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Fractionation of Basic Proteins and Polypeptides

CLUPEINE AND SALMINE

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Interest in the biological action of polypeptides and low-molecular-weight proteins with a high arginine content led to a study of the purity of such compounds. Classical methods of purification have already been used on clupeine, e.g. fractional precipitation or extraction, or both, of the methyl ester hydrochlorides (Felix & Dirr, 1929; Felix & Mager, 1937), of the picrates (Ando, Ishii, Hashimoto, Yamasaki & Iwai, 1952) and of the sulphates (Waldschmidt-Leitz, Kuhn & Zinnert, 1951). Until recently the application to these compounds of methods of purification such as chromatography and countercurrent distribution between solvents had not been described. The present paper describes the application of these techniques to a commercial sample of clupeine leading to the partial separation and identification of the components of clupeine and, to a lesser extent, salmine.

The method of assay used in the present work was based on the modified Sakaguchi-Weber procedure (Weber, 1930) for determination of arginine, but was applied to the whole protein. This method

readily lends itself to the routine analysis of the large number of protamine samples obtained by chromatographic and countercurrent fractionation procedures, and has been used in the fractionation studies (to be published) of another polypeptide containing high arginine, namely, licheniformin.

A number of two-phase systems was examined for suitability in countercurrent distribution but only one system, containing *n*-propanol and a concentrated salt solution, was found to give satisfactory fractionations. A similar system for the fractionation of clupeine had been described by Felix (1953), but at the time when our investigations were made we were not familiar with this work. In view of the complexity of the results obtained a brief outline of our main methods and results of fractionation is given here.

A preliminary countercurrent distribution showed the presence of at least two major and several minor components in our crude clupeine sulphate. By the use of a 50 tube machine and a greater number of transfers a distribution curve (Fig. 1) was obtained

Fig. 1. A 150-transfer distribution (50 tube machine) of crude clupeiine in *n*-propanol-3M sodium acetate (1.5:1, v/v).
●—●, Experimental; —, calculated.

which indicated the presence of at least three major components with partition coefficients so close that a much larger machine would be necessary to give a good separation. Two clupeiine fractions L and H containing components of low and high partition coefficient (*K*) respectively were isolated in such a manner that clupeines of intermediate *K* were absent. Whilst these fractions, which were shown by further countercurrent distribution to be complex, were of no great theoretical interest, they proved to be of considerable value in the elucidation of other fractionation techniques.

Chromatographic methods of separation were investigated but efficient fractionation was obtained only by elution chromatography from basic alumina. The elution curve (Fig. 2) shows two minor (*W*, *X*) and two major (*Y*, *Z*) peaks. Clupeiine isolated from each of these peaks gave only a single peak when re-chromatographed, indicating that true fractionation had been obtained. Countercurrent distribution studies of clupeines *W* and *X* showed that both were complex and contained clupeines of low and high *K*. Clupeiine *Y* was also complex, containing a low *K* component (*Y*1) and high *K* components (*Y*2, *Y*3) but none of intermediate *K* value. Clupeiine *Z*, on the other hand, showed the characteristic distribution curve of a homogeneous substance of intermediate *K* value.

The chromatographic behaviour of clupeines L and H supported this picture of the complexity of crude clupeiine and of the *W*, *X* and *Y* fractions. Both L and H showed *W*, *X* and *Y* peaks, and as was anticipated from the method of isolation neither showed the *Z* peak.

The cold-water fractionation method of Waldschmidt-Leitz, Zeigler, Schaffner & Weil (1931)

Fig. 2. Elution of crude clupeiine sulphate (50 mg.) from a column of 40 g. of alumina (type O) in a tube of 1.2 cm. internal diam. Developing solvent: 0.5M- K_2HPO_4 .

failed to yield a pure clupeiine from our crude material, although the minor components *W* and *X* were eliminated.

RESULTS

Countercurrent distribution of clupeiine

Solvent system. Preliminary studies of two-phase systems suitable for partitioning clupeiine showed that, because of the hydrophilic nature of clupeiine, organic solvents with a high solubility for water were necessary.

Anomalous countercurrent distribution patterns of clupeiine sulphate were obtained in a system (see Experimental) containing phenol, citric acid and aqueous phosphate. Investigations showed that during a distribution the sulphate ion was concentrated in the leading tubes, i.e. it moved as a high *K* component and independent of the clupeiine, and also that the *K* of clupeiine in this system was considerably decreased by the addition of SO_4^{2-} ions. These facts offer an explanation of the observa-

tions, obtained by analysis of both phases, of low K values for the clupeine in the leading tubes and of much higher K values for the clupeine remaining in the initial tubes. Whilst this effect might not reduce the degree of resolution obtained with clupeine components, it would invalidate any simple mathematical interpretation of the distribution curve, and hence the system was not investigated further.

Systems in which two phases were produced from the aqueous solution of a water-miscible alcohol by the addition of large amounts of salt were next examined, and a system was selected with the following composition: 1.5 l. of *n*-propanol and 1.0 l. of 3M sodium acetate. The system was equilibrated at 20° and as the phase compositions are somewhat dependent on temperature it was found desirable to control the temperature to $\pm 1^\circ$ during countercurrent distributions.

Distribution of crude clupeine. A 30-transfer distribution in a 12 tube machine in the propanol-sodium acetate system indicated that our crude clupeine contained two main components with partition coefficients (K values 0.4 and 0.8), in addition to minor components with K values below and above these. It was deduced that with a greater number of transfers in a machine with more tubes a complete separation of the main components

should be possible. Fig. 1 shows the distribution curve of crude clupeine after 150 transfers in a 50 tube machine. It will be seen that only partial separation of components has been achieved.

