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THE IMPORTANCE OF METAL SALTS IN IMMUNIZATION AND ESPECIALLY IN THE PRODUCTION OF DIPTHERIA ANTITOXIN AND OF AGGLUTININ FOR B. COLI

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INTRODUCTION

Numerous studies have been made of the conditions of antitoxin production in the animal organism since the introduction of serum therapy. Many tests have been made for these studies relating to weakening of the antigen, decrease or increase in the antigen doses, the use of different toxins, different ways of injecting, the timing of inoculations, the combination of active and passive immunization, the use of smaller or larger bleedings, etc. These tests have, in addition to their theoretical interest, great practical value for the production of therapeutic antitoxins under optimum conditions.

These investigations have yielded much information, but the deeper aspects of the immunity phenomena in general and of the antitoxin formation in particular are still little known.

The experiments confirmed the observations of Brieger and Ehrlich [1], Salomonsen and Madsen [2-3], Madsen and Jorgensen [4], and they indicate that the rules that apply to the formation of different antibodies seem to be the same throughout: Immediately after injection of the antigen a decrease in the antitoxic strength of the serum is noticed (first negative phase), subsequently a rise occurs (positive phase) and finally a gradual decrease (second negative phase); this curve may be considered as the resultant of antitoxin-

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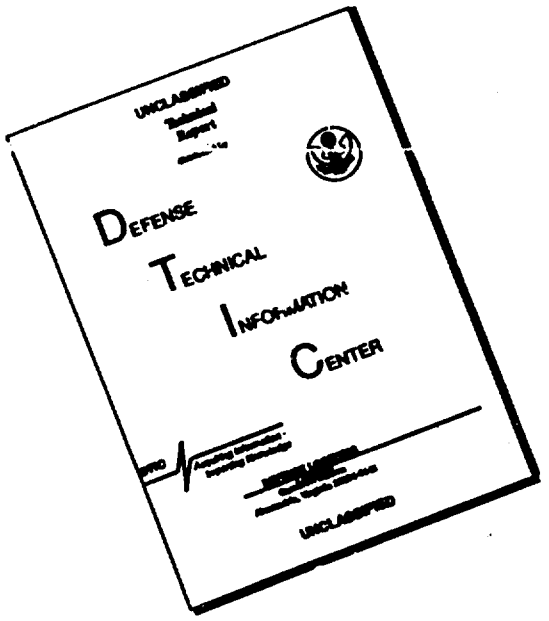

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producing- and destroying-forces in the organism (Madsen [5]).

In the first studies of immunity it was assumed that the appearance of antitoxins in the organism was due to a direct transformation of the injected toxin. Salomonsen and Madsen were the first to suggest that in an actively immunized animal production and destruction of antitoxin are continuous. This opinion has been increasingly confirmed and may be considered generally accepted.

Salomonsen and Madsen [6] showed that pilocarpin increases secretions in general and produces an increase of the antitoxin level of the blood, which is, however, of a transitory nature.

The fact that a large bleeding stimulates antitoxin formation (Roux and Vaillard [7], Salomensen and Madsen [8], Friedberger and Berner [9], Schröder [10], etc.) induced other authors to assume that there is a direct relation between globular blood regeneration and antitoxin production (Pfeiffer and Marx [11], Wasserman and Takaki, Debré, Levaditi, e.a.).

Madsen and Tallquist [12] have followed this idea and they induced formation of red corpuscles in test animals by injecting hemolytic poisons (pyrodin and pyrogallol); simultaneously they observed a parallel rise in the antitoxin curve. Müller [13] found in addition with hetol the existence of a certain relation between the effect of leucocytoses-inducing substances and the formation of antitoxin.

Fürst [14] observed subsequently that injection of methylene blue causes a decrease in the number of leucocytes in the blood and a weak increase in the serum value. Walker [15] succeeded in stimulating agglutinin formation in rabbits by means of salvarsan injections. Hectoen and Corper [16] studied the influence of thorium on the precipitin formation; they found it to be considerably delayed when thorium is injected at the beginning of the immunization process; when precipitin formation has set in the thorium injection has no effect.

Walbum [17] has recently investigated the importance of the action of different metal salts on antitoxin formation from a theoretical standpoint. This is what he writes: "Due to the nature of the problem and our present state of knowledge it is difficult to discuss the mechanism of antitoxin formation in the organism, but whatever the mechanism

(synthetic actions, splitting, intramolecular rearrangements), it may be assumed that enzymatic interactions play a certain part, as in all cellular activity.

Certain conditions have to be fulfilled to enable the full effectiveness of enzyme action, but in most cases the nature of these conditions is insufficiently known. It has, however, been found that the presence of certain metal salts may have a great and sometimes decisive influence (several oxydases) on the effect of many enzymes (catalysts, coenzymes). This induced me to assume that certain metal salts may also play a part in the antitoxin formation; if this is true, it is possible that the sometimes very large individual differences in antitoxin productivity found in animals may be due partly to the type and quantity of metal salts present in the organism. It may be assumed that any animal capable of reacting to toxin injection by the formation of the antitoxin has the necessary specific enzyme, (or enzymes) for such a reaction. These enzymes would determine the qualitative aspects of the antitoxin formation, whereas the type and quantities of metal salts (catalysts) present would to some extent determine the quantitative aspects of this formation. If this be so one may expect that the administration of certain metal salts to the organism during immunization will increase antitoxin productivity and consequently raise the concentration of this antitoxin in the blood."

On the basis of these considerations Walbum has made some experiments on goats immunized against B. coli and on horses immunized against diphtheria toxin, using manganese chloride, nickel chloride, cobalt chloride and zinc chloride with these animals.

All salts studied had a stimulating effect on antitoxin formation; sometimes the effect was very marked.

Encouraged by these results we introduced manganese chloride treatment in practice, and it will be shown that this change in immunization technique resulted in an increase of antitoxin production.

This problem may have both theoretical and practical interest, and therefore we have studied it carefully. We do not, however, consider that the problem has been completely solved, but we believe that our experiments elucidate it to some extent and that is our reason for publishing our results now.

I. EXPERIMENTS WITH ANIMALS IMMUNIZED AGAINST DIPHTHERIA

(Goats and Horses)

1. Goats Immunized Against Diphtheria

The experiments were carried out with three goats immunized by the method in general used at the Serum Therapy Institute (Table I) consisting of alternate injections of toxin and antitoxin during the first immunization period. A relatively fast increase in the quantity of toxin injected is achieved in this way and the immunization time is considerably reduced.

TABLE I

Immunization Schedule for Goats No 1, 2, 3.

DATES	ANTITOXIN AND TOXIN DOSES INJECTED	METHOD OF INJECTION
15 OCTOBER 1920	2000 STANDARD UNITS	INTRAVENOUS
17 —	0.5 cc TOXIN	SUBCUTANEOUS
19 —	2000 S.U.	INTRAVENOUS
21 —	1 cc TOXIN	SUBCUTANEOUS
23 —	2000 S.U.	GOAT 1 INTRAVENOUS GOATS 2, 3 SUBCUTANEOUS
25 —	2 cc TOXIN	SUBCUTANEOUS
27 —	2000 S.U.	"
30 —	3 cc TOXIN	"
1 NOVEMBER 1920	2000 S.U.	"
3 —	5 cc TOXIN	"
5 —	8 cc "	"
9 —	12 cc "	"
13 —	20 cc "	"
17 —	30 cc "	"
22 —	40 cc "	"
29 —	60 cc "	"
9 DECEMBER 1920	100 cc "	"

Goats are known to be poor producers of diphtheria antitoxin and moreover they are rather sensitive to the toxin, and therefore a relatively weak toxin was used (average lethal dose approximately 0.007); the antitoxin used was horse serum that disappeared relatively fast from circulation because it is a heterologous serum, and thus did not cause by itself a measurable increase in antitoxin.

The toxin was injected subcutaneously. In the beginning (on the 15th and 19th of October) antitoxin was injected intravenously, but on the 23rd of October intravenous injection caused a typical anaphylactic shock (sneezing, convulsions, dyspnea and a drop in temperature to 36.8°C) in goat number 1. Subsequent serum injections were therefore given under the skin at the opposite side to that where the toxin was injected.

The metals Mn and Co were used as $MnCl_2 \cdot 4\ H_2O$ and $CoCl_2 \cdot 6\ H_2O$ in 0.01 normal solution in physiological solution. Each dose of either $MnCl_2$ or $CoCl_2$ consisted of 25 cc 0.01 normal solution injected intravenously. The solution was heated to 37° before injection. Blood samples for titration were taken immediately before each toxin injection and determinations were carried out by the customary method.

Goat No 1 (Blank with no metal salt). A two year old male goat, weighing about 40 kg was treated as indicated in Table I. Immunization was free of incidents except for the above mentioned anaphylactic shock. The antitoxin level in the serum rose gradually after each toxin injection, and it was 45 S. U. per cc at the end of the experiment. The weight of the animal was 43 kg at that time.

Goat No 2 (manganese chloride). A two year old male goat weighing 40 kg received daily injections of manganese chloride from the start of immunization, from 15 October to 4 November when injections were stopped for 24 days in order to see whether the shape of the antitoxin curve was influenced; from 29 November to the end of the experiment on 17 December Manganese chloride was again injected daily. The antitoxin strength of the serum rose relatively sharply compared to goat No 1 during the first immunization period, but the rise stopped as soon as the administration of manganese chloride was discontinued. The result was a period when antitoxin concentration did not nearly increase as fast as in goat No 1 (No 1 from 1 S. U. per cc to 15 S. U. per cc; No 2 S. U. per cc to 8 S. U. per cc).

When the manganese injections were resumed they stimulated antitoxin formation so strongly that the antitoxin level at the end of the experiment reached 165 S. U. per cc compared to 45 S. U. per cc for goat No 1. The animal weighed 44 kg at that time.

TABLE II

DATES	GOAT No 1		GOAT No 2		GOAT No 3	
	INJECTIONS	S.U./cc	INJECTIONS	S.U./cc	INJECTIONS	S.U./cc
25 OCTOBER		0,3	15 oct.-4 nov.	0,4	15 oct.-4 nov.	0,2
30 —		0,5	DAILY INJEC-	0,5	DAILY INJEC-	0,25
5 NOVEMBER		1	TIONS OF $MnCl_2$	2	TIONS OF $CoCl_2$	0,5
9 —	NO	2		2		0,75
13 —		5	5 nov.-29 nov.	2	5 nov.-29 nov.	1
17	METAL	8	Pas de	2,5	Pas de	2
22 —	SALT	10	$MnCl^2$.	5	$CoCl^2$.	3
29 —		15		8		8
9 DECEMBER	INJECTIONS	30	29 nov.-17 déc	55	29 nov.-17 déc	50
13 —		35	DAILY INJEC-	100	DAILY INJEC-	65
18 —		45	TIONS OF $MnCl_2$	•	TIONS OF $CoCl_2$	75

Goat No 3 (Cobalt chloride). Two year old male goat weighing 30 kg treated in the same way as goat No 2 but with cobalt chloride instead of manganese chloride. Cobalt chloride injection, does not seem to influence antitoxine formation in the first immunization period, but in the second period the $CoCl_2$ injections caused a sudden and rapid rise in the curve, so that the antitoxic potencey at the end of the experiment was 75 S. U. per cc. The ani al weighed then 30 kg.

The variations in antitoxin level of the serum of these goats are graphically represented in Figs. 1 and 1a.

The dotted parts of the curve indicate (as in all other curves of this paper) the changes in antitoxin level caused by toxin injections only, whereas the parts drawn in solid lines indicate the parts where the metal salt cooperated.

The following conclusions may be drawn from these experiments:

1. Intravenous injection of $MnCl_2$ and also of $CoCl_2$ in goats immunized against diphtheria increases considerably the antitoxin production of these animals.

