L. EFFECT OF ULTRA-VIOLET RADIATION AND HEAT UPON PROTEIN SOLUTIONS OF LOW CONCENTRATIONS.

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PROTEIN solutions of acid or alkaline reaction show a marked increase of absorptive power in the ultra-violet spectrum after prolonged irradiation with the light of a quartz mercury lamp [Spiegel-Adolf and Krumpel, 1927]. The shape of the absorption curve is not altered if the irradiation be carried out in ordinary air, the irradiated product in this case behaving like the original protein at higher concentration [Hausmann and Spiegel-Adolf, 1927; Spiegel-Adolf, 1928]. The protein irradiated under the same conditions but without addition of electrolytes coagulates spontaneously nearly completely, as has been shown in a previous paper [Spiegel-Adolf, 1927]. Becker and Szendrő [1931] working on highly diluted protein solutions (1.5–2.5 mg./100 ml.) found on the other hand that irradiation, especially in presence of oxygen, seemed to destroy the proteins. The degradation products though showing an increased absorption for ultra-violet radiation had lost the absorption band characteristic for proteins and their normal derivatives. In order to explain this apparent discrepancy new experiments were started on protein solutions of different concentrations.

In a first series of investigations measurements of the refraction have been undertaken in order to detect traces of cleavage of the protein irradiated under conditions comparable with those employed by Spiegel-Adolf and Krumpel [1927]. Boiled samples of uncoagulable protein mixtures were studied similarly, since Spiegel-Adolf and Krumpel [1929] have shown that such boiled protein solutions retain their original absorptive power. Changes of the refraction of proteins after denaturation by heat and strong acid have been reported by Obermayer and Pick [1905].

As in the proposed experiments little or no heat change was to be expected and only the effects of heat and light of short wave-length were to be compared, an interferometer (Zeiss-Löwe) was used. Since the interferometric value of a 0.1–0.05 % pseudoglobulin solution attained a stable value after 2 hours of irradiation with the light of a quartz mercury lamp (Hanovia, 220 v. d.c.) and the protein had at the same time practically disappeared from the solution, the irradiation time was limited in this series to 2 hours, the distance of 40 cm. making cooling unnecessary. The protein solutions used were pseudoglobulin and serum-albumin separated from horse serum by fractionation with $(\text{NH}_4)_2\text{SO}_4$, purified repeatedly by refractionation, dialysis and electrodialysis. The stock solution of the pseudoglobulin was 5 %, the c_H was 1.40×10^{-7} , the conductance $\kappa = 1.11 \times 10^{-5}$. The 2 % solution of serum-albumin had c_H 1.17×10^{-5} , a conductance $\kappa = 9 \times 10^{-6}$. The amounts of HCl or KOH added to the protein solutions were within the range of those used in the previous communications.

As in former experiments the heating of the solutions in a water-bath under reflux was limited to 15 minutes. The results are summarised in Table I A and B.

The figures are interferometric units, a 20 mm. cell being used.

Table I.

A. Serum-albumin.

(Final concentration in all samples 0.2 %.)

		Native	Boiled	Irradiated	Boiled and irradiated	Irradiated and boiled
0.001 <i>N</i> KOH	Obs.	325	333	331	326	331
	Calc.	330				
0.005 <i>N</i> KOH	Obs.	375	379	379	380	410
	Calc.	381				
0.001 <i>N</i> HCl	Obs.	320	322	326	329	350
	Calc.	326				
0.005 <i>N</i> HCl	Obs.	356	356	356	382	393
	Calc.	362				

B. Pseudoglobulin.

(Final concentration in all samples 0.1 %.)

0.001 <i>N</i> KOH	Obs.	196	198	199	203	199
	Calc.	201				
0.005 <i>N</i> KOH	Obs.	245	249	245	248	249
	Calc.	252				
0.001 <i>N</i> HCl	Obs.	194	196	199	196	203
	Calc.	198				
0.005 <i>N</i> HCl	Obs.	226	231	228	231	231
	Calc.	235				

Additions of acid or alkali to serum-albumin and pseudoglobulin give lower values than those calculated. These findings agree with those of Thomas and Mayer [1928], whose interpretation seems applicable to all kinds of proteins. The changes in refraction after irradiating or boiling are negligible; they do not exceed 3 % of the interferometric value of the native protein but seem to be outside the limit of error of the method. The influence of heating is most marked in alkaline solutions, *i.e.* under conditions favouring maximum hydrolysis, while irradiation shows its effects most clearly in an acid medium. Nevertheless in most cases there are no differences between the effects of heat and irradiation and when such differences do occur they are so small as to give no support to the suggestions of Becker and Szendrő [1931]. When heating precedes the irradiation the changes if any are much smaller [*cf.* Spiegel-Adolf, 1929].

