On the Nature of the Silver Reaction in Animal and Vegetable Tissues.

By A. B. Macallum, M.A., M.B., Ph.D., Professor of Physiology in the University of Toronto.

(Communicated by W. D. Halliburton, F.R.S. Received March 3,—Read April 6, 1905.)

I. *Introduction.*

Though the use of nitrate of silver as a reagent in histology has now a half century of history, and has been of the greatest service in elucidating the structure of some tissues, it has not yet been determined how the reagent is affected by the tissues, or what compound or compounds in the tissues are responsible for the precipitate which becomes discoloured in the sunlight. There are, indeed, references to these points in the literature of the subject, but these are scanty, and they are not at all in accord, while the majority are only guesses or fanciful explanations of the reaction itself. It may even be said that the investigators who used the reagent directed the whole of their energies to determining what it showed from the morphological side rather than what was involved in the reaction itself, and because of this there has been nearly 30 years of discussion on the question whether the results obtained with the reagent were trustworthy or were of the nature of artifacts.

The principal value of the reagent to the earlier observers appeared to be the fact that, after impregnation with nitrate of silver, and exposure of the preparation to light, cell outlines were revealed, and thereby the structure of lymphatics and of lymph tissue were demonstrated, the outlines being shown through the brown reaction which they manifested in such preparations. Von Recklinghausen,* who was amongst the first to use the reagent for this purpose, held that the silver salt is deposited in what he regards as the cement substance between the cells, and that this cement material under the influence of light reduces the silver. It was pointed that the reaction is not confined to the cell peripheries and intercellular material, for preparations sometimes showed the reaction present in the cytoplasm of the cells, but absent from the membranes and the intercellular structures. This condition was known as the positive reaction or positive image, and was attributed by Schweigger-Seidel† to the decomposition and redistribution of the silver

* 'Die Lymphgefässe und ihre Beziehung zum Bindegewebe,' Berlin, 1862.

† "Ueber die Grundsubstanz und die Zellen der Hornhaut des Auges," 'Berichte d. Kön. Sächs. Gesell. d. Wiss., Math.-Phys. Cl.,' vol. 20, p. 305, 1868.

precipitate which, in the normal cell giving the negative image, is to be found in the intercellular spaces and boundaries, that is, in the cement substance of Von Recklinghausen. Schweigger-Seidel maintained that the decomposition was brought about by chlorides forming silver chloride from the silver albumen precipitate in the intercellar material, the silver chloride so formed dissolving in the presence of chlorides, and thus diffusing into the cytoplasm, where, under the influence of light, it became the subchloride, and he proved this by taking preparations which had been treated with nitrate of silver, but not acted on by light, and placing them away from the light in a solution of sodium chloride. In these, when reduced by the light, the coloured silver salt or compound obtained chiefly in the cytoplasm. According to Hüter* the positive image may arise through diffusion of the cement substance into the cytoplasm and the consequent intracellular precipitation of the silver compound. His,† in 1862, held that the silver compound in the cornea which reduces in light is not an albumin compound, but chloride of silver, for when the cornea was treated with mercuric nitrate, which dissolves chloride of silver, all the silver precipitate was dissolved, which would not have been the case had any of the compound been silver albuminate. In later observations,‡ however, he admits that the silver compounds may be an albuminate as well as a chloride, and that both reduce under the influence of light. A similar view was held by Harpeck,§ Hartmann,‖ Auerbach,¶ and Henle.** Schwalbe†† found that if the serous membrane to be examined be first washed with a 4-per-cent. sugar solution, treatment with silver nitrate will not bring out the silver lines usually obtained, and he concludes from this that the cement substance, such as Von Recklinghausen postulates, has nothing to do

* "Zur Pathologie der Gelenkflächen und Gelenkkapseln, mit einem Kritischen Vorwort über die Versilberungsmethode," 'Arch. für Path. Anat. und Physiol.,' vol. 36, p. 25, 1866.