2. Manganese chloride seems to have a stronger effect than cobalt chloride.

These experiments may have a certain practical value in addition to their theoretical interest. Efforts have been

made to use antidiphtheria serum of the goat in serum therapy, but it has in general not been possible to raise the antitoxin potency of the serum of these animals above 20-30 S. U. per cc by the usual immunization technique and this is insufficient for therapeutic application.

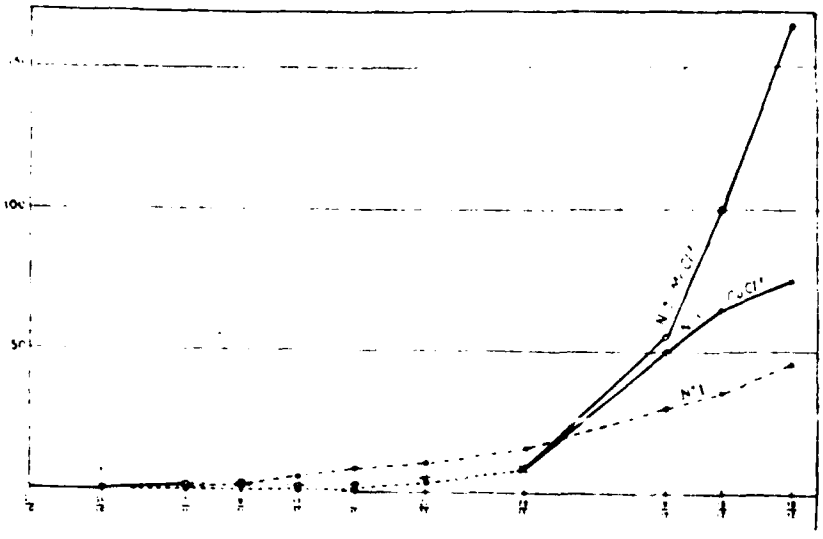

Fig. 1.* Goats Immunized Against Diphtheria. The Numbers Placed Below the Figure Show the Dates.

2. Horses Immunized Against Diphtheria

A series of experiments on horses that produce antidiphtheria serum was carried out in addition to these experiments on goats. The horses were immunized by the same method as the goats and they had supplied serum for various periods. The metal salts were injected either (A) during the period of descending antitoxin curve, or (B) during the period of reinjection with toxin. Finally we tried (C) manganese chloride administered orally.

A. Injection of $MnCl_2$ and $CoCl_2$ During the Period of a Drop in Antitoxin Potency, Without any Toxin Injection

The experimental technique was as follows: at the end of the initial immunization period the horses were bled on the ninth day after the last toxin injection, as usual; the

*In Figs. 1 through 14 the ordinates represent antitoxin units per cubic centimeter.

serum was then titrated. If the antitoxin potency decreased daily intravenous injections were started with 10 cc of a normal solution diluted with the same amount of water (0.99 % $MnCl_2 \cdot 4\ H_2O$ and 1.19 % $CoCl_2 \cdot 6\ H_2O$).

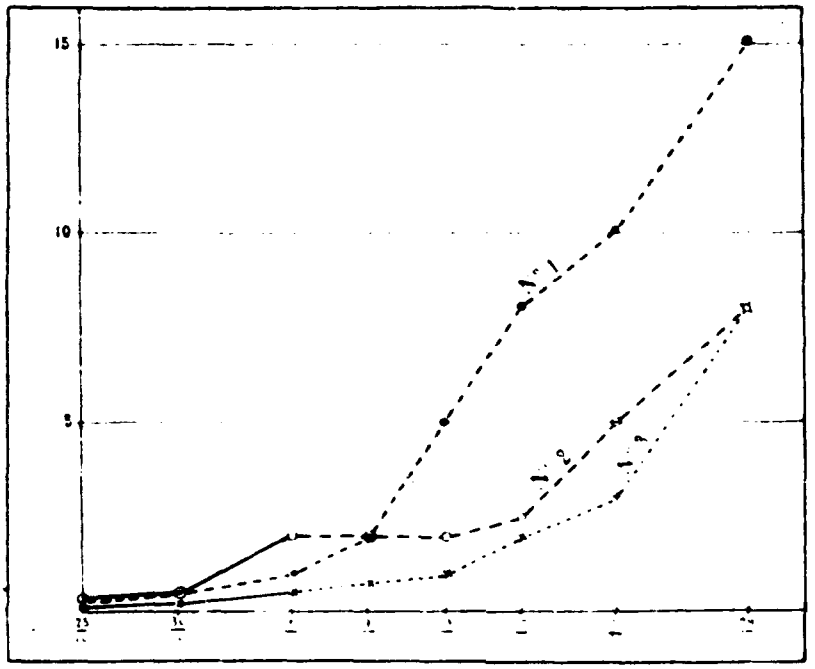

Fig. 1a. Goats Immunized Against Diphtheria. The Figures at Bottom Indicate Dates.

As soon in the test on horse number 30, men tioned subsequently, this is the maximum amount of salt that may be administered without impairing the animal's health. The intravenous injection is always performed in the jugular, and it has to be carried out very carefully to prevent that even a very small amount of the solution enters in the surrounding tissues. In this case extensive hard infiltrations are produced, often accompanied by edema; these can become extremely irritating to the animal, diminishing its mobility and appetite. An injection into the animal's arteries can cause its immediate death.

In the period before the metal salt injections test bleedings were made on alternate days, and during the rest of the experiment every day.

a) Manganese Chloride

Horses Nos 353 and 354. - After immunization these horses were first bled on 1[illegible] August. The blood of horse No 353 contained then 1250 S. U. per cc and that of horse No 354 750 S. U. per cc, but the antitoxin level decreased after the bleeding; it was down to 700 S. U. per cc for the first one, to 450 S. U. for the second on the second day. From this date (18 August) to the end of the experiment $MnCl_2$ was injected daily. The antitoxin curve, instead of falling further, started to rise to such an extent that after four days the serum of horse No 353 reached 1000 S. U. per cc, i.e., 80% of the antitoxin level obtained by energetic treatment with large diphtheria toxin doses for seven weeks. This high antitoxin level was maintained unchanged till 24 August when it dropped in spite of the continued manganese chloride injections. The amount of antitoxin in the serum of horse No 354 increased gradually throughout the manganese treatment and when manganese injections stopped it had reached 675 S. U. per cc, i.e., 90% of the serum potency after injection of 3 liters of strong toxin for seven weeks (see the antitoxin curves Figs. 2 and 3).

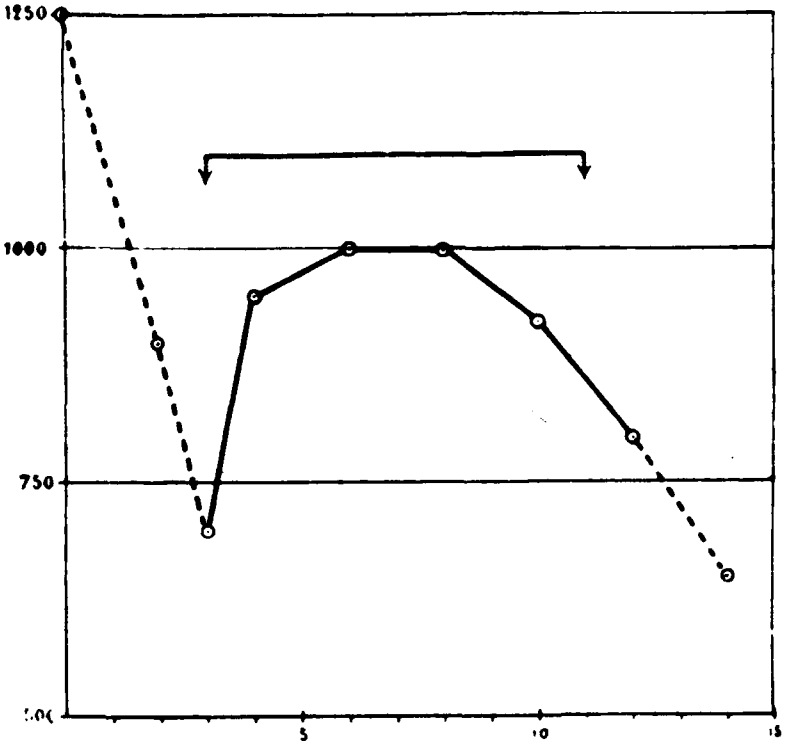

Fig. 2. Horse Immunized Against Diphtheria, No 338. $MnCl_2$. The Figures at the Bottom Indicate Days.

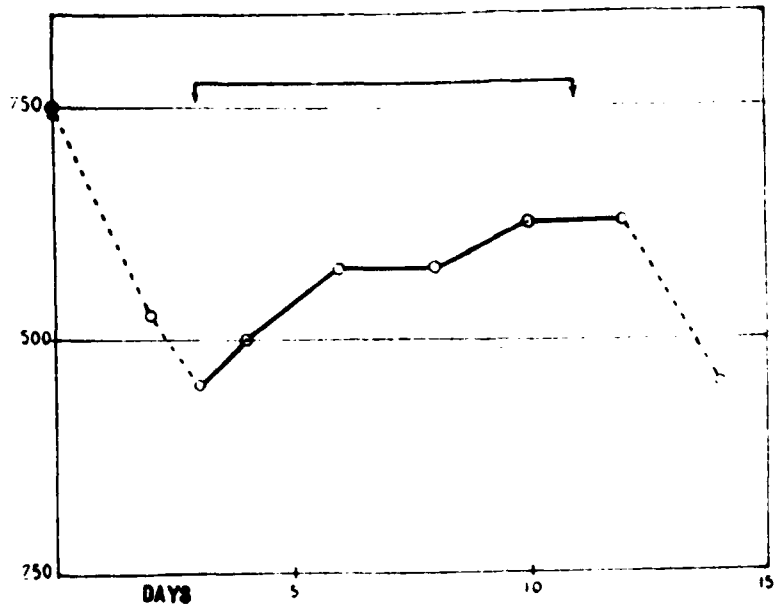

Fig. 3. Horse No 364 Immunized Against Diphtheria. $MnCl_2$.

Horse No 363. - This horse was first bled on 18 August (400 S. U. per cc), for the second time on 15 September (425 S. U.) and for the third time on 23 October (250 S. U.). On 2 November, i.e., nine days after this last bleeding, the animal's serum contained 160 S. U. per cc; from this day to the end of the experiment on 22 November manganese was injected daily. The antitoxin level increased so considerably through this treatment that after 9 injections (11 November) this serum contained 350 S. U., i.e., 40% over its strength after injection of 1200 cc toxin carried out over a period of approximately one month. The antitoxin curve dropped here soon, as with horse No 358 in spite of continued manganese injections (see antitoxin curve, Fig. 4).

Therefore in these three cases daily injections with $MnCl_2$ made the serum of horse 358 rise from 700 S. U. per cc to 1000 S. U., that of horse 364 from 450 S. U. per cc to 575 S. U. per cc, and finally that of horse 363 from 160 S. U. to 350 S. U. per cc. It follows then from these experiments that intravenous injection of manganese chloride in horses immunized against diphtheria without any toxin injection has a very marked stimulating effect on antitoxin production.

These experiments were made by analyzing the antitoxin potency of the serum 24 hours after the injection of metal salt, and immediately before the next injection, and therefore

they do not give information about the rate of antitoxin increase. To examine this we carried out the following experiment with horse No 377.