In a further series of experiments the behaviour of proteins submitted to heat or irradiation at various concentrations without additions of electrolytes was studied. The irradiation and the heating were done in the manner already described, the fluids after filtration through Schleicher and Schüll filter-paper No. 590 were tested for their refraction, protein-content and gold sol reaction.

The results are summarised in Table II A and B.

These results show that both kinds of denaturation are sufficient to precipitate the total protein; only in the most concentrated samples can traces of the latter be detected after irradiation for 2 hours at 40 cm. distance; these disappear on further exposure. Though especially in the highest degree of dilution denaturation seemed to cause but a faint opacity of the solutions it was always possible to get a clear filtrate by using the above-mentioned filter.

Interferometric measurements of the filtrates indicate that not all refractive material has been precipitated with the protein. This may mean that the interferometric method is more sensitive in detecting protein than flocculation by

Table II.

A. Serum-albumin.			Heat			Irradiation			
Concentration %			IU	SSSR	GSR		IU	SSSR	GSR
0.16	<i>a</i>		307	+	+		307	+	+
	<i>p</i>	Coagulation	19	-	-	Coagulation	37	trace	++ +
0.08	<i>a</i>		144	+	+		144	+	+
	<i>p</i>	Coagulation	13	-	-	Coagulation	22	-	++ +
0.04	<i>a</i>		73	+	+		73	+	+
	<i>p</i>	Coagulation	7	-	-	Coagulation	21	-	++ +
0.02	<i>a</i>				-				
	<i>p</i>				+				
0.016	<i>a</i>		27	+	-		27	+	-
	<i>p</i>	Coagulation	4	-	+	Coagulation	13	-	++ +
0.004	<i>a</i>				-				
	<i>p</i>				-				
0.0032	<i>a</i>		6	+	-		6	+	-
	<i>p</i>	Opalescence but filtrable	5	-	-	Clear	8	-	++ +
B. Pseudoglobulin.									
0.2	<i>a</i>		374	+	+		374	++	+
	<i>p</i>	Coagulation	20	-	-	Precipitation	44	trace	+
					R _v				
0.1	<i>a</i>		193	+	+		193	+	+
							28	-	+
	<i>p</i>	Coagulation	8	-	+	Precipitation	43	-	+
0.05	<i>a</i>		91	+	+		91	+	+
	<i>p</i>	Coagulation	5	-	+	Precipitation	42	trace	+
0.02	<i>a</i>		48	+	+		48	+	+
	<i>p</i>	Coagulation	4	-	-	Precipitation	19	-	-
0.004	<i>a</i>		10	+	+		10	+	+
	<i>p</i>	Coagulation	0	-	-	Distinct opalescence	8	-	+
0.002	<i>a</i>		5	+	-		6	trace	-
	<i>p</i>	Coagulation	2	-	-	Doubtful opalescence	7	-	-

IU = Interferometric units in 20 mm. cell.

a = before; *p* = after.

SSSR = Reaction with sulphosalicylic acid.

GSR = Reaction with colloidal gold.

R_v = Red with trace of violet.

sulphosalicylic acid or that precipitation of the protein is accompanied by some cleavage, or finally that the solutions contain something else beside protein. It does not seem possible to exclude any of these possibilities altogether. But the fact that only in the case of heated proteins does the refraction of the filtrates decrease practically to zero with increasing dilution, while in the case of irradiation it reaches finally the refraction value of the original solution, seems to indicate that irradiation of proteins causes not only denaturation but also cleavage. This supposition agrees with the changes in odour and colour.

