AUTOMATED PROTEIN CHROMATOGRAPHY

A COMPARATIVE STUDY OF SOME BALEEN S-CARBOXYMETHYL-KERATEINES

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INTRODUCTION

The demonstration that the baleen of the Sei whale (*Balaenoptera borealis*) is mineralised with apatite-like deposits, closely similar in form and composition to bone mineral[1] and that these deposits are intracellular and variable in quantity from cell to cell[2] has raised new questions for biological calcification and for keratin chemistry.

During the maturation of related mineralised epidermal tissues such as tooth enamel, the total protein content is not only strikingly diminished, but there are changes in the proportions of the constituent proteins[3,4]. It is important, therefore, to establish if similar variations in protein composition occur in baleen cells of widely varying mineral content. Unfortunately, the chemical study of keratinised tissues is made difficult by the insoluble nature of the proteins, although soluble preparations from keratinised tissues (S-carboxymethyl-kerateines) may be obtained by reduction with mercaptoethanol and subsequent alkylation of the free sulphydryl groups. Such preparations may be fractionated by chromatography on columns of DEAE-cellulose in solutions containing 8 *M* urea using step-wise elution with increasing potassium chloride concentrations[5–7].

Baleen fringe fibres may be disintegrated by trypsin treatment into suspensions of single intact cells, and subsequently density fractionated by centrifugation in organic solvents, yielding fractions varying in ash content from some 2 % to 35 % by weight[8,2], providing materials suitable for the study of variation in the proportions of the solublised proteins with the degree of mineralisation of the cells. Whilst it is known that the fractions obtained from "kerateine" preparations by chromatography are generally heterogeneous, this procedure provides a method of detecting gross differences in protein proportions with the degree of mineralisation. In the course of these studies an automated apparatus for protein chromatography has been developed and its accuracy in the analysis of heterogeneous protein preparations examined.

EXPERIMENTAL

Preparation of protein extracts

The method used was based on that of THOMPSON AND O'DONNELL[7]. Samples of

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the dried, density fractionated Sei whale baleen cells (1 g) were suspended in 8 *M* urea/ 0.01 *M* Tris buffer (100 ml, pH 7.4) in 250 ml stoppered flasks. 2-Mercaptoethanol (1 ml redistilled) was added and the pH adjusted to 10.5 with potassium hydroxide solution (5 *N*) using a pH-stat (Radiometer Ltd., Copenhagen). The flasks were flushed with nitrogen, stoppered and shaken at room temperature overnight. A solution of sodium iodoacetate (3 g) in 0.05 *M* Tris buffer (10 ml, pH 8.5) was added and the pH maintained at 8.5 on the pH-stat until alkylation was completed (*ca.* 15 min).

The solublised proteins (some 50 % of initial weight) were separated from the insoluble residue by centrifugation (3,000 *g*, 30 min at 5°), and the extracts were dialysed free of urea and salts, freeze dried and stored at 2°. In this manner soluble protein preparations were obtained from baleen cell samples of 2.8 %, 14.2 % and 27 % ash weight.

DEAE-cellulose

Whatman brand powder (DE. 50) in the chloride form was prepared as described by PETERSON AND SOBER[9] and finally equilibrated with urea buffer (0.01 *M* Tris, 8 *M* urea; pH 7.4).

It was found that the flow resistance of columns of this material in the urea buffer was inconveniently high and the difficulty was overcome by mixing the DEAE-cellulose with an equal weight of Celite (30–80 mesh, "for gas chromatography", British Drug Houses Ltd.).

Urea

Urea; Laboratory Reagent Grade (British Drug Houses Ltd.) was found to contain impurities with a significant ultra violet absorption in the 230–300 mμ region. Urea (A.R. Grade) was therefore used.

Tris

Tris [tris-(hydroxymethyl)-aminomethane], Laboratory Reagent Grade was obtained from British Drug Houses Ltd.

All other reagents were of A.R. quality.

CHROMATOGRAPHIC APPARATUS

The comparative, quantitative analysis of S-carboxymethyl-kerateines by chromatography has not been reported previously, although it has been shown that step-wise elution of these preparations from DEAE-cellulose yields discrete fractions[7].

The known tendency of solubilised keratin preparations to aggregation in solution[10] and the extreme complexity of the chromatographic fractions obtained, as demonstrated by starch gel electrophoresis[7], suggested that rigorously reproducible conditions would be required if useful quantitative data were to be obtained. To this end an automated step-wise elution system was developed.

The apparatus employed is shown diagrammatically in Fig. 1 and comprised:

(a) Recording equipment

A Uvicord flow cell spectrophotometer (L.K.B. Ltd.) reading at 257 mμ coupled to a chopper bar chart recorder (L.K.B. Ltd. Type 6520A) driven at 6 cm/h was used.