† "Ueber die Einwirkung des Salpetersauren Silberoxyds auf die Hornhaut," 'Schweizer Zeitschr. f. Heilkunde,' vol. 2, p. 1, 1862.

‡ "Ueber das Epithel der Lymphgefässwurzeln und über die Von Recklinghausen'schen Saftcanälchen," 'Zeit. f. wiss. Zool.,' vol. 13, p. 455, 1863.

§ "Ueber die Bedeutung der nach Silberimprägnation auftretenden weissen lücken- und spaltähnlichen Figuren in der Cornea," 'Arch. f. Anat.,' 1864, p. 222.

‖ "Ueber die durch den Gebrauch der Höllensteinlösung Künstlich dargestellten Lymphgefässanhänge, Saftcanälchen und epithelähnlichen Bildungen," 'Arch. f. Anat.,' 1864, p. 235.

¶ "Untersuchungen über Blut- und Lymphgefässe," 'Arch. f. path. Anat. und Phys.,' vol. 33, p. 340, 1865.

** "Bericht über die Fortschritte der Anatomie im Jahre 1866," 'Zeit. f. rat. Med.,' 3e Reihe, vol. 30, p. 6.

†† "Untersuchungen über die Lymphbahnen des Auges und ihre Begrenzungen," 'Arch. für Mikr. Anat.,' vol. 6, p. 1, 1869.

with the reaction, that the latter is due to an albuminous layer, "an albuminous cement substance," adhering to the edges of the cells, and which can be washed away.

Legros* was of the view that in the treatment with the reagent the whole cell received a slight stain, and that the dark lines were the contact surfaces of two feebly stained cells. Feltz† held that all the silver lines are artificial products, basing this view largely on the fact that in membranes made from albumin and collodion, similar reticula may be obtained by treatment with silver, and like figures were produced by Severin‡ on surfaces which contained no epithelium. Soboroff§ accepted Von Recklinghausen's view which, however, Reich‖ rejected, holding that the silver lines do not originate from cement substance, but in part from a fluid between the cells, but what the nature of the precipitate is he did not decide. Robinski¶ also does not admit the existence of a cement material, and he found that the cell borders, in *e.g.*, the epithelium of Descemet's membrane, colour first, then the reaction slowly advances into the cytoplasm, and finally the whole cell is stained, which ought not to be the case if Von Recklinghausen's views are correct. A cement substance is unnecessary to bring about contact and adhesion of cells to each other. According to Alferow** the silver precipitate, which colours under the influence of light, is a mixture of the chloride and the albuminate, as the free acids (picric, acetic, lactic, and citric) which he used dissolve all the other precipitates. Adamkiewicz,†† on the other hand, held that the silver lines are albuminate of silver only, and that they are laid down in a cement substance.

The reagent was also employed by Ranvier very much in his histological researches, but he has ventured no explanation of its action.

* "Note sur l'épithélium des vaisseaux sanguins," 'Jour. de l'Anat. et de la Phys.,' 1868 p. 275.

† "Recherches expérimentales sur le passage des leucocytes à travers les parois vasculaires," 'Jour. de l'Anat. et de la Phys.,' 1870, p. 33.

‡ "Beiträge zu der Lehre von Entzündungen," 'Diss.,' Dorpat, 1871.

§ "Untersuchungen über den Bau normaler und ectatischer Venen," 'Arch. für path. Anat. und Physiol.,' vol. 54, p. 137, 1871.

‖ "Einige mikroskopische Studien mit Silbersalpeterlösung besonders an Gefässen des Auges und anderer Organe," 'Sitzber. d. Wiener Akad.,' 1873, vol. 67, p. 81.

¶ "Recherches microscopiques sur l'épithèle et sur les vaisseaux lymphatiques capillaires," 'Arch. d. Physiol.,' 1869, p. 451; also "Die Kittsubstanz auf Reaction des Argent. nitric.," 'Arch. f. Anat.,' 1871, p. 184.

