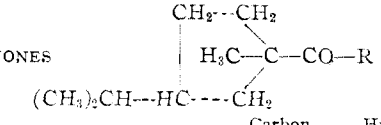
TABLE III

KETONES



R	B.p., °C. (mm.)	d^{20}_4	$n^{20}D$	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found
n-Butyl	97–99 (1)	0.8793	1.4543	79.93	79.95	12.46	12.46
Isobutyl	85–87 (1)	.8763	1.4527	79.93	79.53	12.46	12.34
s-Butyl	104 (6)	.8791	1.4539	79.93	79.59	12.46	12.37
Isoamyl	120–122 (6)	.8769	1.4544	80.29	80.26	12.58	12.44
Phenyl[a]	..	..	..	..	..	..	..

[a] The amount of the phenyl ketimine on hand was insufficient to prepare an isolable quantity of the ketone. A few milligrams of the imine was hydrolyzed and the product yielded a 2,4-dinitrophenylhydrazone, m.p. 138°, calcd. for $C_{22}N_{26}N_4O_4$: N, 13.65; found: N, 13.50.

hydrogen uptake was the calculated amount. Data on the amines are given in Table II. Isopropyl 1-methyl-3-isopropylcyclopentyl ketimine could not be reduced.

Ketones.—Each of the ketimines was refluxed with 6 *N* hydrochloric acid for three hours. The ketones were ether extracted, dried and distilled. The products are given in Table III. Isopropyl 1-methyl-3-isopropylcyclopentyl ketimine showed no signs of hydrolysis after 48 hours, being recovered quantitatively as the hydrochloride.

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[CONTRIBUTION FROM GARVERINÄRINGENS FORSKNINGSINSTITUT]

The Effect of Esterification of the Carboxyl Groups of Collagen upon its Combination with Chromium Compounds

BY K. H. GUSTAVSON

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In comparing the irreversible fixation of chromium complexes by intact collagen in the form of hide powder and by modified collagen with its carboxyl groups esterified by means of methanol, it is found that collagen loses its affinity for *cationic* chromium by the inactivation of the carboxylic groups. Since the methylated collagen fixes large amounts of *non-cationic* chromium complexes from solutions containing predominantly anionic and non-ionic chromium (sulfito-sulfato-chromium compounds), protein groups other than carboxyls must be involved in this type of reaction which probably is located to non-ionic protein groups (coördination). The lack of reactivity of the carboxyl ions of the ion exchange resin Amberlite IRC-50 for solutions containing non-cationic chromium solely provides further evidence for the interpretation of the fixation of the various chromium complexes given, *i.e.*, the carboxyl ions of collagen are responsible for the binding of electro-positive chromium complexes, whereas the combination of non-ionic and electronegative complexes with collagen is located to protein groups other than the carboxyls, probably by coördination on peptide links and hydroxy groups.

Introduction

Modern investigations of the important reaction of collagen with basic chromium salts, which has been extensively studied from the point of view of the chrome tanning process,[1] indicate the carboxyl ions of collagen to be specific for its irreversible binding of cationic chromium complexes. The initially ionic linking of the electropositive chromium complex to the anionic protein groups (carboxyl) is probably changed into a covalent–coördinate bond by the penetration of the carboxyl group into the complex, establishing direct multipoint attachment of the polynuclear chromium complex to the carboxyl groups of adjacent peptide chains (multipoint cross-linking). The great stability of the chrome–collagen compound formed and many other facts regarding its behavior are in harmony with this concept.[2] As to the mechanism of the binding of non-cationic chromium complexes by collagen, evidence for the function of groups other than the carboxyl in this reaction has been presented.[3] However, it has been postulated that the carboxyl group is instrumental for the fixation of chromium compounds generally, irrespective of their electrochemical state.[4]

It has earlier been demonstrated[5] that by complete discharge of the carboxyl ions of collagen by H ions (at *p*H 1.0), its fixation of cationic chromium complexes from dilute solutions of basic chlorides and sulfates of chromium is completely blocked. However, this method is limited to the low *p*H mentioned. By permanent inactivation or blockading of the carboxyl groups, for instance by their esterification, investigation of these reactions in a more desirable *p*H range, such as *p*H 2.5–5.0, should be feasible.

