SOME OBSERVATIONS ON THE ANTIGENIC STRUCTURE OF PSITTACOSIS AND LYMPHOGRANULOMA VENEREUM VIRUSES. II. TREATMENT OF VIRUS SUSPENSIONS BY VARIOUS REAGENTS AND THE SPECIFIC ACTIVITY OF ACID EXTRACTS.

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In a previous paper (Barwell, 1952) it was shown that fresh unheated suspensions of psittacosis and lymphogranuloma venereum (L.G.V.) viruses often reacted weakly in complement-fixation tests using sera from immunized guinea-pigs, rabbits and pigeons and from human patients. Those unheated preparations which reacted strongly with the homologous serum showed slight degrees of partial fixation with heterologous sera or with sera made against the boiled washed virus. This type of reactivity and its irregular occurrence in different unheated suspensions of the same virus were in contrast with the regularly potent group activity of boiled virus. That the serological behaviour of active unheated suspensions is almost entirely due to the heat-labile specific antigen was confirmed by the monospecific results obtained after absorbing sera with the heat-stable group antigen.

While a variety of techniques has been employed in attempts to obtain sharply specific effects with antisera and viruses of this group, the treatment of the virus materials used as antigens for *in vitro* tests has received relatively little attention. Where different reagents have been used in the preparation of complement-fixing antigens the products have been found to possess enhanced group reactivity. Thus Nigg and her associates (Nigg, 1942; Nigg, Hilleman and Bowser, 1946; Bowser and Nigg, 1946; Hilleman and Nigg, 1946) investigated various means for the inactivation of L.G.V. virus as alternatives to formalin which made suspensions anti-complementary. They showed that urea, ether, phenol or heat killed the virus, and that the complement-fixing potency of such preparations was due to the group component. A highly purified group-reactive material was later obtained by fractionation with acetone and alcohol from ether extracts of yolk-sac suspensions of L.G.V., meningopneumonitis and mouse pneumonitis viruses (Hilleman and Nigg, 1948).

In the experiments to be described suspensions of psittacosis or L.G.V. virus were treated by different reagents and the complement-fixing activity was compared with untreated fractions. Largely on the basis of these results further experiments were done in attempts to demonstrate, by means other than absorption, the specific antigen in these viruses.

METHODS.

The virus strains, the preparation of partially purified suspensions from mouse spleens and yolk-sacs and the technique of complement fixation are described in the previous paper (Barwell, 1952). Human sera and those from immunized guinea-pigs and pigeons were used. As the guinea-pig sera contained antibodies to normal yolk-sac they were used only in studies of mouse spleen suspensions of psittacosis virus. Two sorts of serum from these animals were employed: a " complete " serum obtained after immunization with living psittacosis virus and a " group " serum after inoculations with the boiled washed virus.

RESULTS.

Effect of Different Methods of Treatment on the Antigenic Components of Psittacosis and L.G.V. Viruses.

Heat.—Heating suspensions rich in these viruses at 100° for 20 min. regularly increased their complement-fixing potency as group antigens; the associated destruction of the specific antigen was further confirmed by the behaviour, already described, of guinea-pig antisera prepared against boiled washed psittacosis virus. In the preparation of antigens suitable for serological tests it was often necessary to re-suspend the heated material thoroughly by means of a grinding tube, particularly for yolk-sac suspensions which had become partially coagulated.

No difference in complement-fixing activity was detected between a fraction of psittacosis virus suspension placed in a boiling water-bath for 20 min. and one heated in an autoclave at 135° for 30 min. Bedson (1936) by means of complement-fixation tests on centrifuged fractions did not find any activity in the supernatant fluid of a boiled suspension of psittacosis virus from mouse spleen, although some activity could be demonstrated by intradermal tests in sensitized guinea-pigs. In the present studies the major part of the group factor was deposited in the angle centrifuge at least as readily as unheated elementary bodies; the supernatant fluid of boiled or autoclaved yolk-sac suspensions of virus gave feeble complement fixation with heterologous or homologous sera, suggesting that after such treatment a small amount of the group component is finely dispersed or in solution.

Phenol.—Enhanced activity of the group antigen has been observed after treatment of yolk-sac suspensions of L.G.V. virus with urea or phenol or by boiling untreated or phenolized virus (Nigg, 1942; Nigg *et al.*, 1946; Bowser and Nigg, 1946). In this laboratory treatment of psittacosis virus with 0·5 to 2·0 per cent phenol was found to yield suspensions with a full degree of group activity. After angle centrifugation the supernatant fluid was separated from the virus and could be shown to possess slight or moderate group activity.

