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Experimental Studies in Metal Cancerigenesis. III. Behavior of Chromium Compounds in the Physiological *p*H Range

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Investigation of the behavior of Cr^{III} and Cr^{VI} compounds, represented by potassium chromate and chromic acetate, in the *p*H range 4–8 and concentration range 10^{-6}–10^{-2} molar has been made by spectrophotometric, zone and moving boundary electrophoretic and dialysis techniques. Cr^{VI} was completely diffusible under these conditions. Chromic acetate undergoes changes at the normal *p*H of blood, 7.35, hydrolysis, olation, aggregation and eventual precipitation of chromic hydroxide and basic salts, similar to those occurring in solutions of mineral acid salts below and near the precipitation *p*H of chromic hydroxide. These changes are accompanied by significant shifts in absorption maxima and minima and changes in absorbance. The corresponding changes in charge and mobility of the complexes thus formed were followed electrophoretically. The diffusibility of chromic acetate solutions as a function of *p*H, concentration and aging was studied.

Our interest in chromium and its compounds stems from the facts that workers employed in the handling and processing of chromite ore, chromates and chromium pigments have a highly excessive liability to cancer of the lung[1–3]; abnormal amounts of chromium have been found in the blood, urine and tissues of workers in such occupations[3]; the use of chromium and its compounds has steadily increased during the last two decades. The present status of the controversy over the form, valency and solubility of such a chromium containing etiologic factor, complicated by lack of correlative and confirmatory animal data, is presented in several reviews.[1–3]

As part of a general program for the investigation of the metabolism of chromium, the interaction of trivalent and hexavalent chromium compounds with proteins was studied by purely chemical and immunochemical means. Because of the very complex behavior of trivalent chromium compounds at *p*H's approaching neutrality, investigation of the behavior of certain chromium compounds became necessary. The present paper will be confined to investigations concerning tri- and hexavalent chromium compounds in "the physiological *p*H range," *i.e.*, *p*H 4–8. Results of immunochemical and chromium protein studies will be reported separately.

An excellent review of earlier work concerned with the complex behavior of trivalent chromium salts in aqueous media has been compiled by Stiasny.[4] More recent treatments of comprehensive nature are those of Herfeld[5] and Grassmann.[6] Investigation of the composition and charge of chromium complexes in aqueous media containing chromium salts, acid, neutral salts and masking agents, chiefly with the view of correlating such findings with the tanning properties of such solutions, has been carried out by titrimetric, conductometric,[7,8] spectrophotometric[9] and electrophoretic[10] methods as well as in recent years by numerous ion exchangers.[11,12] Of particular significance to our problem in considering the vast literature of the leather chemists is the fact that most of these investigations have been carried out at low *p*H's. As stated by Green[13] the total commercial tanning range lies within the *p*H limits 1.8–3.3.

Experimental

Materials. Potassium Dichromate.—A reference standard stock solution of potassium dichromate, primary standard grade, was prepared to contain 100 μg. Cr/ml.

Potassium Chromate, Chromic Chloride.—Stock solutions of potassium chromate 10.39 mg. Cr/ml. and chromic chloride 10.65 mg. Cr/ml. were standardized by iodometric titration.

Chromic Acetate.—The choice of chromic acetate as representative of trivalent chromium salts was made largely for technical reasons. Britton[14] presents considerable data showing that the precipitation of chromic hydroxide, phosphate and basic salts occurs from dilute solutions in the *p*H range 5.2–5.65. This precluded the use of salts of mineral acids to study the higher *p*H range. A stock solution of chromic acetate containing 12.10 mg. Cr/ml. (0.2326 molar) was prepared and standardized. Solutions prepared by dilution of this stock solution in the range 0.8×10^{-4}–2.1×10^{-3} molar could be stored in phosphate buffer *p*H 7.35 up to two months at room temperature and 1 year at 5–8° without precipitation. Other "fresh" solutions of chromic acetate were prepared from the same solid reagent sample, the chromium content of which had been established by analysis.

Buffers.—The question of buffers for use in the trivalent chromium experiments was complicated by the "masking" effects on trivalent chromium salts of many ions employed in buffer mixtures. This was true of most universal buffers that would cover the *p*H range 4–8. Since chromic acetate was employed, the use of acetate buffers for the range 4–5.6 was the least objectionable of many possibilities. Sodium and potassium phosphate buffers were used for the *p*H range 6–8. Both of these buffers have the advantage of containing only ions normally present in physiological media.

Dialysis Membranes.—Visking seamless clear Cellophane tubing of various diameters was used for the dialysis experiments. Sections of the membranes were soaked in

(1) Public Health Service Publication #192, "Health of Workers in Chromate Producing Industry," U. S. G. P. O. 1953.