Esterification of collagen by means of methyl sulfate and methyl bromide has been attempted by Bowes and Kenten.[6] In view of the non-specificity of these agents for the carboxyl group and the marked hydrolytic breakdown of the protein incurred by the large number of treatments necessary for complete esterification, precaution must be taken in interpretation of the data. The methylating agents mentioned as well as diazomethane have been found unsuitable. The most promising method appears to be esterification by means of methanol in the presence of small amounts of hydrochloric acid, according to the procedure of Fraenkel-Conrat and Olcott[7] originally devised for globular proteins. This method has been employed in the present investigation.

Experimental

Esterified Hide Powder.—Twenty grams of collagen in the form of acetone-dehydrated hide powder (18.0% N) was shaken intermittently for 7 days in 2 l. of methanol made 0.1 *N* in respect to hydrochloric acid (15 ml. of concentrated

(1) For a general survey, see K. H. Gustavson, *Adv. Protein Chem.*, **5**, 353 (1949); S. G. Shuttleworth, *J. Soc. Leather Trades Chem.*, **34**, 410 (1950).

(2) K. H. Gustavson, *Adv. Protein Chem.*, **5**, 353 (1949).

(3) K. H. Gustavson, THIS JOURNAL, **48**, 2963 (1926); *J. Am. Leather Chem. Assoc.*, **42**, 201 (1947); G. Otto, *Leder*, 1, 81, 133, 153 (1950).

(4) S. G. Shuttleworth, *J. Soc. Leather Trades Chem.*, **32**, 116 (1948); **34**, 410 (1950).

(5) K. H. Gustavson, *Svensk Kem. Tidskr.*, **52**, 75 (1940).

(6) J. H. Bowes and R. H. Kenten, *Biochem. J.*, **44**, 142 (1949).

(7) H. Fraenkel-Conrat and H. S. Olcott, *J. Biol. Chem.*, **161**, 259 (1945).

TABLE I

ELECTRO-CHEMICAL COMPOSITION OF SOLUTIONS OF CHROMIUM COMPOUNDS

No.	Composition	pH	% chromium present as: Cationic	Non-ionic	Anionic	Electrophoretic migration of chromium
1	$Cr_2(OH)_2(SO_4)_2$	2.9	96	4	0	Only cationic
2	$Cr_2(OH)_2(SO_4)_2$ + Na_2SO_3	3.4	63	37	0	Mainly cationic; some non-migrating
3	$Cr_2(OH)_2(SO_4)_2$ + 2.0 Na_2SO_3	4.0	12	79	9	Mainly non-migrating; slight cationic and anionic
4	$Cr_2(OH_2)(SO_4)_2$ + 2.5 Na_2SO_3	4.8	(2)	86	12	Mainly non-migrating; some anionic
5	$Cr_2(OH)_2(SO_4)_2$ + 3.0 Na_2SO_3	5.7	0	85	15	Mainly non-migrating; some anionic

acid). After completed reaction an equivalent amount of sodium hydroxide was added and the suspended hide powder was shaken for 30 minutes. The methylated stock was freed from the solution by suction filtering and then shaken two consecutive times with 2 l. of water for 10 minutes. It was then dehydrated in acetone and dried at 60°. The yield was 85%. The modified collagen which resembled the intact hide powder contained 17.3% N on ash-free dry-substance and 0.3% ash (mainly NaCl). Its content of methoxyl was 2.54% on ash-free dry substance, which after correction for the methoxyl content of collagen (methionine) corresponds to 0.80 milliequivalent esterified carboxyl groups per g. of collagen. The stock reacted neutral (a final *p*H of 5.4 was recorded in shaking 2 g. of hide powder for 6 hours in 100 ml. of 2 *M* solution of sodium chloride). The binding capacity for H-ions was 0.11 milliequiv. per g. of collagen (1.0 g. of collagen in 25.0 ml. of 0.1 *N* hydrochloric acid, made 0.5 *M* in sodium chloride for 2 hours). The corresponding value for the intact hide powder was 0.90. The amount of irreversibly fixed anions of acidified sodium polymetaphosphate (at final *p*H 2.0) was 1.73 milliequivalents P per g. of methylated collagen, and 1.59 for untreated collagen. The methoxyl content indicates a degree of inactivation of the carboxyl groups of about 90%, provided the methylation is restricted to these groups, since the content of free carboxyl groups of this type of collagen is of the order of 0.9 milliequivalent per g. of protein. The H-ion binding capacity shows further that only about one-tenth of the carboxyl groups are ionogenic. The modified collagen did not bind chromium from solutions of a basic chromic chloride containing all its chromium in the form of cationic complexes (1 g. of methylated collagen shaken 2 hours in 25 ml. of a solution of 66% acid chromic chloride with composition corresponding to the empirical formula: $Cr_2(OH)_2Cl_4 \cdot 2NaCl$, made 0.5 *M* in sodium chloride, containing 0.6 eq./l. Cr). The intact hide powder fixed 2.1 milliequivalents Cr in the corresponding treatment.

