CLXXXIX. THE EFFECT OF LIGHT AND SALTS ON GELATIN.

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ALTHOUGH it has long been known that gelatin which has been treated with potassium dichromate becomes insoluble on exposure to light, the past workers investigating this problem have assumed that the protein molecule was unchanged and have concerned themselves only with the changes in the dichromate. The reactions involved in the production of insoluble gelatin by the action of chromium salts in the absence of light were also investigated on the same lines. The most important work on the changes occurring in the first process has been carried out by Eder [1925, 1926], while the changes involved in the second process have been chiefly investigated by Lumière and Seyewitz [1902, 1905, 1906].

As early as 1891 Eder showed that the action of light on a potassium dichromate-gelatin mixture was to produce chromium chromate. The next important investigation made by Eder was on the action of heat and moisture on dichromated gelatin in the absence of light. He found that if the solution was kept at the boiling point for 1 or more hours, water being added from time to time to make up for loss by evaporation, it lost its consistency, became thick, and solidified while still at a high temperature. This boiled gelatin, after drying, still gave a gel when immersed in cold water although more slowly and with greater difficulty than before. He also found that air-dried dichromated gelatin became insoluble when kept for a long time in the dark at the ordinary temperature, although much less readily than when in the form of a jelly. Returning to the action of light, Eder observed that dichromated gelatin which contained a trace of insoluble material became insoluble much more rapidly than that which was entirely unchanged. Finally he stated that the exposed parts of a dichromated gelatin film became more completely insoluble when subsequently kept in the dark, while the unexposed parts did not, and he considered that this after-effect could only be stopped by washing out the dichromate.

It must be remembered that throughout this work, Eder used ordinary photographic gelatin and some of the changes which he found may well be due to the presence of impurities such as minute traces of reducing agents, since, as will be shown later, immediate insolubility is produced by the

addition of a reducing agent to dichromated gelatin in the dark. As a chemical change of the type suggested will take place only in solution, this would account for the increase in the rate of change observed when the mixture was moist. That heating should hasten such a reaction is well known. The present writer in an attempt to reproduce some of Eder's results has used a highly purified gelatin and finds that when such gelatin is treated with potassium dichromate it will not become insoluble in the dark. Further, this pure gelatin was not rendered insoluble by boiling for 1 hour with potassium dichromate. It is therefore apparent that the changes which Eder found are due entirely to the presence of impurities in his material.

Lumière and Seyewitz [1905] investigated the composition of gelatin made insoluble by treatment with dichromate and exposure to light. They found that gelatin which had been rendered insoluble by the action of dichromate and light contained chromium sesquioxide and potassium oxide, the latter giving potassium chromate by reaction with excess of dichromate.

They repeated [1906] Eder's investigations on dichromated gelatin rendered spontaneously insoluble in the dark and found that insolubility was slowly produced at ordinary temperature but rapidly at 120° F. The present writer attributes these changes to the presence of impurities since Lumière and Seyewitz, like Eder, did not use purified gelatin. In no case do they mention the source and previous treatment, if any, of their gelatin, so the exact conditions prevailing in these experiments cannot be ascertained, but from experiments with pure gelatin to be described later it is clear that their results are incorrect.

Marion [1873] made the discovery that if a dichromated gelatin film which had become insoluble by exposure to light under a negative were placed in contact with a similar but unexposed film and the two were squeezed together under pressure between moist blotting paper, the gelatin in contact with the exposed gelatin became insoluble after 8–10 hours, and a print of the image was so obtained. It was concluded that once action was set up by light in a dichromated gelatin film this could induce the same change in gelatin placed in contact with it, without exposure to light. If this were the true explanation it seems remarkable that the outline of the image is preserved, since one would expect the change to occur in the unchanged material around the exposed parts. It seems more probable that the real change is purely mechanical, the squeezing causing the insoluble material to impregnate the soluble gelatin in contact with it, thus making it more difficult to dissolve. The present writer, therefore, attempted to reproduce this change in the absence of any mechanical influence and found that no such alteration occurred.

More modern workers on this problem are Schiel [1926] and Schommer [1926], but they have considered chiefly the changes involved in the bromol and ozobrome printing processes. Schommer considers that the oxygen

liberated during the ozobrome process is the important agent in producing insolubility. Liesegang [1926] however in a criticism of this work asserts that the reasons for assuming this are invalid. Further, since the addition of a reducing agent would produce immediate insolubility, it is clearly incorrect to attribute the change to the action of oxygen.