Ether treatment and ether extraction.—The observation that some of the group antigen can be extracted with ether from yolk-sac suspensions of L.G.V., meningopneumonitis and mouse pneumonitis viruses (Hilleman and Nigg, 1946, 1948) was confirmed in the case of similar preparations of psittacosis virus. An example of such an experiment is shown in Table I, which illustrates the serological activity of an unheated suspension compared with an ether-soluble fraction and the residual virus after ether treatment. For this purpose a portion of the unheated suspension was extracted with an equal volume of anaesthetic ether for 3 days at room temperature. The ether layer was then separated and shaken over a

volume of saline equal to that of the suspension until the ether had evaporated leaving a milky fluid. The residual ether was also evaporated from the treated virus suspension, and the products were tested by complement fixation, using human psittacosis and L.G.V. sera. The material extracted was found to possess considerable activity in the presence of both sera as compared with the poor fixing ability of the untreated suspension even at four times the concentration. There was very little activity in the residual virus after extraction with ether.

In the preparation of group-active antigens the ether can be removed by evaporation without first separating the ether layer, but treatment with ether was not used except experimentally, since equally satisfactory suspensions were obtained by the simpler method of boiling.

TABLE I.—*The Effect of Ether Extraction on an Unheated Yolk-sac Suspension of Psittacosis Virus.*

Human serum.	Dilution.	Unheated psittacosis virus ST/F7. Untreated suspension. 1/2.	Ether-treated suspension. 1/2.	Ether extract. 1/8.
A. S. M.— (psittacosis)	1/16	++	±	++++
	1/32	++	−	++++
	1/64	+	−	+++
P— (L.G.V.)	1/16	++	+	++++
	1/32	+	−	++++
	1/64	±	−	+++

Treatment with dilute acid.—It was found that treatment of unheated yolk-sac suspensions of psittacosis and L.G.V. viruses with 0·02 N HCl acid for 20 min. at 37° had the effect of enhancing the group antigen as shown by the serological activity of the virus particles which, after treatment, were centrifuged in the cold and re-suspended to the original volume in neutral saline. Acid-treated virus was less easily re-dispersed than suspensions which had been heated, and this was thought to account for the fact that this method did not invariably provide such active group antigens as the boiled preparations. It was also noted that, while the boiled virus was not stained by Castaneda's method, acid treatment did not interfere with staining; in this laboratory cold N HCl is in fact used routinely for fixing yolk-sac smears of viruses of the psittacosis-L.G.V. group.

Enzyme treatment.—Little work has been done on the treatment of these viruses with enzymes. Lazarus and Meyer (1939) used trypsin as a means of obtaining purified suspensions of psittacosis virus and found that the infectivity was not impaired. Electron microscope studies of psittacosis virus before and after treatment with acid and pepsin failed to show any morphological change in the virus particles (Barwell, Dawson and McFarlane, 1950).

Unheated mouse spleen suspensions of psittacosis virus were treated, in different experiments, with varying strengths of crude trypsin at pH 8·0 and with a preparation of crystalline trypsin in a final concentration of 0·1 mg./ml. at pH 7·6 for 1 hr. at 37°. The virus was separated by centrifugation in the cold and re-suspended in the appropriate volume of saline. Neither the heat-stable nor the heat-labile components were affected by trypsin treatment, as shown by

TABLE II.—*Effect of Treatment with Crude Papain Extract on Heated and Unheated Suspensions of Psittacosis Virus from Mouse Spleen.*

Guinea-pig serum.	Prepared against.	Dilution.	Autoclaved psittacosis suspension M19/24.				Unheated psittacosis suspension PB/2.		
			Untreated.		Treated with papain for 2 hr.		Untreated.	Treated with papain for ½ hr.	
			Deposit. 1/2.	S.N.F. 1/2.	Deposit. 1/2.	S.N.F. 1/2.	1/2.	Deposit. 1/2.	S.N.F. 1/2.
GP5	Unheated psittacosis virus (yolk-sac)	1/32	++++	+	++++	+++	+++	++++	−
		1/64	++++	++	++++	+++	++++	++++	−
		1/128	++++	+	++++	+++	+++	++++	−
GP2	Boiled and re-suspended psittacosis virus (yolk-sac)	1/32	++++	+	++++	+++	++	++++	−
		1/64	++++	+	++++	+++	+	++++	−
		1/128	++++	+	++++	+++	+	++++	−

the fact that treated suspensions were never found, in complement-fixation tests, to react differently from the control material.