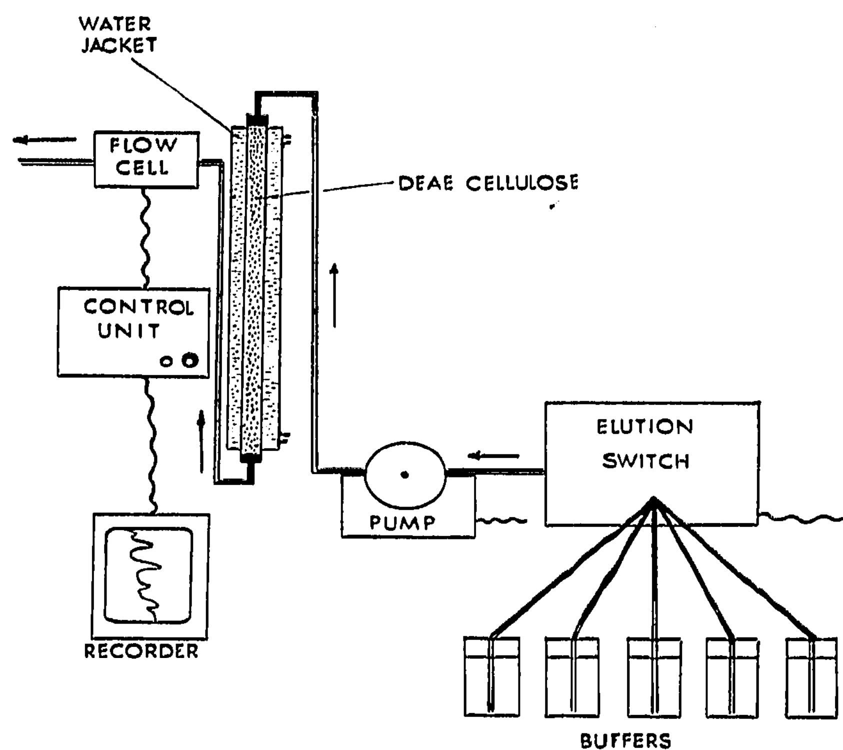

Fig. 1. Diagram of apparatus.

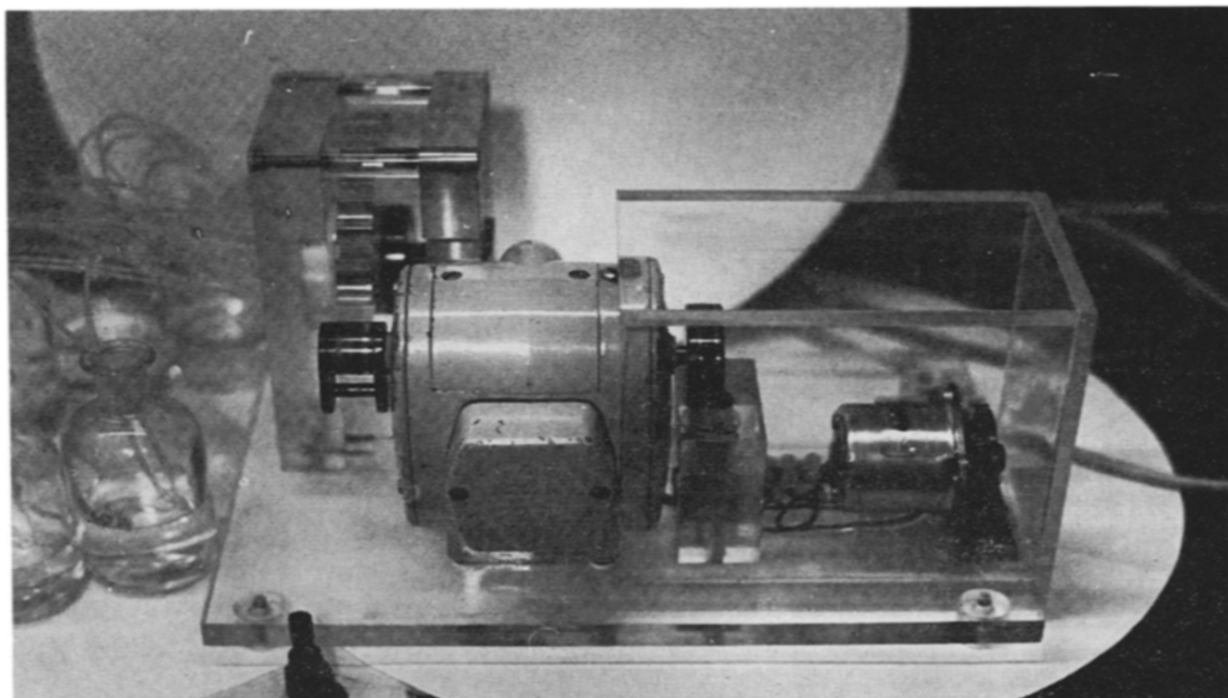

Fig. 2. Elution switch mechanism (see text for description).

