Chemical Modification of the Side Chains of Gelatin

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The reversible gel-forming power of gelatin has been the subject of numerous investigations, without the establishment of any certain knowledge of the molecular linkages involved. If the side chains of gelatin play any important part in the gel-forming property, their chemical modification should affect the latter. The hydrocarbon side chains are susceptible to reaction only under conditions that would cause very severe degradation of the gelatin molecule. They are, however, of only minor interest since they are unlikely sources of cross-linking in the gel. The polar groups present in the side chains are much more reactive, and the present paper describes their modification and the examination of consequent changes in physical properties. For convenience, these groups may be listed as in Table 1.

The carboxyl groups in gelatin (pK 3–5, Cohn & Edsall, 1943) are, with the exception of the phenolic hydroxyl groups (pK 10, Cohn & Edsall, 1943) originating from the traces of tyrosine, the only ones which produce a negative charge on dissociation, and are present in larger amount than any other ionizable group. They were converted into non-ionizable esters by reaction with methanol and an acid catalyst (Fraenkel-Conrat & Olcott, 1945).

Acetylation of gelatin with acetic anhydride in the presence of aqueous sodium acetate was selected as a convenient method of preventing the free amino groups from ionizing. Olcott & Fraenkel-Conrat (1947) claimed that this method, attributed to N. L. Hughes by Herriott (1947), resulted in acetylation of only the amino and, to a less extent, the phenolic groups of proteins. In non-aqueous media, acetic anhydride has been used for acetylation of stable proteins such as insulin (Herriott, 1947); Green, Ang & Lam (1953) have studied such a reaction on collagen; an investigation of the reaction is described here with gelatin under their conditions.

The guanidino groups of the arginine residues in gelatin are responsible for the existence of positive charges on the molecule at all readily measurable pH values, since these groups have a pK assumed to be in the region of 14 (Cohn & Edsall, 1943). The only reaction proposed as specific for the guanidino groups in proteins is that with hypobromite ion in strong alkali (Grabar & Morel, 1950). It was considered that the conditions of reaction would bring about other changes masking those that might be caused by de-guanidation, so the alternative approach of increasing the guanidino content was first investigated. The method adopted

Table 1. *Reactive groups in gelatin*

Quantities are in m-moles/g. of anhydrous ash-free gelatin, all determinations being made on the sample of gelatin used for the work described here.

α-Amino		$0{\cdot}01_5$	Courts (1954): end-groups assay with fluorodinitrobenzene
ε-Amino		0·43	Eastoe (1955)
		0·42	Kenchington & Ward (1954): titration-curve analysis
Carboxyl*		1·26	Eastoe (1955)
		1·23	Kenchington & Ward (1954): titration-curve analysis
Guanidino		0·48	Eastoe (1955)
		0·48	Kenchington & Ward (1954): titration-curve analysis
Hydroxyl:			
Hydroxyproline	1·09		
Other aliphatic	0·65	1·75	Eastoe (1955)
Phenolic†	0·01		

* In an acid-processed gelatin, this figure will include the amide groups which do not ionize in the range pH 2–12 and which are virtually completely hydrolysed to carboxyl groups in alkali-processed gelatin.

† Confirmed by unpublished work by the method of Udenfriend & Cooper (1952) applied to hydrolysed and unhydrolysed gelatin.

was that used by Hughes, Saroff & Carney (1949), which consists in the reaction of *O*-methyl*iso*urea with amino groups, at pH 10–11 and 0°. The guanidated lysine residues differ from those of arginine in having an extra methylene group, and are referred to as homoarginine residues. In addition, hydroxyhomoarginine is produced from hydroxylysine.

Apart from acetylation in non-aqueous media, no reactions were found which were suitable for modification of the hydroxyl groups. Attempts at sulphation with sulphuric acid (Reitz, Ferrel, Fraenkel-Conrat & Olcott, 1946) led to incomplete reaction and severe degradation; no success attended the use of sulphur trioxide under similar and modified conditions.

Preliminary mention of the work described here has been made by Ward (1953, 1955); the material formed part of a doctoral thesis (Kenchington, 1954).

MATERIAL AND METHODS

The gelatin used was a high-grade commercial material obtained by the alkaline process from animal skins. It had the following properties: viscosity 8·7 centipoises (6⅔%; 40°); logarithmic viscosity number (L.V.N.) 60; rigidity at 10°, 66×10^3 dynes/cm.^{2} [5·7% (w/w) concentration of anhydrous gelatin]; isoionic point pH 4·91.

The titration curve of this material has previously been described (Kenchington & Ward, 1954).

Moisture content. This was determined from the loss of weight on drying the powdered gelatin in a shallow metal dish for 18 hr. in a ventilated oven maintained at 105°. Ash content was measured by incinerating 0·5–1 g. of gelatin in a platinum dish at 510° until the residue was no longer black (1–4 hr.).

Titration curves. These were determined by the method of Kenchington & Ward (1954).

Logarithmic viscosity numbers. Values were obtained as described by Saunders, Stainsby & Ward (1954), and the rigidity of set gels was kindly measured by P. R. Saunders (Saunders & Ward, 1953). The L.V.N. is defined by

$$\text{L.V.N.} = \frac{\ln \eta/\eta_0}{C},$$

where C is the concentration (g./ml.) of solution, η is the viscosity of the solution and η_0 the viscosity of the solvent (International Union of Pure and Applied Chemistry, 1952).

Molecular weights. Dr G. Stainsby kindly determined the values in Table 2 by measurement of light-scattering (Stainsby, 1956).

Esterification of carboxyl groups

Gelatin, ground to pass a 60-mesh sieve, was stirred into 0·002–0·036N-solutions of conc. H_2SO_4 in commercial absolute methanol at room temperature: 100 ml. of solution was used/g. of gelatin. The mixture was shaken at intervals during the first 2 hr. to prevent caking of the gelatin, which became considerably swollen. After standing for 18 hr. or more, with occasional shaking, the methanolic solution was decanted from the solid, which was then washed twice with four times its own volume of methanol before being sucked dry on a sintered-glass filter (grade 3). It was then washed twice with ether and again dried, first on the filter and then in a desiccator at 0·1 mm. Hg pressure. As the ether was removed, the swollen granules shrank to a fine powder indistinguishable in appearance from the starting material. This powder, swollen in ten times its own volume of water, made the supernatant liquor of pH below 2, so the latter was treated with 5N-NaOH soln., with stirring, until pH 6 was reached and maintained, sufficient time being allowed for acid to diffuse out from the gelatin. At no time was the pH allowed to become greater than 6. The swollen granules were melted at 40° to a clear liquid which was set to a gel at 4°. This gel, cut to pieces, was dried in a current of filtered air, which was heated to 30–35° towards the end of the drying. During the drying process, movement of water to the surface of the jelly, followed by evaporation, produced a bloom of salts, some of which could be removed by brushing.

