*The Fundamental Conditions for Countercurrent Distribution Studies of Clupeine**

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The heterogeneity of some protamines, protein moieties in sperm nuclei of fishes, namely clupeine[1-9] from herring, salmine[3,4] from salmon, iridine[3,4,10] from rainbow-trout, and mugiline[11,12] from mullet, has been reported from German[1-7] and Japanese[8-12] laboratories. Nevertheless, it is still uncertain how the heterogeneity of protamines originates and what the biological activities of different molecules are. Thus, as the first step to resolve the problem, clupeine has been examined

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1) A. Kossel and E. G. Schenk, *Z. physiol. Chem.*, **173**, 278 (1928).

2) K. Felix and K. Dirr, ibid., **184**, 111 (1927); K. Felix and A. Mager, ibid., **249**, 111 (1937); H. M. Rauen, W. Stamm and K. Felix, ibid., **291**, 275 (1952).

3) H. M. Rauen, W. Stamm and K. Felix, ibid., **292**, 101 (1953).

4) K. Felix, "The Chemical Structure of Proteins", a Ciba Foundation Symp., J. & A. Churchil Ltd., London (1953) p. 151.

5) K. Felix, *American Scientist*, **43**, 431 (1955); K. Felix, A. Krekels and W. Rick, *Trans. Faraday Soc.*, **53**, 252 (1957).

6) E. Waldschmidt-Leitz, F, Ziegler, A. Schaeffner and L. Weil, *Z. physiol. Chem.*, **197**, 219 (1931); E. Waldschmidt-Leitz and L. Pflanz, ibib., **292**, 150 (1953); E. Waldschmidt-Leitz and K. Gauss, ibid., **293**, 10 (1953); E. Waldshmidt-Leitz and R. Voh, ibid., **298**, 257 (1954).

7) K. E. Rasmussen, ibid., **224**, 97 (1934).

8) T. Ando, E. Abukumagawa, Y. Nagai and M. Yamasaki, *J. Biochem. (Japan)*, **44**, 191 (1957); T. Ando, M. Yamasaki, E. Abukumagawa, S. Ishii and Y. Nagai, ibid., **45**, 429 (1958).

9) T. Ando, S. Ishii, M. Yamasaki, K. Iwai, C. Hashimoto and F. Sawada, ibid., **44**, 275 (1957).

10) F. Sawada and T. Ando, The 31th Annual Meeting of the Japanese Biochem. Soc., Sapporo, July 14, 1958.

11) T. Ikoma, *J. Japanese Biochem. Soc. (Seikagaku)*, **26**, 460 (1954).

12) S. Morisawa, Symp. on the Structure of Proteins, Fukuoka, Oct. 29, 1956.

for heterogeneity by means of Craig's countercurrent distribution (C. C. D.) method[13-15].

The usefulness of C.C.D., using a solvent system consisting of lauric acid in butanol and aqueous sodium acetate, for the fractionation of the methyl ester hydrochlorides of clupeine[3-5] and mugiline-β[11,12] has already been described. This solvent system, however, proved to be unsuitable for these protamine sulfates[3,11]. The sulfate is considered to represent more intact protein without any treatment of methyl esterification and to be suitable for the elucidation of the origin of the heterogeneity of clupeine. Thus, a course of study aiming to obtain desirable conditions for the fractionation of clupeine sulfate by means of C.C.D. is described in this paper.

Experimental

Preparation of Clupeine Sulfate[9]. — Dry powder** (50 g.) of the matured milts of herring (*Clupea pallasii*) obtained in 1951 (at Yoichi facing the Japan Sea, Hokkaido, Japan) was stirred with 0.2 N hydrochloric acid (600 ml.) under ice-cooling for 50 minutes, and clupeine was precipitated as picrate (yield 12.3 g.) by the addition of 0.125 M sodium picrate to the first extract. The clupeine picrate (5.29 g.) was then dissolved into 67% (wt./wt.) aqueous acetone, and converted in to the sulfate by adding sulfuric acid (to pH 2) and ethanol under cooling. The aqueous solution of clupeine sulfate reprecipitated once was passed through the Dowex-2 column (sulfate form) to remove the contaminating picric acid. Thus white glittering powder (yield 2.29 g.) of clupeine sulfate was obtained from the eluate after two reprecipitations.

Preparation of Clupeine *p*-Toluenesulfonate.—An aqueous acetone solution of clupeine picrate (253 mg. in 2.5 ml.) and an aqueous solution (6.8 ml.) of 4% *p*-toluenesulfonic acid (see below) were mixed. The liberated picric acid was removed by means of ether extraction (five times with 10 ml. each) and by means of treatment with Amberlite IRA-410 (*p*-toluenesulfonate form). The resultant solution (pH 2) was neutralized with Amberlite IR-4B (OH-form). White amorphous powder of clupeine *p*-toluenesulfonate was obtained by lyophilization (yield 99 mg.)***.

