CLXIV. GOLD SOL REACTION IN IRRADIATED PROTEINS AND PROTEIN DERIVATIVES.

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It has been previously shown [Spiegel-Adolf, 1934] that the specific protein tests become negative in diluted protein solutions after intensive irradiation with the light of a quartz mercury lamp. At the same time the reaction with colloidal gold becomes stronger and shows some new features. Evidence was supplied which proves that the substance giving this reaction was neither protein nor a typical protein derivative.

These studies have been continued on different lines chiefly with the purpose of obtaining more definite information as to the chemical nature of the substances formed during irradiation of proteins.

Gelatin. The investigations previously limited to water-soluble serum-proteins were extended to gelatin. According to Dakin [1920] 91.22 % of the amino-acid content of gelatin is known, so that it is one of the best characterised proteins. On the other hand, electrolyte-free gelatin [Spiegel-Adolf, 1927, 1], has no flocculating power towards colloidal gold, but only a marked protective power towards it. In this way all possibility that the precipitating effect of irradiated proteins is caused by traces of proteins which have escaped denaturation is excluded.

The gelatin used was made from a commercial brand (Coignet, Père et Fils & Comp., Gold Label). This brand was first purified by dialysis and electro-dialysis. A sample of 2.1 % had an electric conductance of $1.67 \cdot 10^{-5}$ ohm⁻¹ (at 30° without water correction). 0.01 mg./ml. of this preparation protected 1 ml. of gold sol effectively against precipitation by 1.25 final normality of KCl. All the results were re-checked with different kinds of colloidal gold, made according to the method of Bredig or by reduction by formaldehyde. Both original samples and other samples purified by dialysis were used. The irradiations were made with the help of a quartz mercury lamp (Hanovia, 220 v. d.c.) at a distance of 15 cm. from the arc. In former investigations [Spiegel-Adolf, 1934] it had been ascertained that the protein derivatives which give positive gold sol reaction were heat-resistant. The container for the gelatin was a pyrex glass dish of 8 cm. diameter, the fluid layer being 1 cm. deep. This dish was covered by a plate of clear quartz. The gelatin was irradiated at a concentration of 0.04 % in order to get results comparable with those of the previous communication [Spiegel-Adolf, 1934], the greater transparency of gelatin in ultra-violet light permitting the work on a thicker layer.

At intervals of 30 minutes 3 ml. were taken and tested for their reaction with colloidal gold alone and in the presence of various KCl concentrations. Finally, with reference to the observations of Neuberg [1908], the reducing power of the solutions was tested.

The results are summarised in Table I.

Table I.

Period of irradiation	Gold sol reaction	Protective power against KCl
0	0	+1.33 <i>N</i>
30 mins.	V	+0.2 <i>N</i> -0.33 <i>N</i>
1 hr.	Bl	+0.2 <i>N</i>
1 hr. 30 mins.	Bl	-0.2 <i>N</i> +0.1 <i>N</i>
2 hrs.	Bl	+0.1 <i>N</i>
2 hrs. 30 mins.	Bl	0.1 <i>N</i> Rv 0.05 <i>N</i> R
3 hrs.	Bl	0.1 <i>N</i> Rvvvv 0.05 <i>N</i> Rvv
4 hrs.	Bl	0.1 <i>N</i> Bl 0.05 <i>N</i> V 0.025 <i>N</i> V 0.0125 <i>N</i> R 0.0062 <i>N</i> V

R = cherry-red; V = violet; Bl = blue; suffixes v indicate shades of red with tinge of violet.

The findings show first that gelatin through irradiation becomes a precipitant for colloidal gold. It behaves in this respect like the other lyophilic proteins. The second change brought about by irradiation affects the protective power of the gelatin. Genuine gelatin does not need the presence of salts to exercise this power, as do serum- or egg-proteins, but under the influence of short-wave radiation the protective power of gelatin undergoes a change. As is shown in Table I gelatin flocculates colloidal gold after 1 hour of irradiation. Traces of unchanged gelatin if present are unable to prevent this flocculation. Yet no changes occur if a certain amount of salt is previously added to the gelatin after irradiation. With increasing irradiation time the amount of KCl necessary to prevent flocculation drops steadily, until it falls below the salt concentration which alone is able to precipitate colloidal gold. In a similar way Pauli and Singer [1932], studying the equilibria of colloidal Congo red, protein and salt, found that non-flocculating salt concentrations were able to protect the colloid against the precipitating effect of the protein.