The prepared derivative was on occasion freed from salts and low-molecular material by rinsing with water to remove the surface salts, followed by soaking in large volumes of water at 0–4°, while suspended in a muslin bag, to reduce the total salt content. The water was changed three times in 24 hr., after which the swollen pieces of jelly were drained and dried as before, and ground.

Deionization was effected by melting the swollen pieces of jelly at 40° and passing them through a bed of mixed ion-exchange resins (Amberlite IR-120 and IRA-400), as in the determination of the isoionic point (Janus, Kenchington & Ward, 1951).

Estimation of methoxyl groups. The method and apparatus were essentially similar to those of Belcher, Fildes & Nutter (1955).

Acetylation procedures

Acetylation in aqueous solution. Powdered gelatin (59 g., air dry; 50 g., anhydrous) was allowed to swell in 1 l. of water at 10–15° for 1 hr. before being dissolved by warming at 40°. Anhydrous sodium acetate (25–150 g.) was dissolved in the warm solution, which was then cooled to 25° before acetic anhydride (0·5–400 g.) was added to the vigorously stirred solution, either dropwise or in portions of up to 20 ml. regulated to ensure that the temperature did not rise above 35°. When all the reagent had been added, the mixture was stirred for 10–15 min., poured into shallow Pyrex dishes and left to gel at 4° for at least 2 hr. The gel was cut into pieces, placed in a 12 l. glass tank and washed in running tap-water (5–10°) for 48 hr., the supply of water being sufficiently vigorous to stir the contents of the tank. Mechanical stirring was found unsatisfactory, since it broke up the pieces of swollen gel, causing loss of material and greater difficulty in manipulation. The pieces of gel became swollen (approx. 2% gelatin concentration) and of ash content 2–4% on dry weight by this treatment. Further washing in distilled water (3×10 l.) over 12–24 hr. greatly reduced the ash content. The gel was separated, melted, deionized (500 ml. of mixed resin bed), set, cut up, dried and ground as previously described. Measurement of the pH of the deionized liquid gave the isoionic point.

Acetylation in the absence of water. Two methods were

used. In the first, finely ground air-dried gelatin (23·5 g.; 20 g., anhydrous) was mixed with acetic anhydride (67 g.) at room temperature and left for 7 days at 4°. The mixture was then diluted with dry ether (500 ml.) and filtered on a grade 2 sintered-glass filter, the residue being washed with ether (5 × 100 ml.) and sucked dry. It was then kept at 0·1 mm. Hg air pressure for 24 hr. over NaOH (flakes). Half of the resulting powder was dissolved in water and electrodialysed at 35° between cellophan membranes for 24 hr. before being recovered as before.

In the second method, gelatin (59 g.; 50 g., anhydrous), acetic anhydride (350 g.) and acetic acid (240 g.) were mixed in a stoppered flask and left at 4° for 3 days. The greatly swollen mass was broken up and diluted with 2 l. of ether, with vigorous stirring, and filtered, washed with ether and dried as above.

Acetyl estimation. Hydrolysis of the acetylated gelatin (up to 1 g. containing not more than 0·5 m-mole of acetyl) was effected by heating with 20 ml. of 5 N-H_2SO_4 in a sealed Pyrex test tube for 5 hr. at 100° in a water bath. The hydrolysate was steam-distilled and titrated potentiometrically to pH 7 with 0·02 N-KOH, standardized under similar conditions. The solution was stirred by a stream of nitrogen bubbles.

O-Acetyl groups were determined in two ways, based on observations by Synge (1939) and Wolfrom, Konigsberg & Soltzberg (1936) that *O*-acyl and *O*-aryl derivatives are unstable to mild alkaline hydrolysis. In the first method, equal weights of acetylated gelatin and crystalline borax were dissolved in 100 ml. of water at 40° and maintained at that temperature for 1 hr. The product was dialysed, deionized and dried, and its acetyl content determined. The difference between this and the original value gave the *O*-acetyl value. In the second method, by using an apparatus similar to that used for the titration-curve determination (Kenchington & Ward, 1954), a solution of 1–3 g. of acetylated gelatin in 100 ml. of water was maintained at 40° and adjusted to pH 7·00 before being brought to and maintained at pH 9–10 by measured additions of standard alkali. When no further drift occurred (0·5 hr.), the pH was readjusted to 7·00 with a measured volume of standard acid, and the product dialysed, deionized and dried for acetyl determination, the *O*-acetyl content being obtained by difference in total acetyl before and after treatment. The volume of alkali added after adjustment to pH 7·00, less the volume equivalent to the acid used finally in returning to pH 7·00, gave the alkali consumed during deacetylation.

Guanidation procedure

O-Methyl*iso*urea was prepared as a solution at 0° in water. Gelatin powder was then stirred in, the ratio volume of solution:volume of gelatin being 20:1.

The weight of reagent used was varied from 10 to 100 times the theoretical amount required for the amino groups present; there was thus always a sufficient excess to maintain the pH of the gelatin at an alkaline value.

Rapid swelling of the gelatin occurred as soon as it was stirred into the reagent solution, the greater part of which was absorbed. The mixture was left at 0–4° for 7–8 days before being neutralized, with continuous stirring, with 5 N-HCl. The slurry was melted at 40° to a uniform solution, set to a gel, washed, deionized and dried.

Total nitrogen was determined by the method of Chibnall, Rees & Williams (1943), as modified by Eastoe & Eastoe (1954). Amino acid chromatography was performed by the methods of Moore & Stein (1951) in collaboration with J. E. Eastoe (Eastoe & Kenchington, 1954).

Oxidation of gelatin with hypobromite

Three solutions, each containing air-dried gelatin (23 g.; 20 g., anhydrous) dissolved in 1200 ml. of water, were cooled to 22° and treated in one of the following ways.

The first solution (*A*) was vigorously stirred while an alkaline solution of potassium hypobromite was rapidly added (the latter solution was made by dissolving 6 g. of bromine in a solution, at 0°, of 23 g. of KOH in 200 ml. of water). After 150 sec., a mixture of 22 ml. of 33% HCl and 9 ml. of acetic acid was added with stirring, giving approx. pH 4.

