XXXVI. THE ACTION OF HYPOCHLORITES AND ALLIED SUBSTANCES ON PROTEINS, AND THEIR BEHAVIOUR ON INJECTION.

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The recent work of Lorrain Smith [1915, 1, 2], Dakin [1915, 1, 2, 1916] and others has directed attention to the value of substances containing chlorine in "available" form as germicidal agents in the treatment of infected wounds. Although such substances have long been recognised as powerfully germicidal, they were not previously made use of to any large extent owing to the decrease in their efficiency in the presence of albuminous material. When small quantities of such a fluid as blood serum are added to hypochlorite solutions there is, even at comparatively low temperatures, an initial rapid lowering in the "available" chlorine value followed by a much slower rate of loss. The initial rapid loss is evidently due to oxidation changes and to the passage of chlorine into the cyclic rings of such protein derivatives as tyrosine etc., the hydrogen attached to the carbon being replaced. Part of the chlorine however, especially at low temperatures, replaces the hydrogen attached to nitrogen in various primary and secondary amine groups, forming compounds containing $.\mathrm{N}:\mathrm{Cl}_2$ or $:\mathrm{N}.\mathrm{Cl}$ groups. As the chlorine in these groups is still in active form, such compounds, if formed under the ordinary conditions of wound treatment or after intravascular injection, would probably constitute valuable germicidal agents. The significance and value of these chloramino-derivatives of protein must depend in the first place on the rate of formation and concentration of these bodies existing in the wound or in the blood, and, in the second place, on the rate at which these compounds lose their available chlorine either by hydrolysis or by transference to the cyclic rings.

In order to gain some knowledge of the changes which hypochlorites and aromatic substances containing chloramine groups undergo in the presence of proteins, it is necessary to carry out the tests at the temperature to which these bodies would be exposed in wound treatment. A series of experiments was therefore carried out to determine the percentage loss of available chlorine, under the above mentioned conditions, at stated intervals during the reaction. From such a study information may also be obtained as to the order of the chemical reactions concerned, but in this communication very little reference will be made to this part of the subject.

THE ACTION OF HYPOCHLORITES ON ALBUMINOUS SOLUTIONS.

Even extremely weak solutions of free HOCl precipitate albumins but solutions of the hypochlorites in general use have not this effect. At body temperature there is however a rapid disintegration of the protein, nitrogen coming freely off and the amino-acids breaking down into aldehydes or ketones with one carbon atom less than the original acids. The odour of aldehyde formation is very characteristic of the action of hypochlorites on proteins. The rate of change of all hypochlorite solutions in the presence of albumin is approximately the same, and as I was engaged in the study of the action of magnesium hypochlorite as a possibly less irritant antiseptic than sodium or calcium hypochlorite, the majority of the experiments were carried out with this solution.

The method of preparation of $Mg(OCl)_2$ was the following: hypochlorous acid was prepared from bleaching powder and boric acid used in the following proportions, 10 g. bleaching powder, 300 cc. water, and 15–20 g. boric acid. Distillation took place in vacuo and the distillate was collected in a vessel containing an excess of freshly precipitated and washed $Mg(OH)_2$, the receiver being kept in an ice-salt mixture. From 50–100 cc. were distilled over, and the solution so obtained contained 4–5 g. HOCl per litre. These solutions are quite stable and may be kept for at least a month. A similar solution may be conveniently and rapidly prepared by the addition of magnesium sulphate solution to one of bleaching powder, the precipitate being allowed to settle and the fluid siphoned off. Such a solution has been used with advantage as a fairly non-irritant antiseptic by Charles Mayer [1916].

The rate of change of hypochlorite in the presence of albumin was determined in the following way. The hypochlorite solution in a stoppered bottle was brought up to a temperature of 36° in a thermostat which also contained a vessel with blood serum. The "available" chlorine value of the former was estimated and when both solutions had reached the proper temperature, a small fixed quantity of the serum was added to a known volume of the warmed hypochlorite. At definite intervals the available chlorine value of the mixture was determined by running a measured quantity of the mixture into potassium iodide solution acidified with acetic acid and titrating the iodine set free with N/10 thiosulphate. In order to determine the type of the reaction estimations of the chlorine loss were carried out with different concentrations of hypochlorite the quantity of serum remaining constant, and also with hypochlorite solutions of the same strength but with varying quantities of serum.