In order to show that the behaviour of irradiated gelatin is not caused by contamination with the original gelatin, further experiments were started. The reactions towards colloidal gold of various mixtures of undenatured gelatin and of a gelatin that had been irradiated for 4 hours were studied. No salts were present (Table II).

Table II.

Final conc. of non-irrad. gelatin %	Final conc. of irrad. gelatin %	Ratio of non-irrad. : irrad. gelatin	Gold sol reaction	
			Immediate	After 24 hrs.
0.004	0.016	1/4	0	Rv
0.002	0.018	1/9	0	R
0.001	0.018	1/18	0	Rvv
0.0005	0.018	1/36	0	Rvvv
0.0002	0.01	1/50	Rv	Rvvv
0.0001	0.01	1/100	Rv	V

R = cherry-red; V = violet; suffixes v indicate shades of red with tinge of violet.

Only if the ratio of non-irradiated to irradiated material drops below 1/30 is a shifting of the red colour of the gold sol towards violet indicated. In order to get complete flocculation as observed with irradiated gelatin alone the content of original gelatin in the mixture ought not to exceed 1 %.

Though Zsigmondy [1920] has stated that the protective power of gelatin is increased by short heating, nevertheless, in order to refer the changes of gelatin to irradiation, influence of temperature had to be excluded. The temperature during irradiation fluctuated between 45 and 50°; a sample of gelatin of the same concentration and in a similarly covered dish was therefore kept in a thermostat for the same length of time (4 hours) at this temperature. After such an exposure gelatin did not show any marked difference from the original sample. It certainly did not flocculate colloidal gold, and it was able to protect the latter against the precipitating effect of high salt concentrations (1.25 N KCl) and of irradiated gelatin.

The changed behaviour of irradiated gelatin can therefore be considered as effect of denaturation through short-wave radiation. As in other proteins [Spiegel-Adolf, 1927, 2] denaturation by radiation is different from the changes caused by boiling.

But differences in the methods of irradiation influence largely the result. Although Neuberg [1908] refers to the reducing power of irradiated gelatin, similar tests made in our laboratory even after a hundredfold concentration of irradiated gelatin failed to give more than a dark greenish colour with Benedict's solution. Control experiments with dextrose and mixtures of dextrose and non-irradiated gelatin at various concentrations failed to give a similar reaction.

Histamine. Practically nothing is known about the exact chemical formulae of protein derivatives that show either marked protective or precipitating power for colloidal gold. As to the chemical changes brought about by irradiation of proteins there are pharmacological indications that small amounts of histamine are formed [Holtz, 1931]. On the other hand only lately Holtz [1933] has given chemical evidence of histamine formation on irradiation of histidine. Since no references [Feldberg and Schilf, 1930] as to the reaction of histamine with colloidal gold could be found, experiments were started in this direction.

Two different brands of histamine were used, histamine dichloride (W. A. Fritch, Inc., New York) and histamine diphosphate (Burroughs, Wellcome and Co., England). After neutralisation with NaOH the c_H of the first solution was $1.0 \cdot 10^{-6}$, of the second $1.41 \cdot 10^{-6}$ at a concentration of 0.3 and 0.5% respectively.

In experiments re-checking former experiences [Adolf and Pauli, 1924] it was ascertained that HCl solutions causing visible changes in colloidal gold had a c_H 20–30 times higher than those of the neutralised histamine solutions. In Table III are summarised results of a gold sol flocculation test by varying concentrations of histamine phosphate. Histamine chloride gave practically the same results when the concentration of the base was the same.

Table III.

mg./ml. histamine	0.000125	0.00025	0.000375	0.0005	0.00075
Gold sol reaction	0	Rv	Rvv	V	V

R = cherry red; V = violet; suffixes v indicate shades of red with tinge of violet.

By these figures it is shown that histamine is a very powerful flocculating agent for colloidal gold, visible changes being elicited by 0.00018 mg./ml. of the former. As histamine diphosphate contains only 36 % of free histamine the figures obtained are to be divided by about 3. The flocculating power of

histamine exceeds therefore not only the flocculating power of proteins but also that of irradiation products of proteins. In terms of normality it is 8 times stronger a precipitant for colloidal gold than AlCl_3 , even though the latter is more acid [Adolf and Pauli, 1924]. When the gold sol reaction was made according to Lange's clinical liquor test with a neutralised 0.5 % histamine the diphosphate following results were obtained after 24 hours: 666666543211.

Experiments were now made in order to test the effectiveness of protecting material against gold sol flocculation by histamine.