It can be seen from the foregoing that the results of past workers on the action of light on dichromated gelatin have really contributed little to our knowledge of the essential changes. They have shown that the dichromate is reduced and that chromium as the sesquioxide is fixed by the gelatin, but these workers have not investigated changes in the gelatin itself or shown whether such changes do occur. The present writer having pure gelatin at her disposal undertook the following investigations, repeating only such work on the changes in the dichromate as was necessary.

(1) Insoluble gelatin was prepared by three methods and the ash in several samples of each form was estimated. If insoluble gelatin were a definite compound all samples should contain the same percentage of ash. (2) The rate of the reaction proceeding when light acts on dichromated gelatin was investigated and this was compared with the rate of reaction when a known small amount of sodium sulphite was added to the mixture. These experiments also showed the maximum change obtainable and suggested the possibility of a reversible reaction. (3) The possibility of a reversible reaction was investigated. (4) The catalytic action of the insoluble material was investigated. (5) The Hausmann numbers of pure and insoluble gelatin were determined.

During the course of this research a new insoluble ash-free gelatin has been prepared. When acetone is added to a solution of gelatin or dichromate-gelatin in water the protein is precipitated, but the precipitate is soluble in water. If however the solution of dichromate-gelatin be first exposed to light, the precipitate obtained by adding the acetone will no longer dissolve in water. It is ash-free, consequently the change produced is not due to any combination of the gelatin with chromium. It is reasonable to suggest that the change is a denaturation, which is of great interest, for, as is well known, gelatin cannot be coagulated by heat and as far as the present writer is aware has not yet been obtained in a denatured form.

The results obtained have led to the conclusion that the first action of the light is to produce a change in the protein itself, either the denaturation referred to above, or a change preparatory to denaturation, which in any case involves no fixation of chromium. After this alteration the gelatin which is still in solution becomes insoluble either by fixation of chromium by prolonged exposure, or by keeping in absence of light for about 2 weeks, or immediately by precipitation with acetone.

The properties of this insoluble ash-free gelatin were investigated as follows.

(1) The Hausmann numbers were determined. (2) The rate of digestion of the insoluble gelatin by pepsin was determined and this was compared

with the rate of digestion of ordinary gelatin. (3) A dichromated gelatin solution was exposed and most of the dichromate was removed by dialysis after exposure. The optical rotation of the solution obtained was determined and was compared with that of a solution of pure gelatin.

EXPERIMENTAL.

The gelatin used was Coignet's Gold Label gelatin purified by electro-dialysis and flocculation [Schryver, 1923]. The ash in the gelatin so prepared was less than 0.02 %.

Percentage of ash in insoluble gelatin prepared by three methods.

(1a) To a solution containing approximately 4 % gelatin, sufficient potassium dichromate was added to give a 3 % solution, and this was spread as a film on a glass plate and exposed to the light from a mercury vapour lamp for 2 hours. The insoluble material formed was scraped off the plate and washed with distilled water, first cold and finally at 30°, until the washings were colourless; after drying at 110° the ash amounted to 5.21 %.

(1b) The gelatin was treated as above, but after the washing with water it was washed with 1 % hydrochloric acid which removed a further quantity of dichromate; the acid was then removed by washing with distilled water, and the material was dried at 110°. The ash was 4.34 % in one experiment and 4.62 % in another.

(2) The gelatin was made insoluble by treatment with potassium dichromate and addition of sufficient powdered sodium sulphite to give a 0.5 % solution. The insoluble product was washed as in (1b) above and the ash determined: (a) 3.68 %, (b) 4.05 %.

(3) The gelatin was made insoluble by adding sufficient chrome alum to give a 3 % solution and allowing to gel. The product was washed and dried as before and the ash determined (3.93 %).

These results do not suggest the formation of a definite compound.

The rate of change of dichromated gelatin on exposure to light.

Potassium dichromate was added to a gelatin solution to give a 3 % solution of the salt. The exact concentration of the gelatin was found by nitrogen determination. A series of exposures of increasing duration was made with 2 cc. samples of this solution spread over the bottom of 100 cc. beakers. After the given time the unchanged gelatin was dissolved by washing first with cold water and finally with water at 30°, the solution was filtered and made up to 25 cc. By estimating the nitrogen in this solution the amount of unchanged gelatin was found. The results are given in Table I.