The second solution (*B*) was treated with KOH (18·8 g.) and KBr (9 g.) dissolved in 200 ml. of water at 0° for 150 sec., before the same acid mixture as was used for solution A was added.

The third solution (*C*) was treated with KBr (9 g.), KCl (17 g.), potassium acetate (12 g.) and acetic acid (2 g.), all dissolved in 200 ml. of water.

All three resulting solutions were thus of identical salt content, it being assumed that the hypobromite was all reduced to bromide. All three were concentrated by distillation at reduced pressure to a volume of 300 ml., when they were found to be at pH 7 ± 0·5. After determination of rigidity and viscosity, the whole of the first two solutions were separately dialysed against distilled water (two changes of 10 l.) at 4° for 24 hr. The products were dried in polythene dishes in a current of air, yielding 20 and 21 g. respectively. A portion of each was redissolved and deionized to measure the pI (pH at isoelectric point). Paper chromatography of a portion of a hydrolysate of each was performed, after the methods of Consden, Gordon & Martin (1944).

Attempts were made to use milder reaction conditions, by lowering the pH of reaction from 14 to 8 in a series of experiments, and also by working with 0·5–1% gelatin solutions at 4°, in the hypobromite-oxidation stage.

RESULTS

Methylated gelatin

The methoxyl contents of gelatin methyl esters increased according to (*a*) the concentration of the acid in the methanol used for esterification and (*b*) the time of treatment with the reagent, until a maximum value of 1·27 m-moles/g. was achieved, the constant value of 0·03 m-mole/g. obtained for the untreated material being allowed for. Washing in water at 4° for 24 hr. reduced the methoxyl content of the most highly methylated gelatins by up to 20%. Except with slightly methylated gelatins, deionization greatly reduced the methoxyl content, the maximum value after deionization being 0·4 m-mole/g. Only a few of the cooled deionized solutions showed the characteristic isoelectric turbidity of gelatin solutions. Table 2 illustrates these results. The L.V.N. of the methylated gelatin fell with increase either in time of

Table 2. *Esterification of gelatin carboxyl groups*

L.V.N. is the logarithmic viscosity number; M_w is the weight-average molecular weight.

Sulphuric acid (m-moles/ 500 ml. of ethanol/5 g. of anhydrous gelatin)	Time of reaction (hr.)	Methoxyl content (m-mole/g.)	L.V.N.	M_w	$10^3 \times$ Rigidity (dynes/cm.^{2}) (10°)*	Methoxyl content (m-mole/g.)	After deionization: pH of liquor†	After deionization: L.V.N.
1·8	18	0·05	63	—	65	0·06	4·9	—
3·7	18	0·11	61	200 000	—	0·11	5·0	—
7·4	18	0·39	53	—	—	0·29	8·7	—
11·0	18	0·71	45	—	—			
18·4	18	0·81	39	80 000	—	0·26	9·7	—
1·8	66	0·19	58	—	—	0·13	5·4	—
3·7	66	0·45	50	—	57	0·26	8·9	—
7·4	66	0·77	38	—	41	0·39	10·0	29
11·0	66	0·97	34	—	23	0·42	10·0	23
18·4	66	1·00	24	—	—	0·39	10·0	24
1·8	196	0·25	34	—	—	0·19	5·8	—
3·7	196	0·81	26	—	—	0·32	9·7	—
7·4	196	1·10	29	—	—	0·39	9·9	22
11·0	196	1·27	26	55 000	—	0·39	10·0	23
18·4	196	1·27	16	—	—	0·42	10·0	20
0·0 (control)	—	0·3	60	220 000	66	0·0	4·91	64

* Concentration: 5·7% (w/w) anhydrous gelatin.
† This will be pI only for gelatins of low methoxyl content.

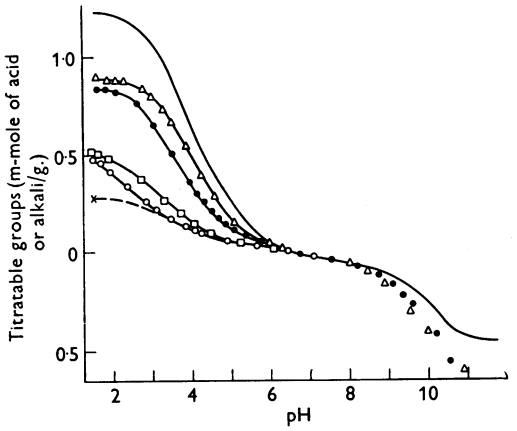

Fig. 1. Titration curves of gelatin methyl esters. Experimental points are not shown on the curve for the original gelatin (Kenchington & Ward, 1954). Gelatins of differing degree of esterification gave samples A (△), B (●), C (□), D (○). Point X is the corrected value for acid end-point of D; the broken curve from X indicates the probable true shape of the titration curve. Points on the alkaline side of A and B show the effect of alkaline hydrolysis; the positions of these points on the pH scale are a function of time of exposure to alkali. (See text for details.)

contact with the reagent or of catalyst concentration, corresponding to increase of methoxyl content. The deionized materials, however, of considerably reduced methoxyl content, showed no corresponding increases in L.V.N. as would have been expected were the original fall in L.V.N. attributable to esterification rather than degradation. The molecular weights confirmed that degradation had occurred.

The titration curves of the original and esterified gelatins are shown in Fig. 1, carboxyl titrations in the pH range 1·5–6·5 showing reductions corresponding to the degree of esterification. There were large variable increases in the titration at pH 8·5 and higher, illustrated by two series of points where considerable pH drifts occurred and the titration was non-reversible. With one gelatin, 'S', before and after acid titration (to pH 1·5) the material contained 0·44 m-mole of methoxyl/g., whereas after alkaline titration (to pH 11) the material recovered contained only 0·11 m-mole of methoxyl/g. In the latter, there was thus a loss of 0·33 m-mole of methoxyl/g.; the increase in free acid groups calculated from the non-reversibility of the titration was 0·2–0·4 m-mole/g. In the back-titration, acid exactly equivalent to the total alkali used in the titration was added. Any alteration in the number of ionizing groups titrated was at once indicated if the observed pH differed from the original pH; the amount of base required to produce this pH change could be measured, to give a quantitative measure of the change in the number of ionizing groups. Gelatins containing more than 0·8 m-mole of methoxyl/g. showed instability towards acid, illustrated by 'D' in Fig. 1, and gave a non-reversible titration on the

Table 3. *Comparison of analytical results for methoxyl*

The methoxyl content was determined by a modified Zeisel method (see Methods). The method of calculating the titration correction is described in the text.