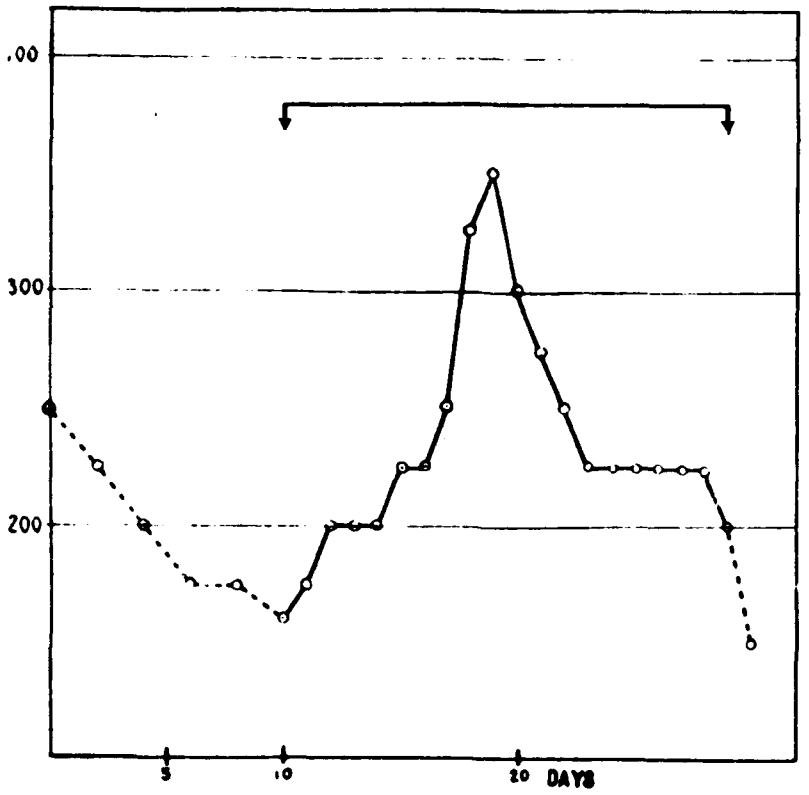

Fig. 4. Horse No 363 Immunized Against Diphtheria. $MnCl_2$. Daily Injection of Manganese.

This animal was bled on 20 April and 22 April (the serum strength was 550 S. U.). On 2 May at 8 o'clock in the morning a sample bleeding was performed, and immediately afterwards 10 cc of a normal $MnCl_2$ solution, diluted by half was injected in the vein, and 5, 15, 30, 45, 60 minutes and 2, 3, 5, 8, 12, 24 and 30 hours after the injection additional sample bleedings were made (Fig. 5).

Examination of Fig. 5 shows that the effect is instantaneous upon injection and that the antitoxin curve rises suddenly. The curve reaches a peak after approximately two hours and the antitoxin increase is then approximately 16 to 17%. The curve then falls off gradually, but 24 hours after manganese injection the antitoxin level of the serum remains about 8% above the level before injection.

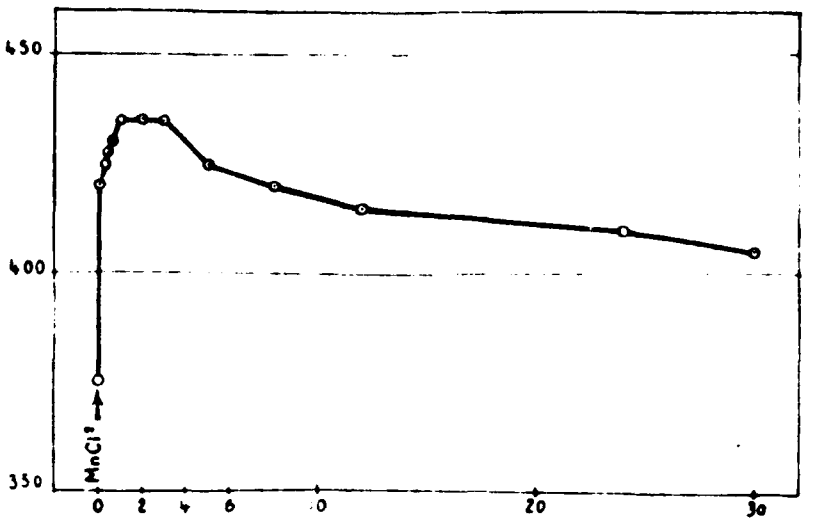

Fig. 5. Horse Immunized Against Diphtheria. One Single $MnCl_2$ Injection. The Figures at the Bottom Indicate Hours.

It is clear that a fundamental difference exists between the effect of a toxin injection and that due to a metal salt injection; the former is often accompanied by a drop in the antitoxin curve (first negative phase) the latter seems to have the opposite effect.

A problem like the one investigated here has obviously much theoretical interest, it may profitably be applied to practice, since a manganese chloride injection one hour before bleeding makes it possible to increase the antitoxin potency of the serum.

b) Cobalt Chloride

Horse No 356. - The blood of this horse contained at the bleeding of 30 November 275 S. U. per cc. On 1 November the antitoxin level had fallen to 175 S. U. It was then wounded at the level of the foot (from a nail in the street) with a fairly high fever; an increase of antitoxin level followed, reaching 200 S. U.; when the $CoCl_2$ injections started this increase had yielded to a decrease (175 S. U.) From 12 November daily injections of $CoCl_2$ were given; the antitoxin level rose gradually to 225 S. U. per cc or approximately 28% increase (Fig. 6).

Cobalt chloride has thus also a stimulating effect on diphtheria antitoxin formation in immunized horses, but it is much

less pronounced than the effect of manganese chloride, similar to what was found for goats.

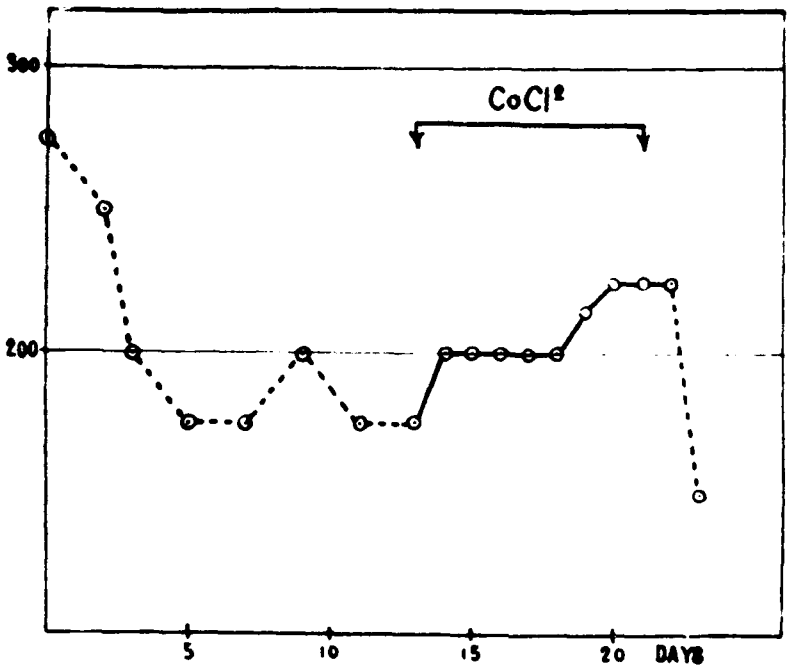

Fig. 5. Horse Immunized Against Diphtheria, No 355. $CoCl_2$.

B. Injection of $MnCl_2$ and of $CoCl_2$ in Serum-Producing Horses During Toxin Reinjection, After the Initial Immunization Period

Antidiphtheria-serum-producing horses at the Serum Therapy Institute are subjected to the following bleeding and reinjection schedule after the initial immunization period.

After bleeding the animal is rested for eight days then 200 cc toxin is injected, eight days later 400 cc and again after eight days 600 cc. On the ninth day after this last injection a first bleeding of 8 liters is carried out, and two days later another bleeding of 7 liters.

It is known what fluctuations the antitoxin curve can go through after these successive reinjections and bleedings. It is clear that the best choice of time to evaluate the effectiveness of the manganese injections, for example, from the standpoint of increase in antitoxin potency would be the moment when the curve is either stationary or descending. This has been done in the experiments reported here. (The plots of the antitoxin potency before these experiments may be seen in Figs. 7 and 8)

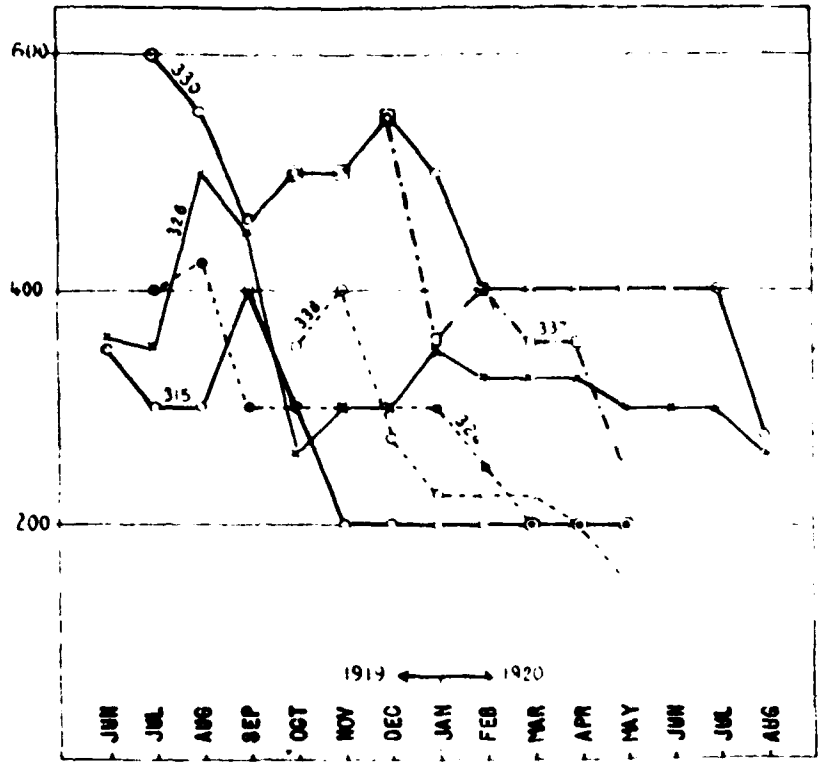

Fig. 7. Horses Immunized Against Diphtheria.

The values of the antitoxin levels given subsequently represent the mean of the two bleedings carried out after a period of toxin reinjection.

a) Manganese Chloride Injections

Horses Nos 309, 348, 334 and 347 were subjected, each at different periods, to intra enous injections of 10 cc of a normal solution diluted to half strength, for each dose. Tests made on horse 309 had shown that this dose could not be exceeded without serious upsets, shivering, excessive sweating, fever, etc...

Horse No 309 (Fig. 9). At each bleeding performed on 29 January, 3 March, 7 April and 8 May, the antitoxin level of this horse was 200 S. U.; it stayed thus in a stationary state that was very favorable for an experiment of the type that we intended to carry out.

From 12 to 15 May, 10 cc normal $MnCl_2$ solution diluted by half were injected, on 19 May 25 cc, on 20 May 30 cc; the

animal did not stand up well to these injections and it was allowed to rest till 26 May. From 26 May to 12 June daily injections of 18 cc of $MnCl_2$ without any incident. On 12 June a sample bleeding, and the antitoxin level was 325 S. U.

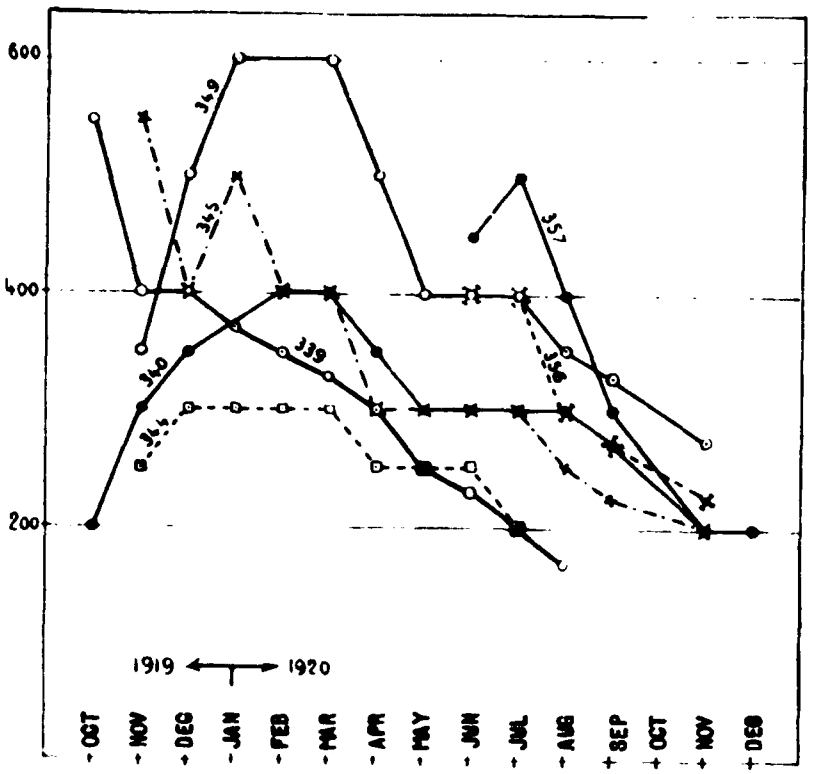

Fig. 6. Horses Immunized Against Diphtheria.