(2) W. C. Hueper, "Occupational Tumors and Allied Diseases," C. C Thomas, Springfield, Ill., 1942.

(3) T. F. Mancuso and W. C. Hueper, *Indust. Med. Surg.*, **20**, 358, 393 (1951).

(4) E. Stiasny, "Gerbereichemie," Steinkopff, Dresden and Leipzig, 1931.

(5) H. Herfeld, "Grundlagen der Lederherstellung," Steinkopff, 1950.

(6) W. Grassmann, "Handbuch der Gerbereichemie," Springer, Vienna, 1939.

(7) A. Küntzel, H. Erdmann and H. Spahrkäs, *Das Leder*, **3**, 73, 102, 148 (1952); H. Erdmann and H. Spahrkäs, *Angew. Chem.*, **64**, 500 (1952).

(8) S. G. Shuttleworth, *J. Intern. Soc. Leather Trades' Chemists*, **24**, 115, 166 (1940); **27**, 104 (1943); **29**, 3 (1945).

(9) K. H. Gustavson, *J. Soc. Leather Trades' Chemists*, **34**, 259 (1950).

(10) K. H. Gustavson, *ibid.*, **35**, 160 (1951).

(11) R. S. Adams, *J. Am. Leather Chemists' Assoc.*, **41**, 552 (1946).

(12) K. H. Gustavson, *Svensk. Kem. Tidskr.*, **56**, 14 (1944); **58**, 2, 274 (1946); *J. Intern. Soc. Leather Trades' Chemists*, **29**, 11 (1945). K. H. Gustavson and B. Holm, *Svensk. Kem. Tidskr.*, **64**, 137 (1952).

(13) R. W. Green, *Biochem. J.*, **54**, 187 (1953).

(14) H. T. S. Britton, "Hydrogen Ions," Chap. XVII–XX, D. Van Nostrand Co., New York, N. Y., 1932.

several changes of distilled water before use. The bottom end of the membrane was best tied with undyed buttonhole twist or #30 pure linen thread, which were small enough to effect a good seal but not to cut the membrane.

Methods. Moving Boundary Electrophoresis.—Moving boundary electrophoretic studies of chromic acetate in phosphate buffer $\mu = 0.10$, *p*H 7.35 in the concentration range 3.53×10^{-3} to 7.05×10^{-2} molar were performed on both freshly prepared solutions and solutions aged up to 15 days. Somewhat higher concentrations of chromium than were employed in dialysis and other studies had to be employed in this method in order to see and photograph the pattern. These experiments were carried out using the Aminco-Stern Electrophoresis Apparatus equipped with the Thovert-Philpot-Svensson system of observation. The measurements were made in the standard analytical cell (9-ml. capacity) at 2–3° using a current of 10 ma. for periods of 2–3 hours. It was found that the use of Dow DC-4 silicone grease[15] *in lieu* of the recommended Tiselius grease resulted in sealing the component parts of the cell for at least 5–6 runs and did not cause abnormalities in the patterns or later ones with proteins. Stern and Reiner[16] reported that the use of Celloseal grease caused many abnormalities in the electrophoretic patterns of sera which they attributed to the formation of lipoprotein complexes with the grease.

Zone (Paper) Electrophoresis.—By the use of zone electrophoresis techniques a much wider range of conditions could be more readily investigated with very small amounts of materials. The limit was determined largely by the analytical method employed for chromium. The electrophoresis apparatus was similar to that described by Durrum[17] Ten strips of Whatman 3MM filter paper one inch wide could be run simultaneously. The amount of solution streaked across the apex of each strip was usually 25 μl. The high voltage supply of the Aminco–Stern apparatus was used to carry out the electrophoresis at a constant current of 0.59 ma. per cm. width of strip. Individual runs were carried out for periods of 5–6 hours. The effects of aging, temperature of aging, and aging more concentrated solutions in phosphate buffer and then diluting them and comparing with dilute solutions aged at the final concentration, were investigated.

Dialysis Experiments.—Two dialysis systems were employed: one for dialysis of combined sample and dialysate sizes up to 125 ml. and the other for total volumes up to 30 ml. The larger size system was constructed from 4 × 10 cm. thick wall specimen bottles. The bottle was closed with a #9 rubber stopper through which passed a 5-cm. length of 10–12 mm. glass tubing. This tubing, which extended 1–2 cm. into the bottle, provided for attachment of the membrane and permitted removal of samples from inside the membrane at any time. A 3–5 mm. hole near the edge of the stopper permitted removal of samples exterior to the membrane. Stirring external to the membrane was provided by variable speed magnetic stirrers. In some cases when studying trivalent chromium, it was necessary to stir the liquid on both sides of the membrane in order to attain equilibrium with respect to chromium. In such experiments internal stirring was also provided by glass stirrers and small 3-watt motors.[18]

The smaller system was constructed by flattening the bottoms of 25 × 100 mm. Pyrex test-tubes. The same stirring and mounting arrangements could be employed with this system using $^9/_{16}$" inflated diameter membranes.