Sample	Methoxyl content (m-mole/g.) After Zeisel	From titration curve
A	0·31	0·33
B	0·44	0·42
C	0·77	0·77
D	0·88	0·76 uncorrected 0·9 corrected

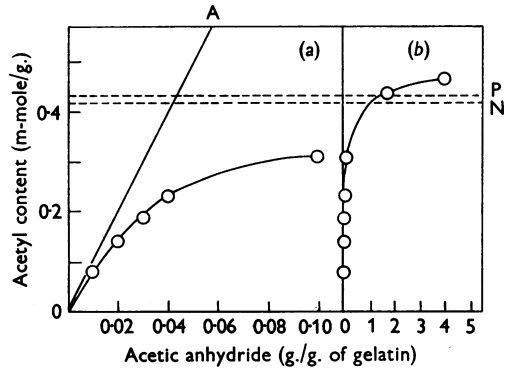

Fig. 2. Acetylation of gelatin, in aqueous sodium acetate solution, with acetic anhydride. Broken lines N and P show calculated contents of ε-acetamido and ε-acetamido plus α-acetamido of fully *N*-acetylated gelatin. (*a*) Line 0A is theoretical for 100% reaction of the anhydride with gelatin. Effect of small amounts of acetic anhydride. (*b*) Effect of larger amounts of acetic anhydride.

acid as well as the alkaline side, and a different shape in the titration curve on the acid side. When the end point of the titration curve on the acid side was corrected by deducting the titration due to additional carboxyl groups liberated by hydrolysis of ester groups (obtained as above from the non-reversibility), the anomalous form of the curve was explained. The methoxyl content calculated from the titration curve value after this correction was in good agreement with that obtained by the Zeisel estimation, as were the values for the other materials where no correction was necessary (Table 3).

Acetylation of gelatin

In aqueous sodium acetate solution, the degree of acetylation of gelatin depended on the amount of acetic anhydride added, as shown in Fig. 2 (*a*) and (*b*). A maximum acetyl value of 0·47 m-mole/g. was found. On treatment with alkali by either of the methods described, this was reduced to 0·43 m-mole/g. Acetylated gelatin of acetyl content not exceeding 0·43 m-mole/g. was stable to alkali under the conditions of the titration-curve determination, and showed a reduction in the titration of α- and ε-amino and histidine groups between pH 6·5 and pH 11·5, corresponding fairly well with the acetyl content (Fig. 3 and Table 4). The values calculated for amino substitution by the change in isoionic point, however, were not in good agreement, being low. The minimum isoionic point recorded was at pH 4·28. There was a small reduction in the carboxyl titration, of 0·06 m-mole/g.

The physical properties of the *N*-acetylated

Table 4. *Acetylation of gelatin in acetate buffer solution*

L.V.N. is the logarithmic viscosity number. For determination of acetyl content, see Methods. Acetyl content and titration values are expressed as m-moles/g. of anhydrous ash-free derivative.

Material	Acetic anhydride (g./g. of gelatin)	Acetyl content by chemical analysis	Titration values: pH 6·5–8·0	pH 8·0–11·5	Difference from original between pH 6·5 and 11·5	pI	Acetyl content calculated from pI*	L.V.N.	10^3 × Rigidity (dynes/cm.^{2}) 0°	10°	20°
Original gelatin	0·0	0·0	0·06	0·42	0	4·91	0·0	60	101	66	26
U	1·7	0·44	0·05†	‡	‡	4·28	0·33	58	85	53	25
T	4·0	0·47	0·06†	‡	‡	4·29	0·33	46	75	45	13
T_a (after KOH)	—	0·42	—	—	—	4·39	0·31	—	—	—	—
T_b (after borate)	—	0·43	0·04	0·06	0·38	4·39	0·31	40	66	40	8
S	0·10	0·31	0·05	0·10	0·33	4·31	0·32	51	98	61	20
R	0·03	0·19	0·04	0·22	0·22	4·58	0·20	62	90	61	22
P	0·01	0·08	0·05	0·36	0·07	4·76	0·10	58	100	63	23
Q	0·02	0·14	0·05	0·28	0·15	4·65	0·13	56	98	61	22

* Allowance was made for decrease in carboxyl titration.
† May be high owing to the beginning of instability to alkali.
‡ No value was obtainable owing to pH drifts.

gelatin (Table 4) were very similar to those of the starting material.

The material produced by acetylation in acetic acid–acetic anhydride solution, of acetyl content corresponding to both *N*- and *O*-acetylation, swelled in water, but the swollen particles would not melt even at 100°. Similar behaviour was observed with a 50% (w/v) urea solution used as a hydrogen-bond breaker; after suspension for 2–3 hr. in a borax solution at pH 10, the swollen gel melted slowly at 30–40°, presumably owing to decomposition of alkali-labile intermolecular cross-links of ester type.

The material of low acetyl content produced by treatment with acetic anhydride alone was similar to the original gelatin in solubility, solution viscosity, and gel rigidity.

Guanidation of gelatin

The reaction products from *O*-methyl*iso*urea and gelatin were of increased nitrogen content, but unchanged pI. The titration curves differed from the original gelatin only in the range from pH 8 to 11·5, where the content of titratable groups was reduced as the nitrogen content increased, corresponding with increase in amount of the guanidating reagent used (Table 5). The viscosities showed a slight decrease, as did the rigidities. The most highly guanidated material, whose titration curve is given in Fig. 4, was analysed for basic amino acid content (Eastoe & Kenchington, 1954), and the chromatogram showed the emergence of two new peaks attributed to homoarginine and hydroxyhomoarginine. The analytical values are reproduced in Table 6.