Before referring to the curves of fall in concentration of active chlorine in the presence of serum, it is necessary to gain some idea of the amount of hypochlorite which can be used up at room temperature by a given amount of protein when an excess of the former is used and when sufficient time is given to complete the reaction. Effront [1905] has calculated that 1 mgm. protein (egg white) uses up 3·055 mgm. active chlorine, or for 1 mgm. nitrogen of the protein 18·629 mgm. chlorine. Using the same method as Effront, I have found that 1 mgm. nitrogen of blood serum uses up 15·87 mgm. $Mg(OCl)_2$. Therefore when 5 cc. serum are taken containing 61 mgm. nitrogen about 0·968 g. $Mg(OCl)_2$ can be used up. 200 cc. $Mg(OCl)_2$ $m/20$ contain 1·2722 g. $Mg(OCl)_2$, so that if 5 cc. serum were added to this solution there would be at the end of the reaction a slight excess of hypochlorite, while with the weaker hypochlorite solutions or with higher protein concentrations, the protein would be in excess[1].

Fig. 1 shows the available chlorine fall when 200 cc. of $m/20$ $Mg(OCl)_2$ (Graph I) and 200 cc. $m/40$ $Mg(OCl)_2$ (Graph II) respectively are mixed with 5 cc. blood serum (containing 0·061 g. nitrogen) the mixture being kept at 36°. Control solutions of $Mg(OCl)_2$ of the same strength without the addition of serum were also kept under the same temperature conditions and the available chlorine determined at the stated intervals. The control solutions showed practically no alteration during the period of the experiment.

The curves show a fall down to 30–35 % of the original value within 15 minutes, but after the first half hour the rate of loss of available chlorine

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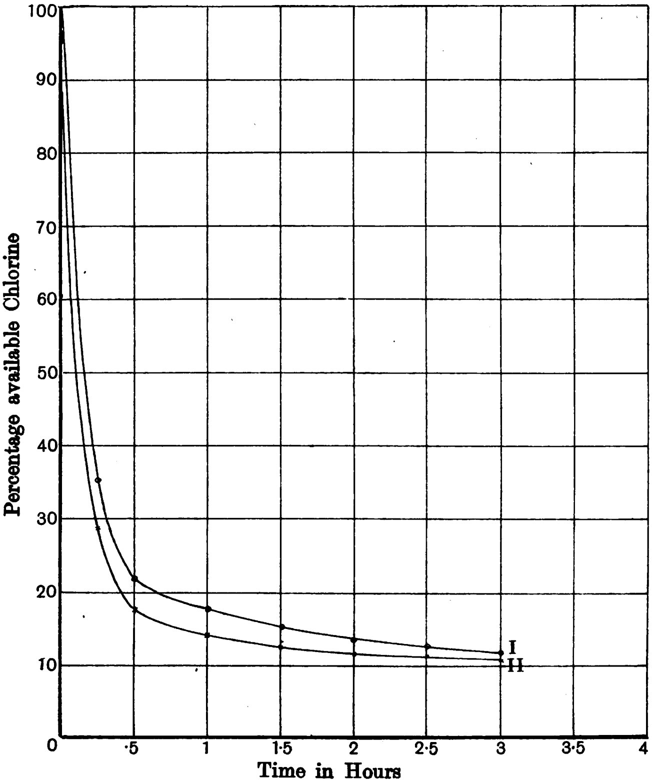
[1] The interesting observations of Dakin [1916] published after the completion of my work show that with higher protein concentrations than I used with hypochlorite solutions, namely from 1 cc. up to 10 cc. serum added to 5 cc. neutral hypochlorite containing 0·0126 g. HOCl, more active chlorine disappears in a given time with the smaller amounts of serum than with the larger.