(*b*) *Chromatographic column*

The older type of "cone and socket" chromatographic columns are unsuitable for flow analysis systems and a 30 × 0.6 cm flanged column with high pressure connections was obtained from Technicon Instruments Ltd. (Chertsey, Surrey). The "dead space" above the packing was largely eliminated by packing with Celite (30–80 mesh), after application of the sample. Temperature control was maintained by a water jacket connected to the mains supply; (*ca.* 10°). This column was entirely satisfactory in use and was easy to re-pack when required.

(*c*) *Peristaltic pump*

A simple peristaltic pump was used which fitted the range of pump tubes available from Technicon Ltd. With a 0.02 in. internal diameter pump tube and a rotor speed of 18 r.p.m. the flow rate was maintained at 32–35 ml per h. Pump tubes became distended after 24 h running and were therefore changed after every three chromatographic runs.

(*d*) *Elution switch mechanism*

A variety of devices for the automatic delivery of solutions to chromatographic columns have been described. Many of these have been designed for use with gravity-fed systems in which the passage of the meniscus from one eluant is "sensed" by an electrode system which activates transfer to the next eluant[11–14]. This system has been termed "depletion sensing"[13]. In systems in which the eluant flow rates are determined by metering pumps, chromatographic reproducibility may be attained by the use of time-determined eluant changes[15,16]. Generally, these mechanisms have made use of solenoid-operated valve assemblies dependent on complex electronic devices for their activation.

However, KARL *et al.*[17] have recently described the use of four and five channel mechanical switching mechanisms based on the "Geneva Mechanism" principle for the programmed elution and regeneration of automated amino-acid analyser columns. For the purpose of the automated step-wise elution chromatography, described in this paper, the use of the "Geneva Mechanism" has been extended to the time programmed application of as many as sixteen eluant changes. The apparatus is simple, extremely robust and has been in constant use over a period of a year. Details of the construction of the apparatus are shown in Figs. 2 and 3.

A geared motor (Drayton Type R.Q., 250 V, 50 ~, 4 r.p.m.) drives a sixteen position "Geneva Mechanism" which is coupled to a sixteen channel P.T.F.E. cylindrical tap (7) rotating it through one position for each movement of the mechanism. In this manner each of the 1/16 in. diameter holes of the tap mechanism is located exactly. The Drayton motor is activated by a micro-switch (19), operated by a sector cam driven by a synchronous motor (Sangamo-Weston, 1 r.p.h.). Interchange of the sector cams allows any time period for the elution steps of up to one hour, giving a total elution time of sixteen hours; alternative timing mechanisms allow elution/regeneration/loading sequences of longer duration and complexity to be used.

The Drayton motor is coupled also to a second cam (14) which switches off the motor after one rotation, leaving the indexing cam (21) in position to make the next change. Each changing operation, from channel to channel, is thus of only some 5 sec duration. The input and output channels of the tap barrel are tapered to accept