Tests were also done with papain although only a very crude preparation was available. An extract containing papain was prepared by suspending 1 g. papain powder (B.D.H.) in 100 ml. saline. After leaving the suspension overnight in the refrigerator, a clear yellow solution was obtained by filtration through paper and this was neutralized to pH 7·2 with 0·1 N NaOH. In experiments with unheated and heated psittacosis virus from mouse spleen, a 1/10 volume of the enzyme solution was added to the suspension and activated by 0·01 M sodium thioglycollate. The reaction was allowed to proceed for varying periods at 37° and was stopped by the addition of 0·001 M sodium iodoacetate. The virus was centrifuged and re-suspended to volume in saline. Control tubes received sodium iodoacetate before the other reagents, and the activity or inactivation of the enzyme was checked by testing samples for the ability to clot milk.

TABLE III.—*Complement Fixation and Skin Reactions with a Boiled Yolk-sac Suspension of L.G.V. Virus of which One Fraction was Treated with* 0·005 M *Potassium Periodate.*

Serum.	Dilution.	Boiled suspension L.G.V. 65. Untreated. 1/4.	Boiled suspension L.G.V. 65. Untreated. 1/8.	Boiled suspension L.G.V. 65. Treated with periodate. 1/4.	Boiled suspension L.G.V. 65. Treated with periodate. 1/8.	Normal yolk-sac suspension 1/4.
Serum P. human L.G.V.	1/8	++++	++++	±	—	—
	1/16	++++	++++	—	—	
	1/32	++++	+++	—	—	
	1/64	+++	++	—	—	
Skin test patient:						
T26370			12 mm.*		15 mm.	0 mm.
T31854			14 „		12 „	0 „

* Diameter of indurated lesion measured two days after intradermal inoculation.

With a boiled or autoclaved virus suspension, no loss of serological activity was detected after treatment for 2 hr. at 37°. The supernatant fluid of a sample volume withdrawn at 1/2 hr. was inactive, but some partial fixation of complement was shown by such fractions after incubation for 1½ and 2 hr. Unheated psittacosis virus suspensions were altered by similar treatment, so that after ½ hr. they became fully group-reactive. The supernatant fluids gave no reaction. Examples of these results are presented in Table II.

Periodate.—The destructive effect on the group component of high dilutions of KIO_4 was reported in a preliminary note (Barwell, 1948), and experiments with this reagent are described here in greater detail.

A stock solution of 0·02 M KIO_4 was added in varying quantities to measured amounts of boiled or autoclaved virus suspensions. After incubation at 37° for 10 to 30 min. the periodate was inactivated by the addition of an excess of glucose, and the products were tested by complement fixation and, in a few experiments, by skin reactions in Frei-positive patients.

In an experiment of this sort the effect on a boiled psittacosis mouse spleen suspension of periodate at three different concentrations was examined. Equal volumes of the suspension were diluted to the known complement-fixing titre of 1 in 4 by the addition of saline buffered to pH 7·6 and of KIO_4 so as to give

final concentrations of 0·001, 0·0005 and 0·0002 M. After 10 minutes at 37°, each tube, which contained a volume of 1·0 ml., received 0·05 ml. of 5 per cent glucose in saline. A control tube without periodate was included. It was found that the serological reactivity had been entirely destroyed by 0·001 M periodate, while twice this dilution produced only a slight reduction. The minimum amount of periodate required for full inactivation of the group antigen may in fact be less than that estimated, since it is possible that tissue components present in partially purified suspensions also combine with the reagent.

Similar results were obtained in experiments with boiled or autoclaved yolk-sac suspensions of psittacosis and L.G.V. viruses. A volume of boiled L.G.V. virus was treated for 30 min. at 37° with 0·005 M KIO_4, and was found to be serologically inactive when compared with an untreated fraction in a complement-fixation test using human serum and dilutions of the antigens at the titre of 1 in 4 and at 1 in 8. In this experiment intradermal tests were also done in two positive reactors using 0·1 ml. of 1 in 8 dilutions of both the untreated and treated virus suspension. The results are shown in Table III, and show that destruction of the complement-fixing activity of the heat-stable antigen by periodate was not accompanied by any gross loss of skin reactivity.