Chromium Compounds.—For the present purpose, it was advisable to employ solutions of basic chromium salts containing entirely cationic chromium complexes, on one hand, and those with non-cationic chromium as the dominant constituent, on the other hand, as well as solutions containing a mixture of cationic, non-ionic and anionic chromium complexes, since the problem concerns the mechanism of the binding of chromium complexes generally by hide protein. Series of solutions of basic chromium sulfate containing increasing amounts of sodium sulfite meet these requirements, since the solution of the pure chromium sulfate contained practically all its chromium in the electropositive form and with increasing contents of sulfite the percentage of cationic chromium decreased in proportion to the increasing formation of non-ionic and anionic chromium compounds; the latter being the main constituents of the solutions containing the largest amounts of sodium sulfite. Hydroxo-sulfito-sulfato complexes of chromium are formed.

A stock solution of 66% acid chromic sulfate, free from neutral salts, with composition corresponding to the empirical formula: $Cr_2(OH)_2(SO_4)_2$ with a final concentration of 8.0 equiv./l. Cr, was prepared by reducing chromic acid (CrO_3) in the presence of an equimolar amount of sulfuric acid by means of sucrose,[8] under conditions practically eliminating the formation of organic acids by incomplete oxidation of sucrose. The stock solution was diluted to 0.8 equiv./l. Cr. Further, solutions of this concentration containing increasing amounts of sodium sulfite were prepared. The solutions were not heated but aged for three weeks before use. The complex composition of these solutions, given in Table I, was determined by the ion exchange method, employing the column method with Dowex-50, operated in the sodium cycle, and Amberlite IRA 400, in the form of the hydrochloride.[9]

The electrophoretic behavior of the chromium complexes was studied by paper-electrophoresis according to a method described elsewhere,[10] using 0.1 *M* sodium chloride solution as electrolyte. The duration of the electrophoresis was limited to 2 hours on account of the instability of the highly complexed solutions under the experimental conditions employed.

Reactions.—Intact hide powder and methylated hide powder in quantities equal to 0.5 g. of protein were shaken with 20.0-ml. portions of these solutions for 2 hours. The reaction was limited to 2 hours in order to eliminate hydrolysis of the methylated carboxyls. The contents of chromium and collagen were determined in the treated specimens, which had been washed free from sorbed chromium compounds.

Since it should be of interest to ascertain the behavior of a substrate with carboxylic ions as the unifunctional group toward these chromium salts, series with a cation exchanger containing carboxyl ions as the functional group (Amberlite IRC 50) were included. After equilibration with sodium ions at *p*H 7.0, in a 2 *M* solution of sodium chloride, an amount of 45% of its carboxyl groups were ionized (4.8 milliequiv. cation (Na) per g. of dry resin). Portions equal to 0.5 g. of dry resin were shaken for 2 hours in 10.0 ml. of the solutions. The uptake of chromium by the resin was obtained indirectly by analysis of the combined exhaust and washings (100 ml.).[9]

Results and Discussion

The results of the chroming experiments of the hide powders and the resin are graphically given in Fig. 1.