Analysis of the curve showed that an oversimplified picture of the complexity of crude clupeine had previously been obtained, and indicated that a mixture of three major components and at least four minor components was the simplest that would fit the experimental results. As the ratios of the K values of adjacent components were less than two in all cases it was obvious that something considerably larger than the 50 tube machine at our disposal would be necessary to effect a worthwhile fractionation of the major components.

Isolation and distribution of clupeines L and H. In view of the above findings further preparative fractionation of crude clupeine by countercurrent distribution was limited to the isolation of two fractions, L and H, which would be enriched in the major components of lowest and highest K (subsequently identified by elution chromatography as the components Y 1 and Y 2), and at the same time contain none of the third major component of intermediate K (subsequently identified as the chromatographic component Z).

This was achieved by the scheme given in Table 1, which was based on the analysis of the distribution

Table 1. *Isolation of clupeines L and H by countercurrent fractionation of crude clupeine*

Crude clupeine sulphate (1.0 g.) in <i>n</i> -propanol system				
		12 tube (50 ml. phase capacity) distribution machine. 30 transfers applied		
Fundamental fractions (0-11) pooled and clupeine recovered as the hydrochloride L1. Yield: 490 mg. $K = 0.5^*$		Withdrawn fractions (12-30) pooled and clupeine recovered as the hydrochloride H1. Yield: 420 mg. $K = 1.0^*$		
L1		H1		
		Fraction H1 was added to tube 1 of a 50 tube machine (10 ml. phase capacity) and after 14 transfers L1 was introduced into tube 0 and distribution continued for a total of 165 transfers		
Fundamental fractions 0-25	Fundamental fractions 26-41	Fundamental fractions 42-49 and withdrawn fractions 165-115	Withdrawn fractions 114-73	Withdrawn fractions 72-50
Discarded	Pooled and clupeine recovered as the hydrochloride L. Yield: 129 mg. $K = 0.4^*$	Discarded	Pooled and clupeine recovered as the hydrochloride H. Yield: 259 mg. $K = 1.1^*$	Discarded

* K = concn. in top phase/concn. in bottom phase; obtained by direct measurement.

curve of crude clupeine (Fig. 1). After partial fractionation and isolation of the clupeine (see Experimental) the two fractions were fed back into the initial tubes of a 50 tube machine in such a way as to make use of the reflux principle, thus enhancing the final degree of separation of clupeines with low and high K values.

The two fractions were examined by counter-current distribution (150 transfers in the 50 tube machine). From an analysis of the distribution curves fraction L appeared to consist mainly of two components (K values 0.33 and 0.43) and several minor components, whilst H appeared to consist mainly of two components with K values 0.8 and 1.0, and several minor components.

Countercurrent distribution of clupeine fractions W, X, Y and Z. Crude clupeine was split into four components (W, X, Y and Z) by elution chromatography from basic alumina (Fig. 2) and all these components were examined for homogeneity by analytical countercurrent distribution in the n -propanol-sodium acetate system. The distribution curves of clupeines W and X indicated that each of them was complex and contained several components with K values ranging from 0.25 to 1.7, i.e. a similar range to that found with crude clupeine.

The distribution curve of the main chromatographic fraction, Y, showed that it also was complex, the theoretical analysis of the curve being given in Table 2.

Table 2. *Theoretical analysis of countercurrent distribution curve of clupeine Y*

A 30-transfer distribution was carried out in a 12 tube machine on 25 mg. of clupeine Y.

Clupeine component	...	...	Y1	Y2	Y3
Estimated partition coefficient (K) of the component			0.43	0.96	3.13
Amount (%) of component			40	50	10

Fig. 3. A 49-transfer distribution (50 tube machine) of 15 mg. of clupeine Z in n -propanol-3M sodium acetate (1.5:1, v/v). —, Experimental; - - -, calculated.

It will be seen that Y1 and Y2 correspond with one of the main components in each of the fractions L and H, and it has been confirmed (see below) by elution chromatography that both L and H do contain a main component belonging to the Y complex.

The distribution curve of clupeine Z, shown in Fig. 3, corresponded to that of a homogeneous compound of partition coefficient 0.7.

Partition chromatography

The use of the n -propanol-aqueous sodium acetate system in countercurrent distribution studies suggested that it might be of value in partition chromatography. The complete phase diagram (Fig. 4) of this system at 20° was determined (see Experimental), and from these data a number of two-phase mixtures were selected and the correlation between the partition coefficient of clupeine and phase composition was determined. No suitable phase mixture was found, however, as the K values lay between 0.2 and 2.0, whereas K values in the range 0.1–0.02 or 10–50 are desirable for successful partition chromatography (Porter, 1953).

Adsorption chromatography

Various chromatographic adsorbents were examined and, whilst no adsorption of clupeine was obtained on calcium carbonate, acid-washed alumina, or on anion-exchange resins, almost complete adsorption took place with powdered cellulose, various Celite products, Decalso F (sodium

Fig. 4. Phase diagram of the n -propanol-aqueous sodium acetate system at 20°. The partition coefficients (K) refer to crude clupeine in the systems defined by the tie-lines.

aluminosilicate), Amberlite IRC50 and basic alumina. Conditions for successful elution, however, were established only with Amberlite IRC50 and basic alumina. Fractional elution from Amberlite IRC50, buffered at pH 4.4, with 0.2M sodium acetate-hydrochloric acid buffers of decreasing pH in the range 2.6–2.0 showed promise, and some clupeine fractions were isolated. When, however, these fractions were re-chromatographed, elution patterns identical with that of crude clupeine were obtained, showing that the apparent fractionation was only an increased removal of clupeine with increasing hydrogen-ion concentration and not a separation into components.