Salt concentrations in a range of 0.1–0.04 *N* KCl were ineffective. Gelatin or combinations of serum-proteins and neutral salts when added previously to the colloidal gold prevented flocculation by histamine subsequently added. The correspondence of this behaviour to the one observed in irradiated proteins is rather obvious. It becomes still more striking when one considers that histamine, like the potent principle in irradiated proteins, shows heat-resistance when boiled in watery solutions, and also shows deterioration on standing, owing to growth of bacteria.

It is known that histamine formed through irradiation of histidine is destroyed by further irradiation. Experiments were started to investigate if the destruction of biological potency of histamine is accompanied by an analogous decline of the gold sol-flocculating power.

The biological potency of histamine was measured by its power to lower the blood pressure of a cat previously atropinised. 1 ml. containing 0.00005 mg. of histamine still gave a distinct reaction in a cat of 3 kg. This histamine concentration is far beyond the range giving positive gold sol reactions.

In order to obtain marked changes the neutralised histamine solution was brought to a concentration of 0.05 % and irradiated in the usual way for 4 hours. The reactions of irradiated histamine with colloidal gold showed that the flocculating power of the former was not completely destroyed, but that it had dropped to 1/100 of its original value. But when the irradiated histamine was examined as to its effect upon blood pressure, evidence could be given that its potency had shrunk to 0.00001 of its original value and came within the range of concentrations which yield positive gold sol reactions. These experiments show that histamine, though it is the most powerful gold sol precipitant known, owes these properties not exclusively to the molecular arrangement which causes its biological potency.

On the other hand, even if we suppose that the total histidine contained in gelatin—according to Kestner [1925] gelatin of mol. wt. 20,000 contains in 100 molecules 1 molecule of histidine—is changed by irradiation into a derivative of histamine having the full flocculating power of genuine histamine, the entire flocculating power of irradiated gelatin could still not be accounted for. The conclusion is therefore reached that gelatin and probably other proteins contain other constituents besides histidine which on irradiation are able to undergo such changes as to acquire gold sol-flocculating properties.

Amino- and imino-acids. A number of amino-acids are known to yield derivatives which are chemically analogous to histamine but more or less devoid of its biological potency. It was therefore supposed that those products might be formed by irradiation in the same way as histamine is formed from histidine. As a control histidine was included in a number of amino-acids which were subjected to irradiation.

Table IV contains a survey of the eight preparations examined, of the respective concentrations used and of the reactions with colloidal gold before and after irradiation.

Table IV.

Name	K_a and K_b	mg./ml.	c_H	Before irradiation	After 1 hour of irradiation	After 2 hours of irradiation
Histidine	$3.9 \cdot 10^{-10}$	0.15	$2.14 \cdot 10^{-7}$	0		0
	$1.2 \cdot 10^{-8}$	0.208			0 After 24 hrs. V After 72 hrs. W	0-72 hrs. 0 0-24 hrs. V After 72 hrs. Bl
		0.625	$1.69 \cdot 10^{-7}$	0-24 hrs. 0 After 72 hrs. W After 3 weeks W		
		3.5				
Arginine	$2 \cdot 10^{-10}$	2.5	$c_{OH} = 1.1 \cdot 10^{-6}$		V V-Blw V-Blw Min. dos. > 0.1 mg./ml.	Blw Min. dos. = 0.17 mg./ml
	$2 \cdot 10^{-6}$	2.5				
Lysine	$3 \cdot 10^{-11}$	5.0		After 72 hrs. 0		
	$2 \cdot 10^{-5}$	0.6	Neutral	After 72 hrs. 0	0-24 hrs. 0 After 48 hrs. V	
Proline	$2.5 \cdot 10^{-11}$	0.6	$c_{OH} = 1.22 \cdot 10^{-7}$	0-48 hrs. 0	Bl Min. dos > 0.06 (alkaline)	
	$1 \cdot 10^{-12}$					
Glycine	$1.8 \cdot 10^{-10}$	1.0	$K = 2.3 \cdot 10^{-5}$	0-48 hrs. 0		0-48 hrs. 0
	$2.6 \cdot 10^{-12}$					
Phenylalanine	$7.5 \cdot 10^{-10}$	0.25	Neutral	0		0-24 hrs. 0
	$4 \cdot 10^{-13}$	0.5		0		0-24 hrs. 0
Tyrosine	$7 \cdot 10^{-11}$	0.16	Neutral	0-48 hrs. 0	0-42 hrs. 0	
	$1.7 \cdot 10^{-12}$	0.23		9-72 hrs. 0	0-42 hrs. 0	
		0.46				0-72 hrs. 0
		0.6				0-66 hrs. 0
Tryptophan	$4.1 \cdot 10^{-10}$	0.075	Neutral	0-64 hrs. 0		
	$2.2 \cdot 10^{-12}$	0.15				0-72 hrs. 0
		0.3		0-64 hrs. 0	0-64 hrs. 0	

0 = no reaction with colloidal gold. V = violet. Bl = blue. Blw = blue-white. W = white.