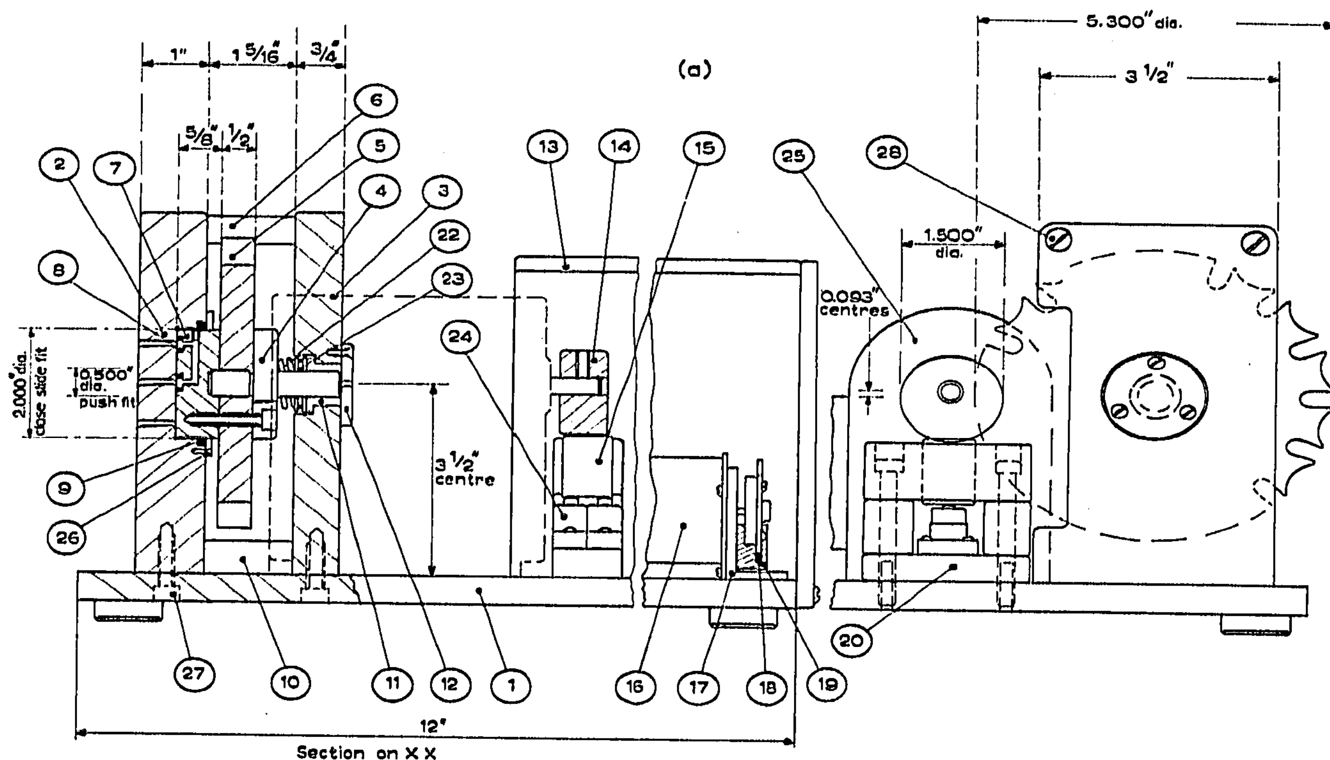

TABLE I

KEY TO NUMBERS IN FIG. 3a

No.	*Item*	*Material*
1	Base plate	Perspex
2	Valve plate	Perspex
3	Back plate	Aluminium alloy
4	Spindle	Brass
5	Indexing wheel	Perspex
6	Support bar	Perspex
7	Valve spiggot	P.T.F.E.
8	"O" ring	Neoprene
9	"O" ring	Neoprene
10	Support plate	Perspex
11	Bush	Brass
12	Cover plate	Brass
13	Cover	Perspex
14	Switch cam	Brass
15	Switch plunger	Brass
16	Synchronous motor	1 r.p.h. "Sangamo-Weston"
17	Angle bracket	Brass
18	Micro-switch cam	Brass
19	Micro-switch	240 V, 2 A
20	Plunger switch bracket	Perspex
21	Indexing cam	Brass
22	Spring	Stainless steel
23	Thrust bearing	½ in.
24	Castelco push-pull switch	250 V, 1 A
25	Drayton motor	4 r.p.m.
26	Flange plate	Brass
27	Screw	Brass, ¼ in. Whit
28	Screw	Brass, 2 B.A.
29	Foot pad.	Perspex