Elution chromatography from basic alumina. It was found that clupeine could be eluted in good yield from basic alumina, i.e. chromatographic alumina without pre-treatment, by means of dipotassium hydrogen phosphate. The degree of elution varied from 0%, with 0.1M phosphate, to nearly 100% with 1M phosphate. The optimum concentration for maximum efficiency of resolution of the clupeine was found to be 0.5M with the alumina O used in the preliminary experiments, and 0.45M with the alumina H used subsequently. A typical elution curve for crude clupeine is shown in Fig. 2, separation into two minor peaks W and X and two main peaks Y and Z being obtained. The relative distribution of crude clupeine between the four peaks is given in Table 3.

The maximum loading of clupeine which would give efficient separation of the components was found to be 3 mg./g. of alumina, and this was used in the preparative work. For analytical purposes column loadings as small as 70 µg./g. of alumina for each clupeine peak could be employed, the lower limit of loading being dependent only on the sensitivity of the clupeine-assay method.

In view of difficulties experienced in the isolation of clupeine from the column effluents, which appeared to be due to the presence of K^+ or HPO_4^{2-} or both, the use of other eluting agents was tried. Various sodium salts were effective in elution but of those examined none was of value in fractionating clupeine.

Isolation of clupeine from column effluents. It was found that whilst clupeine could be quantitatively precipitated with phosphotungstic acid, recovery of clupeine from the complex was poor. Similar

behaviour had been observed in the recovery of clupeine from sodium acetate solution and was solved by the removal of the acetate ion before complex formation. In this case, however, removal of the phosphate ion did not yield solutions from which clupeine could be isolated via the phosphotungstate.

Phosphomolybdic acid was found to effect complete removal of clupeine from phosphate solution at pH 5–6, and the clupeine was recovered from the precipitate in reasonable yield by extraction with aqueous acetone containing hydrochloric acid. As a result of occasional failures to obtain good recoveries by this technique (see method I in Experimental) the concentration of acid in the aqueous acetone was increased from 0.4 to 1.7N, thus ensuring clupeine recoveries of at least 80%. The use of the more concentrated acid may, however, have resulted in degradation of at least one of the clupeine components, as some samples of clupeine Z isolated by method II (see Experimental) were found when examined by countercurrent distribution to contain, in addition to the Z component (K 0.7), a second component with a K greater than that of any of the main components of crude clupeine.

Chromatographic behaviour of clupeines W, X, Y and Z. To obtain clear evidence that true fractionation had been obtained by elution chromatography the main fractions Y and Z were rechromatographed, the results being given in Figs. 5a and 5b. W, X and Z were shown to be absent from the Y component

Fig. 5. Elution of two clupeine fractions from columns of 40 g. of alumina in tubes of 1.2 cm. internal diam. with 0.5M- K_2HPO_4 as developing solvent. (a) 5.5 mg. of clupeine Y; (b) 2.6 mg. of clupeine Z.

Table 3. *Elution chromatography of crude clupeine from basic alumina*

Clupeine component	...	...	W	X	Y	Z
Amount of component in effluent (%)			6	6	59	25
Amount of component isolated (%)			4	5	45	17

and components W, X and Y were absent from the Z component. Further, the eluate volumes corresponding with the emergence of the clupeine components, and the position of the peaks, agreed with those observed for the Y and Z components from the fractionation of crude clupeine (Fig. 2).

Chromatographic behaviour of clupeines L and H. The evidence of complexity of composition of clupeines W, X and Y afforded by countercurrent distribution studies suggested that further evidence of the composition of crude clupeine might be obtained by examination of the chromatographic behaviour of clupeines L and H. Peaks corresponding to components W, X and Y were found in the elution chromatogram of both L and H, but component Z was absent from both. These results confirmed the interpretation of the countercurrent distribution curves of components W, X, Y and Z, namely, that each of W, X and Y contained at least one low and one high *K* component whereas Z was the component of intermediate *K* eliminated during the isolation of clupeines L and H and is possibly a single substance.

Purification of crude clupeine by a cold-water-extraction technique

It has previously been reported (Waldschmidt-Leitz *et al.* 1931, 1951) that clupeine can be purified by repeated extraction with cold water, extraction being continued until no further removal of low-molecular-weight material occurs as measured by the approach of the ratio (total nitrogen/ α -amino nitrogen) to a constant high value.

Crude clupeine sulphate (10 g.) was dissolved in de-ionized water (140 ml.) at 35° and kept at 0° overnight. The mother liquor was removed and the residual oil redissolved in water (140 ml.) as before and again kept at 0° overnight. The extractions were repeated five times in all. After the last extraction the residual oil WE1 and the fifth mother liquor WE2 were dissolved in de-ionized water (5 l. and 600 ml. respectively), and the clupeines WE1 (4.4 g.) and WE2 (0.9 g.) isolated by freeze-drying.

The ratio (total nitrogen/ α -amino nitrogen) was found to have increased only from 101 for crude clupeine to 106 for clupeine WE1 (cf. Waldschmidt-Leitz *et al.* 1931, 1951). WE1 was analysed by elution chromatography and found to contain only Y and Z components in the proportion 70:30, the W and X components having been eliminated. Examination of clupeine WE2 by elution chromatography confirmed that five extractions were necessary, as WE2 contained a trace of W in addition to components X, Y and Z.

The heterogeneity of WE1 was confirmed by countercurrent distribution in the *n*-propanol-sodium acetate system, the curve obtained by 150

transfers in a 50 tube machine indicating the composition given in Table 4.

It will be observed that reasonably good analytical agreement is obtained from the distribution and chromatographic techniques, especially in estimating the Z content of the mixture.

Application of the fractionation methods to salmine

The analytical techniques and the methods of elution chromatography and countercurrent distribution were found to be applicable to a sample of commercial salmine (British Drug Houses Ltd.) with only minor modifications.