** "Nouveaux procédés pour les imprégnations à l'argent," 'Arch. de Physiol.,' 1874, p. 694.

†† "Ueber die Behandlung von Gefässen mit Silbernitratlösung," 'Berl. Klin. Woch.,' 1874, p. 355.

Amongst the more recent observers, Boveri* holds that the material in which the silver precipitate occurs is not a specific substance, either cement or otherwise, but only the contact surfaces of two neighbouring cells. He claims that he observed, inside of blood vessels, red blood corpuscles lying in contact with each other, and between the contact surfaces the silver precipitate occurred as between endothelial cells. What the precipitate itself is he does not say. Rabl† regarded it as probable that the reagent combines with the albumin to form a silver-nitrate proteïd, through union of the molecules, analogous to that process which operates in the precipitation of urea with mercuric salts. That the brown granules of the precipitate are not metallic silver is shown by their solubility in sodium thiosulphate. According to Mann‡ the precipitate is probably a proteïd in combination with chlorides and carbonates.

From this review it will be seen that there is as yet much uncertainty as to the nature of the reaction which silver nitrate undergoes in tissues under the influence of light, some observers holding that the silver compound is a mixture of a chloride and an albuminate, both of which become coloured when exposed to the light, while others postulate the presence of an albuminate compound only.

The cause of this confusion lies in the fact that our knowledge of the action of organic compounds on silver salts under the influence of light is very indefinite and fragmentary. It is, of course, known that when a silver salt is added to a solution of one of certain organic compounds, and the mixture exposed to light, a more or less coloured product soon appears, the formation of which is supposed to be due to reduction. This is a term which applied to a salt of silver, has a wide meaning, one application comprehending that decomposition of the salt in which metallic silver is set free, another involving that change of the salt in which the quantity of the element or substance combined with the silver is diminished. Both of these types of reduction are illustrated in the case of a salt of silver in association with organic compounds.

The reduction to the metallic condition occurs when alkaline silver solutions come in contact with living protoplasm (Loew and Bokorny),§ and it occurs also when certain organic compounds in alkaline solution—*e.g.*, uric acid, levulose, dextrose, hydroxylated benzol derivatives, hydrazine and aldehyde compounds—are heated with nitrate of silver. In neutral solutions

* "Beiträge zur Kenntniss der Nervenfasern," 'Abhandl. d. Kön. Bayer. Akad., Math.-Phys. Cl.,' vol. 15, p. 421, 1886.

† "Ueber geschichtete Niederschläge bei Behandlung der Gewebe mit Argentum nitricum," 'Sitzber. Wiener Akad., Math.-Phys. Cl.,' vol. 102, Abth. 3, p. 342, 1893.

‡ 'Physiological Histology, Methods and Theory,' Oxford, 1902, p. 266.

§ 'Chemische Ursache des Lebens.,' München, 1881.

the hydroxylated benzol compounds have the same effect on silver salts. In these cases the metallic silver is black, and it is not soluble in the solutions (sodium thiosulphate, etc.), which dissolve argentic or argentous chloride.

The reduction involving diminution in the quantity of the element combined with the silver is, of course, well known in the case of the haloid salts, the chloride AgCl, for example, becoming converted by the action of light into the subchloride Ag_2Cl, which is violet, reddish-violet, blueish-violet in mass, but it may also be reddish-brown when occurring in thin layers, membranes, or deposits, the shade of colour apparently depending on the presence of the subhaloid in a finely divided form or otherwise. The quantity so converted is, according to Carey Lea,* not more than 1 per cent. of the total haloid salt, with a portion of which it combines, the compound formed not containing more than 9 per cent. of the subchloride. Hodgkinson,† however, regards the salt formed by the action of light on silver chloride as an oxychloride, probably of the composition represented by the formula Ag_4OCl_2, in which case there is no reduction, the change merely involving a substitution of oxygen for half the chlorine. There is no doubt about the loss of chlorine, as simple experiments indicate such a loss, but whether the coloured salt is an oxychloride or a simple subchloride does not matter for the present, although Carey Lea is pronounced in the view that the compound is a simple subhaloid, while Guntz‡ was able to prepare the subfluoride of silver Ag_2F, from which, through the action of chloride of carbon, of silicon, and of phosphorus he obtained Ag_2Cl. He further obtained by the same method the subiodide Ag_2I, the subsulphide Ag_4S, and the suboxide Ag_2O.

