[CONTRIBUTION FROM THE UNIVERSITY LABORATORY OF PHYSICAL CHEMISTRY RELATED TO MEDICINE AND PUBLIC HEALTH, HARVARD UNIVERSITY, AND THE DEPARTMENT OF DERMATOLOGY, HARVARD MEDICAL SCHOOL]

The Interaction of Human Serum β-Lipoprotein with Certain Small Molecules[1,2]

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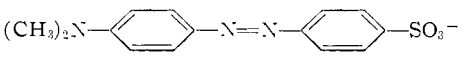
RECEIVED JUNE 11, 1955

The binding of the anionic dye methyl orange by human serum β-lipoprotein has been studied by spectrophotometric and equilibrium dialysis methods. It has been found that, at *p*H 7.4, β-lipoprotein binds methyl orange more strongly than does bovine serum albumin but less strongly than human serum albumin. This is the only reported example of a protein which binds anions on the alkaline side of the isoelectric point as strongly as the albumins. Spectrophotometric evidence is presented for the binding of the uncharged dye chrysoidin by β-lipoprotein.

Introduction

There has been much discussion in the recent literature concerning the configurational requirements for the binding of anions by proteins.[4-7] One way of testing the hypotheses set forth is to examine the binding properties of a wide variety of pure proteins and try to correlate the relative strength with which a protein binds anions with other properties or characteristics of the protein. Klotz[4] has studied the binding of methyl orange (I) by a number of pure proteins and correlated

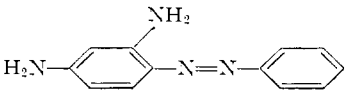


I

the binding strength with the binding index, a function of the amino acid composition, based on the concept of competition of the anion with other groups on the protein for binding sites. Mihalyi[8] has studied the binding of methyl orange by myosin and found that the results support the binding index hypothesis.

In the course of other studies on human serum β-lipoprotein it seemed worthwhile to investigate the binding of methyl orange by this protein in order to provide data to test further the hypotheses which have been proposed.

Since it has been suggested that β-lipoprotein is a transport medium for charged and uncharged lipids in the blood stream,[9] and since the binding of neutral molecules has been attributed to the same sites on the albumin molecule as the binding of anions,[10] spectral studies were also carried out on the binding of the uncharged dye chrysoidin (2,4-diaminoazobenzene) (II) by β-lipoprotein.[11]



II

Experimental

Materials.—The human serum β-lipoprotein was prepared from Fraction III-O of human plasma by the method of Oncley, Gurd and Melin.[9] Fraction III-O was provided by the laboratories of the Massachusetts Department of Public Health as a by-product in the preparation of albumin[13] and γ-globulin[14] from blood collected by the American Red Cross.

Phosphate buffer was prepared from reagent grade salts. The crystalline sodium salt of methyl orange was a product of Merck and Company. Crystalline chrysoidin hydrochloride was obtained from National Aniline Company.

Methods.—The interaction was studied by the method of equilibrium dialysis. A bag of Visking sausage casing containing 5 ml. of approximately 0.5% protein solution in 0.1 *M* phosphate buffer at *p*H 7.4 was placed in a glass vial containing 5 ml. of dye solution in buffer. The vial was then stoppered with a polyethylene cap and allowed to rotate in a constant temperature bath at 0.0 ± 0.1° for 16 hours. The time required to reach dialysis equilibrium was determined by rotating for varying lengths of time a vial in which a solution of methyl orange and a dialysis bag containing only buffer were placed. Sixteen hours were found to be adequate. Such a vial was also carried through with each group of dialysis experiments. The equilibrium concentration of methyl orange in the outside solution was determined by measurement of the absorbancy of the solution at 465 mμ in a 1-cm. cuvette with a Beckman DU spectrophotometer. The total concentration of dye inside the bag was determined by measuring the absorbancy of the solution at the isosbestic point of the spectra of free and bound dye and correcting for the absorbancy of the protein at this wave-length. The free dye concentration inside the bag was assumed to be the same as the free dye concentration outside the bag, since the Donnan effect should be negligible in solutions of 0.1 *M* phosphate for the protein concentration used. The protein concentration was calculated using a known dilution from a stock solution. The concentration of the stock solution was determined by drying an aliquot to constant weight *in vacuo* at 70°, with correction for the weight of salts in the solution. Absorption spectra were determined with a Beckman DU spectrophotometer. *p*H measurements were made with a Beckman Model G *p*H meter.