No chromium was fixed by the methylated collagen from dilute solutions of basic chlorides and perchlorates of chromium of the cationic type. For the chromium fixation by intact collagen any reactive groups of collagen are available, whereas, in the case of the esterified collagen, the carboxylic group which is specific for the cationic chromium is blocked. However, provided the attachment of the non-cationic chromium does not involve the carboxyl group, the latter complexes should be able to combine with the esterified collagen.

The data of Fig. 1 show that the cationic chromium complexes of the blank solution (no. 1) possess a slight affinity for the protein devoid of carboxyl groups only. On the other hand, the intact hide powder binds considerable amounts of chromium. By the addition of sodium sulfite to the solution of the chromium sulfate, the *p*H is increased which connotes increase of carboxyl ions and hence of fixation of cationic chromium. Further, non-cationic complexes are gradually formed. These complexes are fixed by both types of hide powder. Solutions No. 4 and 5, mainly containing non-ionic

(8) K. H. Gustavson and P. J. Widen, *Ind. Eng. Chem.*, **17**, 577 577 (1925).

(9) K. H. Gustavson, *J. Colloid Sci.*, **1**, 397 (1947); *J. Am. Leather Chem. Assoc.*, **45**, 536 (1950); *J. Soc. Leather Trades Chem.*, **35**, 270 (1951).

(10) K. H. Gustavson, *J. Am. Leather Chem. Assoc.*, **47**, 151 (1952).

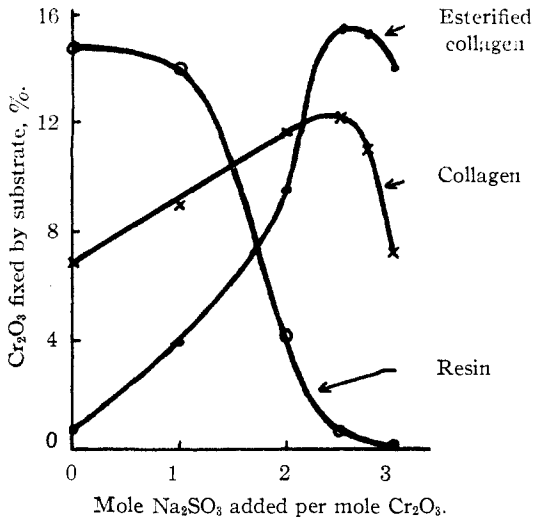

Fig. 1.—The fixation of chromium, in % Cr_2O_3 on the basis of collagen or dry weight of resin from solutions of basic chromium sulfate complexed by means of sodium sulfite by: -X-X-X-, intact collagen; -●-●-●-, collagen with inactivated carboxyls; -O-O-O-, cation exchange resin of carboxylic type.

chromium, react more extensively with the esterified collagen than with the intact protein. The reactivity of the carboxyl ions of the resin is evidently restricted to the positively charged chromium complexes. Comparing the curves of resin and collagens, the predominance of the fixation of non-cationic chromium by collagen in systems containing 2–3 moles sulfite per mole of Cr_2O_3 is evident.

A number of findings point to the binding of the non-ionic complexes of chromium to non-ionic protein groups, such as the peptide link by coördination (H-bonding). The hydroxy group of serine and hydroxyproline may also be involved.[2]

The larger chromium uptake by esterified collagen as compared to intact collagen, shown in the last instances, is not surprising, since a partial rupture of crosslinks, probably H-bonds, evidently is incurred in the methylation process. This has been demonstrated by the increased fixation of typical coördination agents, such as vegetable tannins (mimosa) resulting from the esterification; the increase being of the order of 75 to 100%.[11] The number of coördination active sites of the collagen chains, free to react with any suitable agent, is hence increased, facilitating the fixation of the non-ionic chromium complexes. The deep-going changes of the intermolecular linking of collagen, resulting in drastically lowered intermolecular cohesion, are evident by the great impairment of the hydrothermal stability caused by the esterification. The shrinkage temperature is decreased 25°, and the methylated hide powder swells considerably in water.[11]

It is interesting to note that pretreatments of collagen in concentrated solutions of lyotropic substances such as thiocyanates and urea, as well as hydrothermal shrinkage (denaturation) of collagen lead to a large augmentation of the fixation of the sulfito-sulfato complexes examined, as shown by investigations of some 25 years standing.[12] All these treatments have an effect in common: partial rupture of the stabilizing crosslinks of the protein units; decreasing the stability of the protein and increasing its reactivity toward agents specific for non-ionic protein groups.[13] Hence, the data obtained in the present investigation are in harmony with the theoretical deductions.