Attempts, by Means other than Absorption, to Demonstrate Specific Antigenic Activity.

It has been shown (Barwell, 1952) that exclusively specific reactions of fresh unheated virus can be demonstrated by complement fixation in the presence of immune sera from which the group antibody has been absorbed. This method has disadvantages, and depends on the presence of the specific antigen in a fully active state, a property possessed by only a proportion of fresh suspensions rich in virus.

The possibility of demonstrating, *in vitro*, specific reactions with unabsorbed sera might be realized (i) by the use of a reagent which eliminates only the group reactivity, (ii) by treating virus suspensions with some reagent which would preserve and enhance specific activity, or (iii) by separation of the virus components with the production of a specific fraction. These possibilities were investigated particularly with reference to some of the effects of treatment with different reagents already described above.

Specific activity of unheated virus treated by periodate.—Potassium periodate was used in the treatment of unheated virus because of its rapidly destructive effect on the serological activity of the group antigen. In one such experiment an unheated suspension of psittacosis virus from mouse spleen was divided into two fractions, one of which was treated with 0·002 M periodate for 30 min. at 37°. The two suspensions were then tested against the " complete " and " group " guinea-pig sera with the results shown in Table IV. In order to demonstrate a full degree of fixation with the serum containing specific antibody, it was necessary to use the treated suspension undiluted instead of at 1 in 2 per unit volume. Even with this concentration the " group " serum failed to react. The effect of boiling the periodate-treated virus was to reduce considerably its fixing power with the " complete " serum and to produce a small degree of activity with the " group " serum. This confirms further the destructive effect of heat on the specific antigen, and shows that some of the group antigen in elementary body suspensions is, as was found by skin tests, unaffected by periodate.

TABLE IV.—*The Effect of Potassium Periodate on Unheated Psittacosis Virus and the Behaviour of a Fraction Subsequently Heated to* 100°.

Guinea-pig serum.	Prepared against	Dilution.	Psittacosis suspension 116P. (mouse spleen). Untreated. Unheated. 1/2.	Untreated. Boiled. 1/4.	Periodate-treated. Unheated. 1/1.	Periodate-treated. Boiled. 1/1.
GP5	Unheated psittacosis virus (yolk-sac)	1/64	++++	++++	++++	++
		1/128	++++	++++	++++	++
		1/256	+++	+++	++	−
GP3C	Boiled and re-suspended psittacosis virus (yolk-sac)	1/16	+	++++	−	++
		1/32	++	++++	−	+
		1/64	+	++++	−	−

This reagent was also used to treat unheated yolk-sac suspensions of psittacosis and L.G.V. viruses. It was again found that, although the treated material gave feeble reactions except when used undiluted, L.G.V. virus reacted in a uniformly specific manner with pigeon and human sera. Psittacosis virus gave reactions which were of this character or which still showed some, though diminished cross-reactivity. In some instances treated yolk-sac suspensions of psittacosis virus failed to give satisfactory results, and this was probably due to a lack of active specific antigen in the particular batch of virus.

Attempted preservation of the specific component.—The very irregular serological activity of the specific antigen and its rapid deterioration in suspensions stored in the refrigerator suggested a search for some method of treatment which might have a preservative and perhaps enhancing effect on this component. The effect of formalin in this connection has not been investigated, although a diagnostic complement-fixing antigen has been prepared from yolk-sacs infected with psittacosis virus using prolonged formalin treatment followed by extraction with ether (Smadel, Wertman and Reagan, 1943). Nigg (1942) noted that formalin treatment of L.G.V. virus in crude yolk-sac suspensions made them markedly anti-complementary and for this reason investigated alternative methods of inactivating the virus ; all of these had the effect of enhancing the activity of the group antigen.

In this laboratory different virus preparations were treated with 0·5 to 2·0 per cent formalin for varying periods. In some cases psittacosis or L.G.V. virus suspensions showed, when tested within a few days of treatment, specific complement-fixing activity equal to but never better than the control untreated virus. Often such suspensions, whether from mouse spleen or yolk-sac, were intensely anti-complementary when first tested ; they invariably developed this property when kept for further study. This effect could not be removed by washing the virus in saline, nor could it be prevented by removal of excess formalin in the supernatant fluid obtained after centrifuging the suspension as soon as the period of treatment had ended.