Results

In the presence of 0.5% human serum β-lipoprotein the peak in the absorption spectrum of methyl orange was shifted from 465 to 420 mμ,

(1) Supported by a research grant from the United States Public Health Service (RG-3091).

(2) Presented before the Division of Biological Chemistry at the 124th meeting of the American Chemical Society, Chicago, September, 1953.

(3) Hall Laboratory of Chemistry, Wesleyan University, Middletown, Connecticut.

(4) I. M. Klotz and J. M. Urquhart, THIS JOURNAL, **71**, 1597 (1949).

(5) J. M. Luck, *Discs. Faraday Soc.*, **6**, 44 (1949).

(6) F. Karush, THIS JOURNAL, **72**, 2705 (1950).

(7) J. Schellman, *J. Phys. Chem.*, **57**, 472 (1953).

(8) E. Mihalyi and B. N. Ghosh, *Arch. Biochem. Biophys.*, **41**, 104 (1952).

(9) J. L. Oncley, F. R. N. Gurd and M. Melin, THIS JOURNAL, **72**, 458 (1950).

(10) I. M. Klotz and J. Ayers, *ibid.*, **74**, 6178 (1952).

(11) With the possibility in mind that calcium binding by lipoproteins might account for a substantial part of the serum calcium which is bound by globulin,[12] exploratory experiments were carried out on the interaction of calcium with β-lipoprotein, α-lipoprotein, fraction IV-4[13] and γ-globulin. It was found that the lipoproteins and fraction IV-4 bind approximately the same amount of calcium, while the binding by γ-globulin is substantially smaller. The amounts bound were such as to indicate that none of the globulins tested accounts for a disproportionate amount of the serum bound calcium.

(12) F. C. McLean and A. B. Hastings, *J. Biol. Chem.*, **108**, 285 (1935).

(13) E. J. Cohn, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin and H. L. Taylor, THIS JOURNAL, **68**, 459 (1946).

(14) J. L. Oncley, M. Melin, D. A. Richert, J. W. Cameron and P. M. Gross, Jr., *ibid.*, **71**, 541 (1949).

as indicated in Fig. 1. This shift is in the same direction as found by Klotz for bovine albumin[15] and β-lactoglobulin.[4] When equilibrium dialysis experiments were carried out with β-lipoprotein, the results shown in Fig. 2 were obtained. For purposes of comparison the results are plotted as number of moles of bound dye per 10^5 grams of protein, and some data of Klotz and co-workers for binding of methyl orange by bovine serum albumin,[16] human serum albumin[4] and β-lactoglobulin[4] have been plotted on the same graph. It may be seen that β-lipoprotein binds methyl orange much more strongly than β-lactoglobulin does and is comparable in binding ability to bovine and human albumin. The experiments with β-lipoprotein were carried out in higher concentrations of phosphate buffer than those with bovine and human albumin and β-lactoglobulin. Any errors in the comparison would therefore be in the direction of minimizing the binding ability of the lipoprotein. In the case of the β-lipoprotein, only about 23% of the weight of the lipoprotein is due to peptide,[9] and if the results of Fig. 2 were expressed in terms of peptide (or amino acid) content, the binding by β-lipoprotein would be stronger than any of the other proteins mentioned here.

The results of spectrophotometric studies with chrysoidin are shown in Figs. 3 and 4. The change in the absorption spectrum of the dye in the presence of β-lipoprotein is presumptive evidence for the interaction of this uncharged dye with the protein. For reasons which will become clear below, the spectra of chrysoidin in the presence of serum albumin, in strongly acid solution, in petroleum ether and in dodecyl alcohol are also shown.

Discussion

Up to this time no other globular protein has been found to bind anions on the alkaline side of the isoelectric point as strongly as do the serum albumins. The explanations advanced for the heretofore unique position of the serum albumins in anion binding have been primarily of two kinds. Klotz[4] has correlated the binding ability of various proteins with the amino acid composition, while Karush[17] has invoked the "configurational adaptability" of serum albumin to account for its affinity for a wide variety of anions.