Since the blocking of the carboxyl group of the protein will tend to increase its positive charge, the possibility of the cationic chromium complexes being repelled by the additional positive charge merits attention.[14] However, this effect appears to be of secondary importance only, compared with the primary function of the carboxyl as a complexing group for chromium as the following facts will show.

Beforehand, it should be stressed that the effect of *p*H upon the chrome fixation by collagen from solutions of cationic basic salts cannot be evaluated simply from the point of view of the charge of the protein, since a number of factors of the system are *p*H-dependent, besides the charge and the number of carboxyls ionized at a given *p*H, also the complex composition, molecular size, coördination potency and ionic equivalent weight of the chromium salt. Moreover, solutions of these chromium salts are unstable at *p*H values exceeding 3.5.

The principal function of the carboxyl group is as a complexing agent. This is evident from investigations of reactions between collagen, on one hand, and the hydroxo-aquo-chromium and hexa-urea-chromium cations, on the other hand, at *p*H values about 3.5. Collagen fixes large amounts of the hydroxo-cations, whereas only insignificant amounts of hexa-urea-chromium cations are combining with collagen. The hydroxo-chromium complex contains weakly coördinated aquo groups, which are easily displaced by the penetrating carboxyl group. On the other hand, all coördination sites on the chromium atom of the hexa-urea-chromium cation are occupied by the strong coördinated urea; obstructing the coördination of the carboxyl group on chromium. Hence, an additional requisite for tanning potency is the presence of coördinated groups in the complex, which are easily displaced by the carboxyl of collagen.

An illustration of the unique power of collagen of maximum positive charge to combine with cationic chromium complexes gives the following example. Collagen, equilibrated with moderately concentrated solutions of 67% acid chromium chloride, which contained all its chromium in the form of cations, fixed very large amounts of chromium (10–12% Cr_2O_3, on the weight of protein) at final *p*H values of 1.3–1.8, in which *p*H range collagen acquires its maximum positive charge.[15]

Final proof of the postulate that for the fixation of cationic chromium by collagen carboxyl ions are an absolute requirement and that the binding of

(11) K. H. Gustavson, *Acta Chem. Scand.*, in press.

(12) K. H. Gustavson, *Colloid Symp. Monograph*, **4**, 79 (1926); W. Grassmann, "Handbuch der Gerbereichemie," Vol. II/2, J. Springer, Vienna, 1939, pp. 185–193.

(13) K. H. Gustavson, *Biochem. Z.*, **311**, 347 (1942).

(14) A suggestion of the referee.

(15) K. H. Gustavson, *Ind. Eng. Chem.*, **17**, 945 (1925); **19**, 1015 (1927). K. H. Gustavson and P. J. Widen, *Collegium*, 153 (1926).

non-ionic chromium takes place independently of the carboxyl group of collagen, is provided by unpublished data on the interaction of modified polyamides with the two forms of chromium complexes. A hydrated, modified polyamide was prepared from a copolymer of hexamethylene–adipic acid salt and caprolactam, by turbining a 3% hot methanol solution of the polyamide into water. The content of anionic groups (carboxylic end groups) was 0.02 milliequiv. per g. of polyamide or less than 2% of the number of carboxyls of collagen. The main functional group is the –CO–NH– link, part of the same possessing free coördination sites. Its coordination potency is evident from the fact that the modified polyamide exceeds collagen in its binding capacity for polyphenols (tannic acid). The sulfito complexes are not suitable reactants on account of their high degree of aggregation, their diffusion into the polyamide being obstructed. Solutions of chromium perchlorates were used. From solutions of the 33% acid chromium perchlorate, which contained 32% cationic and 68% non-ionic complexes, the polyamide fixed 7.2% Cr_2O_3, figured on its original weight. In corresponding series with purely cationic chromium complexes of the 75% acid perchlorate, no reaction was evident, neither with the basic chromium sulfate (no. 1 of Table I); thus proving the –CO–NH–link to be the functional group of the polyamide for its combination with non-ionic chromium complexes.