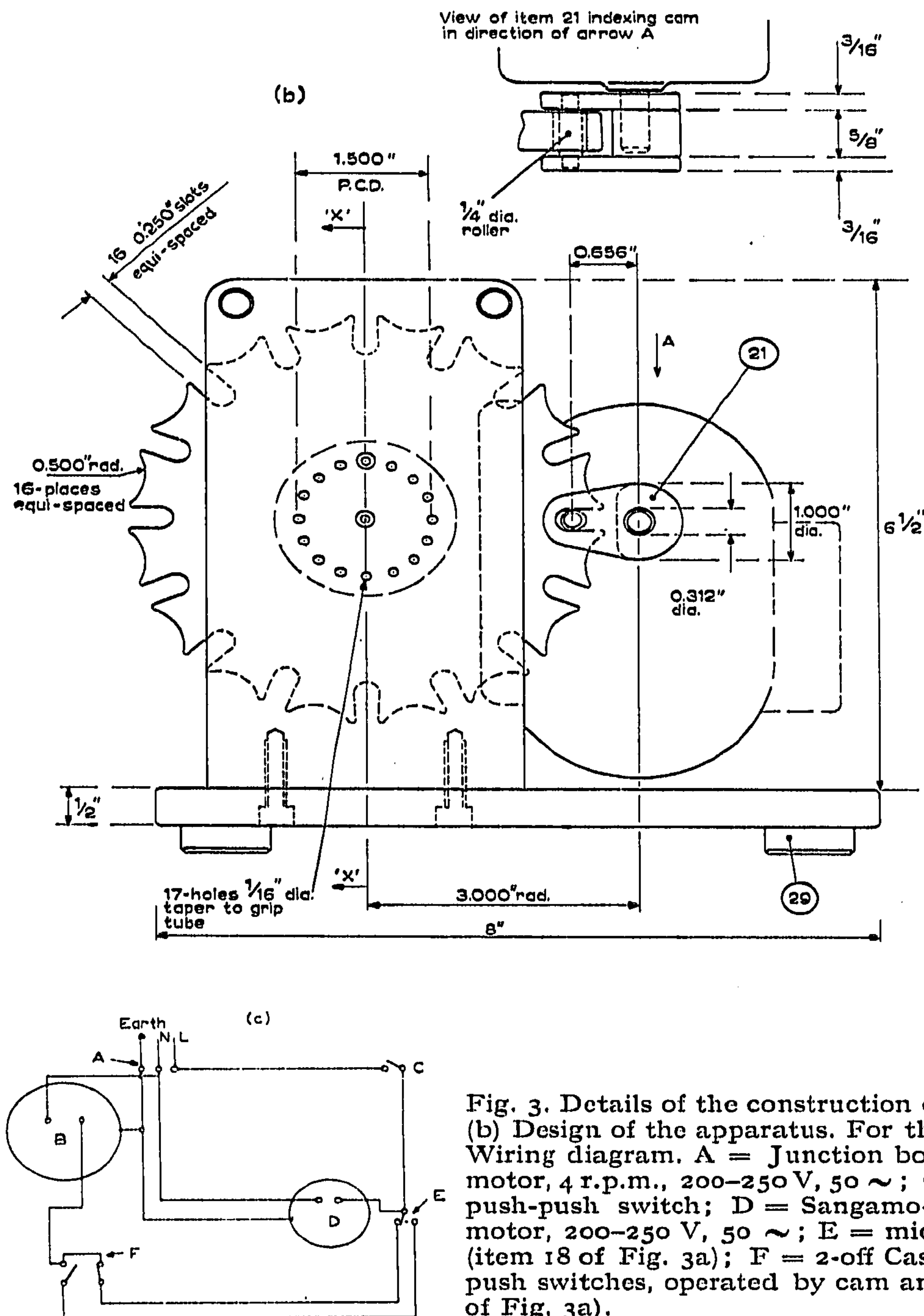

Fig. 3. Details of the construction of the elution switch. (a) and (b) Design of the apparatus. For the numbers, see Table I. (c) Wiring diagram. A = Junction box; B = Drayton type R.Q. motor, 4 r.p.m., 200–250 V, 50 ~; C = Castelco 200–250 V, 1 A push-push switch; D = Sangamo-Weston type S7 synchro-motor, 200–250 V, 50 ~; E = micro-switch operated by cam (item 18 of Fig. 3a); F = 2-off Castelco 200–250 V, 1 A, push-push switches, operated by cam and plunger (items 14 and 15 of Fig. 3a).

2 mm O.D. polyethylene tubing with which the connections to the buffer containers and metering pump may be made.

This system of eluant switching has the advantage of simplicity and reliability and may readily be extended to any number of channels. EVELEIGH AND THOMPSON[18] have described recently a multichannel mechanism using the peristaltic pump principle although their method of obtaining intermittent change is by timed pulses to the activating motor.

Application of the sample

Solutions of the freeze-dried total S-carboxymethylated baleen preparations were made up in Tris/urea buffer at a concentration of 50 mg/ml. It was found that the solution of such preparations was incomplete, and a slight residue remained insoluble, even in the 8 *M* urea solution. The solutions were, therefore, clarified by centrifugation at 25,000 *g*, 30 min at 5°.

0.4 ml (20 mg protein) was applied to the column with a pasteur pipette, the upper surface of the ion exchanger being protected with a circle of filter paper; Whatman No. 541 (No. 1 paper tends to disintegrate in the urea solution). The sample was washed into the column with nitrogen (15 lb./sq. in.). "Starting buffer" (0.01 *M* Tris, 8 *M* urea, pH 7.4) was then layered on to the column and displaced with Celite until the dead space was filled. The upper pressure connection was then made, care being taken to exclude air bubbles, and the joint sealed with two Thomas clips (Technicon Ltd.).

Elution of the proteins

The column was eluted with a step-wise gradient of increasing concentration of potassium chloride in Tris/urea buffer, dispensed automatically to the column with the apparatus described. Significant "step-gradient" mixing being eliminated by the limited "dead space" above the column. Regeneration of the exchanger was carried out on the column using 1 *M* potassium chloride in Tris/urea buffer. The column was then equilibrated with Tris/urea "starting buffer" (*ca.* 50 ml) before the application of the next sample.

Recovery of protein from the column

Recovery was determined from the absorption at 280 mμ of the total pooled eluant from the column. The protein concentration being read from a calibration plot for baleen S-carboxymethyl-kerateine in urea buffer. Recoverties were in the range 91–95 % of the sample weight.

Quantitative comparison of the chromatograms

The chromatograms were analysed by tracing the chart profile on to writing paper of good quality, cutting carefully round the outline and estimating the proportion present in each fraction by weight. It was found that this procedure gave reproducible analyses and was a satisfactory method for the analysis of chromatograms with non-gaussian peaks. The reproducibility of the method is shown for duplicate tracings and "cuts" from a typical analysis (Table II).