Dialysis experiments were generally of three types: determination of minimal time for equilibration, determination of diffusion rate and percentage of diffusible chromium. These were carried out at 5–8° in $\mu = 0.1$ buffers. Known amounts[19] of Cr^{III} and Cr^{VI} were added either interior or exterior to the membrane. Samples were withdrawn[20] for analysis either during the dialysis or after dialysis for definite time intervals. The rate of diffusion and time for equilibration of Cr^{VI} at the various *p*H's was determined. Experiments with chromic acetate, aged at 5–8° at *p*H's from 4–7.35 for periods up to 60 days, were run to study the diffusibility of chromium complexes thus formed.

Spectrophotometric.—Spectrophotometric studies of the behavior of chromic acetate in the concentration range 10^{-3}–10^{-2} molar were carried out using 1-cm. Corex cells and a Beckman model DU spectrophotometer. Positions of and shifts in maxima and minima were determined for solutions in water and in phosphate buffer for periods of aging up to 91 days at room temperature. Measurements were made on fresh and aged solutions adjusted to various *p*H's from 4–11. The spectra of chromates have been extensively studied[21,22] and were not further investigated. All *p*H measurements were made with a Beckman model G *p*H meter and are accurate to ±0.02 *p*H.

Analytical.—Spectrophotometric microdeterminations of chromium during this work were based on the pink 1,5-diphenylcarbohydrazide–chromium complex formed at *p*H 1.3–1.8.[23–25] A simplified procedure has been worked out and will be published later.

Results and Discussion

Moving Boundary Electrophoresis.—The derived mobilities of the various components in phosphate buffer of *p*H 7.35 (a) at constant time of aging and varying chromium concentration and (b) at constant concentration and varying times of aging are shown in Table I. At constant age and increasing chromium concentration predominantly negative components were present the mobility of which decreased with increasing chromium concentration. At constant concentration and varying age, positive and negative components existed simultaneously.

TABLE I

MOVING BOUNDARY ELECTROPHORESIS OF CHROMIC ACETATE SOLUTIONS IN PHOSPHATE BUFFER *p*H 7.3–7.4 AND IONIC STRENGTH 0.1

$Cr^{III} \times 10^3$, *M*	Mobility, × 10^5 cm.^{2} volt^{-1} sec.$^{-1}$ Ascending boundary	Descending boundary
(a)	Constant age, 1.5–2 hr., varying concn.	
3.53	−1.79	−2.64
5.64	−1.49	−2.01
14.1	−1.40	−2.00
28.2	−1.37	−2.06
70.5[a]	−0.46, neutral, +4.2	−0.91, neutral, +5.35
(b)	Constant concn., 14.1 × 10^{-3} *M*, varying time of aging	
Time of aging, hr.		
17	−1.14, +4.70	−1.82, +4.85
65	−1.09, +5.21	−2.37, +5.39
137	−0.99, +5.09	−2.36, +5.09
305	−1.83, −2.97, +5.33	−2.33, −5.28, +4.57
356	−1.45, −2.72	−1.90, −5.18, +4.92
	+0.42, +2.01, +5.07	

[a] Very small amounts of positive components appeared to be present at concentrations lower than this (particularly the values 28.2 and 14.1 × 10^{-3} molar) but the evaluation of their mobilities was not practical.

Zone Electrophoresis.—A series of chromic acetate concentrations in the range 4.36×10^{-3} to 1.09×10^{-2} aged 10 hours at 5–8° gave typical patterns as shown in Fig. 1. These diagrams all

(15) The bulk of the silicone grease could be removed by washing or soaking the cell parts in trichloroethylene. The rest was removed by an aqueous solution of 10% sodium hydroxide and 5% borax.

(16) K. G. Stern and M. Reiner, *J. Electrochem. Soc.*, **97**, 213 (1950).

(17) E. L. Durrum, *J. Colloid Sci.*, **6**, 274 (1951).

(18) M. Reiner and R. L. Fenichel, *Science*, **108**, 164 (1948).

(19) The chromium solutions were added in all cases from a Gilmont ultramicroburet, 992 scale divisions = 0.100 ml.

(20) All aliquots up to 500 μl. were withdrawn with Kirk type micropipets (Micro-Chemical Specialties Co., Berkeley, Calif.). These pipets are calibrated to contain and were rinsed several times with water, buffer or acid to ensure transfer of the aliquot.

(21) C. T. Kasline and M. G. Mellon, *Ind. Eng. Chem., Anal. Ed.*, **8**, 463 (1936).