Neutralization of formalin by excess ammonium hydroxide followed by adjustment of the pH with sulphuric acid has been used in the preparation of stable non-infectious influenza virus antigens (Polley, Burr and Gillen, 1951). Similar treatment of formolized psittacosis virus successfully removed the anti-complementary effect but, in a limited number of tests, these products were not found to be more stable or more reactive than fractions untreated with formalin.

It was thought possible that certain enzyme inhibitors might have a useful effect in preventing or slowing the loss of specific antigenic activity. The reagents tested in this laboratory had already been shown to reduce *in vitro* the infectivity of psittacosis virus as shown by subsequent titration in eggs (Burney and Golub, 1948). In the present experiments no definite preservation or improved specificity resulted from the exposure of psittacosis virus to 0·0005 M sodium *p*-chloromercuribenzoate, 0·001 M mepacrine methanesulphonate or 0·001 M proflavine sulphate.

Soluble fractions containing specific antigen.—The improved activity of the group antigen which resulted from treatment of fresh virus suspensions with papain, dilute HCl or heat suggested that the process involved might be one in which the specific antigen was dissolved from the virus particles, thus exposing an increased amount of the group component. After these methods of treatment all, or almost all, the group antigen remained in association with the elementary bodies. The possibility that specific antigen might be detectable in virus-free fluids after acid treatment was therefore investigated; some of the results obtained in these experiments have already been reported briefly (Barwell, 1949).

After treatment with 0·02 N HCl at 37° for 20 min., unheated yolk-sac suspensions of psittacosis and L.G.V. virus were angle-centrifuged in the cold at 4500 r.p.m. for 1½ hr., and the faintly opalescent supernatant fluid removed. This was neutralized by the cautious addition of NaOH to pH 7·5. At about pH 5·0 a large precipitate began to appear and usually flocculated during the addition of further alkali. The tube was left in the refrigerator until flocculation appeared to be complete, after which the precipitate was removed by centrifugation and the almost clear supernatant extract reserved for use. In order to equate as far as possible the quantities of material which might be present in these extracts of the two viruses the initial L.G.V. virus deposit, obtained during the preliminary purification process, was re-suspended in half the volume of saline used in the case of psittacosis virus. From infected yolk-sacs the yield of L.G.V. virus is regularly about half that of psittacosis virus. Heated suspensions of the viruses made in these relative quantities give parallel antigen titres in complement-fixation tests, and a further check was made by titrating the residual acid-treated virus after re-suspension in the appropriate volume.

With these extracts positive results were never obtained by complement fixation. In some tests more than one unit volume of undiluted fluid was used, and in others a long period (18 to 24 hr. in the refrigerator) was allowed for fixation. Other *in vitro* methods which gave negative results included attempts to agglutinate specifically erythrocytes of various animals or collodion particles which had been exposed to the extracts and then washed. Precipitin reactions also were not obtained with antisera from different sources.

Trials of these fractions were made by intradermal tests in patients whose Frei and complement-fixation tests were positive, and in a small number of subjects in whom there had been clinical and serological evidence of infection with psittacosis or ornithosis virus.

The results of these tests are shown in Table V, where the reactions to the standard heated and diluted antigens and to the undiluted acid extracts are recorded as the mean diameter of the area of infiltration 2 days after intradermal inoculation. Any zone of erythema extending beyond or occurring in the absence of a palpable lesion was disregarded. In none of the results shown in the table

TABLE V.—*Skin Test Reactions Measured 2 Days after Intradermal Inoculation of Boiled Virus and of Acid Extracts.*

	Patient.	Diagnosis.	Heated suspensions.		Acid extracts.	
			L.G.V.	Psittacosis.	L.G.V.	Psittacosis.
A	T33986	L.G.V. Inguinal adenitis	(6)	—	25	<5
A	T34684	L.G.V. Inguinal adenitis	(5)	(5)	17	0
A	H7265	L.G.V. Rectal stricture	(10)	12	20	0
A	H5779	L.G.V. proctitis	8	15	25	0
A	T18132	? Latent L.G.V.	17	—	12	0
A	A. S. M—	Psittacosis	0	20	0	15
B	T8930	L.G.V. penile ulcer	10	8	23	0
B	F. O. M—	Psittacosis 15 yr. previously	0	12	0	0
C	T34738	L.G.V. Treated case.	(13)	—	30	0
D	T25730	Early L.G.V.	37	—	36	15
D	T47432	L.G.V. Inguinal adenitis	(12)	—	20	10
D	H12869	L.G.V. Inguinal adenitis	9	—	10	0
D	R. M—	Atypical pneumonia	<5	9	11	27
E	T50612	L.G.V. Old case	16	—	32	6
E	T50940	L.G.V. Old case	20	—	20	<5

The figures represent the mean diameter in millimetres of the indurated lesion. No reaction = 0. Not tested = —. Where tests were done on separate occasions in one patient, earlier results are shown in brackets. All patients gave positive complement-fixation reactions.