From 22 June to 15 July, after a 200 cc toxin injection daily injections of $MnCl_2$. On 15 July the antitoxin level is 350 S. U.

From 30 July to 15 August, after 400 cc toxin manganese chloride is injected daily and the bleeding on 16 August gives 375 S. U.

From 10 to 20 September after 600 cc toxin renewed daily injections of $MnCl_2$ are given. On 20 September: 350 S. U. The horse shows at this time infiltrations on either side of the neck, due to the manganese injections.

From 12 to 16 October, after 400 cc, $MnCl_2$ is given daily. The injections are interrupted on 16 October as the infiltrations near the jugular have increased. The injections

are started again [illegible] continued t[illegible] October, on [illegible] this date the [illegible]al is bled. The antitoxin level is [illegible] S. U.

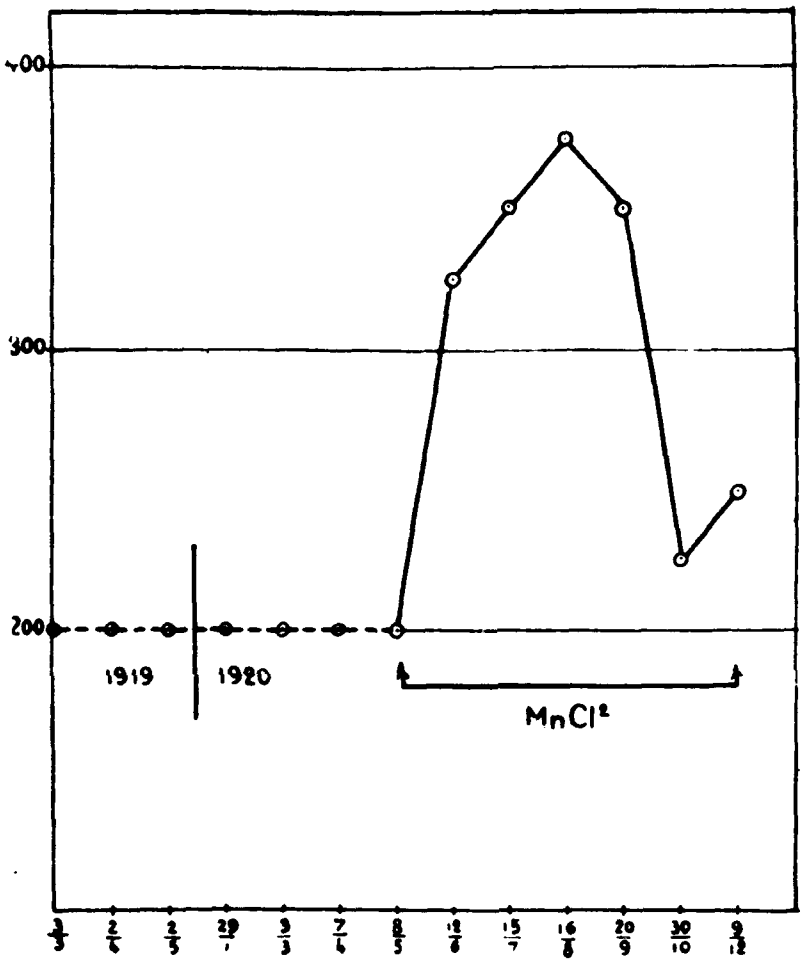

Fig. 9. Horse Immunized Against Diphtheria No 309. The Figures at the Bottom Indicate Dates.

From 1 to 9 December renewed daily injections of $MnCl_2$. On 9 December: 250 S. U.

This horse had thus originally a steady antitoxin level in the serum of 200 S. U. and under the influence of manganese chloride injections it rose to 375 S. U.

In the case of horse 309 the manganese chloride injections could not prevent the subsequent fall in the antitoxin potency, but this was possibly due to the interruptions of the manganese treatment that the state of health of the animal made necessary.

Horse No 348 (Fig. 10) - The antitoxin level before the manganese chloride injections was 325 S. U. per cc.

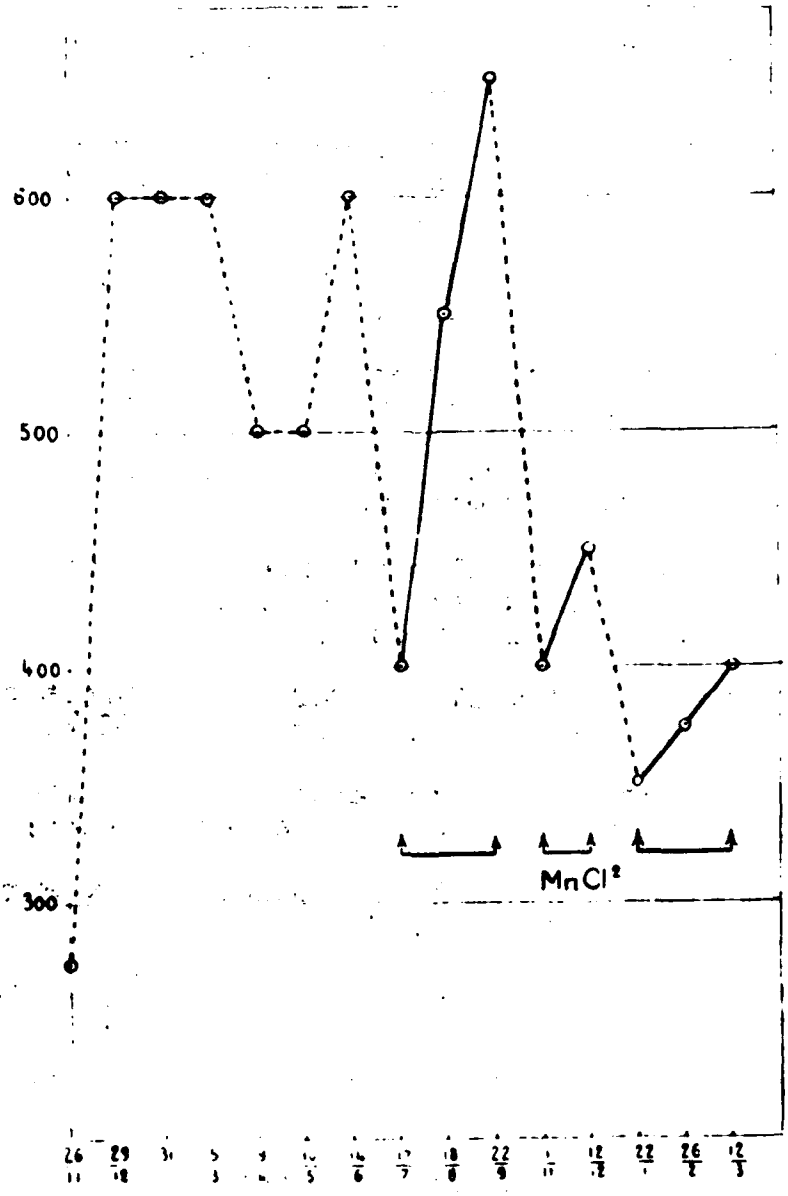

Fig. 10. Horse Immunized Against Diphtheria, No 348. The Figures at the Bottom Indicate Dates.

This horse underwent the same treatment as No 309 from 12 May to 12 June; consequently on 22 June his serum reached 575 S. U.

From 12 June to 15 July toxin only is injected without manganese: drop of the serum to 325 S.U.

From 30 July to 16 August, after 400 cc toxin, daily injections of $MnCl_2$. On 16 August, 450 S. U.

From 16 August to 20 September, injections of toxin only. On 20 September, 300 S. U. As the animal showed infiltrations, it was rested till 27 October.

From 27 October to 30 October renewed daily injections of $MnCl_2$. On 30 October, 225 S. U.

From 1 December to 9 December manganese is given daily. On 9 December 250 S. U. The animal is killed.

The increase in serum level in this animal shows clearly the stimulating effect of manganese.

The decrease that followed after this rise can probably be blamed on the small number of metal salt injections and on the animal's state (infiltrations).

Horse No 316 (Fig. 11). - Before the treatment with manganese chloride the antitoxin level was 300 S. U. per cc.

From 14 June to 23 June, after 600 cc toxin, daily injections of manganese chloride. Level on 23 June: 350 S. U.

From 25 June to 24 July no toxin, but manganese chloride every day. On 24 July: 400 S. U.

From 12 August to 30 August, after 400 cc toxin, daily treatment with manganese. On 30 August: 500 S. U.

From 17 September to 4 October, the same treatment. On 4 October: 600 S. U. The horse, a very old one, is killed.

The antitoxin level in the serum is thus increased in this horse from 350 S. U. to 600 S. U. When this curve is compared to those of test horses that received only toxin, and where the serum dropped to 200 S. U., it is realized how exceedingly favorable the effect of manganese injections is on the rise of the antitoxin level.

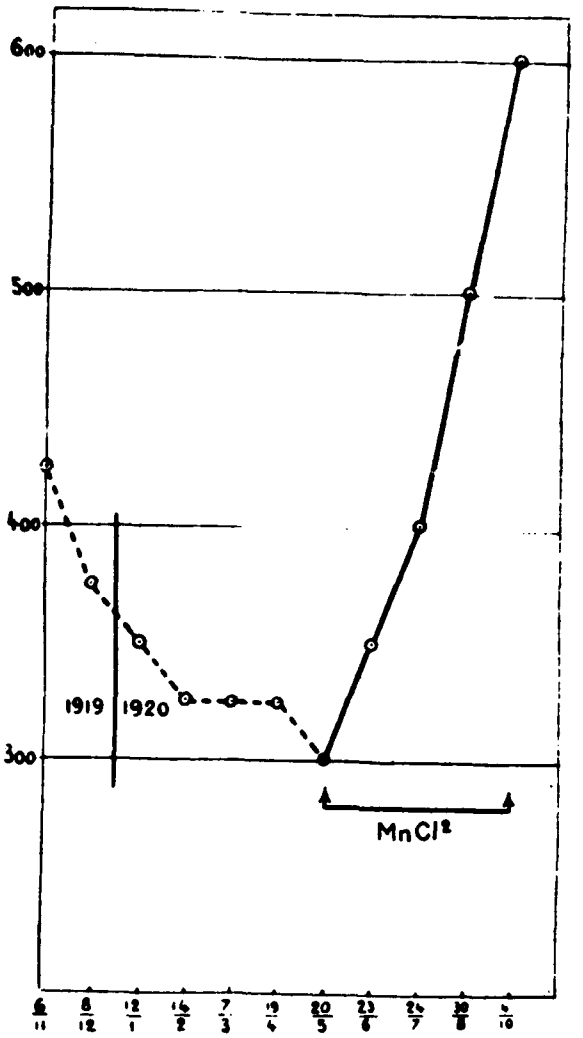

Fig. 11. Horse Immunized Against Diphtheria, No 316. The Figures at the Bottom are Dates.

Horse No 334 (Fig. 12). - Before treatment, 350 S. U.

From 14 June to 23 June, after 600 cc toxin, manganese chloride is injected daily. On 23 June, 400 S. U.