This rate of change was compared with the rate of formation of the insoluble products obtained by adding 0.2 cc. of a 5 % sodium sulphite solution to 2 cc. of the dichromated gelatin at the beginning of the exposure, the results of which are given in Table II.

Table I. *Rate at which dichromated gelatin was rendered insoluble by exposure to light.*

Time exposed (min.)	% changed	Time exposed (min.)	% changed
10	3.1	90	65.0
20	7.1	100	89.4
40	13.2	120	88.3
60	21.7	200	88.5
80	46.5	220	89.7

Table II. *Rate at which dichromated gelatin in the presence of sodium sulphite was rendered insoluble by exposure to light.*

Time exposed (min.)	% changed	Time exposed (min.)	% changed
2.5	7.0	10	66.4
5.0	16.0	15	79.2
7.5	46.4	20	85.1

Although the second change is far quicker than the first, it is significant that in both cases the curves constructed from the data given in Tables I and II are similar in shape, and also that the rate suddenly increases after about 20 % of the gelatin has been changed. The maximum change produced by a prolonged exposure with the sodium sulphite was found to be 88.8 %. This is in close agreement with the maximum change produced by simple exposure with dichromate, *i.e.* 89.7 %. The maximum change produced in dichromated gelatin in the absence of light by addition of sodium sulphite is 88.2 %; the maximum change produced by 3 % chrome alum is 89.0 %. That the residual nitrogen in each case is due to the presence of unchanged gelatin is shown by the fact that the filtrates give gels on evaporation, which can be made insoluble by exposure. It is obviously not possible to obtain a complete change to the insoluble form although former workers have made no mention of this fact.

To determine whether the reaction is reversible.

From the above results it seemed probable that the reaction was reversible, equilibrium being attained when about 90 % of the material had become insoluble. To determine this, known weights of the insoluble gelatin, which had been well washed with water and dried in a vacuum desiccator over sulphuric acid, were moistened with water and exposed for one hour to the mercury vapour lamp. After exposure they were well washed with water and the nitrogen in the washings was determined.

In the first experiment 0.106 g. of insoluble material, containing 0.006 g. of chromium trioxide and adhering dichromate was used. The filtrate contained 0.34 mg. N, corresponding to 2.0 mg. of gelatin. In the second experiment the insoluble material amounted to 0.172 g. and contained 0.009 g. of the chromium trioxide and dichromate. The filtrate contained 0.52 mg. N, corresponding to 3.3 mg. of gelatin, *i.e.* approximately 2 % of the gelatin had gone into solution.

If the nitrogen in the filtrate really represents gelatin which has become soluble, these results would suggest that the action is slowly reversible; but the amount of material in the filtrate is too small to give a gel, and although a precipitate is obtained by adding acetone, other tests showed the presence of amino-acids only and did not confirm the presence of a protein. The reversibility of the reaction was therefore not definitely proved.

Catalytic action of the insoluble material.

Since the rate of formation of the insoluble compound increases with time the effect of the addition of minute traces of the insoluble product was investigated. When 0.05 g. of the insoluble gelatin was exposed with 2 cc. of an approximately 4 % solution of dichromated gelatin, the extent of the solution of the insoluble form completely masked the reverse change occurring in the 2 cc. of soluble gelatin. To overcome this, 2 cc. of dichromated gelatin of known concentration was exposed for 2 hours to insure complete insolubility, after which it was well washed and the nitrogen in the filtrate was determined. A similar exposure was made with another 2 cc. sample but after exposure a further 2 cc. was added to this and the exposure was continued for 1 hour. The product was washed with water, filtered and the nitrogen in the filtrate was determined. The ammonia derived from 2 cc. of the gelatin solution in a Kjeldahl determination neutralised 57.5 cc. of $N/70$ acid. Exposure for 1 hour rendered insoluble gelatin equivalent to 15.5 cc. of acid or 27 % of the total. When 2 cc. of the gelatin after exposure for 2 hours was added to another 2 cc. and the whole was exposed for another hour, the nitrogen in the filtrate was equivalent to 39.5 cc. The gelatin which had been exposed for 2 hours contributed to this only 5.0 cc., so that the 2 cc. of gelatin exposed for only 1 hour—but in the presence of gelatin already rendered insoluble—contributed 34.5 cc., showing that an equivalent of 23.0 cc. or 40 % of the gelatin had been rendered insoluble. These changes definitely prove the catalytic action of the insoluble material. That the catalysis was not due to the small amount of gelatin which remained soluble was proved by the inability of this fraction to catalyse the reaction after it had been separated from the changed material.