(22) G. Kortüm, *Z. physik. Chem.*, **33B**, 243 (1936).

(23) B. E. Saltzman, *Anal. Chem.*, **24**, 1016 (1952).

(24) P. F. Urône and H. K. Anders, *ibid.*, **22**, 1317 (1950).

(25) H. J. Cahnmann and R. Bisen, *ibid.*, **24**, 1841 (1952).

show the predominance of slow negatively charged particles and small amounts of positively charged particles. Aging for 1–2 days gave only broad peaks similar to graph 1 of Figs. 1 and 2. Aging up to 27 days decreased the total positive components and divided the broad peak of negative components into a large component of no net or slightly negative charge and a smaller component of pronounced negative charge as shown by graph 2 of Fig. 2.

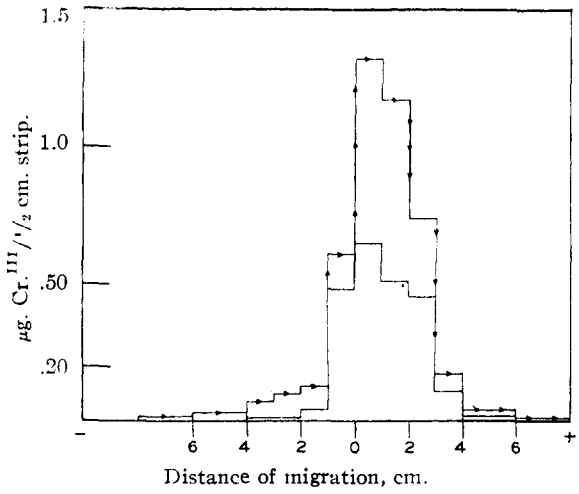


Fig. 1.—Paper electrophoresis of chromic acetate in μ = 0.1, phosphate buffer pH 7.35: I (—), 4.36 × 10^{-3} M; II (→→), 6.44 × 10^{-3} M.

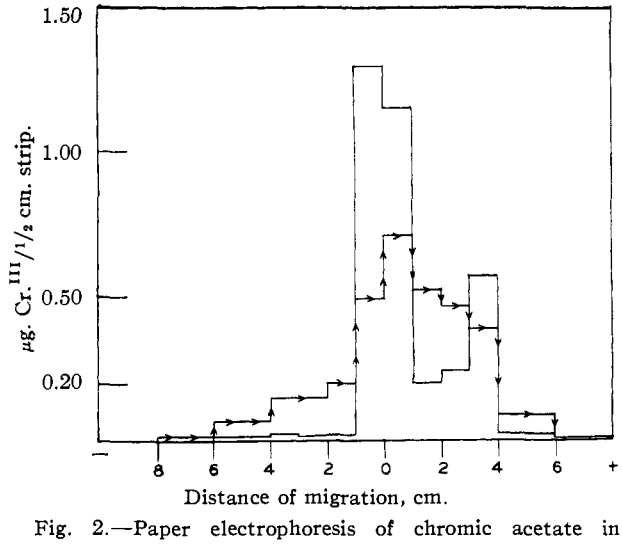


Fig. 2.—Paper electrophoresis of chromic acetate in μ = 0.1, phosphate buffer pH 7.35: I (→→), 6.44 × 10^{-3} M by dilution of 27-day old 0.655 M solution; II, (—), 6.44 × 10^{-3} M, 27 days old.

Dilute solutions aged at the final concentration gave patterns similar to graph 2, Fig. 2, while solutions differing by a concentration factor of 10–100 aged and then diluted gave patterns of broader more general charge distribution containing more positive components. Solutions aged at room temperature generally showed most chromium with no net or slightly negative charge while identical solutions aged at 5–8° showed more over-all positive components and subdivision of negative components.

In most experiments with trivalent chromium recoveries of 95–105% were had by cutting up and analyzing the entire strip. A quantitative comparison of positive, negative and neutral components on this basis is made difficult by electroösmosis effects which have been reported to cause some shift of negative components (*e.g.*, plasma γ-globulin) to the cathode side of the strip.[26]

Under similar conditions potassium chromate migrated off the strip in about 80 minutes. Even after passage of current for 5–6 hours residual chromate was found all along the strip. Most of the retained chromium was fairly uniformly concentrated on the anode side except at the origin. Much smaller amounts were also on the cathode side. With increasing amounts of applied chromium in the range 12–76 μg. the percentage retention dropped from 17 to 6% while the maximum concentration per 1/2 cm. strip increased from 0.08 to 0.21 μg.

Dialysis.—Dialysis experiments with potassium chromate in the concentration range 10^{-5}–10^{-2} molar throughout the pH range 4–8 in μ = 0.1 buffers demonstrated that the chromate ion (and di and acid chromates formed at lower pH's) was completely diffusible through Cellophane membranes. Equilibrium at all pH's and concentrations was attained in 2–6 hours at 5–8°.