TABLE II

QUANTITATIVE ANALYSIS OF A CHROMATOGRAM. DUPLICATE "CUTS"

Fraction	*Cut 1*		*Cut 2*	
	Weight (mg)	*% total*	*Weight (mg)*	*% total*
1	8.3	4.3	9.1	4.6
2	61.8	31.9	62.4	31.5
3	42.1	21.8	42.6	21.5
4	38.9	20.1	39.3	19.9
5	42.3	21.9	44.5	22.5
Totals:	193.4		197.9	

RESULTS

The effect of variation in the elution sequence

It is generally considered that the resolution of complex mixtures of macromolecules by ion exchange column chromatography is achieved most readily by the use of a continuous gradient of ionic strength or pH (for a review of this topic, see SNYDER[10]). However, it has been shown[20] that the elution of proteins from ion exchange materials without "trailing" occurs only at R_F values close to unity, a condition which is met by step-wise elution procedures. O'DONNELL AND THOMPSON[6] showed that the elution of both α- and γ-keratoses[21] of wool from DEAE-cellulose in buffers containing 8 M urea, with a continuous gradient of potassium chloride, failed to produce separation of discrete fractions, whereas the application of a step-wise gradient produced fractions which could be shown to differ in their amino acid composition.

In a similar fractionation of S-carboxymethyl-kerateine-A from wool[7] using the step-wise procedure, the fractions obtained were found, by starch gel electrophoresis, to be very heterogeneous, although marked variation in the proportions of fractions and of the constituents within each fraction was found. The fractionation of soluble keratin preparations by these methods is essentially arbitrary, and it has been suggested[6] that the number of fractions obtained is closely related to the number of elution steps applied to the system. This is verified by the present experiments on baleen kerateines. The effect of variation in the number of elution steps is shown in Fig. 4.

Fig. 4a showing a five step elution is similar to the result obtained in a five step elution of wool α-keratose (O'DONNELL AND THOMPSON[6]) who obtained three major and two minor fractions. Fig. 4b illustrates the result of adding a further step to the elution sequence, showing a more complex pattern with indications of heterogeneity in some of the peaks. Fig. 4c is an example of the application of a fourteen step sequence yielding at least thirteen distinct peaks. These data are in close agreement with those of THOMPSON AND O'DONNELL[22] for wool kerateines and it is clear that the number of fractions obtained will depend on the number of elution steps applied. The equivocal results obtained in gradient elution experiments appear to result from the complexity of S-carboxymethyl-kerateine preparations.

Accuracy of the analytical system

The accuracy of reproducibility of the chromatographic analysis was determined by the repeated analysis of a sample of baleen kerateine preparation, using an eight step elution sequence of 0, 0.1, 0.15, 0.2, 0.3, 0.4, 0.5 and 1 M potassium chloride in Tris/urea buffer. A typical chromatogram is shown in Fig. 5. It can be seen that eight principal "peaks" are produced. The data for thirteen analyses of the same preparation of material are presented in Table III.

Comparison of S-carboxymethyl-kerateine preparations from baleen density fractions

Since the complexity of the chromatogram obtained from these soluble keratin preparations is a function of the number of elution steps applied, in order that the chromatograms should not be so complex as to render comparison of preparations unduly difficult, a standard elution sequence of six steps in the potassium chloride concentration of 0, 0.05, 0.1, 0.2, 0.3, 1.0 M was adopted. A typical chromatogram is