Since no amino acid analyses are available for serum β-lipoprotein, it is not yet possible to use the data reported here as a test for the "binding index" of Klotz. Furthermore, the presence of 75% by weight of lipid in the lipoprotein molecule[9] may make inapplicable a test based on the analysis of the 25% of the molecule composed of peptide chains.

In discussing the "configurational adaptability" of serum albumin Karush[17] correlated the binding ability of albumin for anions with its solubility. β-Lipoprotein is a euglobulin, insoluble in the absence of salts at the isoelectric point,[9] but still binds anions as strongly as does albumin. This would seem to throw some doubt on the correlation between solubility and anion binding.

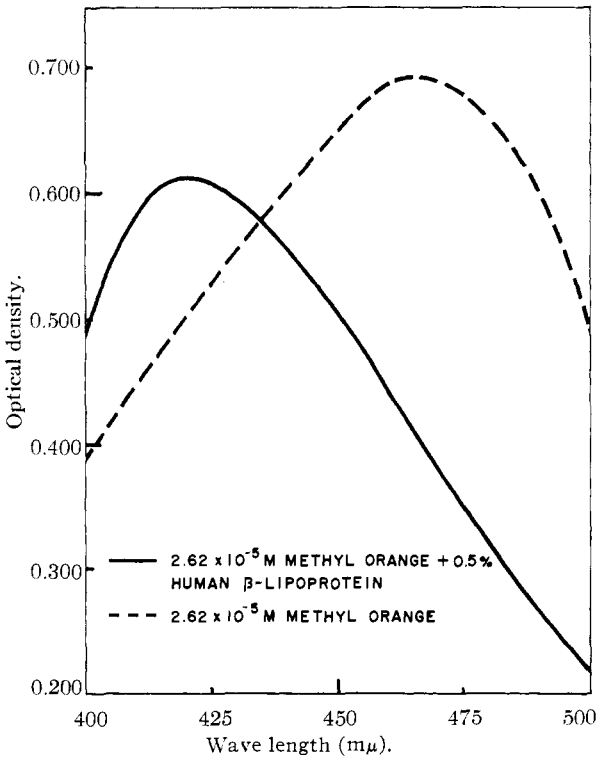


Fig. 1.—Spectrum of methyl orange in the presence and absence of β-lipoprotein; 0.1 *M* phosphate buffer, *p*H 7.4.

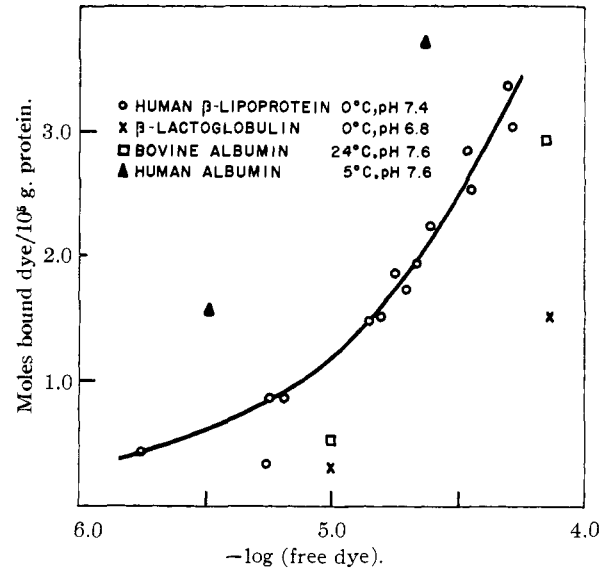


Fig. 2.—Binding of methyl orange by β-lipoprotein; 0.1 *M* phosphate buffer, *p*H 7.4.

In order to obtain additional information about the binding sites on the lipoprotein molecule, the results were calculated in terms of $\bar{\nu}$, the average number of dye ions bound per mole of lipoprotein, at each concentration, c, of free dye. The molecular weight of the lipoprotein was taken to be 1,300,000.[18] If there are n binding sites with

(15) I. M. Klotz, THIS JOURNAL, **68**, 2299 (1946).

(16) I. M. Klotz, H. Triwush and F. M. Walker, *ibid.*, **70**, 2935 (1948).

(17) F. Karush, *ibid.*, **72**, 2705 (1950).