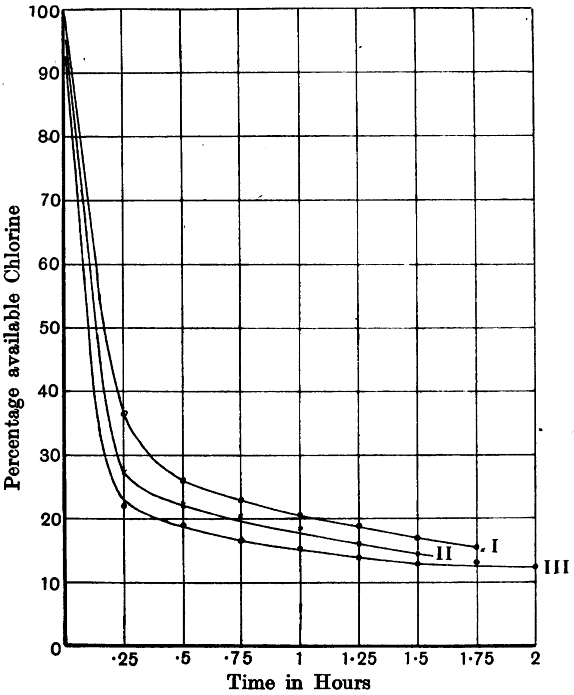
becomes very much slower. The curves are of the same type, slightly the greater fall taking place with the weaker solution. At the end of three hours, the available chlorine value is only slightly over 10 % of the initial concentration and is approximately the same in both cases. The curves show falls which are practically independent of the initial concentration.



200 cc. Mg $(OCl)_2$ *m*/20 + 5 cc. Blood Serum = I
,, ,, *m*/40 + ,, ,, ,, = II

Fig. 1.

Fig. 2 shows the effect of varying the protein and keeping the initial hypochlorite concentration the same. Three 200 cc. portions of *m*/20 Mg $(OCl)_2$ were taken and 5 cc. (Graph I) 10 cc. (Graph II) and 20 cc. serum (Graph III) respectively were added to the solutions. The available chlorine value in the first case fell to 37 % of the initial value, in the second to 28 % and in the third to 22 % within 15 minutes of admixture. This primary rapid fall is succeeded by the characteristic slow fall observed in Fig. 1. The types of curve are the same and at the end of 2 hours the active chlorine is about 12–15 % of the initial value in the three solutions. The fall continues slowly during the next 24 hours.



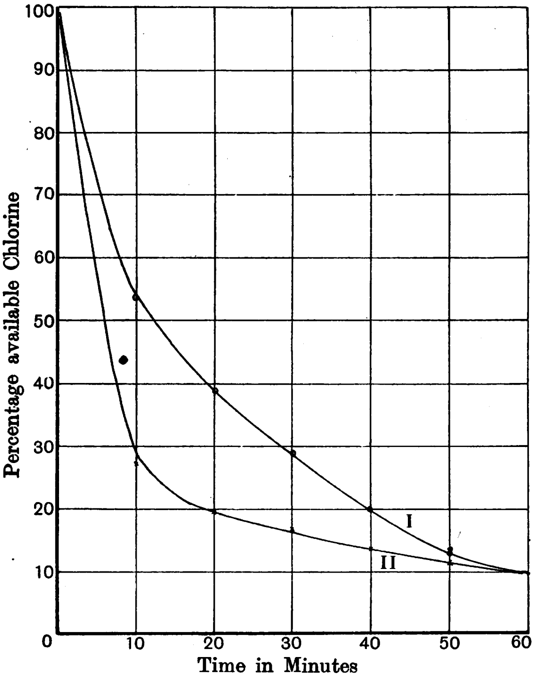
200 cc. Mg (OCl)$_2$ $m/20+$ 5 cc. Serum = I
" " " +10 cc. " = II
" " " +20 cc. " = III

Fig. 2.

In Fig. 3 the fall is plotted out for shorter intervals and for solutions of eusol (Graph I) of a strength which is commonly used as an antiseptic (0·315 % "available" chlorine) and Mg (OCl)$_2$ (0·556 % "available chlorine") (Graph II).

In the case of the former 5 cc. serum were added and in the latter 9 cc.

In the case of Graph I, Fig. 3 apart from the fall during the first 10 minutes, the logarithm of the concentration is practically proportional to the time—a reaction of the first order. At the close of the first hour the chlorine value in both cases is only 10 % of the initial figure. From this point onwards the fall becomes very slow in both cases so that 18 hours later the chlorine value is still 3 % of the initial figure.