Repetition of Marion's experiments in the absence of any mechanical influence (see p. 1707).

A known amount of dichromated gelatin was exposed until maximum insolubility had been produced, an equal amount of the same gelatin was added and the whole was left in the dark for 24 hours. After this time the mixture was washed and the nitrogen in the filtrate was determined. It was seen from this that no change had occurred in the added gelatin. The experiment was repeated but the mixture was allowed to remain in the dark for a greater length of time; again no change was found. The results obtained were as follows.

	N (cc. N/70 acid)
2 cc. of the gelatin used contained	18.5
Soluble fraction from 2 cc. of this gelatin after exposure for 3 hours	2.0
Filtrate from 2 cc. of gelatin exposed for 3 hours and left for 24 hours with an additional 2 cc. of gelatin in the dark ...	20.5
Above mixture after leaving for 2 weeks	20.3
Above mixture after leaving for 4 weeks	20.4

Determination of Hausmann numbers.

The Hausmann numbers of pure gelatin, gelatin made insoluble by exposure with dichromate, and insoluble ash-free gelatin were determined by the micro-method of Thimann [1926]. The latter has since shown [1930] that in order to obtain results truly comparable with one another the estimations should be carried out on solutions containing equal concentrations of nitrogen, since the percentage of nitrogen precipitated by phosphotungstic acid varies continuously with the total concentration of nitrogen. Several estimations were therefore made with the three forms of gelatin and curves were constructed showing the relation between the percentage of nitrogen precipitated by phosphotungstic acid, and the concentration of nitrogen in the solution used. The values for pure gelatin agreed closely with the values for the insoluble ash-free gelatin, but were in all cases 1-2 % higher than the values obtained with the gelatin made insoluble by exposure; since it is known that the presence of salts lowers the percentage of nitrogen precipitated by phosphotungstic acid the estimations with pure gelatin were repeated, but sufficient potassium dichromate was added to the hydrochloric acid used for the hydrolysis to correspond approximately with the chromic oxide present in the insoluble product. The values obtained for the two forms were then the same, as shown by the results in Table III.

Table III. *Phosphotungstic acid precipitates from hydrolysis products of pure gelatin and insoluble dichromated gelatin after hydrolysis with 20 % HCl for 20 hours.*

Pure gelatin + 5 % $K_2Cr_2O_7$		Insoluble dichromated gelatin	
Total N (cc. N/70 acid)	% of total N in phosphotungstic acid precipitate	Total N (cc. N/70 acid)	% of total N in phosphotungstic acid precipitate
54.0	19.5	20.5	15.9
85.2	21.7	59.0	21.0
130.0	21.0	89.7	21.6
159.2	19.5	141.8	20.9
		152.8	20.0

From the curves constructed it was seen that the change which occurs in the protein molecule whereby it is rendered insoluble is not one which can be detected by an examination of the Hausmann numbers.

Table IV. *Phosphotungstic acid precipitates from hydrolysis products of pure gelatin and insoluble ash-free gelatin.*

Pure gelatin		Insoluble ash-free gelatin	
Total N (cc. <i>N</i> /70 acid)	% of total N in phosphotungstic acid precipitate	Total N (cc. <i>N</i> /70 acid)	% of total N in phosphotungstic acid precipitate
31.5	19.6	25.2	18.4
42.0	20.15	42.0	20.0
50.1	21.0	93.4	23.1
87.5	23.2	121.0	23.0
139.5	22.9		

Preparation and properties of the insoluble ash-free gelatin.