(18) J. L. Oncley, G. Scatchard and A. Brown, *J. Phys. Colloid Chem.*, **51**, 184 (1947).

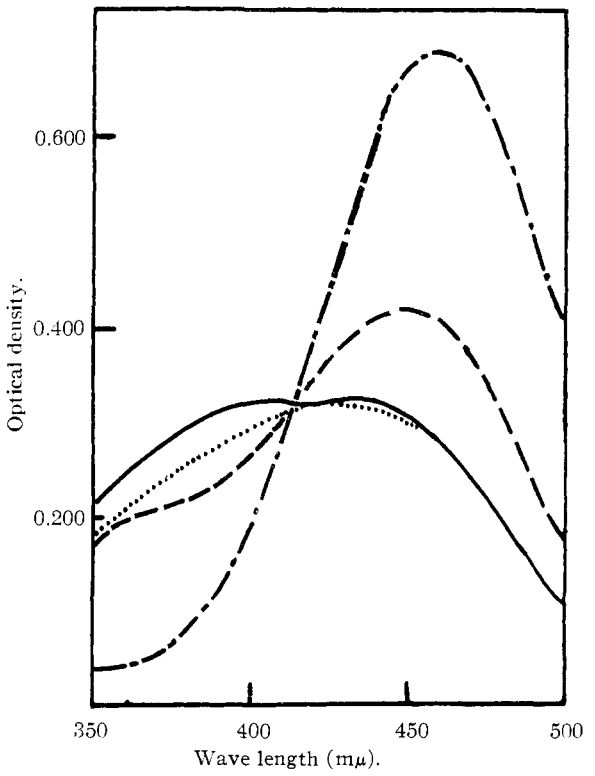


Fig. 3.—Absorption spectra of chrysoidin: —, in 0.1 *M* phosphate buffer, *p*H 7.4; — — —, in buffer with 0.5% β-liopoprotein; . . . . , in buffer with 0.5% human albumin; — · —, in 1 *M* HCl.

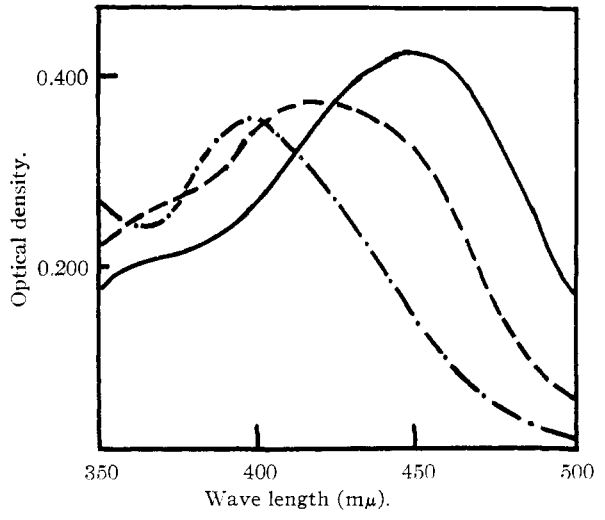


Fig. 4.—Absorption spectra of chrysoidin: –, in 0.1 *M* phosphate buffer, *p*H 7.4, with 0.5% β-lipoprotein; — — — —, in dodecyl alcohol; — · —, in petroleum ether.

intrinsic association constants K_i, Von Muralt[19] has shown that

$$\bar{\nu} = \sum_{i=1}^{n} \frac{K_i c}{1 + K_i c} \qquad (1)$$

The simplest case to treat is that in which all the sites have the same intrinsic constant, k, and there is no interaction between successively bound ions. Then, (1) reduces to

$$\bar{\nu} = \frac{nkc}{1 + kc} \qquad (2)$$

If (2) is rearranged as suggested by Scatchard,[20] the resulting expression is

$$\bar{\nu}/c = nk - \bar{\nu}k \qquad (3)$$

If the condition of equal intrinsic constants is fulfilled, a plot of $\bar{\nu}/c$ against $\bar{\nu}$ should be linear. In Fig. 4, the points are the experimental values for the binding of methyl orange by β-lipoprotein, and the straight line was calculated from the data by the method of least-squares. The least-squares equation leads to a value for n of 69 and a value for k of 3.01×10^4. In the absence of additional data at very low and high values of $\bar{\nu}$ the apparent linearity of the data may be deceptive. This, together with the use of a simple least-squares treatment which assumes equal uncertainty in the ordinates and none in the abscissas, leads to considerable uncertainty in the numerical values of n and k. The difficulties in assigning an absolute significance to the values of n obtained from binding data have been pointed out by other authors.[20-23]

The results with chrysoidin are of interest because albumin has been considered unique in its ability to bind neutral molecules[10] as well as anions. As with albumin, the ability to bind neutral molecules accompanies the ability to bind anions.