Further evidence of the cleavage of proteins submitted to irradiation is afforded by the gold sol reaction. This is more sensitive for pseudoglobulin than for serum-albumin but does not reach the sensitiveness of the sulphosalicylic acid test. Colloidal gold, however, can be used besides for the classification of proteoses, and, according to the explanation of the clinical gold sol tests, is able to reveal pathological changes in proteins where other methods fail.

Table II A and B shows that in the cases of heated proteins with the exception of serum-albumin in a range between 0.02 and 0.01 % the filtrates do not show any gold sol reactions. This confirms the results of Ogiu and Pauli [1932], who made similar observations, and the fact that the filtrates were practically free from protein. But the filtrates of irradiated proteins even when they show no trace of proteins with sulphosalicylic acid give a strong gold sol reaction. The type of the gold sol reaction is somewhat different from the two types of gold sol flocculation by proteins described by Ogiu and Pauli [1932], the change in the present case being directly from pink to blue and white.

The finding of a substance giving a positive gold sol reaction seems to confirm the results of Becker and Szendrö [1931] as to the partial splitting of the protein by irradiation. But the formation of this particular proteose depends upon the intensity and perhaps upon the wave-length of the radiation—the protein solutions themselves acting as selective filters, whose absorption is maximum between 292.7 and 261 $m\mu$. If for instance a 0.2 % serum-albumin solution is not filtered after the first hour of irradiation when it is nearly opaque but is irradiated for another hour, then the sulphosalicylic acid test becomes negative, but the gold sol reaction becomes negative too. If a solution of 0.5 % pseudoglobulin is irradiated in the usual way, after 4 hours it reaches a stage at which the sulphosalicylic acid test is still positive, but the gold sol reaction due to the protein content of the solution has already disappeared. Two more hours of irradiation cause total disappearance of the protein reaction, while the gold sol reaction becomes strongly positive. Nevertheless the solid content of the filtrate is only 1/10 of the initial protein content, and the end absorption starting at 230 $m\mu$ cannot account for the increased absorption of irradiated protein solutions containing acid or alkali within a range of 400–267.5 $m\mu$.

Finally experiments were performed under conditions similar to those used by Spiegel-Adolf and Krumpel [1927]. 0.5 % solutions of serum-albumin and pseudoglobulin containing 0.005*N* HCl or KOH were irradiated during 5½–6 hours at a distance of 30–40 cm. from the light source. The solutions were protected against evaporation by cork stoppers fitting tightly into the quartz test-tubes. (Controls of pure protein solutions were used in order to ascertain the effect of irradiation upon them.) After the exposure the solutions were exactly neutralised. They still contained traces of heat-coagulable proteins proving the existence of small amounts of undenatured protein. After renewed filtration the solutions showed a slight reaction with sulphosalicylic acid but no gold sol reaction. This was taken to show that the effect of irradiation depends greatly on the original protein content of the solution and on the amount of acid or alkali, as these determine the initial protein concentration through the entire experiment. The different kinds of cleavage caused in protein solutions of varying concentration or electrolyte content by irradiation may explain the discrepancy of the spectrographic findings of different authors which have been the starting-

Table III.

	Filtration	Period of irradiation hrs.	Electrolytes	Gold sol reaction
0.2 % serum-albumin	Filtration after 1st hour	2	None	+
0.2 % serum-albumin	No filtration	2	None	—
0.5 % pseudoglobulin	Filtration	4	None	—
0.5 % pseudoglobulin	Filtration	6	None	+
0.5 % serum-albumin or pseudoglobulin	Filtration	5½–6	0.005 <i>N</i> HCl or KOH	— (after neutralisation)

point of this paper. (The above-mentioned experiments are summarised in Table III.)

Becker and Szendrö [1931] have made elaborate investigations of the nitrogen content and certain chemical properties of the diluted protein solutions after irradiation. It seemed desirable to extend the investigation in a more physico-chemical direction, particularly concerning the relations to other proteoses of the substance giving a positive gold sol reaction.

In order to get solutions of this substance of higher concentrations 0.1 % solutions of serum-albumin and pseudoglobulin were irradiated in flat dishes at 15 cm. distance directly under the tube of the quartz mercury arc lamp; the surface of the dish was about 50 cm.², the fluid layer having a depth of 1 cm. After 6 hours of irradiation more than half of the total protein content, *i.e.* 0.026–0.03 g. of the initial 0.05 g. was precipitated; the fluid was then concentrated to 1/10 vol. on the boiling water-bath.