All patients in any one of the five groups were injected with the same two batches of extracts.

was there any reaction to normal yolk-sac suspension but the readings were vitiated by such a reaction in one other patient.

The number of infections with psittacosis or closely related virus was very limited. Six patients having this form of atypical pneumonia and not available in London were tested, through the co-operation of local practitioners and pathologists, with acid extracts only of the two viruses ; no positive reactions occurred. Three other patients were tested with both heated viruses and extracts ; A. S. M—, six months after psittacosis, reacted to both preparations of the homologous virus and not to either made from L.G.V. virus. Patient F. O. M—, fifteen years after psittacosis, reacted only to the heated homologous suspension. R. M— was tested within a few weeks of recovery from this form of atypical pneumonia and reacted to heated psittacosis virus, but not to heated L.G.V. virus ; both extracts produced lesions, but that in response to the homologous material was more than twice the diameter of the reaction to L.G.V. virus extract.

Of the 12 patients with a diagnosis of L.G.V., one showed no evidence clinically of infection but gave a positive Frei and negative serum test ; he was suspected of having a latent infection with this virus. All 12 developed conspicuous lesions in response to extracts of L.G.V. virus, and, in some cases these were two or three times the diameters of those produced in the same individual by the diluted boiled virus. A similar extract of psittacosis virus had no effect in 7 of these patients, produced insignificant reactions in 2 (less than 5 mm. in diameter),

and in the remaining 3 produced lesions which were half or less than half of those elicited by the homologous extract.

DISCUSSION.

It seems probable that heating removes from the virus particles the specific antigen and possibly some inert tissue component so that an increased amount of group antigen becomes available. Electron micrographs have shown that a peripheral zone of low absorbing power disappears from human pneumonitis virus as the result of boiling (Kurotchkin, Libby, Gagnon and Cox, 1947), but this effect was not seen after acid treatment of psittacosis virus (Barwell *et al.*, 1950).

A similar enhancement of group activity was produced by treatment with phenol, ether, dilute hydrochloric acid and crude papain. After exposure to heat or any of these reagents a small amount of group antigen was often detectable in the supernatant fluid after removal of the virus by centrifugation. Prolonged treatment with anaesthetic ether resulted in more complete disruption of the virus, since the major part of the group-active material was found in the ether-soluble fraction. Unlike papain, trypsin was not found to alter the antigenic activity of living psittacosis virus, and this conforms with the observation of Lazarus and Meyer (1939) that the infectivity of this virus is unaffected by similar treatment.

The marked heat-stability of the group antigen suggests that it might be polysaccharide in nature, while the solubility in ether of this antigen from yolk-sac suspensions might indicate a lipid component. In this connection Hilleman and Nigg (1946) have suggested that the antigen might be bound with a lipid derived from the yolk itself, and it was subsequently found (Hilleman and Nigg, 1948) that highly purified antigenic material from L.G.V. virus was inactive unless lecithin were added. The non-protein nature of the group antigen is further supported by its resistance to trypsin and papain.

The destructive effect of high dilutions of potassium periodate on red cell receptors for influenza virus led Hirst (1945; 1948) to conclude that the substance was, at least in part, carbohydrate. It is known that this reagent attacks chemical linkages which are also present in other classes of compound, for example the amino-acids serine and threonine. Goebel, Olitsky and Saenz (1948) demonstrated the destruction by periodate of the biologically active protein substances ribonuclease and an immune globulin; the virus of Western equine encephalomyelitis was also rapidly inactivated. These workers were using considerably higher concentrations of periodate than were employed by Hirst or in the present experiments, and it may be reasonable to regard the effect on psittacosis and L.G.V. viruses as supporting the other evidence in favour of the carbohydrate nature or content of the group antigen.