Solvents for the C.C.D.—*n*-Butanol, commercial grade; acetic, dichloroacetic, trichloroacetic and picric acid, commercial grade.

p-Toluenesulfonic acid—The commercial reagent was treated with charcoal and recrystallized from a hot solution in concentrated (1 : 1, by volume) hydrochloric acid, or by saturation of the aqueous solution with gaseous hydrogen chloride. The melting point was 104~105°C (cor.).

Principles of Method. — To the C.C.D. of proteins, only a few types of solvent systems, especially the so-called "butanol systems"[16] are applicable. The author tested, therefore, various systems consisting of *n*-butanol and aqueous solution containing some reagents such as hydrochloric, acetic, dichloroacetic, trichloroacetic, *p*-toluenesulfonic**** or picric acid, sodium *p*-toluenesulfonate and sodium chloride. In every case, the partition coefficients of clupeine in these systems were measured, and the system satisfying the conditions necessary for C. C. D.[14] —no irreversible effects on the solute and a linear partition isotherm***** with a partition coefficient not much shifted from unity—was searched for.

Determination of Partition Coefficient.—A mixture of 1 ml. of *n*-butanol and 1 ml. of an aqueous solution containing the known concentrations of a clupeine sample and a reagent or reagents (see above) were put into a test-tube (1×12 cm.) with a glass stopper, and left to stand at a constant temperature in an incubator for 30 minutes. Then the test-tube was vigorously shaken for about 30 seconds at room temperature, and allowed to stand again in an incubator for 10 minutes. After the phases separated, the amounts of clupeine in both of the phases were measured by the retention analysis using an acidic dye, ponceau 6RF[9]. The average value from three to six measurements was used. The ratio $K=C_1/C_2$, where C_1 and C_2 are the equilibrium concentration of the sample in the upper and the lower phases respectively, was defined as a partition coefficient.

Results and Discussion

Dependence of the Partition Coefficient upon Concentration of the Reagents in the System.—In the systems consisting of *n*-butanol and aqueous solutions of various concentrations of hydrochloric, acetic or picric acid, the *K*-values of clupeine sulfate were found to be almost zero. Therefore these systems were discarded.

In the case of the systems containing

13) L. C. Craig, *J. Biol. Chem.*, **155**, 519 (1944).
14) L. C. Craig, C. Golumbic, H. Mighton and E. Titus, ibid., **161**, 321 (1945).
15) J. R. Weisiger, "Organic Analysis", Vol. 2, Interscience Publisher, New York (1954).
** The preparation of the dry powder of the milts was performed by Dr. M. Yamasaki according to the Rasmussen's method[7].
*** An atempt to obtain clupeine *p*-toluenesulfonate by elution of clupeine adsorbed on Amberlite XE-64 (IRC-50) with aqueous *p*-toluenesulfonic acid was unsuccessful.
**** Dichloroacetic, trichloroacetic and *p*-toluenesulfonic acids are known to be good complexing agents for peptides or proteins[16,17].
16) F. Sawada, *Protein-Nucleic Acid-Enzyme*, (*Tampakushitsu-Kakusan-Koso*), **2**, 167 (1957).
17) L. C. Craig, *Anal. Chem.*, **28**, 723 (1956).
***** A plot of the concentration of solute in one phase in equilibrium with the concentration in the second one is called a partition isotherm. When a partion isotherm remains constant over a wide range of concentration, the isotherm is said to be linear (Ref. 15, p. 277).

dichloroacetic, trichloroacetic or *p*-toluenesulfonic acid, the *K*-values of clupeine sulfate at a definite concentration increased as the concentration of these acids increased (Fig. 1). Because of the low *K*-value for dichloroacetic acid and of the difficult solubility of the sample in trichloroacetic acid, only the system containing *p*-toluenesulfonic acid was found to be favorable for the distribution of clupeine.

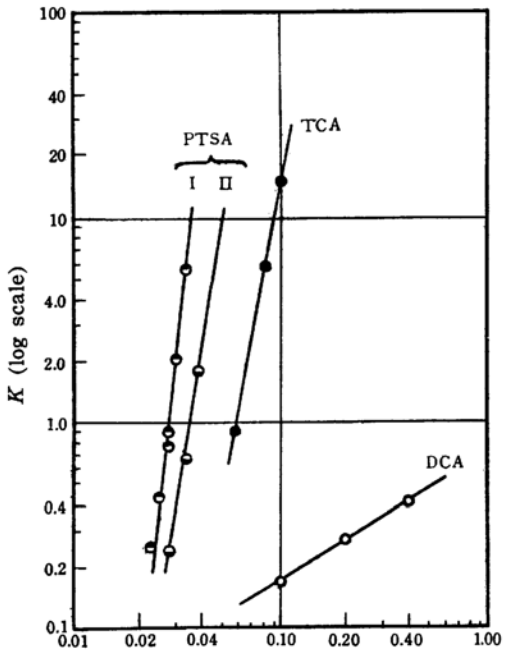


Fig. 1. Dependence of *K*-value of clupeine sulfate upon the concn. of acid in the system of *n*-butanol vs. aq. acid (18~23°C).