Deguanidation of gelatin

The gelatin solution *A* which had been treated with alkaline hypobromite was yellowish brown and possessed an odour reminiscent of hypobromite, which was not lost on treatment with urea or sulphite solutions. The values for rigidity

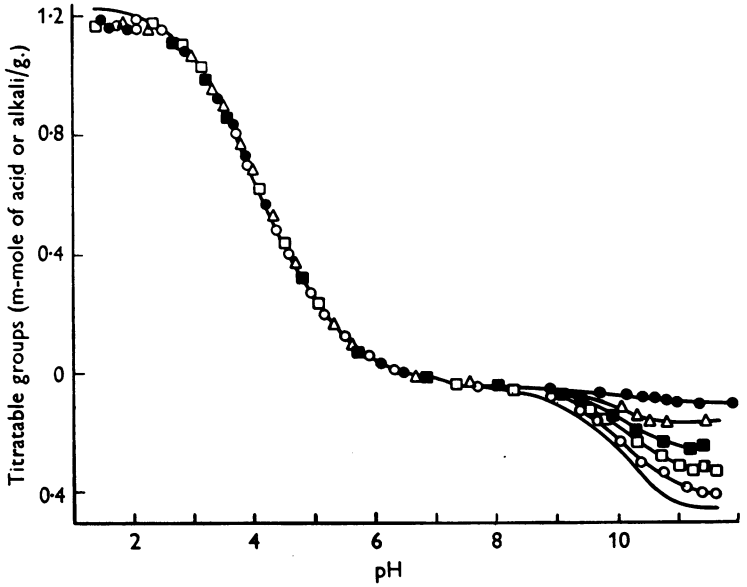

Fig. 3. Titration curves for acetylated gelatins. Experimental points are not shown on the curve for the original gelatin (Kenchington & Ward, 1954). Effect of increasing acetyl content is shown by curves for P (○), Q (□), R (■), S (△), T_b (●).

Table 5. *Analysis and physical properties of guanidated gelatin*

L.V.N. is the logarithmic viscosity number.

Material	% Total nitrogen	Decrease in titration pH range 8·0–11·5 (m-mole/g.)	L.V.N.	10^3 × Rigidity (dynes/cm.^{2}) 0°	10°	20°
Original	17·9	0	57	101	61	28
X	18·5	0·10	51	96	58	26
Y	18·5	0·33	51	97	55	27
Z	18·7	0·36	48	95	49	16

and viscosity are given in Table 7, and indicate severe degradation. The influence of the salts on the rigidity is seen to be large, as is the effect of alkali alone (solutions *B* and *C*). Similar results were obtained by treating 8 % gelatin solutions at 40° with hypobromite at 0°.

Attempts were made to use milder conditions by reducing the pH of reaction and by working at 4° with dilute gelatin solutions to prevent gelation. It was found that at any pH below about 12, addition of hypobromite produced an immediate precipitate of what appeared to be cross-linked gelatin: physically this precipitate resembled that produced by organic solvents such as acetone or ethanol, but differed in that it appeared immediately on the addition of a small volume of reagent. It was insoluble in hot water but, if freshly prepared, could be redissolved in warm sulphurous acid solution.

When the reaction was carried out at pH 12–13, precipitates were formed initially but soon redissolved. Above pH 13 no precipitates were seen. Observations were impeded by the generation of large numbers of minute gas bubbles, and the necessity for high-speed stirring to ensure homogeneity in the short reaction time. The gas liberated was not investigated; it was assumed to be nitrogen (Chinard, 1948). Attempts to work at pH 8 or less brought about precipitation accompanied by liberation of bromine from the hypobromite under these less alkaline conditions.

The viscosity of solutions treated under these modified conditions, and which had not precipitated, was considerably reduced, as was the gelling power. The volume of ethanol required to bring about precipitation of the 'gelatin' from solution was increased by 200–300 %; pI was variably reduced although the carboxyl titration appeared largely unaltered. Paper chromatograms showed that considerable amounts of arginine were still present in these materials.

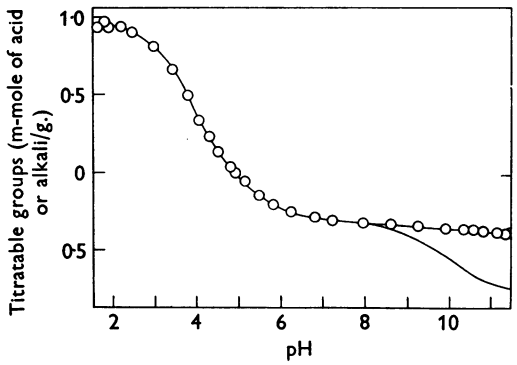

Fig. 4. Titration curves for original gelatin (—) and guanidated gelatin Z (○—○).

Table 6. *Amino acid content (m-moles/g.) of original and guanidated gelatin*

Residue	Material: Original		Material: Z
Lysine	0·312	0·34	0·01
Ornithine	0·027		
Hydroxylysine	0·077		0·003
Histidine	0·056		0·053
Arginine	0·476		0·532
Homoarginine*	0·0		0·280
Hydroxyhomoarginine	0·0		0·067

* Cf. Greenstein (1938).

DISCUSSION

Methylation of gelatin

The maximum methoxyl content achieved (1·27 m-moles/g.) agrees closely with the values of 1·23 m-moles/g. for free carboxyl in the gelatin used, obtained from the titration curve, and of 1·28 m-moles/g. from chromatographic analysis. Complete esterification of the carboxyl groups of gelatin can be considered to have been achieved. The blank of 0·03 m-mole/g. of 'methoxyl' is attributed to the thio-methyl group of methionine (Baernstein, 1932), compared with 0·05–0·06 m-mole/g. obtained by Eastoe (1955) using the method of Moore & Stein (1951).

The instability of the methyl esters to alkali is typical, and is responsible for the irreversible alkaline titration. It prevents any conclusion being drawn about the effect of the reaction on ε-amino groups, although the absence of any

Table 7. *Physical properties of hypobromite-treated gelatin*

Solution *A* was treated with hypobromite, solution *B* with the corresponding amount of alkali. Solutions *A*, *B* and *C* all had the same final concentrations of salts and protein. L.V.N. is the logarithmic viscosity number.

Solution	Untreated: L.V.N.	Untreated: 10^3 × Rigidity (dyncs/cm.^{2})	Deionized: L.V.N.	Deionized: 10^3 × Rigidity (dynes/cm.^{2})
A	30	No gel	30	8
B	58	10	48	73
C	56	35	58	100

change in the α-amino plus histidine titration indicated little, if any, reaction of α-amino groups. The agreement between the Zeisel methoxyl value and that calculated from the titration curve is good.

The instability to acid, and even to water, of gelatin methyl esters appears to be confined to those of high methoxyl content, and may be due to the different carboxyl groups involved.