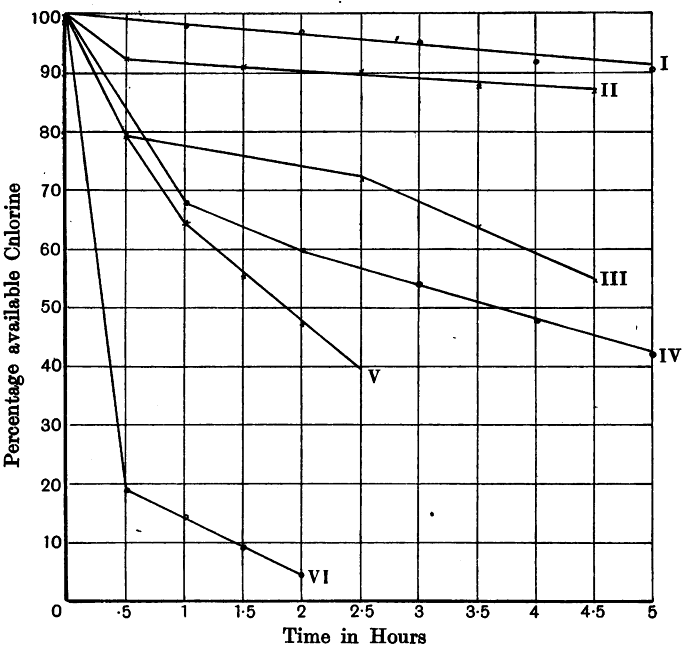
200 cc. "Eusol" (0·315% Chlorine) + 5 cc. Serum = I
„ $Mg(OCl)_2$ (0·556% „) + 9 cc. „ = II

Fig. 3.

THE ACTION OF CHLORAMINE-T ON ALBUMINS.

The valuable work of Dakin and his collaborators [1916] has directed attention to substances containing chlorine attached to nitrogen in primary and secondary amine groups. The chemical characters and bactericidal properties of a number of aromatic chloramines prepared originally by Chattaway have been studied by these investigators. As the action of hypochlorites on albumins probably results, in small part at least, in the formation of bodies of this type, it is probable that from a comparison of the velocity of reaction of such a body as sodio-*p*-toluene-sulphochloroamide ("chloramine-T") in albuminous fluids with the reaction velocity of hypochlorites in the same fluids, one may arrive at some knowledge of the nature of the latter reaction. When chloramine solutions are mixed with varying quantities of blood serum, the reaction is not only a very much slower one but also differs in many other particulars from the hypochlorite-serum reaction. Thus the fall varies directly with the volume of serum added.

The percentage fall in available chlorine when chloramine solutions are mixed with different quantities of serum was determined in the same way as in the hypochlorite-serum mixtures. All the experiments were carried out at 36°. When small quantities of serum, similar to those used in the hypochlorite experiments, are added to chloramine solutions the rate of fall in available chlorine is extremely slow. Fig. 4 shows a series of graphs with varying concentrations of chloramine and albumin:



200 cc. Chloramine-T 1·338 % + 5 cc. Serum = I
" " 1 % + 10 cc. " = II
" " 1 % + 20 cc. " = III
" " 0·354 % + 5 cc. " = IV
" " 0·1 % + 10 cc. " = V
100 cc. " 2 % + 100 cc. " = VI

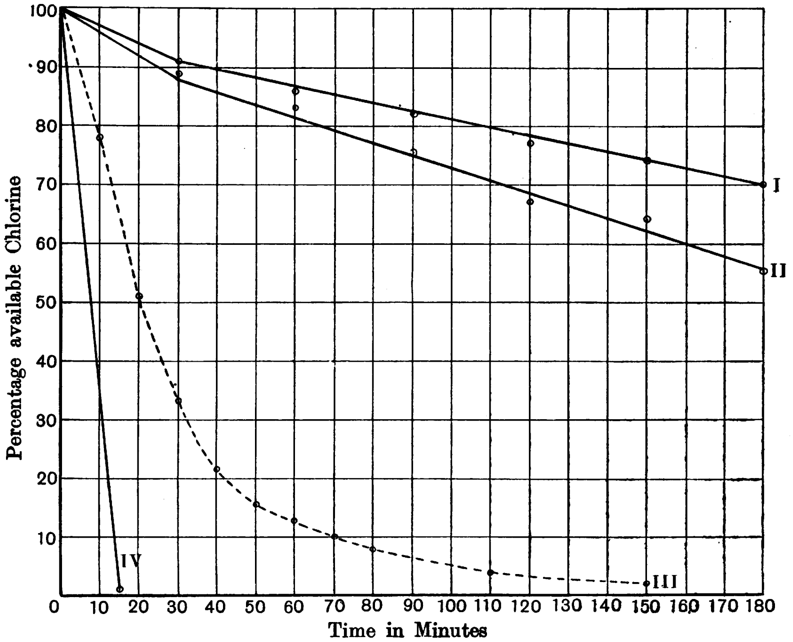
Fig. 4.