Symbol	Acid	Concn. of clupeine sulfate (%)
○—○	dichloroacetic	0.025
●—●	trichloroacetic	0.025
I ◑—◑	*p*-toluenesulfonic	0.02
II ◑—◑	*p*-toluenesulfonic	0.04

Dependence of the Partition Coefficient upon the Concentration of the Sample.— In the solvent system consisting of *n*-butanol and an aqueous *p*-toluenesulfonic acid at a definite concentration, the partition isotherm was not linear; in other words the *K*-value of clupeine sulfate decreased as its concentration increased (curve I in Fig. 2). The same relation was observed when either clupeine *p*-toluenesulfonate was taken for its sulfate (curve II in Fig. 2) or *p*-toluenesulfonic acid was replaced with its sodium salt (curve III in Fig. 2); only in the last case *n*-butanol in the system was replaced by *n*-butanol-*n*-propanol mixture (3 : 1, v./v.) from the viewpoint of miscibility.

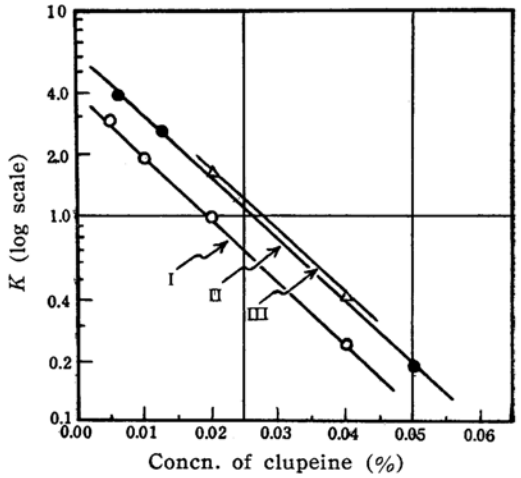


Fig. 2. Dependence of *K*-value of clupeine salt upon its concn. in the system of *n*-butanol* vs. *p*-toluenesulfonic acid or its Na salt.

Symbol	Concn. of the acid or the salt	Clupeine	Temp. (°C)
I ○—○	0.029 M acid	sulfate	20
II ●—●	0.020 M acid	*p*-toluenesulfonate	27
III △—△	0.021 M salt	sulfate	30

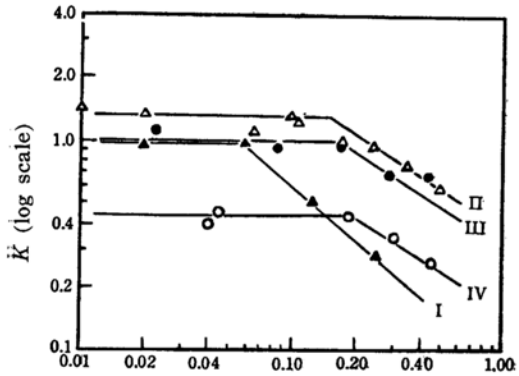
* In the case of III, *n*-butanol-*n*-propanol (3 : 1).

The non-linearity of an isotherm is generally observed in the partition of inorganic cations, and the favorable partition can be performed by the use of a solvent system containing a large excess of a complexing agent at a constant pH[18]. The same situation is expected in the case of clupeine which is a strongly basic protein.

Thus, the higher concentration of *p*-toluenesulfonic acid may bring a linear isotherm but with an undesirably large *K*-value and a very low pH. However, its salts, for example sodium *p*-toluenesulfonate, would have the same effect at neutral pH as the acid has, and the addition of sodium chloride in the system would make the *K*-value decrease because of its "salting-in" effect. Therefore, in the solvent system consisting of *n*-butanol and an aqueous solution containing a high and suitable concentration of both sodium *p*-toluenesulfonate and sodium chloride, a

18) E. Hecker, "Verteilungsverfahren im Laboratorium", Verlag Chemie, GMBH, Weinheim/Bergstr. (1955), pp. 18, 107.

linear isotherm with a suitable K-value would be obtained at a neutral pH range. In fact, these considerations were proved to be the case as shown in Fig. 3.



Concn. of clupeine sulfate (% in log scale)

Fig. 3. Dependence of K-value of clupeine sulfate upon its concn. in the system of n-butanol vs. aq. salts.