Although various methods of formalin treatment of virus suspensions from mouse tissue or embryonated eggs were tested, a marked degree of anti-complementary activity invariably developed. It is possible that more highly purified suspensions would not present this difficulty and, in any case, such preparations might repay full investigation by the method of agglutination. Formolized suspensions of psittacosis virus from allantoic fluid were agglutinated by antisera from pigeons, but not by a limited number of human L.G.V. sera which fixed complement in the presence of the heated antigen (Labzoffsky, 1946).

Acid treatment of virus suspensions was investigated especially for the

presence of soluble specific antigen in the supernatant fluids, but the neutralized extracts were never found, by a variety of *in vitro* methods, to contain detectable amounts of group or specific antigen. They were, however, active in the skin sensitized patients and produced sharply specific reactions in 9 out of 12 with L.G.V. while, in the remaining 3, considerably larger reactions resulted from the homologous extract. In 2 patients recently recovered from psittacosis the reaction to these extracts was wholly or mainly specific, but they did not react to Frei antigen although they did to heated psittacosis virus. This contrasts with the findings in L.G.V. patients, in whom the reactions to boiled psittacosis and Frei antigen were equally good.

Apparently skin sensitivity always develops in patients with L.G.V., while in human infections with viruses of the psittacosis group this occurs much less regularly. Rake, Eaton and Shaffer (1941), for instance, found the Frei test to be positive in only 5/8 cases of atypical pneumonia due to this cause, but phenolized psittacosis virus was not used as well in these tests. It is possible that this difference is due to the more acute nature of psittacosis, although a very chronic carrier state can occur (Meyer and Eddie, 1951). In rabbits previously infected with meningopneumonitis, psittacosis, mouse pneumonitis, feline pneumonitis or L.G.V. virus, Kilham (1948) demonstrated cross intradermal reactions in the majority to phenolized antigens. There were however some negative results with heterologous antigens, and one rabbit, infected with mouse pneumonitis virus, reacted only to the homologous antigen. Virus suspensions are not usually washed after treatment with heat or phenol, and it seems probable that the specific antigen is sometimes still capable of playing a major rôle if the antibody to it predominates.

Further work is required in order to define the properties of the specifically reactive component mainly present in acid extracts of psittacosis and L.G.V. viruses. Investigation is also needed into the possible relationship between the specific toxic factor studied by Rake and Jones (1944) and the specific antigen demonstrable by the skin reactions and *in vitro* tests described here.

Where fowl sera have been used in inhibition complement-fixation tests for studying viruses of this group the antigens were prepared in such a way as to destroy, or considerably reduce, the activity of the specific antigen (Karrer, Meyer and Eddie, 1950; Hilleman, Haig and Helmold, 1951). However, Downie and MacDonald (1950) found that sera from hyperimmune chickens revealed specific differences and affinities between the pox viruses more clearly than mammalian sera. It might well be profitable to use chicken sera in this way with unheated suspensions of the Castaneda-positive viruses, and similar concentrated and purified preparations might be found to agglutinate specifically with sera from the same source.

SUMMARY.

An enhanced complement-fixing activity of the group antigen, comparable with that produced by boiling or autoclaving, resulted from the treatment of unheated psittacosis or L.G.V. virus with phenol, dilute hydrochloric acid or papain. In these and in similar tests on boiled virus the group antigen was resistant to these reagents, but slight group activity was sometimes detected in virus-free supernatant fluids. In experiments with acid-treated suspensions

these traces of soluble group antigen were precipitated on neutralizing the centrifuged fluid.

Ether treatment of unheated yolk-sac suspensions of psittacosis virus also produced enhancement of group activity. Ether extracts, separated from the aqueous layer after prolonged exposure, contained most of the group antigen.

Both heat-labile and heat-stable components were unaffected by trypsin.

High dilutions of potassium periodate rapidly and completely destroyed the complement-fixing power of boiled suspensions of both viruses. Such preparations were still capable of eliciting positive Frei reactions. This reagent effectively eliminated the relatively weak group activity of unheated virus suspensions, and specific complement fixation was then obtained although high concentrations of virus were required for this purpose.

Various attempts to prepare suspensions of regular and stable specific reactivity were unsuccessful.

Extracts of psittacosis and L.G.V. viruses prepared by treatment with dilute hydrochloric acid did not react *in vitro*, but produced specific or largely specific skin reactions in psittacosis and L.G.V. patients.

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