The fall in "available" chlorine appears to be a linear one of slow velocity usually with a short initial period with a more rapid rate of change. It is evident that with albumin concentrations such as were tested with hypochlorites the rate of fall in available chlorine is much slower in the case of chloramine and varies directly with the serum concentration. It is in the later stage of the hypochlorite-albumin reaction that one obtains a rate of change similar to that observed with chloramine. Even with chloramine-albumin mixtures, especially in the case of the higher albumin concentrations there is also however an initial more rapid rate of change but there is not the same tendency for the chlorine to pass into the cyclic rings in the protein, as is evident from a comparison with the hypochlorite-albumin curves. It is interesting to note that with 50 % serum in a 1 % chloramine mixture, there is a rapid initial fall of a little over 80 % chlorine during the first half hour followed by a slower rate of loss, so that at the close of 2 hours the active chlorine is about 5 % of the initial concentration. Dakin, Cohen, Daufresne and Kenyon [1916] used 50 % serum concentrations for their bacteriological tests, and as they used 1–1000 to 1–2000 chloramine concentrations the available chlorine must have been brought down to an extremely low level shortly after admixture with the serum.

THE ACTION OF HYPOCHLORITES AND CHLORAMINE-T ON AMINO-ACIDS.

Langheld [1909] has studied the action of NaOCl on various amino-acids, glycine, alanine, valine, leucine etc. He found that the α-amino-acids readily react with the hypochlorite in the same way as simple amines, forming in the first place chloramino-derivatives of the acids which, on warming, readily break down forming aldehydes or ketones, ammonia, carbonic acid and sodium chloride. The products obtained from the decomposition of glycine are formaldehyde, ammonia and carbonic acid. This change takes place very rapidly. Thus when equimolecular solutions of $Mg(OCl)_2$ and glycine (1·151 g. of the former and 0·68 g. of the latter in 200 cc.) are kept at 36°, within 15 minutes over 99 % of the "available" chlorine is lost (Fig. 5 IV). A similar mixture of equimolecular chloramine and glycine, $m/20$ in each case, shows a much less rapid fall in available chlorine (Fig. 5 III). Amino-acids such as hippuric acid, which have an acid group attached to the nitrogen offer much greater resistance to the action of hypochlorite, but at 36° slow decomposition occurs as is shown in Fig. 5 I and II. Fig. 5 I shows the fall in available chlorine when 0·812 g. hippuric acid (as sodium salt) is added to 200 cc. $Mg(OCl)_2$ $m/20$ and Fig. 5 II shows the fall when $m/40$ $Mg(OCl)_2$ is taken. The nitrogen value of the added hippurate was equal to that of 5 cc. serum. The type of fall in these cases is similar to that seen in chloramine-serum reactions and differs greatly from the hypochlorite-serum reaction. Thus an increase in the hippurate-hypochlorite ratio leads to a regular displacement downwards of the curve. Chloramine-T on the other hand shows no loss in its available chlorine even after prolonged warming with sodium hippurate.

Hypochlorite solutions therefore most readily attack the amino-acids when the N is not attached to an acid group, less readily proteins, and with greatest difficulty hippuric acid. The same order holds for chloramine-T solutions but in each case these solutions are much less effective than the hypochlorites. It seems most probable that the later slower fall in available chlorine occurring in protein-hypochlorite mixtures exhibits really the rate of change of chloramino-compounds formed by the action of the hypochlorite on the protein molecule.