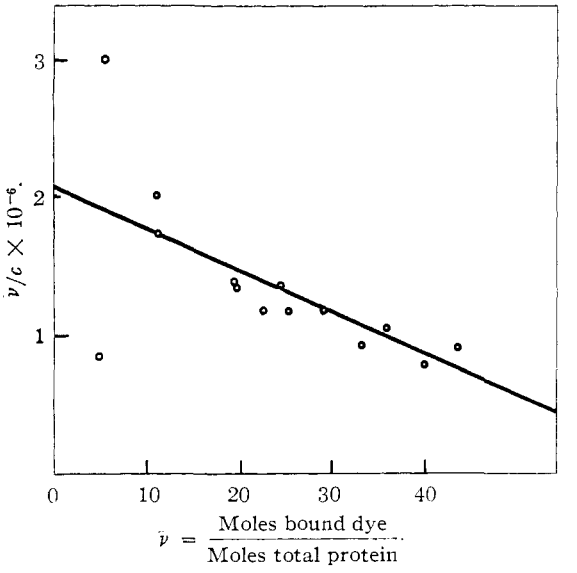


Fig. 5.—Binding of methyl orange by β-lipoprotein, 0.1 *M* phosphate buffer, *p*H 7.4, 0°.

The spectra of chrysoidin shown in Figs. 3 and 4 were obtained to gain some insight into the nature of the binding of this dye by β-lipoprotein. In Fig. 3 are the spectrum of chrysoidin in phosphate buffer at *p*H 7.4, the spectrum in the presence of β-lipoprotein in the same buffer, and the spectrum

(19) A. L. Von Muralt, THIS JOURNAL, **52**, 3518 (1930).

(20) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).

(21) I. M. Klotz and J. M. Urquhart, THIS JOURNAL, **71**, 847 (1949).

(22) F. Karush and M. Sonenberg, *ibid.*, **71**, 1369 (1949).

(23) I. M. Klotz, *Cold Spring Harbor Symposia Quant. Biol.*, **14**, 97 (1950).

in acid solution. These data support the suggestion that the uncharged dye is bound to the protein by hydrogen bond formation in which the dye acts as an electron donor, as proposed for binding by albumin.[10] The hydrogen bonded dye would be expected to have a spectrum intermediate between that of the free base and that of the hydrochloride. We cannot explain at the present time why albumin, which has been shown by equilibrium dialysis methods to bind chrysoidin,[10] does not produce such a spectral shift.

To test the possibility that the dye is bound to the lipoprotein by "solution" in the lipid portion of the protein a spectrum of the free base in petroleum ether was obtained. It can be seen from Fig. 4 that this spectrum is quite different from that obtained in the presence of the protein, indicating that the environment of the dye is not entirely hydrophobic when bound. It has also been suggested[24] that the spectral shift could be a result of hydrogen bonding with the lipid portion of the protein molecule. The similarity of the spectrum in dodecyl alcohol to that in the presence of lipoprotein supports this point of view.

Acknowledgments.—The authors gratefully acknowledge the generous advice and the encouragement of Drs. J. L. Oncley and F. R. N. Gurd. Thanks are also due E. Virginia Rosenberg for some of the spectrophotometric data.

(24) By a referee.

BOSTON, MASS.

NOTES

Non-exchange of F^{18} between HF and Fluorinated Methanes

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RECEIVED AUGUST 3, 1955

The exchange of isotopic chlorine between HCl and the series of compounds CH_3Cl, CH_2FCl, CHF_2Cl and CF_3Cl in the gas phase recently has been studied[1] in an attempt to obtain information regarding the effect of fluorine on the reactivity of such compounds. We have now attempted to obtain similar data for fluorine exchange between HF and the compounds CH_3F, CH_2F_2, CHF_3, CF_4, and CF_2Cl_2.