As was found with clupeine the rate of elution and degree of fractionation of salmine from a basic alumina column were critically dependent on the nature and concentration of the eluting salt solution. Thus with basic alumina H columns prepared as for clupeine fractionation (see Experimental), and with salmine sulphate loadings of 0.25–4 mg./g. of alumina, elution was incomplete with 0.45M dipotassium hydrogen phosphate, whereas with 0.55M solution very little separation into fractions was observed. Elution with 0.5M dipotassium hydrogen phosphate, however, was practically complete and gave an elution pattern qualitatively similar to that obtained with clupeine (Fig. 2), although there was a slightly greater overlap of the two major fractions.

A 50-transfer countercurrent distribution of salmine in the *n*-propanol-sodium acetate system also gave a partial resolution of salmine into several components. An analysis of the distribution curve indicated the composition shown in Table 5.

Amino acid composition of clupeine components

The amino acid composition of clupeines L, H and Z was determined by starch chromatography (Moore & Stein, 1948, 1949) on acid hydrolysates.

Table 4. *Theoretical analysis of distribution curve of clupeine WE1 obtained by cold-water extraction*

Clupeine component	...	Y'	Y1	Z	Y2
Partition coefficient of the component		<0.45	0.47	0.7	1.0
Amount of component (%)		10	38	32	20

Table 5. *Theoretical analysis of distribution curve of crude salmine*

A 49-transfer distribution was carried out in a 50 tube machine on 15 mg. of crude salmine sulphate in the *n*-propanol-sodium acetate system.

Estimated partition coefficient of component	0.32	0.64	1.0	1.44
Amount of component (%)	4	60	20	12

Quantitative recoveries of arginine from synthetic mixtures containing more than 80 % of arginine were not obtained by this method, and so arginine was determined in the hydrolysate by the modified Sakaguchi technique. The results are shown in Table 6.

N-Terminal amino acids. Dinitrophenyl (DNP)-clupeines were prepared by reaction of clupeines with 1-fluoro-2:4-dinitrobenzene (FDNB) (Sanger, 1945), the technique of isolation (see Experimental) being modified from that normally used for DNP-protein, as DNP-clupeines are water-soluble substances. The DNP-clupeines were hydrolysed by heating with acid and the ether-soluble materials from the hydrolysates were analysed on two paper chromatogram systems (see Experimental), which were based on the general procedure of Blackburn & Lowther (1951).

DNP-clupeine Z. After hydrolysis with 6N-HCl in a sealed tube at 100° for 16 hr. DNP-clupeine Z yielded DNP-alanine, the latter being identified on paper chromatograms and also by hydrolysis of the DNP-amino acid with ammonia (Lowther, 1951) and identification of alanine on a paper chromatogram. It was found that the yield of alanine could be increased threefold if the time of hydrolysis was increased from 2 to 20 hr.

DNP-clupeine Y. After hydrolysis under the conditions used for DNP-clupeine Z, DNP-clupeine Y yielded an amount of coloured ether-soluble material which was markedly smaller than was anticipated from the DNP content of the starting material.

As proline was considered to be a possible *N*-terminal amino acid (cf. Felix, Fischer, Krekels & Rauen, 1950) and in view of the reported instability of DNP-proline (Porter & Sanger, 1948; Bowes & Moss, 1953; Courts, 1954) to acid hydrolysis, the conditions recommended by Porter & Sanger for use with DNP-prolyl derivatives, namely, hydrolysis for 16 hr. in a sealed tube with 12N-HCl instead of 6N acid, were tried but without success. DNP-salmine, which has been stated (Porter & Sanger,

1948; Ando *et al.* 1952) to contain only proline as the *N*-terminal amino acid, was prepared for reference purposes. Once more after hydrolysis at 100° for 16 hr. with 12N-HCl very little coloured ether-soluble material was obtained and paper chromatography showed no DNP-proline but only the breakdown product, 2:4-dinitrophenol, and unidentified yellow material.

As a result of a study of the stability of DNP-proline under acid conditions, which will be reported separately, the conditions of hydrolysis for suspected DNP-prolyl compounds were amended to heating for periods of 2 and 4 hr. with 12N-HCl in an evacuated sealed tube at 100° (cf. Schramm & Braunitzer, 1953). Under these conditions DNP-salmine and DNP-proline both yielded ether-soluble yellow-coloured material which was shown by paper

Fig. 6. Absorption spectra of DNP-clupeine Y (—), DNP-salmine (---) and DNP-proline (.....), in 1% NaHCO₃.

Fig. 7. Absorption spectra of DNP-clupeine Z (—), DNP-alanine (---) and clupeine Z (.....), in 1% NaHCO₃.

Table 6. *Amino acid analysis of clupeine fractions*

Results are given as g. of amino acid residue/100 g. of clupeine base (water and ash free).

Amino acid	Clupeine fraction		
	L	H	Z
Alanine	3.6	3.7	4.2
Arginine	81.5	85.5	83.1
Glycine	1.4	0.1	Nil
Leucine and/or isoleucine	1.2	0.2	Nil
Proline	6.2	8.3	4.7
Serine	6.3	4.7	5.9
Threonine	3.9	2.8	Nil
Valine	0.4	4.3	3.4

chromatography to contain dinitrophenol, a trace of DNP-proline and two other DNP derivatives. Further acid hydrolysis of the ether-soluble material, after removal of dinitrophenol, yielded more dinitrophenol and proline, the latter being identified by paper chromatography and by the isatin test (Grassman & Armin, 1934).

The ether-soluble material from DNP-clupeine Y appeared to contain DNP-alanine in addition to the breakdown products of DNP-proline. This was confirmed by further acid hydrolysis of the ether-soluble material, when proline and DNP-alanine were obtained, the latter being identified as alanine after hydrolysis with ammonia. The yield of DNP-alanine from DNP-clupeine Y was about one-fifth of that obtained from DNP-clupeine Z and also about one-fifth of the total DNP-amino acid content indicated by the spectral absorption curve of DNP-clupeine Y.