200 cc. Mg$(OCl)_2$ $m/20$ + Hippuric Acid ·812 gm. = I
„ „ $m/40$ + „ „ „ = II
200 cc. containing $m/20$ Chloramine-T + $m/20$ Glycine = III
„ „ 1·151 gm. Mg$(OCl)_2$ + 0·68 gm. „ = IV

Fig. 5.

If the germicidal action of the chloramines be a function of the available chlorine, as is probably the case, then there may be an advantage in the employment of chloramine solutions in the treatment of infected wounds when it is desired to maintain the available chlorine concentration in the presence of albumin at a moderately high level. Thus in cases where fairly long intervals elapse between the dressing of wounds, the slower rate of change of the solutions of chloramine compared to those of hypochlorite might prove of advantage. In the primary disinfection of septic wounds, however, the greater rate at which the hypochlorite gives up its available chlorine in the attack on the protein molecule will undoubtedly result in a more efficient penetration and oxidation of necrosed material. This solvent action on necrosed tissue material is undoubtedly a valuable feature in the action of alkaline hypochlorites. The hypochlorite solutions will also act more rapidly on the products of bacterial action than the chloramine solutions of a similar strength.

INTRAVENOUS INJECTION OF HYPOCHLORITES AND CHLORAMINE-T.

At Professor Lorrain Smith's request the physiological effects produced by the intravenous injection of hypochlorite solutions were investigated. From the preceding study of the changes which hypochlorites undergo in albuminous solutions, one recognised that on injection into the blood there would be a very rapid fall in available chlorine taking place near the seat of injection, but there was always a possibility that the more resistant chloramine derivatives might enable the action to be sufficiently prolonged to render the blood germicidal. There was also the possibility that the powerful oxidising action of the hypochlorites might render innocuous certain products of bacterial action. This seemed much more likely than the former hypothesis, as in the cases, which have been described by Lorrain Smith and his co-workers [1915, 2], of benefit resulting from intravenous injection of comparatively small quantities of hypochlorite in the treatment of septic conditions in man, the available chlorine concentration of the blood must have been much below the germicidal level.

Hypochlorite solutions however attack the red blood corpuscles and this action must be borne in mind. Solutions of extremely low hypochlorite concentration injected in isotonic saline solution give rise to haemolysis and the pigment so set free is with greater concentrations broken down finally to colourless derivatives. The following table shows the concentrations at which haemolysis takes place when a 4 % blood corpuscle emulsion is mixed with isotonic hypochlorite and chloramine-T solutions. The tubes were left overnight at 37°.

	"Available" chlorine concentrations		
	1–20,000	1–7000	1–2500
Eusol	Very slight haemolysis	Reddish brown opaque solution	Reddish brown
Dakin's solution	,, ,,	Clear deep red solution	Pigment bleached yellow
$Mg(OCl)_2$ ...	,, ,,	,, ,, ,,	Clear deep red solution
Chloramine-T*	No haemolysis	No haemolysis	Very slight haemolysis

* (Concentrations in terms of chloramine-T not chlorine.)

On injection into the blood laking does not occur quite so readily as in the test tube experiments, but it was met with in every case of hypochlorite injection where the available chlorine concentration of the blood had been raised to 1–3000. Probably it occurs with much lower concentrations. In practically no case did chloramine injections give rise to laking. Great care requires to be taken in the case of hypochlorite injections to prevent prolonged contact of the solution with the vessel wall as there is a gradual disintegration of the lining endothelium with a possible subsequent leakage and irritation at the seat of injection. Hence as soon as the injection is finished saline ought to be used to wash away the irritant. It is advisable to use therefore a vaselined double-way cannula, so that the saline may be injected without delay after the hypochlorite.

The intravenous injections were carried out with a view to determine the point to which the available chlorine concentration of the blood might be raised without seriously affecting the circulation and respiration. The actions of eusol, Dakin's solution, $Mg(OCl)_2$ solution and chloramine-T were investigated. As the results are much the same in the case of the various hypochlorites a short general summary will be sufficient.

The experiments were carried out on rabbits, cats and in one case on the dog. When concentrations are specified, they refer to cats of approximately 2½ kgm., unless otherwise stated.