The choice of amino-acids was determined by the wish to include the most important constituents of gelatin. But in consideration of the previous work done on serum-albumin and pseudoglobulin tyrosine and tryptophan were also examined.

The concentrations of the solutions to be irradiated were chosen according to the different spectroscopic behaviour of the preparations which has been ascertained in every case.

As the reaction is supposed to influence the results of irradiation [Feldberg and Schilf, 1930], all solutions have been tested in this respect. If necessary the preparations were carefully neutralised and the results controlled. In all cases showing a reaction with colloidal gold p_H determinations were made with the hydrogen electrode.

The same kind of solution of colloidal gold and the same method of irradiation were used as were used for gelatin. The optimum irradiation time (1-2 hours) was determined by experiments which showed that poorer results were obtained if the time was shorter ($\frac{1}{2}$ hour) or longer (3 hours).

Not one of the preparations flocculated colloidal gold before irradiation. Only the sample containing colloidal gold and histidine showed a change of colour after 72 hours at room temperature. A histidine solution which had been kept for 3 weeks in the ice-box flocculated colloidal gold at once. This behaviour may be explained by the bacterial formation of histamine [cf. Feldberg and Schilf, 1930].

Irradiation affects the preparations variously. While a 1 % solution of glycine remains colourless, the cyclic amino-acids, phenylalanine, tyrosine and tryptophan become dark brown. The other preparations turn more or less yellow. The dark brown colour interferes with the gold sol reaction. In order

to eliminate it the irradiated samples were acidified, whereupon a black-brown substance was precipitated. This substance could easily be redissolved in alkali, showing in this respect the same behaviour as melanic acid [Hoppe-Seyler and Thierfelder, 1924]¹. After some hours' standing, centrifuging or filtration a colourless fluid was separated. This was exactly neutralised.

Of the eight irradiated preparations four gave a positive gold sol reaction (histidine, arginine, lysine and proline), while the other preparations brought about no changes in the colloid. The type of reaction was the same as that described with irradiated proteins or with histamine.

The amount of irradiated histidine necessary to flocculate colloidal gold agreed approximately with the findings of Ellinger [1929], who proved that in the presence of oxygen 1/500 of the histidine is changed by irradiation into histamine. The flocculating powers of irradiated arginine and proline seem to come within a similar range. These facts suggest that the precipitating factor in irradiated histidine is for the greatest part histamine. The corresponding derivatives of arginine, lysine and proline would be putrescine, cadaverine and perhaps succinimide [Ciamician and Silber, 1913]. As has been shown above the great biological differences between those substances and histamine would not necessarily affect the gold sol-precipitating powers in the same degree.

Some suggestions will now be offered as explanations of the changed behaviour of these amino- and imino-acids towards colloidal gold after irradiation. As colloidal gold is a negative colloid, every increase of the basicity of a substance should increase its gold sol-flocculating power. In the case of proteins or amino-acids an increased positive charge can be conferred either by destruction of carboxyl or by increase of amino-groups. The three amino-acids, histidine, arginine and lysine, which give a positive reaction with colloidal gold after irradiation, are originally stronger bases than acids. The apparent dissociation constants given in Table IV are quoted from Kirk and Schmidt [1929]. The chemical difference between histamine and histidine consists in loss of the carboxyl group from the latter. Analogous changes can be supposed to take place in irradiated arginine and lysine. There is therefore some reason to suppose that irradiation enhances the positive charge of amino-acids by decarboxylation. Even when using an irradiation which destroyed more than 50 % of the histidine, Lieben and Getreuer [1933] failed to detect the formation of new amino-groups.

The same explanation can possibly be applied to the behaviour of irradiated proline. According to Bachér [1928] a solution of pyrrole when oxidised by irradiation becomes black. No such changes were observed in irradiated proline. Neither have Lieben and Getreuer [1933] been able to show a marked effect of irradiation on the cyclic ring of proline as measured by the amount of NH_3 set free. The fact that the solution becomes slightly alkaline on irradiation is accounted for by decarboxylation. The end-product may be identical with or closely related to succinimide.

The cyclic amino-acids, phenylalanine, tyrosine and tryptophan, behave in a different way. But it has been mentioned already that they show signs of deeper changes, *e.g.* formation of melanic substances. Results of spectroscopic measurements seem to point in the same direction.