Absorption spectra of DNP derivatives

The absorption spectra of the DNP derivatives of clupeines Y and Z, salmine, alanine and proline were determined and compared; the DNP-amino acids were examined at a concentration of $20\ \mu\text{M}$ in 1% sodium bicarbonate solution, and the DNP-proteins at concentrations which gave approximately the same peak absorption. The absorption spectra which are shown in Figs. 6 and 7 fall into two groups: DNP-clupeine Y and DNP-salmine exhibit the characteristic DNP-proline pattern (Fig. 6), whereas DNP-clupeine Z and DNP-alanine show (Fig. 7) the spectral characteristics of DNP-amino acids other than DNP-proline. Clupeine Z, in common with the other unsubstituted clupeine components, shows a negligible absorption (Fig. 7) in the 250–470 $\text{m}\mu$. range.

DISCUSSION

The choice of conditions for successful fractionation of clupeine by elution chromatography appears to be very limited. Small changes in the concentration of the eluting solution affect the rate of elution and degree of fractionation very considerably, and in applying the method it is necessary to examine a range of eluting solution concentrations so as to obtain the optimum conditions for fractionation.

The factors responsible for the resolution of crude clupeine into the four components W, X, Y and Z obviously differ from those involved in the fractionation of clupeine by countercurrent distribution in the *n*-propanol-sodium acetate system, as the complex components W, X and Y are all found to contain clupeines of both low and relatively high partition coefficients, some of which can be fairly readily resolved by countercurrent distribution. Thus the joint application of these two methods of

fractionation provides a powerful tool for the checking of homogeneity of clupeine samples.

Countercurrent distribution of Y shows that this consists of about 40% of one component Y1 (*K* 0.43) and 50% of Y2 (*K* 0.96). The existence of these two components was confirmed by the chromatographic behaviour of the L and H fractions which both showed a major peak in the Y position despite the fact that, by virtue of their method of isolation, they contained no component in common. The presence of about 25% of a component with a *K* between that of Y1 and Y2 is indicated by the countercurrent pattern of crude clupeine. Clupeine Z, which represents about 25% of the crude clupeine, was found by countercurrent distribution to behave as a homogeneous substance of *K* 0.7, and the elution patterns of the chromatograms of L and H, which show a complete absence of Z, confirm that Z was the component of intermediate *K* eliminated in the isolation of the L and H fractions.

Thus it can be seen that our crude clupeine consists of three main components, Y1, Z and Y2, in addition to a minor component, Y3, and two minor fractions W and X, both of which are complex. The partition coefficients of the components of the latter cover such a wide range that separation of these from the main components by countercurrent distribution in the *n*-propanol-sodium acetate system would be very difficult. They can, however, be readily separated from the Y and Z components either by elution chromatography from alumina columns or by the cold-water-fractionation method of Waldschmidt-Leitz *et al.* (1931).

The value of the latter method for the purification of our crude clupeine is mainly limited to the removal of the minor components W and X, although examination by our fractionation techniques also shows some enhancement in the clupeine Z content and a considerable enrichment in clupeine Y1 as compared with clupeine Y2. The failure of this extraction technique to yield a pure clupeine component suggests either that we are dealing with different clupeine components from those investigated by Waldschmidt-Leitz or that the relative proportions of the components in our clupeine are such as to make the technique relatively ineffective.

The techniques developed for the fractionation of clupeine have been successfully applied to another protamine, salmine. A detailed study was not made, but sufficient evidence was obtained to show the presence of at least two main and two minor components in our salmine. It appears probable that these techniques could be applied to other protamines.

The investigations of fractionation by chromatographic or distribution techniques are greatly facilitated by a rapid and reasonably accurate method of analysis, and the assay method described

in this work would appear to be generally applicable to the determination of all proteins which contain a sufficiently high arginine content.

The amino acid analyses of hydrolysates of various clupeine fractions show that crude clupeine contains at least three components which differ qualitatively in their amino acid composition.

The simplest amino acid pattern is shown by clupeine Z, which contains only four monoamino acids, namely alanine, proline, serine and valine. In this respect it resembles the clupeine of Waldschmidt-Leitz *et al.* (1951), but there is no quantitative agreement and it differs markedly in the nature of the *N*-terminal group, as we found alanine whereas Waldschmidt-Leitz reported proline.

Clupeine H, which is known to be a mixture of components, contains threonine and a trace of leucine or isoleucine or both, in addition to the above amino acids. Qualitatively this agrees with the clupeine C of Felix *et al.* (1950), although our material has a considerably higher proline content.

Clupeine L, which also is known to be a mixture, contains glycine and leucine or isoleucine or both, in significant amounts, but a much smaller proportion of valine than has been previously reported in clupeine samples. A clupeine fraction containing both glycine and leucine has been described by Šorm & Šormová (1951).

Clupeine Y contains alanine and proline as *N*-terminal amino acids. The identification of proline as the *N*-terminal residue gave rise to unexpected difficulties but was eventually achieved by comparison of the spectral-absorption curves of the various DNP-derivatives, DNP-prolyl peptides having the spectral characteristics of DNP-proline (Schroeder, Honnen & Green, 1953) and hence being readily detected. By this method salmine and clupeine Y are shown to have proline as an *N*-terminal amino acid, the proportion of *N*-terminal alanine in clupeine Y being insufficient to alter significantly the characteristic shape of the DNP-prolyl peptide curve (Fig. 6).

The spectral-absorption curve of DNP-clupeine Z indicates an *N*-terminal group other than proline and this was identified in the usual manner as alanine.