The present findings prove the exclusive function of the carboxyl ions of collagen for the initial attraction of electropositive chromium complexes and for the final irreversible fixation of these complexes by collagen, the high complexing power of the carboxyl group directing the ultimate attachment. Further, they disprove the supposed versatility of the carboxyl group in binding all types of chromium irrespective of their sign of charge and composition; a view held by Shuttleworth.[4] The great avidity of collagen lacking in carboxyl groups for non-cationic complexes and the array of earlier findings proving this type of chrome fixation to be governed by the degree of availability of non-ionic protein groups, and hence to be a function of the nature of the pretreatments of collagen, unequivocally provide evidence for the participation of groups other than the carboxylic group in the binding of non-cationic chromium complexes by hide protein.

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STOCKHOLM, SWEDEN

[CONTRIBUTION FROM THE LILLY RESEARCH LABORATORIES]

Papaverine Homologs. III. The Preparation of Some 3-Methylisoquinolines[1]

BY EDWIN R. SHEPARD, JOHN F. NOTH, HERSCHEL D. PORTER AND CLAUDENE K. SIMMANS

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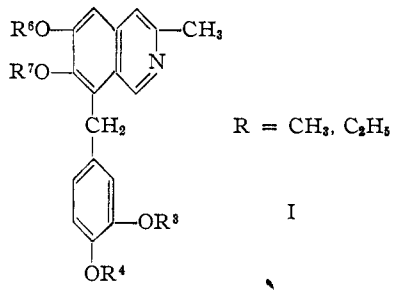
The investigation of papaverine-like isoquinolines has been extended to the 3-methyl compounds (I). During the course of preparing the intermediate phenylisopropylamines, two methods of synthesis were investigated more fully. Three of the isoquinolines have shown unusual pharmacological activity. One of these, 6,7-dimethoxy-1-(4′-ethoxy-3′-methoxy)-3-methylisoquinoline, has been examined clinically.

In view of the results which were obtained upon investigation of the methoxy-ethoxy homologs of papaverine for their coronary dilator action,[2] it was felt to be desirable to prepare the corresponding 3-methyl homologs (I) for comparative

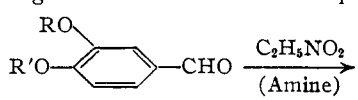


R = CH_3, C_2H_5

I

pharmacological evaluation. The previous literature had indicated that the 3-methylisoquinolines were effective as antispasmodics on smooth muscle.[3] However, since we had found that the antispasmodic action on isolated muscle did not parallel the coronary dilator action in a quantitative sense, there was no sound basis for predicting the activity of the 3-methylisoquinolines as coronary dilators.

The synthetic route followed for the preparation of this group of compounds (I) was essentially the same as that previously reported, with the exception of the preparation of the intermediate amines. For this latter purpose the condensation of the various 3,4-dialkoxybenzaldehydes with nitroethane to yield the corresponding 1-(3,4-dialkoxyphenyl)-2-nitro-1-propenes was quite attractive since the alkoxy substituents could be varied readily to provide ultimately the prerequisite 1-(3,4-dialkoxyphenyl)-isopropylamines. The yields upon examination of the literature seemed promising for the entire series of steps[4–6]



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(2) E. R. Shepard and J. F. Noth, THIS JOURNAL, **72**, 4364 (1950).

(3) F. T. v. Brücke and H. Jesserer, *Arch. exptl. Path. Pharmakol.*, **190**, 515 (1938); H. Kreitmair, *ibid.*, **164**, 509 (1932).

(4) H. Kauffmann, *Ber.*, **52**, 1431 (1919).

(5) A. G. Susie, Ph.D. Thesis, Purdue University, 1939.

(6) H. Adkins and C. F. Winans, THIS JOURNAL, **55**, 2051 (1933).