From 12 August to 30 August, 400 cc toxin; then daily injections of manganese chloride. On 30 August, 500 S. U.

From 17 September to 4 October, 400 cc toxin, then $MnCl_2$ daily. On 4 October, 525 S. U.

From 11 November to 18 November, same treatment.. On 18 November, 550 S. U. The horse dies suddenly.

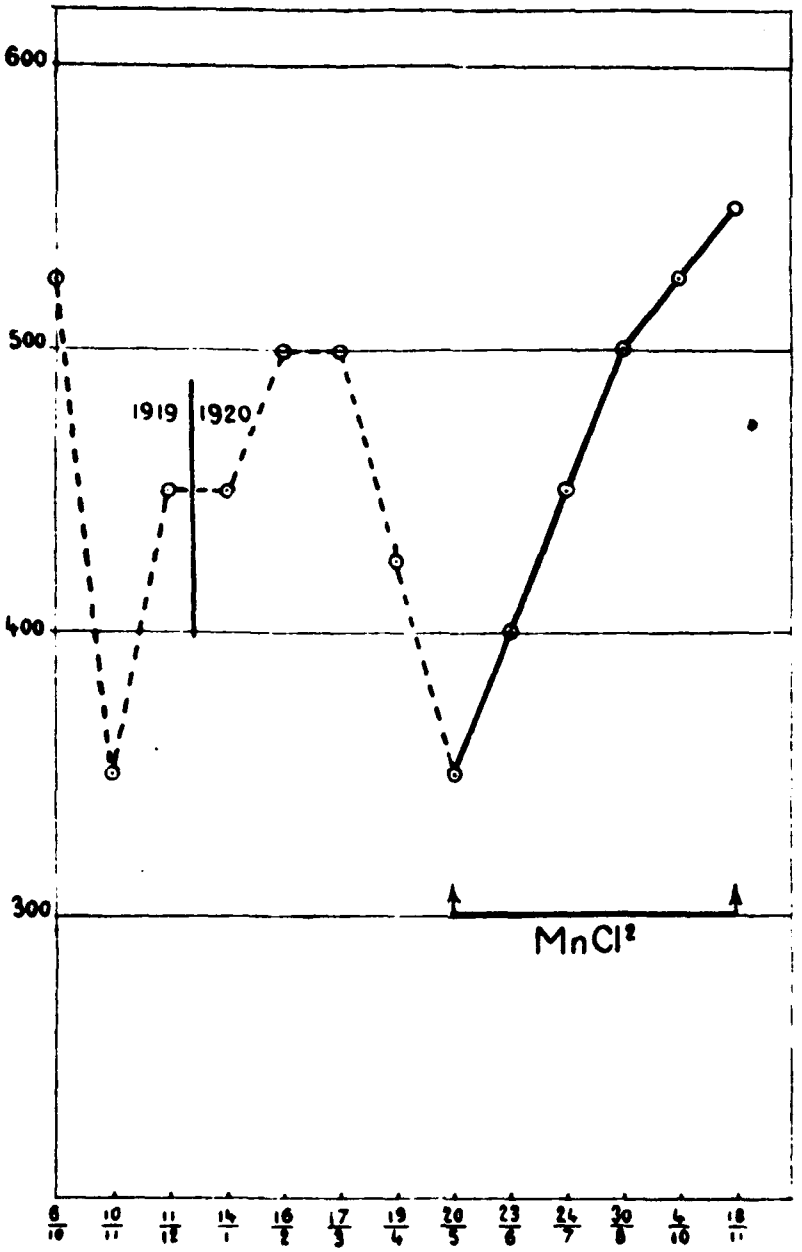

Fig. 12. Horse Immunized Against Diphtheria, No 334. The Numbers at the Bottom Indicate Dates.

In this horse there was thus a gradual and continuous increase in the antitoxin level of the serum.

Horse No 347 (Fig. 13). - The antitoxin curve of this horse that received a manganese treatment with interruptions is similar to that of horse 348. It shows, as did the curve

of 348, that after interruption of the injections of $MnCl_2$, the subsequent effect of these injections decreases.. The first period of injections increases the number of units from 400 to 550, or 44%; the second period from 400 to 450, or 12%, and the third from 350 to 375, or 7%.

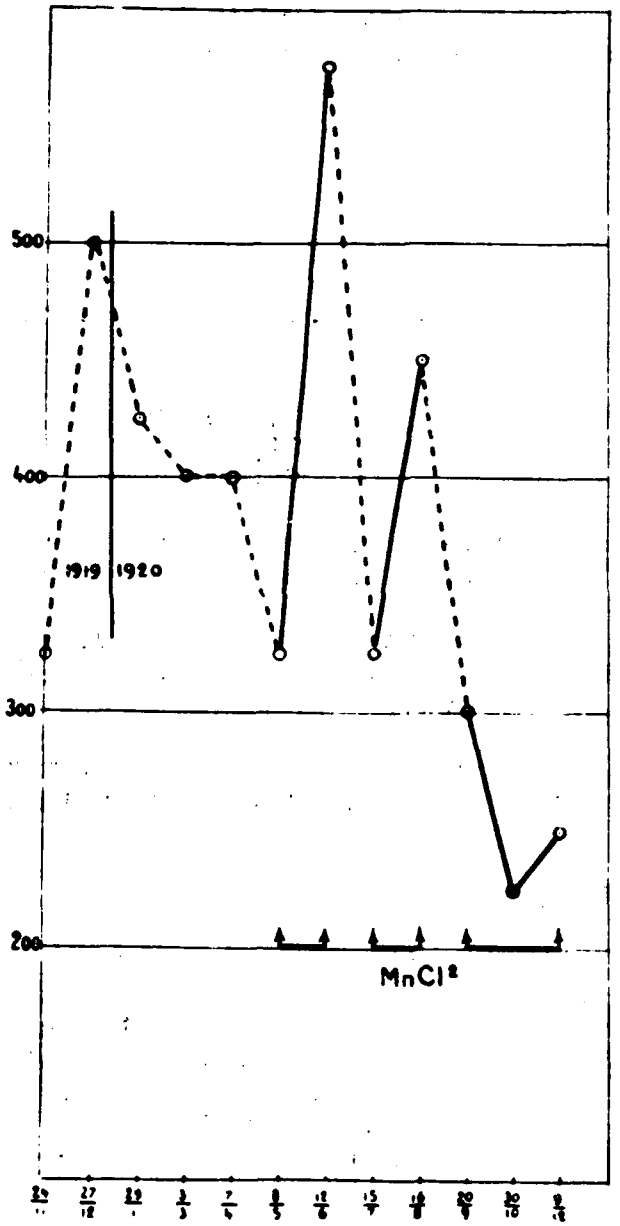

Fig. 13. Horse Immunized Against Diphtheria, No 347. The Figures at the Bottom Indicate Dates.

The conclusion following from these experiments is that the injection of manganese chloride causes a very marked increase in the antitoxin level of serum in serum producing horses.

b) Cobalt Chloride

Horse No 349, 356 and 357. At the last bleedings before the $CoCl_2$ treatment the serum of these horses contained the following quantities of antitoxin:

Number	349	325 S. U. per cc
"	356	275 " " " "
"	357	300 " " " "

From 4 October to 1 November, i.e., after 200 cc of toxin, $CoCl_2$ was injected daily, and at the bleeding of 1 November contained the following quantities of antitoxin:

Number	349	275 S. U. per cc
"	356	225 " " " "
"	357	200 " " " "

The injection of cobalt chloride could thus not prevent in these animals the drop in antitoxin level.

From the previously made experiments (Fig. 1 and Fig. 6) one might have assumed that the salt would here also stimulate the antitoxin formation.

We have not had an opportunity to repeat these experiments and we therefore do not know yet to what this difference in the results should be attributed.

C. Manganese Chloride, Oral

It is possible that part of the manganese chloride administered orally is resorbed and enters into the circulation. We have therefore made some experiments in order to determine whether the amount resorbed would be sufficient to influence the antitoxin production. Four horses, numbers 330, 339, 340 and 345 (Fig. 7 and 8) with an antitoxin level that had been decreasing over a long period were selected for this experiment.

The major part of manganese chloride is able to pass through the digestive tract without being absorbed, and thus the daily dose was set at 10 g of $MnCl_2$. This amount was

added to the drinking water in such a way that the animal received 5 g in the morning and 5 in the evening. The administration of this amount did not seem to influence the animal's health. From 8 August to 25 August, i.e., after 400 cc toxin, the horses were given this dose daily. It is seen from Table III (A) that this treatment had no clear cut effect.

Because of this result the horses were given 20 g $MnCl_2$ from 25 August to 25 September (it is impossible to exceed this dose, as the horses refuse to drink). Table III B shows that the results obtained were no better, on the contrary, the antitoxin content fell even more

TABLE III

Manganese Chloride, Oral

Horse	Before $MnCl_2$	A. After $MnCl_2$	B. After $MnCl_2$
300	300 U. E.	300 U. E.	"
339	200 U. E.	200 U. E.	175 U. E.
340	300 U. E.	275 U. E.	200 U. E.
345	250 U. E.	225 U. E.	200 U. E.

3. The Importance of Manganese Chloride for the Antidiphtheria Serum Production

It follows from the preceding that the injection of the metal salts considered here (especially manganese chloride) causes in general an increase in the antitoxin production, or at least a higher concentration of the antitoxin in the blood of the immunized animals. In order to obtain more exact data on this subject it would be necessary to extend the observations over a longer period. At the Serum Therapy Institute we have used manganese injections for two years, either regularly or at more or less widely spaced intervals. We have compared the results obtained during these two years with those obtained over twenty years with the old immunization method. We have calculated the mean level of the serum produced and also the mean strength of the immunization toxins used during those twenty years (Tables IV and V).

One need only glance at Fig. 14 in order to see how strongly the serum antitoxin level depends on the strength

of the toxin used for the injections; the two curves are perfectly parallel. Considering the large number of animals (300 to 400 horses) and the long period (19 years) that were available for the computation of the preceding statistics, it is no exaggeration to conclude that the more potent the diphtheria toxin available, the more favorable the conditions for the production of an active antidiphtheria serum.

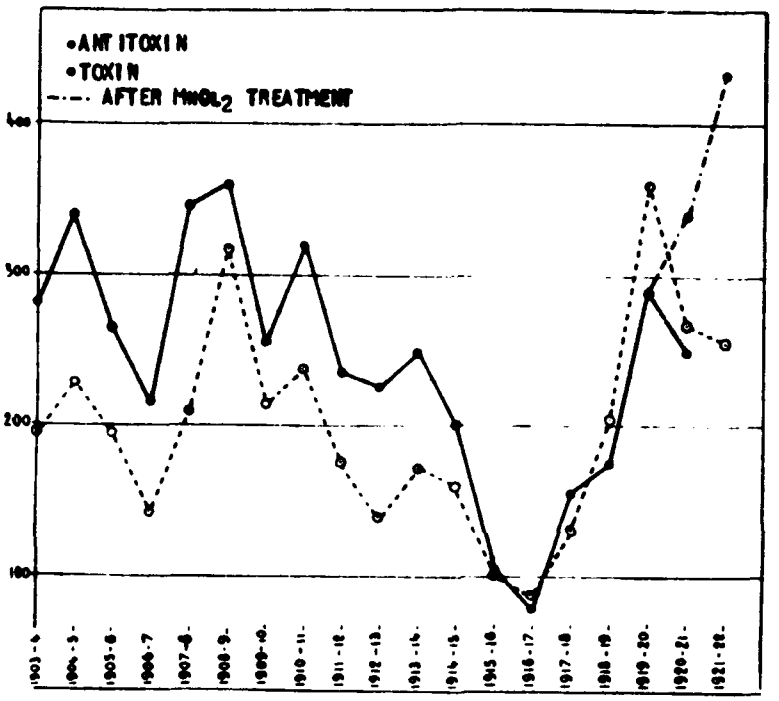

Fig. 14. Statistics of Toxin and Antitoxin From 1903 to 1922.
• antitoxin, · toxin ------ after $MnCl_2$ treatment.