The fate of DNP-proline under acid hydrolysis conditions has been studied and reported separately (Scanes & Tozer, 1956).

The complexity of some clupeine samples may be due to the method of isolation and fractionation, although species variation is obviously responsible for many of the reported differences in clupeine composition (cf. Ando *et al.* 1953). The examination of non-homogeneous material has undoubtedly contributed to the confused picture of the chemistry of the protamines, and it is thought that the fraction-

ation methods described in this paper could be of value in the checking of homogeneity of protamine samples.

EXPERIMENTAL

Materials

The clupeine, which had been extracted from herring roe as the copper complex, precipitated as the picrate and finally isolated as the sulphate, and the salmine sulphate were obtained from British Drug Houses, Ltd., Poole. The alumina used in the chromatographic separation was activated alumina from Peter Spence and Sons, Ltd., Widnes. In the early experiments Type O alumina was successfully used, but later supplies of Type O alumina were found to be unsatisfactory and it was replaced by Type H alumina (100–200 mesh size).

Analysis of fractions

The direct determination of dry weight of protein in the various distribution or chromatographic fractions could not be effected because of the presence of salts. Analysis was therefore carried out by a determination of combined arginine on the whole protein by a modified Sakaguchi-Weber procedure (Weber, 1930). This procedure would be satisfactory if the constituent proteins had similar arginine contents and, in fact, the colour yields from crude clupeine and from partially purified components are almost identical. When purified components were available, however, reference has been made to the appropriate weight-colour calibration curve.

Preparation of samples for analysis. Various substances which can react with hypobromite, such as phenol and alcohols, will interfere with the colour development and must be removed or suitably reduced in concentration before the colour is developed. Samples containing phenol were extracted with chloroform at pH 1–2, and alcohols such as *n*-propanol were removed by evaporation of the sample to dryness in a stream of warm air.

In the case of effluents from alumina columns, interference with colour development was marked, and to minimize this effect samples were diluted at least fivefold before analysis. As the final colour was still dependent on the concentration of column effluent in the sample until dilutions of at least 25-fold had been made, all samples were prepared so as to contain 20% of column effluent after final adjustment of the clupeine content (see below), if necessary by the addition of column effluent containing no clupeine. The latter was obtained from columns identical with the fractionating column but containing no clupeine.

Calibration-curve samples were prepared with known amounts of the appropriate clupeine component, if available, dissolved in solutions similar in composition to the fractions being analysed and prepared for analysis in an identical manner.

Procedure. The sample, treated as above for interfering substances, was adjusted in volume with water to a concentration of 5–20 $\mu\text{g./ml.}$ A portion of this solution (2.5 ml.) was pipetted into an absorptiometer tube and 0.5 ml. of 2*N*-NaOH and 0.1 ml. of 0.1% (w/v) ethanolic α -naphthol were added and well mixed. The optical density of this solution was measured in a Hilger Biochem absorptiometer with an OB2 filter and a 14 mm. diameter tube, and was a blank to be applied to the subsequent measurement. One

drop (0.04 ml.) of NaOBr (prepared daily by mixing equal volumes of water saturated with Br_2 at 0° and $2N\text{-NaOH}$) was then added, the solution well mixed for 5 sec. and 0.5 ml. of 40% (w/v) aqueous urea solution immediately added and well mixed. The optical density was then determined without delay (as even with the use of urea some colour fading takes place after a minute or so) and compared with the calibration curve.

Countercurrent distribution

Solvent systems. A number of two-phase systems containing an organic solvent with a high solubility for water were examined for suitability in fractionating clupeine. The two systems which were studied in detail were as follows: (i) the phenol system, prepared by equilibrating 167 g. of phenol (A.R.) with 450 ml. of 0.1 M citric acid and 50 ml. of $0.2M\text{-Na}_2\text{HPO}_4$ at 20° ; and (ii) the *n*-propanol system prepared by equilibrating 1.5 vol. of *n*-propanol (laboratory grade) with 1 vol. of 3M sodium acetate (A.R.) at 20° .

Apparatus. Glass distribution tubes of the original Craig pattern (Craig & Post, 1949) were used, but connexion between tubes was made with short lengths of thin-walled polythene tubing, thus avoiding the use of ground joints or taps for removal of phases. At the completion of a distribution the polythene tubing from each tube was arranged so as to deliver into a sample bottle which had been attached to the outside of the tube, and then by a single manipulation of the machine the top phase of each tube was transferred to the corresponding sample bottle. After substitution of another set of sample bottles all the bottom phases were simultaneously removed by a single manipulation of the machine.

Tubes with a lower-phase capacity of 50 ml. were used in the early preparative stages and tubes with a lower-phase capacity of 10 ml. were used for the later preparative stages and generally for preliminary investigations and for analytical purposes.

Procedure. Distributions were carried out at $20 \pm 1^\circ$, by both the 'fundamental' procedure (Craig & Craig, 1950) and the single-withdrawal procedure of the upper phase.

Analysis of distribution curves. The distribution curves were interpreted according to the general mathematical formulae of Craig & Craig (1950), but for fundamental distributions involving 49 or fewer transfers theoretical distribution values were obtained from tables of the binomial probability distribution (Way & Bennett, 1951).

The theoretical curves for the withdrawn series were calculated from equation (1) or (2) (cf. Newton & Abraham, 1952):

$$W_{n,R} = \frac{K}{K+1} T_{(n-1),R}, \quad (1)$$

$$W_{n,R} = \frac{(n-1)!}{R!(n-R-1)!} \frac{K^{R+1}}{(K+1)^n}, \quad (2)$$

where K is the partition coefficient of the material, $W_{n,R}$ is the fraction of the total material withdrawn after n transfers from the end tube of a machine containing $(R+1)$ tubes and $T_{(n-1),R}$ is the fraction of the total material present in the end tube after $(n-1)$ distributions, i.e.

$$T_{(n-1),R} = \frac{(n-1)!}{R!(n-R-1)!} \frac{K^R}{(K+1)^{n-1}}.$$

For values of n and R less than 50 the calculations were readily made by using equation (1) and the binomial prob-

ability distribution tables, but for higher values of n and R it was necessary to calculate a series of tables for each machine, i.e. for each value of R , from equation (2).