With chromic acetate, however, the percentage diffusibility under identical conditions depended on the age, concentration, pH, and temperature of aging. Figure 3 shows the percentage diffusibility of 8.40 × 10^{-3} molar chromic acetate as a function of aging. Each point on the curves was reproducible within less than 5%. The greatest loss of diffusibility occurs within 30 days but 1/3 or more of the chromium is still diffusible after 60 days aging at this high pH.

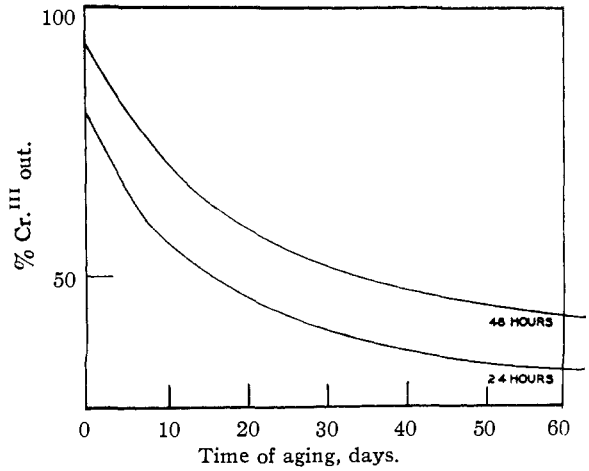


Fig. 3.—Diffusibility of 8.40 × 10^{-3} M chromic acetate in μ = 0.1, phosphate buffer at 5–8° (pH 7.35).

Figure 4 shows the rate of diffusion of 8.40 × 10^{-3} molar chromic acetate aged 50–60 days as a function of pH of aging. The greatest diffusibility, as expected, was found at the lowest pH. How-

(26) E. L. Durrum, THIS JOURNAL, **72**, 2943 (1950).

TABLE II

EFFECTS OF AGING AT ROOM TEMPERATURE ON THE ABSORPTION SPECTRUM OF 6.31 × 10^{-3} MOLAR CHROMIUM ACETATE

Time of aging, days	In *p*H 7.35 phosphate buffer, μ = 0.1			In distilled water		
		Wave length of			Wave length of	
	*p*H	Maxima	Minima	*p*H	Maxima	Minima
0.08–0.17	7.20	425, 570	365, 495	4.92	425, 575	370, 500
1	7.00	425, 575	355, 495			
2	6.98	425, 580	355, 495			
5		425, 575–580	355, 495–500			
9		430, 580–585	355, 495–500			
14		.....	.....		420, 570	355, 490
21	6.90	430, 595–600	360, 505			
41[a]		430, 600	365, 510			
42	..	.....	.....	4.43	415, 570–575	365, 490
51	..	.....	.....	4.48	415, 570	360, 485
70		430, 600	360, 515			
91	6.91	430, 600	370, 515			
0.08–0.17[b]	..	.....	.....	4.60	425, 570–575	365, 495–500

[a] Generally somewhat after 30 days of aging slight turbidity developed in some of the samples and visible precipitation commenced between 40 and 60 days. The absorbance values were thus no longer comparable but the solutions or their supernatants could still be used to locate maxima and minima. [b] Prepared by dilution of 0.2326 molar stock solution 16 months old.

ever, repetition of the experiments at *p*H 4.14 and changing the dialysate buffer every 24 hours for 118 hours did not result in attainment of equilibrium across the membrane.

In further attempts to determine conditions near neutrality under which the chromic acetate might be completely diffusible, experiments were run with 5–10 ml. of buffer inside the membrane into which various concentrations of chromic acetate, aged in 100 ml. of buffer *p*H 7.35, were dialyzed for definite time intervals. The results of a series of such experiments with 4.63 × 10^{-4} molar chromic acetate demonstrate that chromic acetate and complexes formed therefrom by hydrolysis and olation equilibrate across a Cellophane membrane at this concentration for times of aging up to at least 170 hours.

Spectrophotometric.—The absorption spectra of solutions of varying concentration (1 to 16.7 × 10^{-3} molar) of chromic acetate in phosphate buffer freshly prepared by dilution of the stock solution, show two maxima near 420 and 570 mμ and two minima near 365 and 490 mμ. If measured within a few hours after preparation the solutions within this concentration range obey the Beer–Bouquer law at the two maxima. Freshly prepared aqueous solutions either without added base or with sodium hydroxide added to give *p*H 7.35 yielded spectra identical with those in phosphate buffer, *p*H 7.35.