There is also the difficulty presented by the proteïds. The current view held, not only by the chemists, but also by scientific exponents of photography, is that proteïds form coloured reduction products with silver, and that organic matter generally gives similar reduced compounds. Upon this point the evidence has appeared decisive, for if egg "albumen" or serum "albumen" be treated with acid nitrate of silver the result is a precipitate which in the sunlight quickly becomes coloured. Further, if gelatine in solution be similarly treated, a precipitate may not occur, but the colour reaction quickly appears, and is usually of a pronounced character. This summarises the evidence, and, taken in conjunction with the fact that certain organic compounds in neutral or alkaline solution, in sunlight, "reduce" salts

* "On some Reactions of Silver Chloride and Bromide," 'Amer. Jour. of Science,' 3rd series, vol. 15, 1878, p. 189; also "On Red and Purple Chloride, Bromide and Iodide of Silver, on Heliochromy and on the Latent Photographic Image," 'Amer. Jour. of Science,' vol. 33, p. 349, 1887.

† Meldola, 'The Chemistry of Photography,' 1889, p. 56.

‡ 'Comptes Rendus,' vol. 112, pp. 861 and 1212.

of silver, seemed to indicate very distinctly that the coloured silver compound produced in solutions of proteïds is due to the latter, it being supposed that an "albumenate" of silver obtains which in light forms a compound, argentous "albumenate," analogous to the argentous haloid salt of the photographic plate.*

All these facts make it possible to understand how it came about that there was any discussion as to the nature of the silver reaction in tissues, and why it was accepted without much question that proteïds took a part in this reaction. The author has thought the subject worthy of further investigation, on the ground that if the silver reaction is due to the presence of few or many organic compounds, as well as to the presence of haloids, the accurate localisation of chlorine, bromine, and iodine in tissues is possible only under great difficulties, whereas if the reaction is due to haloid salts, or can be applied so as to demonstrate the presence of haloid salts only, the cyto-chemist has a means of determining the presence of not only chlorine, bromine, and iodine, but also to a certain extent of sodium and potassium in tissues.

It has for several years been the view of the author that the reaction of proteïds with nitrate of silver in sunlight is due to the presence of chlorides only, and that if proteïds could be thoroughly freed from chlorides, the former would give no reduction compound with the silver salt. The demonstration of this, when attempted, was beset with difficulties, due partly to the fact that chlorides, and particularly the chloride of sodium, are present everywhere, and, therefore, contaminating, as they do more or less, every reagent and preparation, the absolute removal of chlorides appeared to be impossible of accomplishment, and in part, also, to the extreme sensitiveness of the reaction which demonstrates the presence of the haloid salts. Silver nitrate will demonstrate the presence of one part of chlorine in 1,000,000 parts of water, but I have found, also, that if sunlight is allowed to act on the preparation, one part of sodium chloride may be detected in 1,000,000 parts of water—that is, 1 part of chlorine in 1,600,000. It is manifest that this test is exceedingly delicate, and consequently the reaction, if properly sought for, could be obtained in all fluids, and particularly with colloids, which are very tenacious of the inorganic salts with which they are associated. Because of this, it appeared hopeless to attempt to free proteïds from haloids, and thus demonstrate the inactivity of proteïds towards salts of silver.

The results of researches recently carried out regarding the detection and localisation of potassium salts in animal and vegetable tissues, having indicated how important it is to determine whether anything else than

* Meldola, *op. cit.*, pp. 116 to 119 and 342 to 352.

halogens affect the silver salt in light, I was led to take up the question anew on lines that would promise a definite solution of the problem. The results of these observations seem to be decisive, and therefore these and the methods by which they were reached may now be described.