The partition coefficients (K) were either obtained by mathematical analysis of the distribution curves or by direct measurement of the concentration of clupeine in the two phases.

Phase diagram of the *n*-propanol-aqueous sodium acetate system

Phase diagrams of a number of systems were prepared as described by Martin & Porter (1951). The binodal curve was determined by titrating the aqueous salt solution with organic solvent to permanent opalescence, the end point being more readily recognized in some systems if an oil-soluble dye was included. The tie-lines were determined by analysis of the salt content of both phases.

The phase diagram of the system *n*-propanol-aqueous sodium acetate is shown in Fig. 4 and the partition coefficients of crude clupeine obtained in some of the two-phase mixtures are marked on the corresponding tie-lines.

Chromatography

For analytical and small-scale preparative purposes, with a clupeine sulphate loading of 5–120 mg., a tube of 1.2 cm. internal diameter was used. Alumina (40 g.) was slurried in water and poured into the tube and allowed to settle. The column, 32 cm. in length, was pressed slightly, if necessary, so that a flow rate of 15–18 ml./hr. was obtained. The clupeine, dissolved in 3–5 ml. of water, was added to the column and washed on with 3 ml. of water. The column was developed with aqueous K_2HPO_4 solution and the effluent was collected in an automatic fraction collector.

Re-isolation of clupeine from fractions

The concentration and re-isolation of clupeine from typical pooled fractions obtained by distribution and by chromatography is given below.

***n*-Propanol-sodium acetate solution.** Nineteen withdrawn fractions from a 30-transfer, 12 tube distribution of 1 g. of clupeine sulphate were bulked to give a total volume of 1 l. The solution was adjusted to pH 3.4 with 70 ml. of 6N-HCl and the solids were precipitated with 3 l. of acetone. The solids were removed by filtration, dried *in vacuo* and were dissolved in 250 ml. of water. Analysis indicated a clupeine content of 440 mg. A solution (25 ml.) containing 10% (w/v) phosphotungstic acid (PTA) in N-HCl was then added, until precipitation was complete. The phosphotungstate was filtered off (sintered Pyrex glass filter, porosity 4) and washed, first with 15 ml. of 1% PTA and then with 20 ml. of 0.1% PTA, and sucked dry. It is necessary to free the precipitate from NaCl at this stage. The precipitate was then dissolved in a mixture of 25 ml. of 70% (w/w) acetone and 0.5 ml. of 6N-HCl. The solution was filtered and the filtrate dropped with stirring into 400 ml. of acetone acidified with 0.1 ml. of 6N-HCl. After standing for some hours the solution was filtered, and the clupeine which was distributed as a sticky residue on the walls of the vessel in which the precipitation had been effected, in addition to that on the filter, was dried *in vacuo*. The dried residue was dissolved in 40 ml. of 95% (w/w) methanol containing 0.05 ml. of 2N-HCl and dropped with stirring into 400 ml. of acetone containing 0.05 ml. of 2N-HCl. After standing for 1 hr. the amorphous

precipitate was filtered off, acetone-washed and dried *in vacuo*; yield, 420 mg.

Potassium phosphate effluent from alumina columns.

Method I. From the fractional elution of 420 mg. of crude clupeine sulphate on a column with 140 g. of alumina a pooled fraction containing 105 mg. (by analysis) of clupeine Z in 370 ml. of effluent was obtained. The solution was adjusted to pH 5-6 with 30 ml. of 6N-HCl, any precipitate filtered off, and 5 ml. of 10% phosphomolybdic acid added slowly with stirring. The suspension was allowed to settle and the precipitate filtered off (sintered-glass filter, Pyrex, porosity 4), washed with 30 ml. of water and sucked dry. The precipitate was extracted for 15 min. on the filter with a mixture of 10 ml. of 70% (w/w) acetone and 0.7 ml. of 6N-HCl and the solution filtered, the residue being extracted with another similar mixture of aqueous acetone and acid. The combined filtrates were dropped into 200 ml. of acetone and the sticky precipitate of clupeine hydrochloride was centrifuged, washed with acetone, re-centrifuged and dried *in vacuo*. The residue was dissolved in 10 ml. of 95% (w/w) methanol containing 0.12 ml. of 6N-HCl, and the filtered solution dropped into 20 ml. of acetone. The amorphous precipitate of clupeine hydrochloride was centrifuged off and dried *in vacuo*; yield, 70 mg.

Method II. The procedure was similar to that of Method I except that the phosphomolybdate precipitate was extracted with a total mixture of 15 ml. of 80% (w/w) acetone and 6 ml. of 6N-HCl.

Amino acid analysis

Clupeine (100 mg.) was heated in a sealed evacuated tube with 1 ml. of 6N-HCl at 110° for 24 hr. The HCl was then removed by repeated evaporation of the aqueous solution *in vacuo*. The component amino acids were isolated and determined by the starch-chromatography technique of Moore & Stein (1948, 1949). The estimation of the relatively large amounts of arginine recovered from the columns was not reproducible and therefore the arginine content of the hydrolysate was determined directly on the hydrolysate by the modified Sakaguchi reaction as used for the whole protein determination.

Preparation of DNP derivatives

DNP derivatives of valine, alanine, proline and serine were prepared by the methods of Sanger (1945) and Porter & Sanger (1948).