Table II summarizes the changes in position of the maxima and minima of chromic acetate solutions aged in water and in buffer of initial *p*H 7.35. Progressive aging in both water and buffer was accompanied by decreasing absorbances at both maxima and minima. Spectral shifts in water were less pronounced than observed in phosphate buffer where both maxima and the second minima underwent gradual bathochromic shifts which reached 430, 600 and 515 mμ as precipitation started. Figure 5 compares the absorption spectra of 8.19 × 10^{-3} molar chromic acetate in water *p*H 4.86 (graph 1), adjusted slowly over a period of 3 days with 0.1 *N* alkali to *p*H 6.3 (graph 2) and adjusted rapidly to

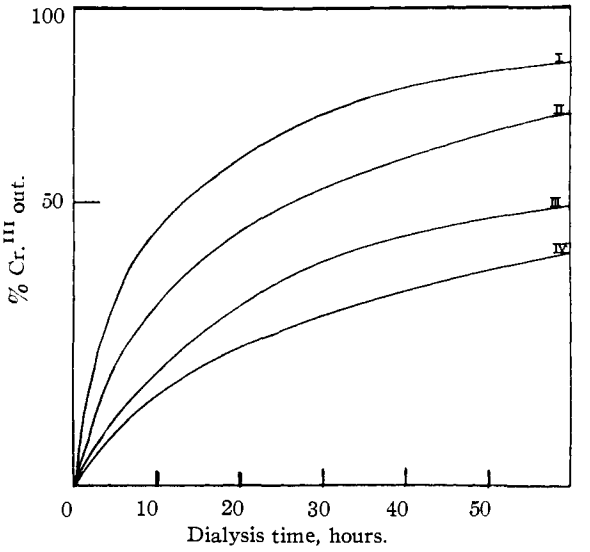


Fig. 4.—Rate of diffusion of chromic acetate in μ = 0.1, acetate and phosphate buffers at 5–8° as a function of *p*H: I, *p*H 4.14; II, *p*H 5.30; III, *p*H 6.40; IV, *p*H 7.35.

*p*H 11.2 (graph 3). The solution at lowest *p*H appeared violet while that at the highest appeared blue-green. The chief differences in the latter spectrum were the hypsochromic shift of the first minimum and the bathochromic shift of the second maximum with large decrease in absorbance.

Early investigations of the spectra of chromic complexes were carried out by Elöd and Schackowsky,[27] Küntzel and Reiss[28] and Ueberbacher and Dröscher.[29] The investigations of Theis, Serfass, *et al.*,[30] dealing with the "penetration" of the coör-

(27) E. Elöd and T. Schackowsky, *Collegium*, **763**, 701 (1933); **772**, 414 (1934); E. Elöd, T. Schackowsky and M. Weber-Schäfer, *ibid.*, **785**, 406 (1935); E. Elöd, T. Schackowsky and R. Sinn, *ibid.*, **805**, 258 (1937).

(28) A. Küntzel and C. Reiss, *ibid.*, **791**, 138 (1936).

(29) E. Ueberbacher and K. Dröscher, *ibid.*, **832**, 433 (1939).

(30) E. R. Theis, E. J. Serfass and A. Clark, Jr., *J. Am. Leather Chemists' Assoc.*, **41**, 401 (1946); E. J. Serfass and E. R. Theis, *ibid.*, **43**, 206 (1948); E. J. Serfass, C. D. Wilson and E. R. Theis, *ibid.*, **44**, 647 (1949).