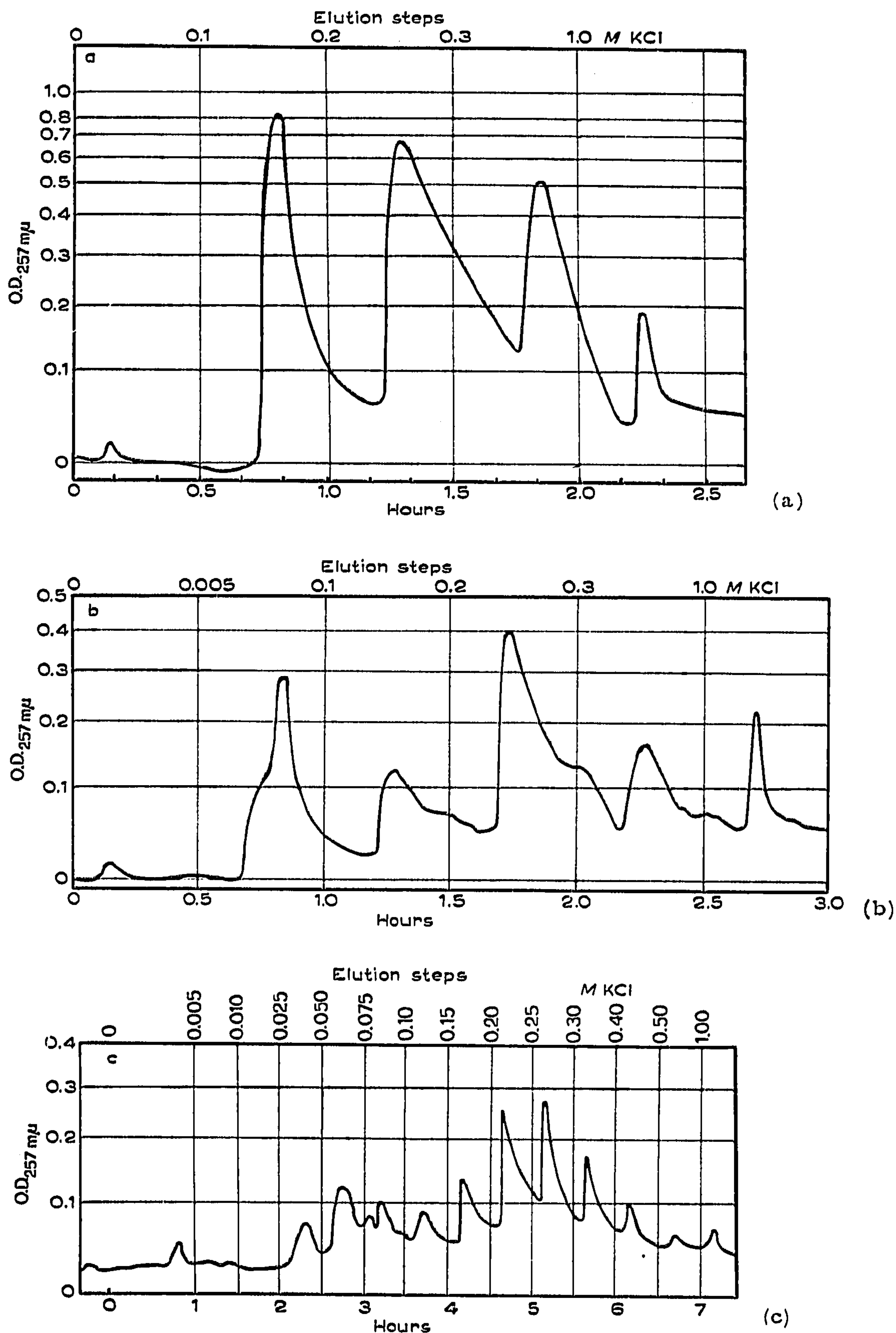

Fig. 4. (a) Chromatogram of baleen kerateine preparation using a five step elution programme. (b) Chromatogram of baleen kerateine preparation using a six step elution programme. (c) Chromatogram of baleen kerateine preparation using a fourteen step elution programme.

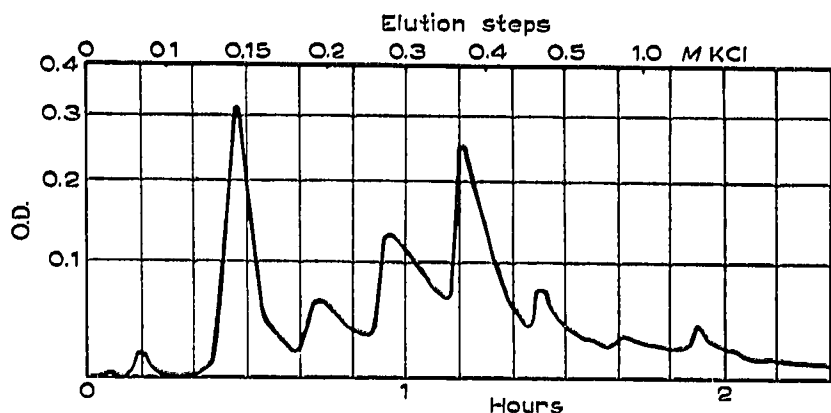

Fig. 5. Baleen kerateine preparation chromatographed using an eight step elution sequence.

TABLE III

MULTIPLE ANALYSES OF BALEEN KERATEINE

	*Chromatogram fractions**							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
1	2.89	23.04	12.10	18.60	21.04	9.07	6.18	7.08
2	4.28	22.13	11.90	19.16	20.60	8.14	6.10	7.69
3	0.88	21.50	9.91	20.44	23.24	10.57	5.69	7.77
4	3.25	16.19	11.60	22.98	22.42	8.85	6.86	7.85
5	3.25	20.47	10.17	19.62	25.30	10.04	5.56	5.59
6	2.17	20.66	10.56	19.30	22.18	10.36	6.76	8.01
7	4.99	20.95	6.51	21.00	25.21	9.21	5.90	6.23
8	2.29	21.40	8.77	19.46	25.08	10.23	6.17	6.60
9	1.98	21.76	10.58	18.80	23.50	8.89	6.67	7.82
10	3.28	14.73	11.78	20.16	24.14	10.06	7.20	8.65
11	2.13	27.91	9.13	17.94	21.75	8.67	6.07	6.40
12	1.93	24.74	10.61	19.65	20.77	8.85	6.20	7.25
13	1.61	25.50	10.90	20.10	18.56	7.62	6.77	8.94
Mean:	2.67	21.61	10.32	19.78	22.60	9.27	6.32	7.37
Standard error of the mean:	±0.31	±0.97	±0.43	±0.36	±0.57	±0.25	±0.14	±0.26

* Expressed as percentage of total.