The investigations of irradiated amino- and imino-acids offer evidence that other than histidine derivatives contribute to the gold sol-flocculating power of irradiated gelatin. According to Kestner [1925] 100 molecules of gelatin contain

It may be interesting in this connection that Risak [1930] was able to trace melanin in the urine of a patient who had undergone an excessive exposure to sunshine with resultant dermatitis.

77 molecules of glycine, 1 of histidine, 10 of lysine and 10 of arginine. If compounds of amino-acids behave in irradiation like the latter alone, only a quarter of the constituents of gelatin are responsible for the gold sol-flocculating power of this protein after irradiation.

Thymonucleic acid. In order to ascertain whether other substances showing originally only protective power towards colloidal gold become flocculating after irradiation, a few experiments were made on thymonucleic acid and some pyrimidines. Zsigmondy [1920] states that nucleic acid has a gold number of 2.5. In two different preparations of thymonucleic acid carefully neutralised under control of hydrogen ion determinations, protective power for colloidal gold against the flocculating effect of salt could be detected. Neutralised thymonucleic acid alone did not apparently change the gold sol. In a previous communication [cf. Spiegel-Adolf, 1934], the importance of the dilution for the results of irradiation has been demonstrated. By spectrographical studies to be reported elsewhere [Spiegel-Adolf and Seibert, 1934] the concentrations penetrable by ultra-violet light were determined. Irradiation with the quartz mercury lamp was tried on thymonucleic acid samples of various concentrations (0.33–0.02–0.0033 %).

Even 6 hours' irradiation of the most concentrated sample did not change the protective power of thymonucleic acid. But in the more dilute samples it was sufficient not only to destroy any trace of protective power but also to produce a substance which could precipitate colloidal gold. (As the solutions became strongly acid on irradiation they had to be carefully neutralised.) These findings agree to a certain extent with references in the literature. Lieben and Getreuer [1933] working on 0.1 % solutions of thymonucleic acid found no changes following irradiation, while Heyroth and Loofbourow [1931] who used solutions of 0.0035 % and worked at 2 cm. distance from the mercury arc describe the disappearance of selective absorption after 2 hours' irradiation.

Though the participation of derivatives of other constituents of the nucleic acid cannot be entirely excluded, only a few irradiation experiments were made on some pyrimidines. Changes of the latter can be easily checked by spectrographical methods. Lieben and Getreuer [1933] have shown that on irradiation uracil is split with the formation of new amino-groups. A sample of uracil in neutralised solution after 2 hours' irradiation showed neither changes in the spectrum nor reaction with colloidal gold, but after 4 hours' further exposure the selective absorption disappeared and the solution precipitated the sol. Cytosine showed a similar behaviour on irradiation. A formation of new amino-groups, associated as it is with an increased basicity of the irradiated solutions as suggested by Lieben and Getreuer [1933], would explain these observations. The loss of protective power and the gain of precipitating power for colloidal gold after irradiation does not seem therefore a characteristic feature of protein colloids, alone, although the increase in positive charge may be reached by other ways.

Spinal fluids. In a previous paper [Spiegel-Adolf, 1934] some similarity was discussed between the behaviour of irradiated proteins and some pathological spinal fluids. The findings that irradiation and bacterial infection lead in some instances to the same result in producing substances which precipitate colloidal gold encouraged some further investigations along these lines.

In a preliminary series of experiments made with E. Spiegel ten pathological spinal fluids were tested according to their depressing effect on the blood pressure of previously atropinised cats, no equally sensitive test being known for the biologically non-active derivatives. Though some of the spinal fluids showed

the characteristic gold sol curve as described in general paralysis, no unquestionable proof of the presence of histamine could be obtained. However, it is a well-known fact that histamine is easily destroyed in the body.

The investigations will be continued in several directions.

SUMMARY.

1. Gelatin in the course of irradiation with a mercury arc lamp loses its protective power towards colloidal gold and gradually becomes a strong precipitant for the latter.

2. Histamine possesses a remarkable power of precipitating gold sol, as little as 0.00018 mg./ml. yielding a reaction. Irradiation does not destroy this property at the same rate as biological activity.

3. Arginine, histidine, lysine and proline flocculate colloidal gold only after irradiation, while glycine and the cyclic amino-acids do not do so even then.

4. Irradiation destroys the protective power of thymonucleic acid, which like cytosine and uracil becomes a gold sol precipitant.

The chemical and some biological aspects of these findings are discussed.

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