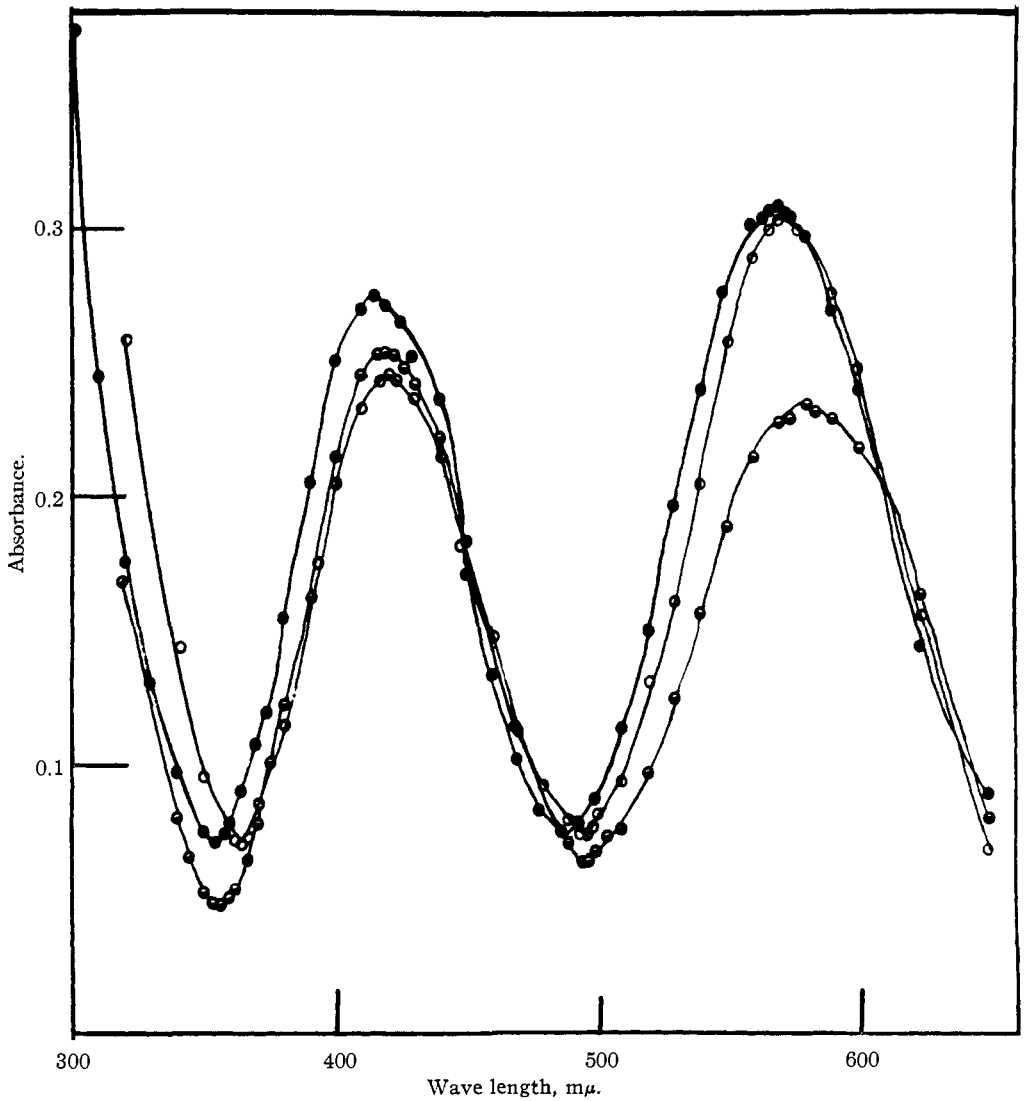


Fig. 5.—Absorption spectra of 8.19×10^{-3} *M* chromic acetate at various *p*H's: (I) ○, *p*H 4.86; (II) ●, *p*H 6.30; (III) ◒, *p*H 11.20.

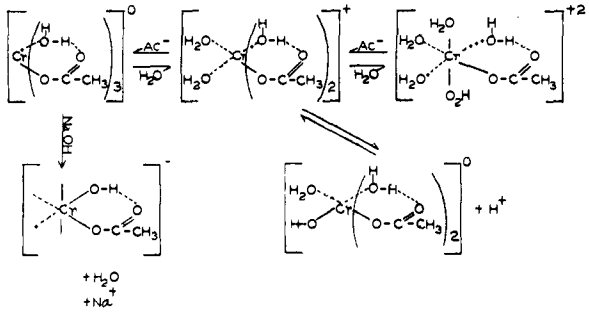
dination sphere of chromium by organic acids have more direct bearing on our problem. On the basis of their work the coördinate stability series of Stiasny has been modified and extended as follows (in order of decreasing stability) OH > glycinate > oxalate > tartrate > citrate > glycolate > acetate > monochloroacetate > formate > sulfate > chloride > H_2O < nitrate. They found a marked hyperchromism as the number of carboxyl groups coördinated to the central chromium atom increased.

On the basis of the data of Theis, Serfass, *et al.*, the general hypochromism which we observed on aging chromic acetate at higher *p*H's indicates a reversal of the "penetration" by carboxyl groups or an exit from the coördination sphere of acetato or aquo groups and replacement by OH. Hamm[31] found that the replacement of the single coördinated water in chromic ethylenediamine tetracetate by OH resulted in large bathochromic shifts in the maximum at 540 (to 580–600) and in the minimum at 450 (to 480) mμ with a hypochromic effect at the maximum. Thus the significant bathochromic shifts with hypochromism observed at 570 and 495 mμ on aging chromic acetate in phosphate buffer are evidence of the entrance of hydroxyl into the coördination sphere.

We observed similar bathochromic shifts (maximum at 555–560 to 590, minimum at 495 to 515 mμ) in chromic chloride solution freshly diluted to 6.31×10^{-3} molar on adjusting the *p*H from 2 to 5.2 a *p*H near the precipitation point where the entrance of hydroxyl is known to occur. In recent studies on acetate (and formate) masking of dilute solutions of hexaaquo chromic chloride in the *p*H range 2.2–6.9 Küntzel, *et al.*,[32] have demonstrated the formation of a violet intermediate diacetatoaquo-hydroxy complex stable to long aging. These authors also postulate the existence of anionic complexes according to the scheme

(31) R. E. Hamm, THIS JOURNAL, **75**, 5670 (1953).

