs at room temperature were recently reported by KNUDSEN *et al.*[8].

The presence of contaminants with molecular weights different from that of insulin was established by incorporating sodium dodecyl sulphate and iodoacetamide in the polyacrylamide gel electrophoresis buffers. In these conditions a linear relationship was found between the mobilities and the logarithms of the molecular weights of the marker proteins, whose molecular weights lie in the range 3,000–66,000. Other workers have found that the linear relationship does not hold over the whole of this range[9]. Staining of the protein bands in these experiments was found to be difficult possibly because of competition between dye and sodium dodecyl sulphate molecules for sites on the protein surface. When the insulin samples were examined, three bands were observed, whose mobilities corresponded to molecular weights of approximately 3,000, 6,000 and 22,000. The second of these figures is close to the

TABLE I

TEMPERATURE VARIATIONS IN ACRYLAMIDE GEL COLUMNS DURING GEL ELECTROPHORESIS

Time after start of experiment (min)	*Temperature (°C)*	
	Ambient temperature 3°	*Ambient temperature 22°*
1.5	16.0	—
3	17.0	25.5
6	19.0	27.0
9	20.5	28.0
12	22.5	29.5
15	25.0	—
18	27.0	31.5
24	31.5	35.0
30	37.0	39.0
36	41.5	43.0
42	42.5	46.0
48	—	46.0
54	—	46.0
60	43.5	46.0

molecular weight (5,750) of insulin itself, but the other two bands represent contaminants which may be quite distinct proteins. No band corresponding to the molecular weight of proinsulin could be detected, perhaps because of the poor staining.

It was concluded that the crystalline insulin samples studied contained both modified insulin molecules and contaminants of very different molecular weights. It was also found that electrophoresis on gelatinised cellulose acetate was a satisfactory method for the analysis of such samples.

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