The averages of the serum potency vary, of course, considerably. The period 1915-1918 was especially bad. In this period, however, the potency of the toxin was also fairly low, and as the two curves over the 10 or 11 preceding years correspond exactly with each other, it is quite justifiable to ascribe this lower value of the serums to the toxin weakness.

In May 1920 manganese treatment of a certain number of antidiphtheria horses was started, the course of which has been described in the preceding pages, the rest of the horses receiving toxin only.

As seen in Fig. 14 the antitoxin curve of the horses that received no manganese showed a drop similar to that of the toxin, whereas the antitoxin curve of the horses treated with manganese rises appreciably.

TABLE IV

Toxin Statistics (1903-1922)

YEARS	AVERAGE OF UNITS OF TOXIN PER cc (*)	MEAN LETHAL DOSE	
		Maximum	Minimum
1903-04	194	0,0025	0,015
1904-05	228	0,0025	0,005
1905-06	194	0,005	0,01
1906-07	142	0,003	0,01
1907-08	209	0,003	0,01
1908-09	317	0,002	0,006
1909-10	213	0,0015	0,01
1910-11	237	0,003	0,007
1911-12	175	0,003	0,015
1912-13	138	0,005	0,02
1913-14	172	0,005	0,007
1914-15	160	0,005	0,008
1915-16	102	0,005	0,02
1916-17	88	0,005	0,02
1917-18	132	0,004	0,015
1918-19	205	0,003	0,02
1919-20	360	0,0015	0,01
1920-21	266	0,002	0,01
1921-22	256	0,0015	0,007

(*) THE FIGURE GIVING THE NUMBER OF TOXIN UNITS PER cc IS THE RECIPROCAL OF THE MEAN LETHAL DOSE.

The toxin curve drops even more in the year 1921-22, but the curve of the horses treated with manganese rises considerably, nevertheless, and the average value of the serum produced in that year exceeds considerably (by 21%) the antitoxin potency of the serums previously produced.

In 1921-1922 the Institute had only horses treated with manganese; if there had been animals undergoing the regular regime without manganese, the level of their serum would probably not have been more than 240 S. U. (the average value from the statistics), whereas the antitoxin level of the serums produced by the former reached 436 S. U.

TABLE V

Antitoxin Statistics (1903-1922)

− / + MnCl²	NUMBER OF HORSES	AVERAGE OF S.U. PER cc	MAXIMUM AND MINIMUM OF ANTITOXIN LEVEL IN S.U. (••)	
			Maximum	Minimum
1903-04 WITHOUT MnCl² (•)	15	280	500	180
1904-05 "	6	340	450	250
1905-06 "	8	265	375	150
1906-07 "	15	215	400	150
1907-08 "	16	365	450	150
1908-09 "	15	360	600	230
1909-10 "	19	255	485	220
1910-11 "	23	320	725	150
1911-12 "	21	335	465	200
1912-13 "	15	255	62[illegible]	50
1913-14 "	6	250	5[illegible]	200
1914-15 "	9	200	430	150
1915-16 "	13	105	200	65
1916-17 "	12	80	200	65
1917-18 "	15	153	200	75
1918-19 "	29	175	335	100
1919-20 "	45	290	550	100
1920-21 "	21	250	375	90
1920-21 WITH MnCl² . . .	13	340	92	125
1921-22 "	36	436	750	200

(•) FROM JUNE 1903 TO JUNE 1904.
(••) SERUM LEVEL PRODUCED BY THE BEST AND THE WORST HORSE DURING THE YEAR.

4. The Fate of Manganese Chloride in the Organism

It is known that heavy metal compounds (Pb, Cu, Hg, Bi, Fe, Mn) are largely eliminated by the muscous membranes of the stomach and the intestines, and a smaller quantity is retained in various organs (mainly in the liver, the spleen and the kidney). It is interesting to know how long this elimination takes in order to make a comparison between the latter and antitoxin production.

We have followed the disappearance of manganese in blood of the horse and the goat and at the same time investigated the elimination in feces and urine.

Finally we determined the concentration of manganese in a number of organs from normal and immunized horses.

Before describing these experiments we have to give an outline of our analytical method.

Analysis of Manganese in Blood and Tissues

Bertrand and Medigreceanu [18] in the first place have studied these analyses, and later C. K. Reiman and A. Minot [19, 20].

This is our method: Blood and fat are carefully removed from the organs, they are wiped with a cloth and cut in thin slices that are weighed and then dried at 50-60° C, and subsequently weighed again, and ground to a coarse powder. The amount of this powder used for an analysis corresponds to 25 g of fresh organ (or blood).

The organ powder is transferred to a quartz Kjeldahl flask of 300 cc and wetted with 20-25 cc concentrated sulfuric acid, briefly heated to eliminate a last trace of water, and after cooling a mixture of 20 cc concentrated hydrochloric acid and 20 cc concentrated nitric acid are carefully added. The mixture is left standing for 24 to 36 hours and then boiled, by heating gently at the beginning and more strongly afterwards until only 1 to 2 cc of a completely colorless liquid is left. After cooling, this liquid is diluted with water to bring the volume to 50 cc and filtered. The manganese, present in the solution as the sulfate is then titrated colorimetrically.

How Fast Does Injected Manganese Disappear From the Blood?

Experiment with horse 338. - The horse weighed 500 kg. At 7 a.m. 10 cc of a 10% manganese chloride solution was injected intravenously, and blood samples were taken at the time intervals indicated in Table VI. The samples were citrated to prevent coagulation. Each analysis was made on 25 g blood.

No trace of manganese was found in the blood before the injections of manganese chloride; however, according to Bertrand's investigations there is 0.02 mg Mn per liter of horse blood, and consequently there should be 0.0005 mg manganese in the 25 g blood that we worked with. Such an extremely small quantity of manganese is not found with the experimental technique used by us; the minimum quantity detected by the colorimetric method is 0.002 to 0.004 mg Mn.

TABLE VI

Disappearance of Manganese From Circulation
(Horse)

TIME AFTER INJECTION	mg OF Mn IN 100 cc BLOOD	TIME AFTER INJECTION	mg OF Mn IN 100 cc BLOOD
0	0,73 (CALCUL.)	12 HOURS	0,04
5 MINUTES	0,56	24 —	0,046
15 —	0,30	2 DAYS	0,036
30 —	0,20	3 —	0,034
1 HOUR	0,11	5 —	0,032
2 HOURS	0,06	8	0,03
3	0,05	12 —	0,02
6 —	0,046		

Experiments with the goat. - The animal weighed 36 kg. Three days before injection the animal was placed in a cage in such a way that feces and urine could be collected separately.

20 cc of a tenth normal solution of $MnCl_2$ diluted to half that concentration were injected intravenously at 11:30 a.m. and sample bleedings were carried out at the intervals indicated in Table VII.

TABLE VII

Disappearance of Manganese From Circulation
(Goat)

TIME AFTER INJECTION	mg OF Mn IN 100 cc BLOOD	TIME AFTER INJECTION	mg OF Mn IN 100 cc BLOOD
0	2.0 (CALCUL.)	2 HOURS	0.15
5 MINUTES	1.8	3 —	0.1
15 —	0.7	6 —	0,08
30 —	0.5	12	0,05
1 HOUR	0,31	24 —	0,05

The amount of manganese found in the samples of feces and urine are listed in Table VIII.

TABLE VIII

Manganese Elimination in the Feces and the Urine
(Goat)

TIME	MG OF Mn ELIMINATED	
	Urine	Feces
3-4 mars	0,65	6,0
4-5 —	0,64	5,0
5-6 —	0,16	1,3
6-7 —	0,06	0,5
7-8 —	0,53	16,0
8-9 —	0,06	9,5
9-10	0,05	9,6
10-11 —	0,05	9,.
11-12	0,05	7,0

The $MnCl_2$ injections were carried out on 6 March at 11:30 a.m.

These experiments show that manganese chloride injected intravenously disappears swiftly from the circulation (see Fig. 15). It is also seen that the rise in the antitoxin curve is as fast as the drop in the curve representing the concentration of manganese in the blood.

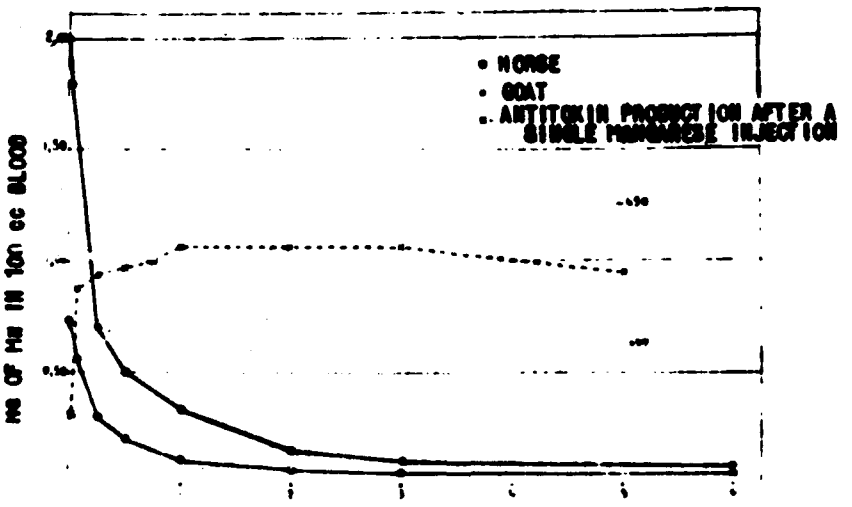

Fig. 15. Disappearance of Manganese from Circulation. The Figures at the Bottom Indicate Hours.

In addition it is seen from Table VIII that the elimination of manganese takes place mainly through the mucous membrane of the intestines, and to a very small extent through the kidney.

It is also seen that the urine and feces of the goat normally contain considerable amounts of manganese, the amount of manganese found in the urine and feces decreases gradually in the period before the manganese chloride injection. This is due to the change in the food of the animal when it was placed in the cage.

The results obtained must therefore be evaluated with a certain reservation.

The Manganese Content of the Organs of Normal Immunized Horses

The results of the analyses performed on the organs of twelve horses (liver, kidney, spleen, heart, lung, lymph glands) are tabulated in Table IX below; the figures indicate the manganese content in milligrams per 100 gram of fresh organ.

The two first horses of the table are normal animals, i.e., they were not immunized. The two next horses No 380 and 393 are, in that order, diphtheria and meningococci immunized; they did not receive manganese.

The eight last horses are immunized against diphtheria, except for number 369 that was used for the production of antitetanus serum. These eight animals received intravenous injections of manganese chloride for a longer or shorter period, and in the table they are arranged according to the amount injected, calculated per kg of horse.

A comparison of the two normal horses with the two immunized horses that did not receive manganese show that the manganese content of the various organs did not undergo any change in immunization, except for the liver. The amount of manganese in the liver, however, has been reduced to one quarter of the normal quantity.

A glance at the analyses made of the organs of the manganese-treated animals shows immediately an increase in the manganese content of all organs (except the liver), and the manganese content increases with the amount of metal the animal was given.

TABLE IX

HORSE No	HORSE'S WEIGHT KG	TOTAL MHOL2 INJECTED c	MHOL2 INJECTED PER KG HORSE	Mn OF Mn IN 100 c ORGAN						TYPE OF HORSE AND SERUM LEVEL
				LIVER	LYMPH GLANDS	KIDNEY	SPLEEN	HEART	LUNG	
				0.15	0.15	0.12	0.09	0.09	0.012	NORMAL HORSES
				0.12	0.12	[illegible]	0.08	0.09	0.009	
[illegible]	150			0.10	0.12	[illegible]	0.06	0.09	0.01	D 150 S. U.
[illegible]	350			0.14	0.14	[illegible]	0.07	0.08	0.01	M BAD
[illegible]	450	20	0.04	0.10	0.28	[illegible]	0.09	0.08	0.06	D 600 S. U.
[illegible]	350	35	0.03	1.12	0.12	0.32	0.1	0.09	0.06	F 10 S. U.
[illegible]	400	50	0.1	0.2	0.14	0.28	0.12	0.14	0.12	D 150 S. U.
[illegible]	500	75	0.15	0.2	0.12	0.16	0.13	0.14	0.16	D 200 S. U.
[illegible]	400	80	0.2	0.16	0.20	0.22	0.12	0.12	0.18	D 600 S. U.
[illegible]	450	60	0.13	0.18	0.80	0.18	0.13	0.13	0.18	D 175 S. U.
[illegible]	400	100	0.2	0.12	0.90	0.12	0.11	0.24	0.22	D 700 S. U.
[illegible]	475	100	0.21	0.18	1.04	0.20	0.10	0.2	0.2	D 175 S. U.