The hydrolysis on deionization is to be expected since the pI for gelatins of even moderate degree of methylation would be in the alkaline range, from pH 9 upwards, under which conditions ester hydrolysis is rapid, with a consequent fall in pI as carboxyl groups are freed. The loss of methoxyl groups would cease as the pI, and hence the pH, approached 7.

Physical measurements on the esterified gelatins indicated that severe degradation had taken place during their production. The fall in viscosity with time of reaction and amount of reagent, although corresponding to increased methoxyl content, appears due to increased hydrolysis. Thus there is no trace of any viscosity increase on reduction of methoxyl content by deionization. The rigidity of set gels of these materials is greatly reduced, which would be expected from the degradation indicated by the lowered viscosity and by reduced molecular weight. There is no evidence of a direct effect on the gelling power, i.e. on the 'rigidity factor' of Saunders & Ward (1955).

Acetylation of gelatin

The stability to alkali of the acetyl groups of acetylated gelatin, and the close relation of the maximum content of such groups to the free amino content of the original gelatin, indicate that the action of acetic anhydride on sodium acetate solutions of gelatin is to produce preferential acetylation of the amino groups. No other groups react until a large excess of reagent is used in order to complete the amino acetylation, when some acetylation of hydroxyl groups occurs. These *O*-acetyl groups are easily removed by virtue of their instability to mild alkaline hydrolysis.

The pH of the reaction was found to change very rapidly from an initial value near pH 8 to pH 4, as would be expected from the use of an acetate buffer. To maintain a reaction pH of 7–8, as claimed by Olcott & Fraenkel-Conrat (1947), it was necessary to add alkali continually. The specificity of the reaction under the conditions used cannot therefore be ascribed to hydrolysis of *O*-acetyl groups in a slightly alkaline medium, but must be due to the more rapid reaction of the amino groups.

Lea, Hannan & Rhodes (1951) acetylated casein by this method and found that neither the rate of addition of the anhydride nor the time of standing had any significant effect on the amino nitrogen; similar results have been obtained in this Laboratory with gelatin. The same authors considered that 94–97% acetylation of amino groups was achieved, but found a discrepancy of 20% more acetyl groups introduced than amino groups lost. Green *et al.* (1953) found it necessary to add alkali to maintain pH 8 when acetylating collagen by this method, and then obtained *N*-acetylation without *O*-acetylation; when no alkali was added some *O*-acetylation occurred.

The maximum of *N*-acetyl groups (0·43 m-mole/g.) reported here is in excellent agreement with the total amino content of 0·42–0·44 (Table 1), and with the maximum *N*-acetyl figure of 0·39 m-mole/g. of Green *et al.* when allowance is made for the absence of 0·02 m-mole/g. each of α-amino groups and ε-amino groups from ornithine. Attempts to measure the *O*-acetyl content by determination of the alkali neutralized during mild alkaline hydrolysis gave values (0·08–0·09 m-mole/g.) in disagreement with the results of total acetyl estimations on the purified material before and after the hydrolytic treatment (0·47–0·43 = 0·04 m-mole/g.).

Comparison of the acetyl values from chemical estimation with those from determinations of the titration curve and the isoionic point shows that there is agreement until the acetyl content rises above 0·3 m-mole/g., when discrepancies appear. The tyrosine content of gelatin should give a titration of 0·02 m-mole/g. from pH 8 to 11·5, whereas the minimum value obtained was 0·06 m-mole/g. A similar result was obtained with guanidated gelatin (cf. Figs. 3 and 4); no satisfactory explanation can yet be put forward. The titration curves of acetylated gelatins show a slight reduction in the carboxyl titrations (mean 0·06 m-mole/g.), which is of unknown origin and significance. The acetylations under anhydrous conditions support the findings of Green *et al.* (1953) that there is little acetylation unless acetic acid is added to the reaction mixture, when a high degree of *O*- and *N*-acetylation is achieved. These authors consider acetic acid to act as a catalyst; in view of the concentration required and of the fact that acetic acid has considerable swelling and solvent power for gelatin, whereas acetic anhydride has none and is soluble in acetic acid but not in water, it would seem probable that the function of the acetic acid is that of a solvent rather than a catalyst. The maximum acetyl content obtained by Green *et al.* for collagen was 1·75 m-moles/g.; with gelatin the same method gives 2·0–2·1 m-moles/g. The theoretical value for complete acetylation of amino groups, phenolic and other hydroxyl groups in gelatin is 2 m-moles/g., calculated from the amino acid composition found by Eastoe (1955). It is possible that the different values for collagen and

gelatin might be due to the presence of ester cross-links in collagen, as suggested by Gustavson (1955). The ability of highly acetylated gelatin to swell in water but not to dissolve on warming corresponds to the behaviour of cross-linked material. The solubility on treatment with dilute alkali suggests that cross-links of carboxylic ester type are involved, being readily hydrolysed by alkali. The use of anhydrides, including acetic anhydride, to promote condensation reactions has been reviewed by Tedder (1955), and there are in gelatin many carboxyl and hydroxyl groups which might undergo condensation.

It is possible that cross-linking reactions of this type may reduce the total acetyl content obtainable with collagen by immobilizing some of the reactive centres by internal cross-links, artificially introducing the type of bond suggested by Gustavson (1955) to occur naturally. The more ordered structure of collagen may facilitate such cross-linking reactions and render the blocking action more effective.

The fall in pI on acetylation is not as large as would be expected from the work of Hitchcock (1923) on deamination of gelatin, which gave pI 4 for completely deaminated gelatin. From the titration curve it can be calculated that since the removal of 0·4 m-mole of amino nitrogen/g. is equivalent to the addition of 0·4 m-mole of acid, the pI should move from pH 4·91 to 4·0, in agreement with Hitchcock's value. Since the conditions used by Hitchock are known to cause reaction of groups other than amino, the position is far from clear. Deamination of the gelatin used in this work under mild conditions (20°, pH 4, 30 min.) gave gelatin having a pI of 4·26, the same as that of acetylated gelatin. The position is yet more complicated because Hitchcock (1923) used a different gelatin for his deamination work from the Eastman Kodak gelatin he used for his titration-curve determinations (1931). It was suggested from titration-curve analysis (Kenchington & Ward, 1954) that in the Eastman Kodak material a considerable amount of arginine had undergone conversion into ornithine, giving high amino nitrogen content. This suggestion has been confirmed by amino acid analyses of gelatin from the same source by Hamilton & Anderson (1954).

The effect of acetylation on the physical properties of gelatin is very small, there being a slight fall in viscosity and jelly strength with highly acetylated gelatins, attributable to slight degradation.