II. *Methods and Results.*

There are, beside the haloid salts of silver, other compounds of the same metal which undergo in the presence of light a change which is termed reduction. Some of these are important, but only those were examined which appeared likely to affect directly the question of the nature of the silver reaction in tissues.

The phosphate when precipitated darkens on exposure to light, but if free nitric acid is present, the precipitate does not occur and the dark reaction fails to appear. The carbonate also darkens, but in the presence of nitric acid the nitrate is formed, and this does not undergo reduction. The sulphate is unaffected, but the sulphocyanide is "reduced," undergoing a slight change in colour, even in the presence of nitric acid. The hippurate, the oxalate, the valerate, the palmitate, the oleate and the stearate are unaffected, while the glycero-phosphate acts like the phosphate in the presence of nitric acid, and lecithin also does not affect the silver salt. The tartrates and citrates in the presence of nitric acid are unaffected. Further, the acetate, the amido-acetate, the amido-propionate, the succinate and the lactate, when pure, give no reaction in the same acid medium. Of the more strictly physiological compounds, taurine and creatine act on the acid solution of the silver salt, producing in the light a coloured reduction compound, and cyanuric acid acts similarly, yet less readily, while alloxan and alloxantin immediately reduce the silver to the metallic condition, but purins, urea, leucine, tyrosine, indol, skatol, and their derivatives exercise no effect.

This makes it certain that only a few compounds of the extractive class may affect the silver reaction in tissues, and, with the exception of creatine, their presence may be disregarded, for they are excessively small in amount when occurring at all in tissues. Creatine, on the other hand, is abundant in the striated muscle of vertebrates, while it is absent wholly from invertebrates. One may consequently avoid the difficulties presented by the occurrence of creatine simply by using for investigation the muscle tissue of invertebrates.

That part of the result of the reaction in tissues is due to haloid salts of silver formed there seems to be beyond doubt. Sodium and potassium chlorides are constituents of all tissues, animal and vegetable, and it is

possible to extract them with water free from haloids, and thereby greatly affect the silver reaction of the tissues so treated. In the case of vegetable stems also, the salts may be extracted with alcohol of 98-per-cent. strength, which, of course, leaves in the preparations their proteïds and carbohydrates and sections made from vegetable tissues (*e.g.*, *Tulipa*), and at once treated with alcohol for 24 hours, usually give no reaction with the silver reagent. This is true also of animal tissues, thin sections of which, made from fresh material frozen with carbon dioxide spray, after lying in alcohol of 90 per cent. for some days, are practically unaffected in a silver solution placed in the sunlight. Stronger alcohol, even of 98-per-cent. concentration, also removes the chlorides if the pieces of tissue be small or in thin sections, but if the pieces be of considerable thickness, the alcohol removes only a small portion of the salts and redistributes the remainder throughout the preparations.

The question is, however, not whether the haloids in tissues constitute a factor in the silver reaction, but rather whether apart from the exceptions referred to they are the only compounds which contribute to the reaction.

The proteïds, as already pointed out, are regarded as possessing the property of forming reducible compounds with silver, and to determine whether this view is correct, the purification of a number of typical proteïds and of an albuminoid was undertaken.

For this purpose it was necessary, first of all, to have every fluid and reagent that was used free from chlorides to the extent that these were below the minimum limit of detection, that is, if chlorine should be present as chloride it would be less than 1 in 1,600,000. This is possible in the case of carefully distilled water, but it is not quite as easy in the case of the precipitating reagents employed, namely, anhydrous sodium sulphate* and ammonium sulphate. In the case of the former, the "chemically pure" material had to be repeatedly crystallised before it was obtained in a form sufficiently free from chlorides. Many of the "chemically pure" preparations of ammonium sulphate put on the market are not of the standard of purity required, and different quantities of the salt from the same manufacturer were found to vary considerably as regards relative purity. In every case, however, only those preparations of the salt were used which reached the standard exacted.