D. DIPHTHERIA HORSE; F. TETANUS HORSE; M. MENINGOCOCCUS HORSE

The faculty for retaining manganese seems to vary widely with the organs. In the horses that received the largest amounts of manganese the following relationship holds approximately:

The manganese content of the heart has about doubled.

The manganese content of the spleen has about doubled.

The manganese content of the kidney is about 5 times as high.

The manganese content of the lymph glands is about 8 times as high.

The manganese content of the lungs is about 20 times as high.

It is seen (Table X) that for the liver there exists a certain relationship between the manganese retertion and the antitoxin productivity of the animal, in other words, the good antitoxin producers show an increase in the manganese content of the liver.

TABLE X

NUMBER OF HORSE	Mn CONTENT OF LIVER IN Mg.	S. U. PER cc	
[illegible]	0.48	600	GOOD ANTITOXIN PRODUCERS
316	0.56	600	
362	0.72	750	
369	1.12	10 TETANUS	
372	0.2	150	BAD ANTITOXIN PRODUCERS
360	0.2	200	
368	0.18	175	
309	0.18	175	

A confirmation of the close connection between manganese retentivity of the liver, and the animal's ability to produce a good serum would justify the assumption that antitoxin production is connected with the presence of this depot of catalytic substance in the liver.

II. EXPERIMENTS ON ANIMALS IMMUNIZED AGAINST B. COLI

For further experiments on the importance of metal salts in immunization, it seems preferable to use antigens that can be tested for activity without tests in vivo.

Walbum has shown (1918) that the chlorides of manganese, nickel, cobalt, and zinc have a considerable stimulating effect on the formation of B. Coli agglutinin, and we have consequently selected the latter for the following experiments. Goats and rabbits were used as agglutinin-producing animals. For our study it was of prime importance to prepare a sufficiently large amount of experimental material to make a comparison of the effects of the various metal salts possible: individual differences in productivity of antibodies are actually very large in animals, and in addition it is necessary to find the suitable moment for proceeding with the metal salt injections.

The same strain of a B. Coli (named Inge) derived from a urinary infection in man was used in all experiments. The agglutinin content of the blood samples was determined in the usual way (Madsen and Jorgensen).

A. What Quantities of Various Metal Salts May be Injected Intravenously in a Rabbit Without Causing Symptoms of Poisoning?

0.001 cc of a normal solution of the salt to be tested per kg rabbit was injected, and if the animal did not show any signs of poisoning after half an hour a new injection of 0.002 cc was given; these injections of 0.004, 0.013, 0.04 and 0.08 cc successively were continued at half hour intervals until the animal died or until it showed definite signs of poisoning.

The results of these tests are shown in Table XI below; the metal salts are arranged in the order of decreasing effectiveness.

It is seen that cadmium chloride is the most toxic salt of those investigated, because 0.002 cc normal solution per kilogram rabbit causes very definite poisoning.

It is obvious that for daily injection over a long period the salts may be used only in amounts that do not cause noticeable poisoning.

TABLE XI

	CUBIC CENTIMETER OF NORMAL SOLUTION					
	0.001	0.002	0.004	0.012	0.04	0.08
$CdCl^o$	0	X	XX	+ 20 min.	"	"
$BaCl^o$	0	0	XX	XX	+ 5 min.	"
$FeCl^o$	0	0	X	XX	+ 10 —	"
$AlCl^o$	0	0	X	XX	+ 10 —	"
$PbCl^o$	0	0	X	XXX	+ 1 h.	"
$AgCl$	0	0	X	XXX	+ 24 h.	"
$HAuCl^o$	0	0	0	XXX	+ 24 h.	"
$H^{ep}PtCl^o$	0	0	0	XXX	+ 48 h.	"
$OsCl^o$	0	0	0	XXX	+ 72 h.	"
$SrCl^o$	0	0	X	XX	XX	+ 1 min.
$CuCl^o$	0	0	0	XX	XX	+ 3 —
$MnCl^o$	0	0	0	X	XX	+ 2 h.
$HgCl^o$	0	0	X	XX	XX	+ 3 h.
$BeCl^o$	0	0	0	XX	XX	XXX
$ZnCl^o$	0	0	0	X	XX	XXX
$CoCl^o$	0	0	0	0	X	XXX
$NiCl^o$	0	0	0	0	X	XXX
$CrCl^o$	0	0	0	X	X	XX
$LiCl$	0	0	0	0	X	XX
$MgCl^o$	0	0	0	0	X	X
$CaCl^o$	0	0	0	0	0	0

0: NO SYMPTOMS OF POISONING
X: WEAK SYMPTOMS OF POISONING
XX: FAIRLY STRONG SYMPTOMS OF POISONING
XXX: PRONOUNCED SYMPTOMS OF POISONING

0.001 cc of a normal solution per kilogram rabbit or goat were used for the following experiments.

B. Comparison Between the Effects of Various Metal Salts

The tests were carried out by taking for each metal salt one goat and two rabbits; the animals were new. Agglutinin formation was stimulated by injecting subcutaneously in each goat 10 cc of a 24 hour old B. coli culture (in broth) heated before injection to 80° for 5 minutes, and by injecting in each rabbit intravenously 0.1 cc of a live B. coli culture (in broth) diluted to 1 cc with a sodium chloride solution.

The first bleeding was performed seven days after the injection of the antigen, and they were repeated daily until the end of the experiment (5 to 10 cc with the goat, 0.5 to 1 cc with the rabbit). The decanted serum was preserved under refrigeration till titration.

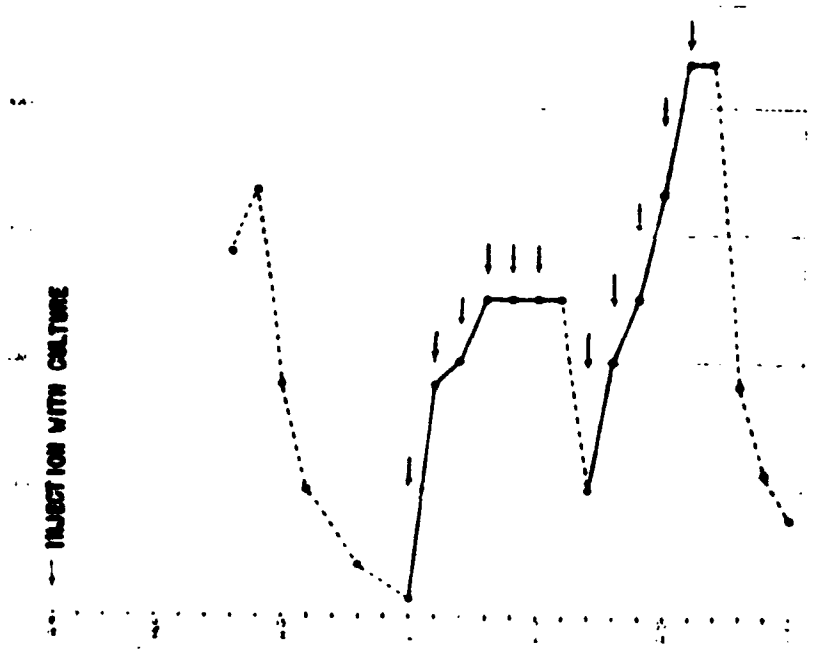

Fig. 16. Rabbit Immunized Against B. Coli. The Arrows Indicate $MnCl_2$ Injections. The Figures in the Ordinate Indicate Agglutinin Units per cc. The Figures at the Bottom Indicate Dates.

The first metal salt injection was given on the 14th day after the culture injection when the agglutinin curve was falling. One cc of a hundredth normal solution diluted tenfold per kilogram of animal was injected intravenously after adding 0.9% sodium chloride to the liquid to make it isotonic. This dose was injected daily for the first six days; on the seventh day (Sunday) no injection was given, but on the six following days injections were repeated daily. The lack of an injection on the seventh day caused always a drop (more or less pronounced) in the agglutinin content (Fig. 16).

The effect of a metal salt is determined by the increase in agglutinin content in proportion to the maximum agglutinin content obtained initially with the antigen by itself.

The results of all these experiments are given in Table XII, the metals being arranged in order of their effectiveness. The table obviously does not contain all the analytic determinations but only those that are relevant to the subject under consideration.

TABLE XII

SALT		WEIGHT IN kg		AGGLUTININ UNITS PER cc			RISE IN %	MEAN RISE	-24
		BEFORE	AFTER	FIRST PEAK	BEFORE SALT INJECTION	FIRST + SECOND RISE			
BeCl²	GOAT	30	30	222	125	.225	1 457,2		
	RABBIT I	"	"	66,7	43,5	1.109	1.662,6	1.174	1.147
	RABBIT II	"	"	125	43,5	491	392,8		
MgCl²	GOAT	38	40	133	66,7	669	525,8		
	RABBIT I	3,05	3,28	333	222	2.330	700,3	589	565
	RABBIT II	2,80	2,83	154	71,4	831	539,6		
PbCl²	GOAT	36	38	333	154	1 628	488,8		
	RABBIT I	2,89	3,05	1.250	286	5 251	420,3	436	402
	RABBIT II	2,70	2,80	333	143	1.226	368,4		
CuCl²	GOAT	26	29	125	50	307	245,6		
	RABBIT I	2,75	2,80	333	125	1.396	419,2	358	334
	RABBIT II	3,00	3,20	250	125	1.024	409,6		
CdCl²	GOAT	60	60	309	250	1.092	218,4		
	RABBIT I	3,8	3,8	176	100	602	346	292	268
	RABBIT II	3,7	3,7	250	100	799	311,6		
BaCl²	GOAT	39	40	435	267	1.816	417,5		
	RABBIT I	2,20	2,35	1.050	769	2.221	211,5	257	233
	RABBIT II	2,50	2,67	625	351	895	113		
ZnCl²	GOAT	30	32	500	46,7	1.108	221,6		
	RABBIT I	2,80	2,80	500	167	1.080	215,9	212	188
	RABBIT II	2,66	2,55	222	125	442	199,1		
HgCl²	GOAT	32	34	465	286	1.333	266,6		
	RABBIT I	2,10	2,11	250	76,9	433	173,2	205	181
	RABBIT II	2,22	2,20	267	125	515	176,5		
AlCl³	GOAT	33	34	556	385	527	94,7		
	RABBIT I	2,02	2,2	154	76,9	358	232,5	190	166
	RABBIT II	2,3	2,27	250	100	601	251,6		
MnCl²	GOAT	39	39	333	16,3	571	171,5		
	RABBIT I	2,9	3,1	333	125	550	165,1	166	142
	RABBIT II	2,5	2,8	200	66,7	333	161,7		
AgCl	RABBIT I	2,35	2,35	1.000	133	1.169	116,9	119	95
	RABBIT II	2,37	2,17	267	76,9	321	121,3		

TABLE XII [Continued]