The solution was clear with a marked yellow tinge, the solid content was in several samples between 0.21 and 0.27 %, the specific conductivity was 3.53×10^{-4} ohms⁻¹, the c_H 6.22×10^{-6} . The latter was measured with a hydrogen electrode, the whole outfit having been carefully checked before and after the determination. The absorptive power in ultra-violet radiation was different from that of protein, being, for the same solid content, rather higher than those of serum-albumin or pseudoglobulin. Using 5 mm. quartz cells a 0.4 % solution of serum-albumin absorbed all light from wave-length 245 $m\mu$, while the absorption of the filtrate from the irradiated sample started approximately at 320 $m\mu$ (Table IV).

Table IV. *Extreme non-absorbed wave-lengths.*

Concentration %	Serum-albumin $m\mu$	Filtrate of irradiated serum-albumin $m\mu$	Proteose of Witte peptone $m\mu$
0.4	245	320	—
0.2	235	275	245
0.1	231	240	—

Pseudoglobulin shows a similar behaviour. But contrary to the findings of Spiegel-Adolf and Krumpel [1927] for concentrated protein solutions irradiated in the presence of acid or alkali, the filtrate did not give any special absorption band beside the end absorption, confirming in this respect the findings of Becker and Szendrö [1931].

The filtrate gave a positive biuret reaction with a slight red-violet tinge and positive reactions with Millon's reagent and trichloroacetic acid, while the test with sulphosalicylic acid was negative and no H₂S could be traced after heating with alkali. Alcohol causes a slight opalescence when its concentration reaches 80 %. The filtrate shows a slight opalescence when saturated with ammonium sulphate but no change on half saturation. If the test is made according to the method of Nonne-Appelt-Schumm for spinal fluid it is negative or doubtful.

The filtrate flocculates gold sol in concentrations equivalent approximately to 0.0005 mg. in 1 ml., the derivatives of serum-albumin and pseudoglobulin giving practically the same figures which are smaller than those given by the native proteins (Table V).

But while the flocculating power of the native proteins can be inhibited by the presence of neutral salts no such effect can be detected with the filtrates obtained by denaturation by radiation, indicating a difference in the nature of the flocculation.

Table V. *Gold sol reactions. The figures indicate concentrations %.*

	0.02	0.01	0.005	0.002	0.001	0.0005	0.00025
Serum-albumin	R _{VVV}	R _V	R	R	R	R	R
Pseudoglobulin	colourless	colourless	colourless	V	V	R _V	R
Filtrate from irradiated serum-albumin	colourless	colourless	colourless	V	R _{VVV}	R _{VV}	R _V

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

After dialysis and electrodialysis there is a nearly complete loss of the conductance, absorption and refractive power of the fluid which no longer gives the biuret reaction and shows only traces of its original gold sol-flocculating power.

Taking into consideration the properties of the filtrate here described it does not seem possible to identify the fraction or fractions with any known cleavage-product of proteins according to the classification of Pick [1925]. Nor does the gold sol reaction give any help. According to Zsigmondy [1920] both secondary proteoses and peptones are able to flocculate colloidal gold with blue colour without additional salt. Zunz [1906] has determined in 13 different fractions of secondary proteoses and peptones the amount necessary to change the colour of red gold to violet. His most potent fraction (Albumose BI) has only 1/10 of the flocculating power of the filtrate obtained by irradiation. Zunz [1907] also stated that secondary proteoses are unable to flocculate mastic sol; the filtrate obtained by irradiation of proteins did precipitate mastic sol though in somewhat higher concentrations (Table VI).

Table VI. *Mastic sol reactions. The figures indicate concentrations %.*

	0.086	0.043	0.021	0.01	0.005	0.002	0.001	0.0005	0.0002
Serum-albumin	×	×	×	×	+	0	0	0	0
Pseudoglobulin	×	×	×	×	×	×	+	0	0
Filtrate from irradiated pseudoglobulin	×	×	+	0	0	0	0	0	0

(× = flocculation, + = cloudiness.)

It is only possible to state that the filtrate from irradiated protein is practically free from proteins and contains some secondary proteoses and probably peptones. But considering the very low acidity of the filtrate it seems scarcely likely that the flocculation of colloidal gold is caused by peptones.