The material employed consisted of egg "albumen," serum "albumen," and gelatin. In the case of egg "albumen" the solution was first of all made

* On the employment of anhydrous sodium sulphate as a precipitant of proteïds from their solutions, see Pinkus: "On the Precipitation of Proteïds," 'Jour. of Physiol.,' vol. 27, p. 57, 1901 to 1902.

in a large quantity of distilled water with the consequent separation of globulin, from which, on sedimentation, the supernatant solution was removed by decantation, and from this the albumins were precipitated on the addition of enough ammonium sulphate to saturate the solution. Tested with silver nitrate solution,* a precipitate was obtained which gave a deep reaction in light in less than 20 minutes. The precipitate obtained with the sulphate was dissolved and again precipitated in the same way, the precipitate again dissolved and re-precipitated, this process being repeated in one case 9 times, in another 14 times, and in a third 11 times before the desired degree of purity was attained. It was subsequently found that if in each case when the proteïd precipitate is re-dissolved the solution be allowed to stand for some time, for example, 10 to 12 hours, before the subsequent precipitation is brought about, the standard of purity may be reached before the eighth precipitation occurs. It would seem as if the chlorides are in intimate union with the particles of the colloid, and that when precipitation immediately follows solution, salts are largely retained in the interior of the colloid molecule groups, but when the latter are acted on for hours by water free from chlorides, the latter pass into the water, with the result that when precipitation occurs much less of the chlorides is carried down. The effect of this may be seen when the filtrates are tested for chlorides. When the precipitations followed each other at very short intervals the chloride reactions of the filtrates were slight, but when the precipitated proteïd was dissolved and allowed to remain in solution in each case some hours before being again precipitated, the filtrate gave a much deeper reaction and of the typically chloride character. When the silver precipitate from the earlier filtrates was allowed to settle, the supernatant fluid decanted, the precipitate then washed several times in water free from haloids, finally collected in a porcelain crucible, dried at 120° C. for three to five hours, weighed, then fused and once more weighed, there was found to be practically no loss of weight, this fact showing that organic compounds do not enter into the composition of the silver precipitate.

The albumins and globulins of serum were not separated from each other, it having been found easier to carry out one series of precipitations for both classes of proteïds in order to free them from chlorides. As a rule, it required more precipitations to purify the proteïds of serum than was the case with egg albumins. What the reason for this difference is is not clear.

The globulins which separated when egg "albumen" was dissolved in distilled water were freed from chlorides only by extraction with distilled

* The solution of silver nitrate used was exactly decinormal, and it contained 25 c.c. of nitric acid (60 per cent. strength) to the litre.

water, and this was continued for two weeks or more, the water being frequently removed by decantation and as often replaced by fresh fluid. Portions of the globulin thus undergoing extraction were from day to day dissolved in dilute solutions of ammonium sulphate, free from chlorides, and, after the addition of some of the silver reagent, placed in the sunlight for days to determine how far the purification had advanced.

It is important not only in the case of globulins, but also in that of all albumins to have them in solution when the reagent is to be added. In the precipitate which silver nitrate produces, the latter is intimately mixed throughout with the proteïd. This is an advantage, for when the reagent is added to the undissolved proteïd it penetrates very slowly, and, consequently, the superficial reaction, if any occurs, may be so slight as to escape observation.

The egg albumins and the serum albumins and globulins which had undergone the indicated number of precipitations as well as the egg globulins which had been extracted for over a fortnight with distilled water, did not yield any reaction whatever with the silver nitrate reagent, even after weeks of exposure to bright sunlight, although the original unpurified material in every case gave an intense reduction effect.*

The purification of gelatin is more easily attained. For this purpose one dissolves the gelatin in water at 45° C. and adding to the solution, while it is maintained at that temperature, anhydrous sodium sulphate till the mixture is saturated. Stirred with a glass rod, the greater part of the gelatin collects on it and it can be thus lifted out of the solution and transferred to, and dissolved in, a fresh quantity of distilled water at 45° C., to which also anhydrous sodium sulphate is added till saturation obtains. The precipitated gelatin is once more in the same way transferred to a fresh quantity of distilled water at 45° C. The process of precipitation and solution was repeated many times, but each stage required only a few minutes, and consequently as many as twelve precipitations and as many solutions of the gelatin were obtained in two and a half hours. The purified product was found to set firmly and was in every case very clear and transparent.