SALT		WEIGHT IN KG		AGGLUTININ UNITS PER cc			RISE IN %	MEAN RISE	-24
		BEFORE	AFTER	FIRST PEAK	BEFORE SALT INJECTION	FIRST ◆ SECOND RISE			
[illegible]Cl³	GOAT	55	55	588	435	381	66,8		
	RABBIT I	"	"	535	200	270	62,1	72	16
	RABBIT II	"	"	2 0	100	180	80		
CdCl³	GOAT	31	33	252	167	270	180		
	RABBIT I	2,0	1,8	511	100	214	39,3	68	15
	RABBIT II	2,5	2.63	200	28,6	111	55,5		
SrCl³	GOAT	50	51	1.000	400	x 0	85		
	RABBIT I	2,2	2,43	222	50	143	50,9	67	13
	RABBIT II	2,12	2,2	370	100	263	65,6		
HgNO³	GOAT	50	50	500	308	321	64,8		
	RABBIT I	1,82	2,26	588	200	270	56	60	36
	RABBIT II	1,9	2.2	588	222	308	67,7		
HAuCl³	GOAT	38	38	500	200	150	30		
	RABBIT I	2,3	2,3	535	250	338	77,7	54	30
	RABBIT II	2,7	2,8	435	200	235	56		
NiCl³	GOAT	25	28	2.222	50	1.111	51,5		
	RABBIT I	3.50	3,80	535	167	263	60	54	30
	RABBIT II	2,51	2,5	500	250	250	50		
CuCl³	GOAT	36	37	500	118	263	58,6		
	RABBIT I	2,82	2,90	200	100	102	51	51	27
	RABBIT II	2,75	2,90	588	266	317	53,9		
CoCl³	GOAT	25	25	333	100	157	57,1		
	RABBIT I	2,85	3,10	769	200	287	37,3	11	20
	RABBIT II	3,35	3,15	266	100	136	47,5		
KCl	GOAT	50	51	200	100	111	55,5		
	RABBIT I	2,65	2,67	200	76,9	NO RISE	0	31	7
	RABBIT II	3,15	3,10	250	100	93	37,2		
LiCl	GOAT	60	60	667	154	146	21,9		
	RABBIT I	3,15	3,28	333	143	90	27	25	0
	RABBIT II	3,35	3,17	267	167	66	25,3		
NaCl	GOAT	53	53	314	125	76	21,7		
	RABBIT I	3,50	"	125	100	30	25	25	0
	RABBIT II	3,25	"	1 540	667	397	25,7		
FeCl³	GOAT	30	30	870	535	269	30,9		
	RABBIT I	2,4	2,3	500	300	38	5	21	— 3
	RABBIT II	3,1	3,25	250	138	71	28,1		

The values indicating the mean increase in agglutinin in the last column of the table have been corrected by a subtraction of 24; this was necessary because an injection of 0.9% sodium chloride (present in all injected solutions of metal salts) by itself caused an increase of 24% in the agglutinin concentration of the serum.

TABLE XIII

	ATOMIC WEIGHT	EFFECT %
THE ALKALI METALS		
LiCl	6.9	0
NaCl	23	0
KCl	39.1	7
THE MAGNESIUM GROUP		
$BeCl^2$	9.1	1.147
$MgCl^2$	24.3	465
$CuCl^2$	63.6	334
$ZnCl^2$	65.4	188
$CdCl^2$	112.4	44
$HgCl^2$	200.6	181
THE CALCIUM (BARIUM) GROUP		
$CaCl^2$	40.1	27
$SrCl^2$	87.6	43
$BaCl^2$	137.4	233
$PbCl^2$	207.2	102
THE SILVER GROUP		
$CuCl^2$	63.6	334
AgCl	107.9	1
$HAuCl^4$	197.2	30
THE IRON GROUP		
$CoCl^2$	59	20
$NiCl^2$	58.7	30
$MnCl^2$	54.9	152
$FeCl^2$	55.8	3
$CrCl^2$	52	48
THE PLATINUM GROUP		
$OsCl^4$	190.1	208
H^2PtCl^6	195.2	56
AlCl	27.1	106

We have tried to compare the results obtained in the production of agglutinin with the atomic weight of each of the metals used (Table XIII). Based on this table we make the following observations:

The alkali metals.- Their effect seems to be extremely small, but there seems to exist a certain proportionality between the atomic weight and the agglutinin content of the serum.

The magnesium group. - The effectiveness of the various metals in this group seems inversely proportional to the atomic weights.

The calcium group. - Here, on the other hand, there seems to exist a direct proportionality.

The silver group. - In spite of the variable valency and solubility of the metals in this group it may be concluded that the effectiveness of these metals relative to agglutinin production is inversely proportional to their atomic weights. The same applies to the iron group.

C. Relation Between Dose and Effect

The amount of metal salt used for the intravenous injections, 0.001 cc of a normal solution per kg of animal was selected because it was approximately the largest amount of the most toxic salt ($CdCl_2$) that can be injected in a rabbit without causing visible symptoms of poisoning. This was a very small amount but one could not a priori reject the possibility that a larger effect could be obtained with smaller quantities.

In order to verify this we treated four goats as described in the previous chapter (B), with one of the goats receiving daily injections of 1 cc of a normal $MnCl_2$ solution diluted 100,000 times, per kg, during the indicated periods, the second 1 cc of a normal solution diluted 10,000 times, the third 1 cc of a normal solution diluted 1,000 times and the fourth 1 cc of a hundredth normal solution (this last quantity is ten times the amount used in the main experiment described above), without any untoward effect on the goat.

The result of these experiments is given in the following table:

TABLE XIV

Goat No	$MnCl_2$ per kg	Rise, %	- 24
1.....	1 cc of a normal solution diluted 100,000 times	71	0
2.....	10,000 times	35	11
3.....	1,000 times	171	147
4.....	100 times	85	61

The experiments in the table are too few to permit us to draw conclusions with complete confidence.

In any case it seems that the injection of the solution diluted 10,000 times is ineffective, whereas the same solution diluted 1,000 times is capable of causing a very definite effect.

D. The Effect of a Single Injection of Metal Salt

We have shown in the preceding experiments (Fig. 5) that the effect of an injection of metal salt is apparent immediately after the injection, that the antitoxin curve rises immediately, and that it reaches its maximum after about one hour. These experiments were done on a horse immunized against diphtheria, but it was interesting to find out its effect in animals immunized against B. coli.

We have carried out a single experiment on a rabbit. It seems that the result (Fig. 17) is the same as that of the horse immunized against diphtheria: the increase in agglutinin follows immediately upon the injection. The maximum in this case is however, reached after about four and a half hours only, but during these few hours the agglutinin concentration in the blood reached exactly the same value as nine days after the antigen injection.

The rabbit received one single injection of 1 cc of a normal magnesium chloride solution diluted 1,000 times per kg.

Summary

The results of the experiments in this report can be summarized as follows:

1. Intravenous injection of manganese chloride ($MnCl_2$) as well as of cobalt chloride ($CoCl_2$) during immunization increases considerably the antitoxin level of goats immunized against diphtheria toxin.

Manganese chloride seems to have a stronger effect than cobalt chloride.

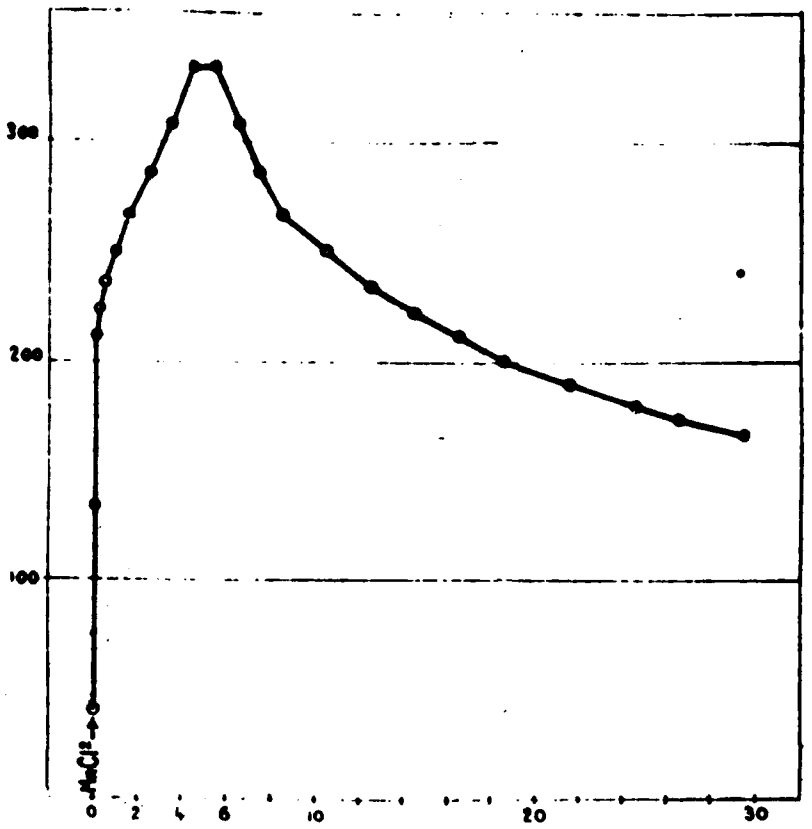

Fig. 17. Goat Immunized Against B. Coli. One Single $MnCl_2$ Injection. The Figures in the Ordinate Indicate the Agglutinin Units per cc. The Numbers at the Bottom Indicate Hours.

2. Intravenous injection of manganese chloride and of cobalt chloride in horses that are immunized against diphtheria causes a very marked increase in the antitoxin production, when given during the period of antitoxin drop and without any toxin injection. The increase follows immediately upon the metal salt injection, but it reaches its peak only after approximately one hour. There is thus a fundamental difference between the effect of a toxin injection and that of a metal salt injection, the former being frequently associated with a drop in the antitoxin curve (first negative phase), whereas the latter seems to have a completely opposite effect.

3. Injection of manganese chloride in horse immunized against diphtheria at various periods causes a very noticeable increase in the level of antitoxin units in the serum.

The complete suspension of these injections during the entire period between two bleedings causes a rapid decrease in the antitoxin curve; the resumption of injections induces a new rise in the curve, but after several successive suspensions and resumptions the effect of manganese becomes less and less noticeable.

4. Manganese chloride, administered orally has no influence on antitoxin production.

5. The annual averages of the antitoxin level of antidiphtheria serum are considerably higher since the introduction of manganese treatment in practice.

6. The curve of these averages is, considered from another point of view, parallel to that of the toxin used to immunize the animals.

7. The injected manganese disappears quickly from the circulation. The elimination of the metal occurs mainly through the mucous membrane of the intestines. Immunization in itself does not cause changes in the normal manganese content of the different organs; only the liver undergoes a reduction to one fourth of its normal manganese content due to the immunization. Immunized horses that receive manganese show a very clear increase in manganese in the organs, and this increase is proportional to the amount of metal injected. There is a close parallel relation between the liver's capacity for the retention of injected manganese and the aptitude of the organism to produce antitoxin.

8. The effect of metal salt injections on the production of agglutinin for B. coli varies widely according to the nature of the salts. There seems to be a certain relation in the majority of cases between the atom number of the injected metal and the magnitude of the effect, either in the form of a direct proportionality or an inverse proportionality, depending on the group to which the metal belongs.

We think that these studies may have considerable theoretical interest in addition to their practical value. In particular, the observation that the metals have an effect on the production of antibodies that has a close relation to their atomic weight may possibly give new information in the field of immunity.

If definitive studies prove that the occurrence and production of antibodies in general are greatly enhanced by metal salt injections, one may ask whether the use of such catalysts may not have considerable significance in the treatment of infectious diseases.

Appendix

Walbum, in collaboration with K. G. Dernby studied the possible influence of metal salt injections on passive immunity. They injected in rabbits (subcutaneously) a fairly large quantity of agglutinin for B. coli, and followed by means of serum titrations the resorption and disappearance of this agglutinin.

During the entire course of the experiments half of the rabbits were subjected to manganese injections, the other half served as comparisons; no difference was observed between the two groups of animals; the injection of metal salts have therefore no effect on the passive immunity. This result was predictable because the experiments reported here tend to show that the metal salts play the part of directly stimulating the antitoxin-producing cells in active immunity.

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