(32) A. Küntzel, H. Erdmann and H. Spahrkäs, *Das Leder*, **4**, 73 (1953).



Scheme 1.

This scheme satisfactorily explains the long stability we observed for violet chromic acetate solutions in the neutral zone as well as the predominance of neutral and negative complexes observed in the electrophoresis experiments.

Volshtein[33] has shown that very stable complexes are formed between Cr^{III} and amino acids. Neuberg and Mandl[34] have demonstrated the solubilization of "insoluble" inorganic compounds by widespread biological compounds. In view of the many agents known to complex chromium (carboxylic acids, amino acids, peptides, hydroxy-carboxylic acids) found in the mammalian body, attention is directed to the possible role of these agents in the metabolism of trivalent chromium compounds which are considered insoluble at the *p*H of blood.

(33) L. M. Volshtein, *Izvest. Akad. Nauk. S. S. S. R., Otdel, Khim. Nauk.*, 248 (1952); *C. A.*, **46**, 10035h (1952).

(34) C. Neuberg and J. Mandl, *Arch. Biochem.*, **23**, 499 (1949).

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOPHYSICAL CHEMISTRY, HARVARD UNIVERSITY]

The Non-clotting Component of the Human Plasma Fraction I-1 ("Cold Insoluble Globulin")[1]

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Fraction I-1 of human plasma has previously been shown to contain a non-clottable globulin, very insoluble at temperatures near 0° and much more soluble above 20°. Studies on the physical properties of Fraction I-1 and of the cold insoluble globulin are reported. Electrophoresis, sedimentation and clotting studies indicate that reversible complex formation occurs, in Fraction I-1, between fibrinogen and the cold insoluble globulin. The latter showed well marked double refraction of flow in glycerol–water mixtures, with a rotary diffusion coefficient, Θ, slightly less than that of fibrinogen, the product $\eta\Theta/T$ being approximately 0.8. Electrophoretic studies, carried out at 20° to avoid precipitation of the protein, gave a mobility of -7×10^{-5} cm.²/volt sec. at *p*H 8.6, for 90% of the protein. The sedimentation constant of the principal component ($s_{20} = 15$ *S*) was nearly twice as great as that of fibrinogen; while its intrinsic viscosity (0.15) was considerably lower than that of fibrinogen (0.25). The relation of this cold insoluble globulin to that of other reported plasma proteins with similar solubility properties is discussed.

Previous observations[3] on the sub-fractionation of Fraction I from human plasma have indicated the presence of a non-clottable globulin, characterized by its great insolubility at temperatures in the neighborhood of 0°, its solubility at temperatures of 20° or above being relatively high. This protein has been known as "cold insoluble globulin." Previous measurements of double refraction of flow[4] on Fraction I from which fibrinogen had been removed by clotting with thrombin had suggested that its rotary diffusion coefficient was not very different from that of fibrinogen. The cooling of solutions of Fraction I led to precipitation of the non-clotting protein in the form of a complex containing approximately 50% of fibrinogen by weight. On removal of the fibrinogen by clotting with thrombin, the cold insoluble non-clottable protein was obtained free of fibrinogen.

Studies on the characterization and partial purification of this non-clotting component of Fraction I are reported here. Some information concerning the complex formed between fibrinogen and the non-clotting component is also included. Since the studies here reported were completed a number of years ago (1947), the starting materials used for the sub-fractionation were those obtained by the earlier method of fractionation reported from this laboratory.[5]

Experimental

In the procedure previously described for the purification of fibrinogen from Fraction I,[3] Fraction I-1 was removed at *p*H 6.3 in citrate solution of ionic strength of 0.3 and 0.5% ethanol by cooling to 0°, since this fraction was found to be very insoluble at that temperature. Approximately 20% of the total protein of Fraction I precipitated in this fraction and approximately two-thirds of the fraction was clottable with thrombin.

In the present studies the procedure was modified in one important respect. Fraction I-1 was found to be very susceptible to surface denaturation, and some preliminary experiments indicated that this susceptibility might be enhanced by the precipitation at *p*H 5.0 which was employed as the first step of the previous process. This degree of acidification was avoided in the studies reported here, and Fraction I-1 was precipitated by cooling Fraction I to 0° at *p*H 6.1, leaving the albumin and globulin impurities and most of the fibrinogen in solution.

Source of Material.—The greater part of the experimental work was carried out on dry Fraction I powder which had been prepared by the Lederle Laboratories according to methods developed here[5] and then stored *in vacuo* for three

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