The gelatin of commerce gives an intense reaction with the silver reagent in sunlight, but the gelatin of the ninth precipitation gave not the slightest reaction with silver nitrate solution after two weeks in sunlight, not even producing a precipitate or an opalescence. It mattered not from what crude preparation of commerce the purified product was obtained, the result was in every case the same.

That the compound or compounds in crude gelatin which react with silver nitrate in the sunlight are chlorides only, was demonstrated satisfactorily.

* When a neutral, instead of the acid, solution of nitrate of silver was used the result was the same. This is true also of purified gelatin similarly treated.

For this purpose the process of purification was modified slightly, the gelatin precipitated from the warm solutions was separated by filtration in a hot funnel, the filtrate cooled down to 5° C., with the consequent crystallisation of the greater part of the sodium sulphate. The mother liquor was then concentrated by evaporation, carefully filtered, and on cooling again another quantity of crystal sodium sulphate formed.* The mother liquor of this crystallisation gave, on the addition of a quantity of the silver reagent, a precipitate which, after being kept in a porcelain crucible at 120° C. for five hours, was of the same weight as it gave when it was fused. This could only be a haloid salt of silver and, therefore, organic compounds of silver do not obtain in the precipitate. The filtrates from the first, third, and fifth precipitations of gelatin gave such silver haloid precipitates, but in quantities diminishing in the order named.

Attempts were made to prepare nucleo-proteïds in a pure form, but these were unsuccessful, simply because the one efficient precipitant of these compounds from their solutions is dilute hydrochloric acid and enough of this reagent always adhered to or was united with the precipitates to give a distinct silver chloride reaction. The same difficulty was experienced in the case of nucleic acid.

It was not necessary, however, to prepare purified nucleo-proteïds or nucleic acid, for when animal and vegetable tissues in fresh condition are treated with the silver reagent and then exposed to light, the nuclei, if normal, are never affected and, therefore, not only is haloid chlorine absent from nuclei, but also nucleo-proteïds do not react with the silver salt. Further, as the head of the male element in the frog and *Oniscus* gives no reaction with the reagent, it may be inferred that the simpler compounds of nucleic acid are also unaffected by nitrate of silver.

No a tempt was made to isolate vegetable proteïds in a form free from chlorides but that they also do not give coloured products when treated with nitrate of silver and placed in the sunlight, seems to be clear from simple experiments which can be readily made on vegetable tissues. When thin sections of any succulent vegetable stem (*e.g.*, of *Tulipa*) are treated with the reagent, sunlight brings out a deep reaction in every part of the preparation, not only in the protoplasm, but also very frequently in the cell walls, the nuclei alone, when normal, never exhibiting the slightest reaction. When, however, the sections were first placed for a couple of hours in 99 per cent. alcohol, no colour reaction whatever developed on treatment with the silver

* The filtration had to be done very carefully, in order to remove organic compounds which unite with silver nitrate, and which are thus precipitated, but such silver compounds do not discolour in sunlight.

reagent in sunlight. Alcohol of this strength* dissolves a considerable amount of sodium chloride and a smaller quantity of potassium chloride, and as the amount of each salt present in a section is small, the alcohol is capable of extracting it wholly, but leaving the proteïds at the points where they came in contact with the alcohol. That the complete absence of a silver reaction in sections so treated is due to removal from them of the salts, was shown when the alcohol which had covered for several hours a large number of sections was completely evaporated, the residue giving a very distinct reaction for chlorides.