Guanidation of gelatin

The reaction of *O*-methyl*iso*urea with gelatin has been shown to be confined to the amino groups and to proceed to completion. There were indications that when an alkali such as potassium hydroxide was used to adjust the pH during reaction, instead of allowing excess of reagent in the free base form to maintain the pH, a lesser degree of reaction was achieved. The reaction with ornithine brings about its reversion to arginine, from which it is produced by the alkaline pre-treatment of collagen in the manufacture of gelatin.

The results from total nitrogen, titration-curve and amino acid-composition determinations are all in fair agreement, and the discrepancies are only of the order of the limits of experimental error. Thus from chromatographic analysis the material titrating from pH 8 to 11·5 should not exceed 0·02 m-mole of ϵ-amino groups/g., plus 0·02 m-mole of phenolic groups/g., whereas the experimental value was 0·06 m-mole/g., as with acetylated gelatin. Theoretically, the nitrogen content should increase by a maximum value of 0·87 %; an increase of $0·9 \pm 0·05$ % was observed.

The values of certain physical properties (such as viscosity and gel rigidity) of the products show small decreases, quite in accord with a very small amount of degradation at pH 12–13 and 0°. There was certainly no sign of any increase in gel rigidity.

Deguanidation of gelatin

These experiments show that hypobromite as a specific reagent for arginine in the investigation of protein structures must be considered of limited use; any results based on it should be treated with reserve until confirmed by independent methods. Grabar & Morel (1950) attributed the gelling power of gelatin to guanidino groups, from results based on the use of hypobromite, supported by evidence which would more plausibly be interpreted as favouring a hydrogen-bonded structure for gelatin gels. The work described here shows that hypobromite not only degrades gelatin, but probably also cross-links it. The combination of both processes is closely akin to the controlled cross-linking with glyoxal, followed by degradation, used by Campbell *et al.* (1951) to reduce the ability of gelatin to gel while maintaining a high molecular weight. The original observation of Chinard (1948) was that hypobromite, while liberating nitrogen almost quantitatively from arginine in gelatin, attacked many other sites on the protein molecule.

General effects of side-chain substitution

None of the chemical modifications achieved has shown any significant effect on gel strength except that which could be attributed to the presence in the product of some material of reduced molecular weight, ineffective in forming the gel. For practical purposes, this degradation is indicated by change in viscosity. All the ionizable groups have, in turn,

been altered in some way in the course of the work, but not the unionizable hydroxyl groups which are present in large numbers and which may well play an important part.

It would seem that whatever effect the charged groups have is relatively small in this connexion; this correlates well with the slight dependence of gel strength on pH, except at extreme values.

Saunders & Ward (1955) and Pouradier, Venet & Trigny (1955) have shown that above a molecular weight of approximately 80 000 the rigidity is largely independent of molecular weight and of viscosity, whereas the rigidity of the gel decreases sharply as the molecular weight is reduced from this value. The existence of different gel-forming powers in proteins of the same molecular weight (the 'rigidity factor' of Saunders & Ward, 1955) may be explained if some considerable degree of order in the molecular structure is necessary to attain the maximum gel strength, and if the latter diminishes with loss of this ordered structure. The most probable linkage for joining these structure groups together is the hydrogen bond. There are vast numbers of sites suitable for its formation available in the molecule, including hydroxyl groups and peptide bonds, while the low heat of melting of the gel necessitates little change in energy. Also, all the known compounds which cause liquefaction of gels, without degradation, are active in hydrogen-bond breaking (Courts, 1957).

Subsequent to the completion of the work, a method for selective acetylation of hydroxyl groups in proteins has been published (Bello & Vinograd, 1956) which still, however, produces degradation. Bello (1956) has also introduced a new reagent, thionyl chloride, for esterification of gelatin, which does not appear to produce much degradation. His work was all performed on gelatin produced by an acid process. The results of physical measurements other than those of viscosity are not yet available.

SUMMARY

1. The ionizable groups in gelatin have been chemically modified in order to observe any resultant effects on the solution viscosity and gel rigidity. No effect not attributable to degradation was detected.

2. Complete esterification of carboxyl groups was achieved, since the analytical figures for methoxyl content agreed with the total free carboxyl content before treatment. Highly esterified material was unstable in aqueous solution at all pH values.

3. Complete *N*-acetylation was achieved by the action of acetic anhydride on gelatin dissolved in aqueous sodium acetate. Little *O*-acetylation occurred until the majority of the amino groups were acetylated. The *O*-acetyl groups were easily removed by mild alkaline treatment.

4. Guanidation of amino groups was shown to proceed virtually to completion.

5. Under all conditions of reaction studied, hypobromite was found to produce extensive degradation and other changes before its reaction with guanidino groups was complete.

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REFERENCES

Baernstein, H. D. (1932). *J. biol. Chem.* **97**, 663.
Belcher, R., Fildes, J. E. & Nutter, A. J. (1955). *Analyt. chim. acta*, **13**, 16.
Bello, J. (1956). *Biochim. biophys. Acta*, **20**, 426.
Bello, J. & Vinograd, J. R. (1956). *J. Amer. chem. Soc.* **78**, 1369.
Campbell, D. H., Koepfli, J. B., Pauling, L., Abrahamson, N., Dandliker, W., Feiger, G. A., Larri, F. & Le Rosen, A. (1951). *Tex. Rep. Biol. Med.* **9**, 235.
Chibnall, A. C., Rees, M. W. L. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
Chinard, F. P. (1948). *J. biol. Chem.* **176**, 1449.
Cohn, E. J. & Edsall, J. T. (1943). *Proteins, Amino-Acids and Peptides*. New York: Reinhold Publishing Corp.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1944) *Biochem. J.* **38**, 224.
Courts, A. (1954). *Biochem. J.* **58**, 70.
Courts, A. (1957). *Recent Advances in Gelatin and Glue Research*, p. 145. London: Pergamon Press.
Eastoe, J. E. (1955). *Biochem. J.* **61**, 589.
Eastoe, J. E. & Eastoe, B. E. (1954). *Biochem. J.* **57**, 453.
Eastoe, J. E. & Kenchington, A. W. (1954). *Nature, Lond.*, **174**, 966.
Fraenkel-Conrat, H. & Olcott, H. S. (1945). *J. biol. Chem.* **161**, 259.
Grabar, P. & Morel, J. (1950). *Bull. Soc. Chim. biol., Paris*, **32**, 643.
Green, R. W., Ang, K. P. & Lam, L. C. (1953). *Biochem. J.* **54**, 181.
Greenstein, J. P. (1938). *J. org. Chem.* **2**, 480.
Gustavson, K. H. (1955). *Nature, Lond.*, **175**, 73.
Hamilton, P. B. & Anderson, R. A. (1954). *J. biol. Chem.* **211**, 95.
Herriott, R. (1947). *Advanc. Protein Chem.* **3**, 169.
Hitchcock, D. I. (1923). *J. gen. Physiol.* **6**, 95.
Hitchcock, D. I. (1931). *J. gen. Physiol.* **15**, 125.
Hughes, N. L., Saroff, H. A. & Carney, A. L. (1949). *J. Amer. chem. Soc.* **71**, 2476.
International Union of Pure and Applied Chemistry (1952). *J. Polym. Sci.* **8**, 270.
Janus, J. W., Kenchington, A. W. & Ward, A. G. (1951). *Research, Lond.*, **4**, 247.
Kenchington, A. W. (1954). Ph.D. Thesis, Birmingham.
Kenchington, A. W. & Ward, A. G. (1954). *Biochem. J.* **58**, 202.
Lea, C. H., Hannan, R. S. & Rhodes, D. N. (1951). *Biochim. biophys. Acta*, **7**, 366.
Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.