DNP-clupeine derivatives. Clupeine (100 mg.) in water (15 ml.) containing NaHCO₃ (100 mg.) was shaken at 20° with FDNB (0.1 ml.) in methanol (5 ml.) for 2.5 hr. The solution was acidified with 2N-HCl (0.9 ml.), extracted with an equal volume of ether and the aqueous solution dropped into acetone (200 ml.) with stirring. An oily residue was separated by centrifuging, dried *in vacuo*, redissolved in 90% (w/w) methanol (4 ml.) containing 6N-HCl (0.05 ml.) and dropped into acetone (30 ml.). The solid was centrifuged off and dried *in vacuo*. The yield of glassy solid was 107 mg.

Hydrolysis of DNP-clupeine and DNP-salmine

Method I. DNP-clupeine (30 mg.) was heated for 16 hr. in a sealed Pyrex tube with 1 ml. of 6N-HCl at 100°. The ether-soluble DNP-amino acids were isolated in the usual manner. This method was used for DNP-clupeine Z.

Method II. DNP-protein (50 mg.) was heated in a sealed Pyrex tube with 1 ml. of 12N-HCl at 100°. The effect of

heating for times varying from 1 to 24 hr. was investigated. This method was used for DNP-clupeine Y and DNP-salmine.

Paper chromatography of DNP-amino acids

Two systems, A1 and BE2, were based on the general method of Blackburn & Lowther (1951). In system A1 the developing solvent was the top phase of a mixture of 4 vol. of *tert.*-pentanol and 1 vol. of the phthalate buffer (pH 6) of Blackburn & Lowther. In system BE2 the developing solvent was the top phase of a mixture of 50 ml. of benzyl alcohol-ethanol (9:1, v/v) and 170 ml. of phthalate buffer (pH 6).

The DNP-amino acid in ethyl methyl ketone (3 mg./ml.) was applied (5 μ l.) to Whatman no. 1 paper which had been pretreated with phthalate buffer (pH 6).

Absorption spectra of DNP derivatives

Samples were prepared in 1% NaHCO₃ solutions at a concentration of 20 μ M for DNP-amino acids and at concentrations of 150-300 μ g./ml. for DNP-clupeines and -salmine. Measurements were made with a Unicam SP. 500 spectrophotometer.

SUMMARY

1. The fractionation of clupeine by counter-current distribution and by elution chromatography from basic alumina has been studied.
2. A modified Sakaguchi method is described for the assay of whole clupeine in the samples obtained on fractionation.
3. Crude clupeine has been shown to contain three main components, Y1, Y2 and Z, and a number of minor components.
4. Clupeine Z showed the simplest amino acid pattern, namely arginine, alanine, proline, serine and valine, with alanine as the *N*-terminal amino acid. Other clupeines contain leucine (or isoleucine or both) and glycine or threonine in addition to the amino acids of clupeine Z, and have alanine or proline as the *N*-terminal amino acid.
5. The fractionation and analytical techniques have been shown to be applicable to the study of another protamine, salmine.

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Structure and Composition of Soluble Feather Keratin

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A large proportion of feather keratin can be extracted by urea and reducing agents, and by subsequent oxidation with performic acid converted into a form soluble in dilute salt solution. The extracted material has a narrow distribution of molecular weight and charge, and Woodin (1954) has suggested that it contains the feather keratin monomer, an asymmetric particle with molecular weight 10 000. Rougvié (1954) independently found that solutions of feather keratin contain particles of these dimensions, and made the further observation that fibres reconstituted from feather keratin which had been oxidized with peracetic acid gave X-ray diffraction patterns with much of the detail of the original feather. It is therefore likely that the asymmetric particle of molecular weight 10 000 is responsible for the diffraction characteristics of feather keratin.

In this paper the results of an end-group analysis and some information on the amino acid composition of soluble feather keratin are reported.

METHODS

Preparation of keratin derivatives

White body feathers from hens were extracted with benzene, dried, extracted with 10% NaCl, washed with distilled water and dried over P_2O_5 . Before solubilization the calmus was cut from each feather.

SH-Keratin. This was extracted in 10M urea, 0.1M- $NaHSO_3$, 0.05M phosphate (pH 5–6), for 18 hr. at 40°. The

extract was filtered and dialysed against running tap water and then distilled water. The white gel was freeze-dried.

Cystic acid-keratin. This was obtained by performic acid oxidation of SH-keratin. Formic acid was mixed with 0.115 vol. of 30% (w/v) H_2O_2 , left at room temperature for 1 hr. and cooled to 0°. One-twentieth of its weight of SH-keratin was added and the mixture left overnight in the refrigerator, filtered and dialysed against tap water and then against distilled water. The resultant opalescent solution was freeze-dried. In the experiments to identify the *N*-terminal amino acids by reaction with 1-fluoro-2:4-dinitrobenzene (FDNB), cystic acid-keratin was used after purification to this stage.

In all other experiments the cystic acid-keratin was purified further. After drying, the solid was not completely soluble in water. When the suspension was adjusted to pH 9, more dissolved, but the pH fell during solution. When more alkali was added to keep the mixture at pH 9 all but a little dissolved. The solution was centrifuged at 20 000 rev./min. and the supernatant passed down a column (15 mm. diameter) of Amberlite IR-120 in the H^+ form, and then down a column (15 mm. diameter) of Amberlite IRA-410 in the base form. Quantities of 20 ml. of each wet resin were used/g. of keratin. A solution was obtained which was very opalescent but free from visible particles. Examination in a flame photometer showed it to be free from metal cations. The solution was freeze-dried and gave a solid which readily dissolved in distilled water to give a very opalescent, acid solution.

Cystic acid-keratin concentrations were determined from measurements of the N content. The N content of the dry protein was taken as 15.5% and the molecular weight as 10 000.