When the preparations were covered for two hours with a mixture† of equal volumes of ether and acetone the greater part of chlorides were left in the sections, though not at the very points where they were during life, and such sections treated in sunlight with the silver reagent gave a marked colour reaction.‡ When the sections were carefully dried and then placed in the mixture for two hours their reaction was not perceptibly less marked than in fresh sections placed directly in the reagent. It was also found that sections which were first dried, then kept in a quantity of the acetone-ether mixture for 2 hours, and finally treated for 10 hours more with 99 per cent. alcohol, gave on the addition of the silver solution no colour reaction. The residue left on evaporation of the alcohol consisted of minute crystals of chlorides, apparently of sodium and potassium.

From all this it would appear that vegetable proteïds, like those of the animal kingdom, do not give with nitrate of silver dissolved in a dilute solution of nitric acid a colour reaction under the influence of light, and that the compounds in vegetable tissues which do react with the silver reagent are soluble in 99-per-cent. alcohol, but do not dissolve to any appreciable extent in the acetone-ether mixture. These facts do not exclude the possibility of the participation of other compounds than chlorides in the reaction which vegetable tissues give, but they seem to indicate that if there are such compounds they must occur in excessively minute amounts, and their presence is. when the silver reaction is obtained, masked by that of the chlorides.

* Sodium chloride is soluble in absolute alcohol to the extent of 65 parts in 100,000, and potassium chloride to the extent of 34 in 100,000 (De Bruyns, 'Zeit. für physik. Chem.,' vol. 10, p. 783). 99 per cent. alcohol takes up a much larger quantity of each salt.

† Such a mixture if made from pure anhydrous acetone and ether, according to the author's determinations, dissolves in 24 hours very little of the chlorides, 1,000,000 parts taking up only 2·4 parts of sodium chloride and 3·5 parts of potassium chloride.

‡ The acetone-ether mixture is a very rapid fixative for animal and vegetable tissues. Small pieces of kidney, liver, stomach, pancreas, thyroid and muscle when placed in it were rendered so firm in half an hour that very thin sections could be made from them with the section knife held in the free hand. The mixture is, on account of the rapidity of its action perhaps, not a serviceable histological reagent.

III. *General Remarks.*

The results given in the preceding pages make it quite clear that the reaction which animal and vegetable tissues give with nitrate of silver dissolved in dilute nitric acid may be attributed to halogens in haloid form,* and to taurine and creatine, and that proteïds and gelatin do not, when freed from traces of haloids, give the slightest colour reaction with the reagent. Of the two organic compounds which give the colour reaction, taurine may be neglected, since it obtains in infinitesimal quantities in animal tissues, and creatine, although present to the extent of 0·21 to 0·39 per cent. in frog's muscle, and of 0·4 per cent. in rabbit's muscle,† occurs in inappreciable quantities in other organs and it is absent altogether from invertebrate tissues.‡ One can, therefore, by appropriate selection of tissues of animal and vegetable forms for treatment with the reagent, determine, with a considerable amount of certainty and a very great degree of accuracy, the distribution of chlorides and, perhaps also, of other haloids, in various cytological elements.

This determination has already been made in the case of a number of cellular structures, and the results which have been obtained are of very great interest. These will form the subject of another paper, but two of them, which stand out in special prominence, are *that intercellular material and structures, including the so-called cement substance of Von Recklinghausen, are rich in chlorides, and that normal nuclei of animal and vegetable cells are absolutely free from them.*

* Compounds containing chlorine in a "masked" form as, for example, trichloracetic acid, give the silver haloid and subhaloid reactions after several days only.

† F. Nawrocki, "Ueber die quantitative Bestimmung des Kreatins in Muskeln," 'Zeit. für anal. Chem.,' vol. 4, p. 330, 1865.

‡ Krukenberg, 'Vergleichend-Physiologische Vorträge,' p. 316, Heidelberg, 1886; also Krukenberg's papers in 'Untersuchungen a. d. Physiol. Inst. d. Univ. Heidelberg,' vols. 3 and 4, 1880 and 1881.