Olcott, H. S. & Fraenkel-Conrat, H. (1947). *Chem. Rev.* **41**, 151.
Pouradier, J., Venet, H. M. & Trigny, L. (1955). *Proc. 27th Congr. Int. Chim. Ind., Brussels*, **3**, 709.
Reitz, H. C., Ferrel, R. E., Fraenkel-Conrat, H. & Olcott, H. S. (1946). *J. Amer. chem. Soc.* **68**, 1024.
Saunders, P. R., Stainsby, G. & Ward, A. G. (1954). *J. Polym. Sci.* **12**, 325.
Saunders, P. R. & Ward, A. G. (1953). *Proceedings of the 2nd International Congress on Rheology*, p. 284. London: Butterworths Scientific Publications.
Saunders, P. R. & Ward, A. G. (1955). *Nature, Lond.*, **176**, 26.
Stainsby, G. (1956). *Nature, Lond.*, **177**, 745.
Synge, R. L. M. (1939). *Biochem. J.* **33**, 1913.
Tedder, J. M. (1955). *Chem. Rev.* **55**, 787.
Udenfriend, S. & Cooper, J. R. (1952). *J. biol. Chem.* **196**, 227.
Ward, A. G. (1953). *Nature, Lond.*, **171**, 1100.
Ward, A. G. (1955). *Nature, Lond.*, **175**, 289.
Wolfrom, M. L., Konigsberg, M. & Soltsberg, S. (1936). *J. Amer. chem. Soc.* **58**, 490.

A Study of the Inhibition of Catalase by 3-Amino-1:2:4-Triazole

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Heim, Appleman & Pyfrom (1955, 1956) have shown that the injection of 3-amino-1:2:4-triazole into rats causes a rapid decrease of the catalase activity of the liver and kidney to very low levels, but has no effect on the catalatic activity of blood. These authors found that crystalline catalase was partly inhibited *in vitro* only at high concentrations of 3-amino-1:2:4-triazole (>0·05M); such concentrations were far above those necessary for the inhibition of catalase by 3-amino-1:2:4-triazole *in vivo*. The inhibition *in vitro* was reversible by dilution, whereas that obtained *in vivo* was not.

Sugimura (1956) showed that the incubation of rat-liver suspensions with 3-amino-1:2:4-triazole at 37° caused similar decreases in the catalatic activity of the suspensions, that the incubation of a purified crystalline preparation of ox-liver catalase with 3-amino-1:2:4-triazole did not result in any decrease of activity, but that when a liver extract was incubated with purified catalase in the presence of 3-amino-1:2:4-triazole the inhibition of catalatic activity occurred. In every case the inhibition required the presence of oxygen.

The present paper is concerned with the further investigation of these effects with simple systems *in vitro* and with the properties of the catalase inhibited by 3-amino-1:2:4-triazole.

MATERIALS AND METHODS

Catalase preparations. Two preparations of catalase were used. One was a commercial recrystallized ox-liver catalase (Worthington Biochemical Corp., St Louis, Mo., U.S.A.) with Kat.f. (Euler & Josephson, 1927) 26 000, and the other was a crystalline catalase (Kat.f. 58 000) prepared from human erythrocytes according to Herbert & Pinsent (1948). The concentration of the haemoprotein solutions was determined by the pyridine haemochromogen method of Keilin & Hartree (1951), and throughout this paper is expressed in terms of haemoprotein haematin.

Enzymic activities. Catalatic activity was estimated by the sodium perborate method of Feinstein (1949), at 37°, and the results were expressed in moles of perborate destroyed/μmole of catalase haematin under the conditions of the test. The concentration of catalase haematin required for this determination is of the order of 10^{-9}M. Kat.f. values were determined according to Euler & Josephson (1927). The rate of oxidation of ribose 5-phosphate (R 5-P) (Francoeur & Denstedt, 1954) was determined at pH 8·5, with 0·025M-2-amino-2-hydroxymethylpropane-1:3-diol–HCl (tris) buffer, with 0·02M-R 5-P as hydrogen donor and 0·002M-$K_3Fe(CN)_6$ as hydrogen acceptor in Thunberg tubes under N_2 at 37°. The reduction of the $K_3Fe(CN)_6$ was followed colorimetrically in a Klett–Summerson photoelectric colorimeter (filter 42) in which the Thunberg tubes could fit directly.

Spectrophotometry. Hilger Uvispek and Beckman DU spectrophotometers were used. Anaerobic spectra were measured in 1 cm. quartz cells fused to quartz Thunberg tubes.

Tissue preparations. Male albino rats (100 g.) of a local strain, bred randomly, were decapitated and the liver and kidneys were removed to cooled moist Petri dishes and weighed rapidly. Suspensions (1:5) were prepared in water with a Potter glass homogenizer (Umbreit, Burris & Stauffer, 1949). Erythrocyte haemolysates from rat blood obtained by heart puncture under ether anaesthesia, or from human blood, were prepared by defibrinating the blood with glass beads, washing the erythrocytes with 0·85% NaCl soln. five times on the centrifuge, haemolysing the cells with an equal volume of water and centrifuging