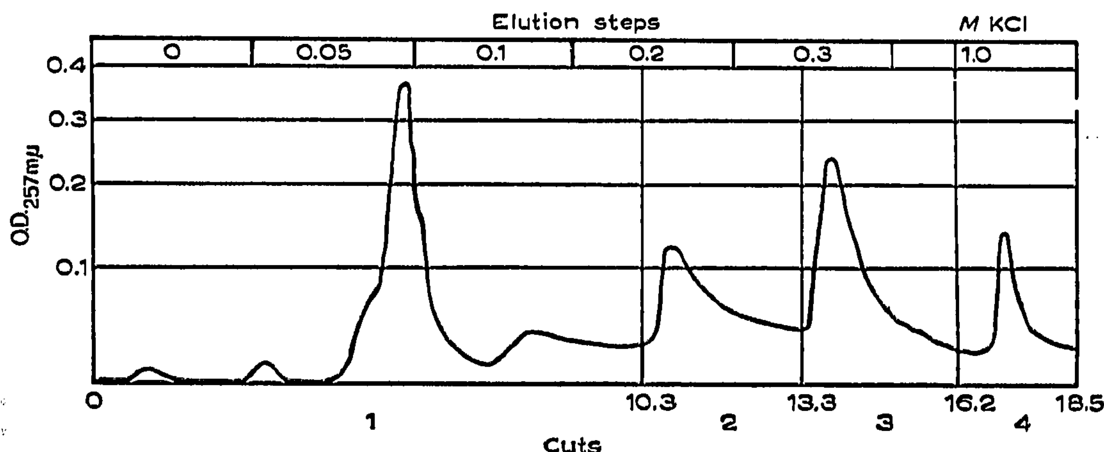

Fig. 6. Typical baleen kerateine chromatogram used for comparative analysis of mineralised preparations. Chromatogram "cuts" shown as described in text.

TABLE IV

MEAN FIGURES FOR THE CHROMATOGRAPHIC ANALYSIS OF SEI WHALE BALEEN TOTAL S-CARBOXY-METHYL-KERATEINE PREPARATIONS*

Chromatogram "Cut"	*% Ash in baleen fraction*		
	2.8%	*14.2%*	*27.0%*
1	41.01 ± 0.07	39.13 ± 1.05	35.47 ± 0.59
2	21.31 ± 0.22	24.40 ± 0.35	20.90 ± 1.90
3	26.10 ± 0.20	25.16 ± 0.69	31.20 ± 1.70
4	11.70 ± 0.28	11.30 ± 0.21	13.57 ± 0.69

* Data are shown as percentage of the total "cut" weight.

shown in Fig. 6. The "cuts" for the quantitative analysis of the chromatograms were made in a manner such that each of the principal peaks was isolated in a single "cut", namely at 10.3, 13.3, 16.2 and 18.5 cm from the origin of the chromatogram. The data from multiple analyses of these preparations are shown in Table IV.

DISCUSSION

The complexity of the baleen kerateine preparations is illustrated by the chromatographic analyses and is in accord with the findings of THOMPSON AND O'DONNELL[23]. However, it appears that the method of analytical chromatography described is capable of resolving these preparations into discrete fractions in a quantitatively reproducible manner. Such methods may be of value for the detailed quantitative analysis of protein preparations from keratinised tissues or other sources.

The comparative analyses of the mineralised baleen preparations suggest that there is a reduction in the proportion of the "initial fraction" (*e.g.* Cut 1, Fig. 6) of the total kerateine preparation with the degree of mineralisation, which is of interest in view of the finding of BURGESS AND MACLAREN[3] for tooth enamel proteins, and the suggested decrease in the α-keratose component of milled, density fractionated, baleen plate[24].

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SUMMARY

A detailed study of the chromatographic fractionation of baleen S-carboxymethyl-kerateines has been made, using a system of automated step-wise elution. A comparison of the total kerateine preparations from baleen cells of widely differing inorganic content suggests a variation in protein proportions with the degree of mineralisation.

The type of chromatographic system used is described and its reliable reproducibility suggests it to be suitable for quantitative analyses of complex protein mixtures.

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