The longest-lived isotope of fluorine, F^{18}, has a half-life of only 112 minutes, and there is only one stable isotope, F^{19}. For this reason we have conducted a few experiments attempting to prepare F^{21} in the hope that it might have a longer half-life, although crude calculations indicate that its half-life may be only on the order of seconds or minutes.

Experimental

Materials.—Methyl fluoride was synthesized by the reaction between KF and CH_3SO_4K.[2] Traces of acetone were removed by repeated distillation from a trap at −100°. The authors would like to express their appreciation to Dr. W. B. McCormack of E. I. du Pont de Nemours and Co. for supplying us with samples of CH_2F_2, CHF_3 and CF_4. The compound CF_2Cl_2 was purchased from the Matheson Co. We are grateful to Dr. H. A. Bernhardt and Dr. W. Davis, Jr., of the K-25 Plant, Carbide and Carbon Chemicals Co., Oak Ridge, for supplying us with highly purified HF and for valuable suggestions relating to the experimental techniques we have used. All compounds were fractionally distilled *in vacuo* and vapor pressure measurements used to establish purity.

Radioactive F^{18}.—Several methods were tried for the production of F^{18}. Irradiation of NH_4F in the Low Intensity Test Reactor for two hours produced about 10 microcuries of F^{18} per gram of NH_4F by the reaction $F^{19}(n,2n)F^{18}$. Irradiation of LiF containing 10 mole % Al_2O_3 in the Graphite Reactor for two hours gave about one microcurie of F^{18} per gram of starting material by the reactions $Li^6(n,\alpha)H^3$, $O^{16}(t,n)F^{18}$. Bombardment of $LiNO_3$ in the Graphite Reactor for two hours produced about 50 microcuries of F^{18} per gram by the same process. There was some chemical decomposition of the nitrate. Bombardment of NaF with 22 mev. protons at low beam intensity in the 86-inch cyclotron for only one minute produced about 50 microcuries of F^{18} per gram by the reaction $F^{19}(p,pn)F^{18}$.

The F^{18} produced in the cyclotron was radiochemically pure, while all of the pile methods produced a mixture of other activities. The F^{18} was identified by its γ-spectrum, γ,γ-coincidence count (from positron annihilation), half-life and chemical separation. The method finally adopted was the $LiF-Al_2O_3$ bombardment in the pile, because of greater convenience for the purpose intended and with the facilities available.

Attempts to Produce F^{21}.—Several attempts were made to produce F^{21} by the sequence $Li^6(n,\alpha)H^3$, $F^{19}(t,p)F^{21}$. Lithium fluoride was bombarded in the Low Intensity Test Reactor for periods ranging from 10 minutes to 45 hours. No activity which could be ascribed to fluorine (other than F^{18}) was observed.

Apparatus and Procedure.—An all-nickel vacuum line was constructed using heliarc-welded joints. This consisted of a manifold with several fixed and removable traps and storage vessels. A Booth-Cromer gage was used to measure pressure.

After the $LiF-Al_2O_3$ mixture was irradiated in the pile, it was placed in a nickel exchange vessel and attached to the vacuum line. Hydrogen fluoride was condensed onto it, allowed to warm to room temperature for a few minutes, then distilled off. The F^{18} in the resulting HF was radiochemically pure and sufficiently active to allow tracer studies to be conducted over 10–12 hours.

Equal amounts of radioactive HF and one of the fluorinated methanes were transferred to a nickel reaction vessel of 70-ml. capacity. The total pressure ranged from 350 to 550 mm. at room temperature. The mixture was heated for the desired length of time in a Marshall furnace, the temperature of which was maintained constant by an Electromax controller. The products were then separated by fractional distillation in the vacuum line and the F^{18} activity in the two products compared with that in the original HF. The gas to be counted was contained in a nickel vessel which could be placed in a well-type scintillation counter.

Results

No exchange of fluorine was observed between HF and the compounds CH_2F_2, CHF_3, CF_4 or

(1) J. E. Boggs and L. O. Brockway, THIS JOURNAL, **77**, 3444 (1955).

(2) E. Moles and T. Batuecas, *J. chim. phys.*, **17**, 537 (1919).