A solution of dichromated gelatin was exposed to the light of a mercury vapour lamp for 2 hours, and was stirred periodically to break up the insoluble film which formed on the surface. After the solution had been filtered from the insoluble particles, acetone was added to precipitate the protein and the mixture was left to stand overnight. The precipitated material was then filtered off and well washed with water to remove any unchanged gelatin and adhering potassium dichromate; it was then dried, first by standing in acetone overnight and finally in a vacuum desiccator over sulphuric acid. This gelatin, after drying, is opaque and greyish white in colour and can be ground to a coarse powder. A whiter and finer preparation may be obtained by allowing the gelatin to stand in water for several weeks before the final drying. The material swells but will not dissolve in either 1 % hydrochloric acid or glacial acetic acid even on warming to 40°. It is insoluble in a solution of sodium salicylate which is a particularly good solvent for ordinary gelatin. The ash amounts to 0.2 % which shows that in this gelatin there is no fixation of chromium. The Hausmann numbers are the same as for ordinary gelatin as shown in Table IV. The rate of digestion by pepsin is the same as for ordinary gelatin as shown by the following experiment.

Rates of peptic digestion of pure and insoluble gelatin.

In these determinations, Willstätter's method was used for the estimation of the liberated amino-acids. Both the insoluble and pure gelatin were used in the granular form obtained by drying in a vacuum desiccator over sulphuric acid after precipitation with acetone. 0.5 g. of material was placed in a 50 cc. conical flask and to this was added 5 cc. of distilled water followed by 5 cc. of an 8 % solution of pepsin in hydrochloric acid. The gelatin was incubated at 20° and after incubation the mineral acid was neutralised by *N*/4 sodium hydroxide, using litmus as an external indicator. The solution was then transferred to a 250 cc. conical flask and to it was added 0.5 cc. of a solution of 0.5 % phenolphthalein in absolute alcohol. The amino-acids were then estimated by titration with *N*/25 alcoholic potassium hydroxide, a further 20 cc. of alcohol being added each time the solution became pink until 100 cc. of

alcohol had been added. The solution then contained approximately 90 % of alcohol which is that required to give the true end-point. The results obtained showed no difference between the two forms, as shown in Table V.

Table V. *Peptic digestion of pure and insoluble ash-free gelatins.*

Time of incubation (hours)	Pure gelatin		Insoluble ash-free gelatin	
	cc. N/4 NaOH	cc. N/25 alcoholic KOH	cc. N/4 NaOH	cc. N/25 alcoholic KOH
1	2.00	18.0	2.08	18.5
2	2.06	19.0	2.10	19.0
4	2.16	20.5	2.15	20.0
24	2.20	22.5	2.18	22.0
48	2.20	23.5	2.20	23.0

To investigate the possibility of racemisation in insoluble gelatin.

To determine whether racemisation had occurred the optical rotations of solutions of pure gelatin and of gelatin which had been exposed with dichromate were examined. Before using the latter solution the greater part of the potassium dichromate was removed by dialysis. The exact concentrations of the solutions were found by nitrogen estimations. The value for the rotation of exposed gelatin was slightly lower than for the pure material, but the differences obtained were too small to allow of the definite conclusion that racemisation had occurred. The use of more concentrated solutions would, no doubt, have given more reliable results but was found to be impossible owing to opacity. The rotations of the products of hydrolysis of pure gelatin and of ash-free insoluble gelatin were next determined. The material was hydrolysed by boiling for 20 hours with hydrochloric acid and the acid was then evaporated under reduced pressure. The residue was taken up in water, and the solution was clarified by boiling with a little animal charcoal, filtered and made up to a known volume. Before measuring the rotations, the solutions were made alkaline by the addition of 10 cc. of 10 % caustic soda to 20 cc. of solution. In the solutions so obtained no difference in rotation was observed.

CONCLUSIONS.

From the experimental results quoted above it is possible to draw the following conclusions.

When gelatin is made insoluble by treatment with dichromate and exposure to light, a change in the protein, allied to denaturation, first takes place but this involves no change in the Hausmann numbers or in the digestibility of the protein. Chromium is then fixed in the form of the sesquioxide but a definite compound is not involved.

The reaction does not proceed to completion and is probably reversible, equilibrium being attained when 90 % of the gelatin has become insoluble.

The reaction is catalysed by the insoluble gelatin.

From the preparation of the insoluble ash-free gelatin, it can be clearly

seen that coagulation is preceded by a change which produces no alteration in the physical state of the solution. This confirms the suggestion first made by Hardy [1899] and supported by Chick and Martin [1910] that coagulation takes place in two stages, the first being a chemical reaction, called by Chick and Martin "denaturation," and the latter, which produces the coagulation, merely physical. In the case of gelatin the denatured protein can be definitely and completely isolated from the coagulated product, and the existence of such a form in the case of gelatin does much to support the theory of denaturation in other proteins.

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