In all cases recovery is complete when the available chlorine concentration of the blood is not raised much above 1–2000, but distinctly higher concentrations may be tolerated, for example 1–1000 or rather more. $NaOCl$ and $Mg(OCl)_2$ may be used in slightly higher concentrations than eusol.

The first injections ought to be weaker than the later ones as the primary effects on circulation are greater than those obtained from the subsequent injections. Hypochlorite solutions containing 0·25 % "available" chlorine produce practically no disturbance until the chlorine concentration of the blood is raised to 1–1500 that is until about 40 cc. have been injected in an animal of 3 kgm. 10 cc. of a 0·5 % solution usually give rise to an initial fall of blood pressure which is quickly recovered from and the second 10 cc. is usually without effect, but on raising the concentration to a higher level the blood pressure begins to fall.

The respirations are quickened after each injection and at least in the case of $Mg(OCl)_2$ are diminished in amplitude. Eusol in concentrations of 0·35 % or higher usually gives rise immediately after injection to slightly irregular respiratory movements. Even when hypochlorite has been added in sufficient quantity to raise the concentration in the blood to 1–600 no hypochlorite or active chlorine reaction can be obtained in a sample of blood withdrawn about half an hour after the injection. If adrenaline however be injected very shortly after hypochlorite is given, care being taken to remove the latter from the seat of injection, the usual rise in blood pressure is not obtained.

This may be due to the laked blood destroying the adrenaline, as is said to occur, or may be due to destruction by oxidation.

In all cases where the chlorine concentration is 1–2000 or more, the blood plasma after centrifugalisation is deep red in tint and the spectrum is one of oxyhaemoglobin. The coagulability of the blood is decreased. The secretion of urine owing to the saline injections is increased and in most cases it is also red in colour due to the presence of free oxyhaemoglobin.

CHLORAMINE-T INJECTIONS.

The blood pressure is more readily affected than by hypochlorite injections. There is always a slight fall in blood pressure after the injection of 30 cc. 0·25 % chloramine into animals of an average weight of 2½ kilogrammes. When 40 cc. 0·25 % solution are injected the fall is not recovered from. This would give a chloramine concentration in the blood of 1–1250. Injections of 10–20 cc. 0·5 % chloramine and over give rise at first to a fairly rapid then a gradual fall in blood pressure which is usually not recovered from. At the same time respirations are slowed and there may be brief apnoeic periods. It is advisable therefore not to inject solutions of greater strength than 0·2 % and the chloramine concentration in the blood ought not to be raised above 1–2000. No haemolysis results from the chloramine injection of this concentration. Adrenaline injections give the usual rise of blood pressure even when given immediately after the chloramine. The coagulability of the blood is apparently not affected. Specimens of blood withdrawn after chloramine injections do not give the reactions of active chlorine.

Dakin and his collaborators have shown that in the presence of 50 % serum the germicidal concentration of chloramine-T is approximately 1–2000. Under the conditions of possible intravenous injection in man when comparatively small quantities of chloramine would come into immediate contact with substances which would rapidly reduce the available chlorine, it is extremely unlikely that anything approaching even a temporary germicidal concentration could be attained without producing dangerous circulatory and respiratory disturbances. Intravenous injections of hypochlorites and allied substances may however prove of value in general septic conditions in some other way than by simply rendering the blood germicidal. The extremely powerful and rapid action of the hypochlorites on the proteins and most of their amino-derivatives makes it probable that a similar action will take place on the products of bacterial action.

REFERENCES.

Dakin (1915, 1). *British Med. J.*, 318.

—— (1915, 2). *British Med. J.*, 609.

—— (1916). *British Med. J.*, 852.

Dakin, Cohen, Daufresne and Kenyon (1916). *Proc. Roy. Soc.*, **89**, 232.

Dakin, Cohen and Kenyon (1916). *British Med. J.*, 160.

Effront (1905). *Ber.*, **37**, 4290.

Langheld (1909). *Ber.*, **42**, 392, 2360.

Mayer (1916). *Paris Méd.*, **8**, 207.

Smith, Lorrain (1915, 1). *British Med. J.*, 129.

—— (1915, 2). *British Med. J.*, 716.