Symbol	Concn. of Na p-toluene-sulfonate	Concn. of NaCl	Temp. (°C)
I ▲—▲	ca. 0.07 M	0.20 M	23～24
II △—△	ca. 0.12 M	0.40 M	ca. 17
III ●—●	0.12 M	0.40 M	22～23
IV ○—○	0.12 M	0.48 M	22～23

The range of the clupeine concentration where the K-value remains constant becomes broader as the concentration of sodium p-toluenesulfonate increases (curves I and II, III or IV in Fig. 3). It seems that the constant K-value ranges depend only upon the concentration of sodium p-toluenesulfonate; temperature (curves II and III in Fig. 3) and concentration of sodium chloride (curves III and IV in Fig. 3) seem not to affect the constant ranges and the gradients of the curves.

Thus, the solvent system consisting of n-butanol and an equal volume of aqueous solution of 0.12 M sodium p-toluenesulfonate and 0.40 or 0.48 M sodium chloride (curve III or IV in Fig. 3) was found to possess at common temperature the linear isotherms with adequate K-values for clupeine sulfate, so far as its concentration was less than 0.2 per cent in the total volume of both phases. In ranges of the higher concentration of clupeine, the K-values suddenly decreased (see also Fig. 3).

Dependency of the Partition Coefficient upon Temperature. — The K-values of clupeine sulfate in the solvent system consisting of n-butanol and aqueous p-toluenesulfonic acid at 0°C and 40°C were equal (Table I). On the other hand, they decreased obviously in the system of n-butanol and an eqeuous solution containing sodium p-toluenesulfonate and sodium chloride as temperature increased (Table II). Therefore, we must do a C.C.D. procedure with the latter system at a constant temperature.

TABLE I

DEPENDENCE of K-VALUE OF CLUPEINE SULFATE (0.02%) UPON TEMP. IN THE SYSTEM OF n-BUTANOL VS. AQ. 0.029 M p-TOLUENESULFONIC ACID

Temp. (°C)	K-value
0	0.7_5
40	0.7_9

TABLE II

DEPENDENCE OF K-VALUE OF CLUPEINE SULFATE (0.022%) UPON TEMP. IN THE SYSTEM OF n-BUTANOL VS. AQ. 0.14 M Na p-TOLUENESULFONATE—0.40 M NaCl

Temp. (°C)	K-value
1	3.9_2
11～12	2.8_3
19～20	1.5_7
34～35	1.0_3

The solvent system described in the above, well satisfies the conditions necessary for C.C.D. procedure (see principles of the method). In such a system of neutral condition, a danger of secondary transformation of protamines would be minimized. In this system the K-value can be easily controlled by changing the concentration of the components. Moreover, the presence of sodium chloride increases the capacity of the system to dissolve the sample and reduces the time necessary for the separation of phases.

The usefulness of the present system was practically proved by a preliminary experiment of C.C.D., in which the heterogeneity of clupeine sulfate was evidently demonstrated[9], contrary to Rauen's result******,[3]. Recently, Scanes and Tozer[19] reported the fractionation of clupeine sulfate by C.C.D. technique, using a unique solvent system, n-propanol-3 M sodium acetate (3 : 2, by volume). The fractionation efficiency of their system seems to be nearly the same as ours.

In connection with the unique effect of p-toluenesulfonate anion in the solvent system, a strange phenomenon was pointed

****** In the Rauen's system, undesirably small K-values were obtained for the sulfates of clupine[3] and mugiline-β[11].

19) F. S. Scanes and B. T. Tozer, *Biochem. J.*, **63**, 565 (1956).

out by Pierce[20] in the case of C.C.D. of an ox pituitary growth hormone. In spite of the fact that the distribution pattern of the hormone protein agreed with the theoretical curve in the solvent system consisting of *sec*-butanol and 0.0044 M aqueous *p*-toluenesulfonic acid, some disagreement was found between the experimental and theoretical curves, when the acid concentration was 0.0022 M. This phenomenon might be explained by such non-linearity of the partition isotherm of the hormone as is shown above in clupeine, though Pierce denied its possibility in his paper.

Summary

The conditions and solvent system suitable for C.C.D. of clupeine sulfate, were systematically studied and the following results were obtained.

1. In the solvent system consisting of *n*-butanol and aqueous hydrochloric, acetic or picric acid, the partition coefficient (K) of clupeine sulfate was almost zero.

2. In the system consisting of *n*-butanol and aqueous dichloroacetic, trichloroacetic or *p*-toluenesulfonic acid, the K-value increased with the increase of the acid concentration at a constant solute concentration. In the last system, at a constant acid concentration, the K-value decreased with the increase of the solute concentration.

3. The system consisting of *n*-butanol and an equal volume of aqueous solution containing 0.12 M sodium *p*-toluenesulfonate and 0.40 or 0.48 M sodium chloride was found to satisfy all the conditions necessary for the C.C.D. of clupeine sulfate.

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20) J. G. Pierce, ibid., **57**, 16 (1954).