In order to ascertain whether the special kinds of proteoses found in the filtrates of irradiated proteins are missing entirely from those of heat-coagulated proteins a 0.2 % solution of pseudoglobulin was heated on the water-bath for 15 minutes. After removing the precipitated protein the filtrate was concentrated to 1/50 vol. The solid content of such a solution amounted to 0.11–0.13 %. Reactions with sulphosalicylic acid and trichloroacetic acid were positive while the gold sol reaction remained entirely negative.

This seems to indicate that in denaturation of proteins by heat and by light of short wave-length, differences can be detected not only in the precipitated fraction [Spiegel-Adolf, 1927] but also in the cleavage products. Changes in odour and colour had already suggested this.

The fact that some proteoses show such a high degree of flocculating power towards colloidal gold seems to be of some interest in connection with the behaviour of spinal fluids in certain pathological cases. Though the results of the reaction of Lange can nearly always be explained by the content of proteins in the fluid and by the ratio between albumin and globulin, nevertheless there are

cases such as the one referred to by Kyrle *et al.* [1920] in which there was a marked gold sol flocculation without an increase in proteins. Besides, Epstein and Rubinstein [1926], using the adsorption method of Willstätter, were able to show that the fractions giving gold sol curves characteristic for general paralysis did not show protein reactions. Though no identity between this substance and the one obtained through irradiation can be reasonably expected, experiments were started in which an equal amount of the latter was added to a spinal fluid and the gold sol reaction of this mixture compared with that of the spinal fluid alone.

The spinal fluids were sterile, Wassermann-negative and did not show any increase in proteins. As in all previous tests two kinds of gold sol were used simultaneously, one made according to the method of Bredig with consecutive purification by dialysis, the other by reduction with formaldehyde. The precipitation was made according to Lange. The results of such an experiment are given in Table VII.

Table VII. *Spinal fluid, "K.M." containing equal amounts of 40 times concentrated filtrate from irradiated protein.*

Diluted ...	10	20	40	80	160	320
Red	.	.	.	.	.	.
Red-violet	.	.	.	.	+	+
Violet	.	.	.	+	.	.
Blue	.	.	.	.	.	.
Bluish white	+	+	+	.	.	.
White	.	.	.	.	.	.

The spinal fluid alone when diluted in the same way did not change the colour of the red gold sol.

These experiments show that the addition of proteoses derived from horse-serum by irradiation elicits in normal spinal fluid a behaviour towards colloidal gold practically identical with the reaction of the same fluid in cases of general paralysis. The test of Nonne-Appelt was negative. Nevertheless there seems to exist a protective power in normal body fluids against the gold sol-precipitating effect of the proteoses, as a much greater amount of the latter was necessary to obtain effects similar to those reached with watery dilutions of the proteoses alone. Solutions of pure serum-albumin or pseudoglobulin did not show this effect.

The behaviour of the proteoses obtained by irradiation seems to suggest the occurrence of other atypical proteoses in spinal fluids in pathological cases as an explanation of a positive gold sol test without increased protein content.

SUMMARY.

1. Protein solutions of 0.1–0.2 % irradiated or heated in the presence of acid or alkali show practically unchanged refraction.
2. Electrolyte-free serum-albumin and pseudoglobulin in concentrations from 0.2 to 0.002 % are practically entirely coagulable by heat or irradiation; in the latter case, only the filtrate shows an increase in refraction and a positive reaction with colloidal gold.
3. Protein solutions of 0.2–0.5 % containing acid or alkali do not show, after irradiation, neutralisation and heat-coagulation, positive reactions with colloidal gold.

4. Filtrates of protein coagulated by heating or irradiation were concentrated 10–50 times and investigated physico-chemically. The results seem to indicate the presence of atypical proteoses in the latter case.

5. Evidence is given that the proteoses are distinguished by strong light absorption. Their power to flocculate colloidal gold exceeds the power of genuine proteins 10–40 times. They also precipitate mastic sol.

When a concentrated solution of the proteoses obtained by irradiation of electrolyte-free proteins is added to normal spinal fluid the mixture gives a gold sol reaction similar to that obtained in cases of general paralysis.

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