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VERVET MONKEY DISEASE:

STUDIES ON SOME PHYSICAL AND CHEMICAL PROPERTIES OF THE CAUSATIVE AGENT

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SUMMARY.—Heat inactivation at 56° for 30 min. failed to inactivate completely the causative agent of vervet monkey disease. Higher temperatures or longer periods are required to bring about complete inactivation.

Ultraviolet light completely inactivates the agent.

There is little if any loss of infectivity on storage at room temperature (+ 20°) + 4° and − 70° over a period of up to 5 weeks.

Filters with an average porosity size of 100 mμ or less were required to produce non-infectious filtrates.

The viricidal effects of a number of chemicals have been tested and chemotherapeutic studies have been carried out with a variety of antibiotics.

Electron micrographs prepared from infected monkey blood reveal particles similar to those seen in monkey and guinea-pig tissue and tissue culture preparations.

SINCE the isolation of the causative agent of vervet monkey disease in guinea-pigs (Smith, Simpson, Bowen and Zlotnik, 1967; Siegert, Shu, Slenczka, Peters and Muller, 1967; May and Knothe, 1968) work has been mainly directed to the study of its pathogenesis in different animals and tissue culture systems. Simpson, Bowen and Bright (1968) experimentally transmitted the disease to monkeys and Zlotnik, Simpson, Bright, Bowen and Batter-Hatton (1968*a*) propagated the agent in tissue culture. Filtration experiments have suggested a size of 450–1000 mμ: larger than most of the known viruses (May and Knothe, 1968). Electron microscopic examination of guinea-pig tissue and tissue culture material has revealed particles of various shapes and sizes (Peters and Muller, 1968; May and Knothe, 1968; Zlotnik, Simpson and Howard, 1968*b*). Kissling, Robinson, Murphy and Whitfield (1968) have shown the agent to be ether sensitive, relatively heat labile and resistant to the metabolic inhibitor 5-bromo-desoxyuridine suggesting RNA as the genetic material. The German workers have provisionally named the agent Marburg virus and opinions have been expressed of similarities to vesicular stomatitis and rabies viruses.

Due to the highly infectious nature of the agent, there was a need for further investigations to be carried out on some of its physical and chemical properties with a view to the safe handling of potentially infected material. We were particularly interested in heat inactivation so that we could carry out serological studies on serum samples taken from infected animals, and also to find out whether those chemicals used in the fixation of infected tissue removed for histological examination were in fact capable of inactivating the agent. Human cases of the

disease in Germany were treated with a variety of antibiotics which had little or no effect on the course of illness. We considered it very necessary to study the effects of a variety of chemotherapeutic agents both *in vivo* and *in vitro* against the causative organism in an attempt to find a satisfactory method of treating the disease.

Finally we examined electron micrographs of the causative agent prepared from monkey blood and compared the structures seen with those seen in guinea-pig and monkey tissue and in tissue culture preparations.

MATERIALS AND METHODS

Vervet monkey agent.—A 10 per cent suspension of infected monkey liver (RH 10) previously passaged 9 times through guinea-pigs and 3 times through monkeys was prepared in borate buffered saline (BBS) pH 7·2 containing 0·75 per cent bovine albumin (Armour Fraction V). This was centrifuged at 2000 rpm to remove large particles and diluted in BBS to contain approximately 10^6 guinea-pig infective doses per 0·1 ml. This was used for all experiments unless otherwise stated and is referred to as RH 10 liver.

Guinea-pigs.—Dunkin–Hartley strain animals (250–300 g.) were used to determine infectivity titres. They were inoculated i.p. with 0·1 ml. volumes, 2 guinea-pigs being used for each dilution.

Filtration experiments were carried out using Millipore cellulose filters of the following average pore sizes 1250 mμ, 450 mμ, 300 mμ, 220 mμ, 100 mμ and 50 mμ.

Ether sensitivity.—Equal volumes of vervet monkey agent and diethyl ether were mixed together and held at 4° for 1 hr.

Desoxycholate sensitivity.—Desoxycholate was added to an RH 10 liver suspension to give a final concentration of 1 : 1000 this was incubated at 37° for 1 hr.

Trypsin sensitivity.—A suspension of RH 10 liver containing 0·5 per cent trypsin was incubated at 37° for 1 hr.

Ultraviolet light inactivation.—RH 10 liver suspension in 1 ml. amounts were dispensed into plastic wells, each well having a diameter of 3·5 cm. and a depth of 1 cm. These were placed on a rocking shelf with a rocking cycle of 17 complete cycles per min. and at a distance of 15 cm. from a 30 watt UV tube giving an output of between 65–80 milliamps. Exposure times of 30 sec. 1, 2, 4 and 8 min. were used.

Thermal inactivation.—Vol. (0.5 ml.) of RH 10 liver suspension were sealed in ampoules, and immersed in a 56° ± 0·5° water bath. Samples were removed at intervals of 10, 20, 30, 40, 50 and 60 min., immediately chilled in an ice bath and titrated in guinea-pigs. The same procedure was adopted for 60° but at this temperature we only used one sampling time of 60 min. Suspensions of infected material were also kept at room temperature (20°), 4° and at 70°, and titrated at intervals over a period of 8 weeks.

The viricidal effect of the following chemicals was tested at room temperature. Tego MHG (1 per cent), cetramide (2 per cent), phenol (0·5 per cent), formalin (1 per cent), Chloros (2 per cent), methyl alcohol (90 per cent) and acetone (90 per cent). Pieces of infected guinea-pig liver measuring 8 × 2 mm. were fixed in 2 per cent osmium tetroxide for 24 hr. The tissues were then rinsed and ground up in diluent to give a 20 per cent suspension and inoculated into guinea-pigs.

Tissue culture.—Monolayer cultures of baby hamster kidney (BHK) cells maintained in the Pirbright modification of Eagle's medium (Capstick, Telling, Chapman and Stewart, 1962) were grown on coverslips in Leighton tubes. Cultures were infected with serial 10-fold dilutions of RH 10 liver suspension and maintained at 37°, medium being changed every 3 days. Cultures were fixed for 2 hr. in 5 per cent picric acid in methyl alcohol and stained with a modification of Picro–Mallory (Zlotnik, 1953) 7–10 days after infection. A reduction in the titre of the vervet monkey agent was indicated by reduction in the number of typical intracytoplasmic inclusion bodies (Zlotnik *et al.*, 1968a).

Chemotherapeutic studies

These were carried out in (a) tissue culture, (b) *in vitro* or (c) *in vivo*.

Tissue culture.—Various drugs were incorporated in the Eagle's medium used to maintain BHK cells. The following drugs and dosages were studied: Penicillin 200 units/ml.,

Streptomycin 200 units/ml., Tylosan 0·5 μg/ml., Marboran 10 μg/ml., M and B 7714 10 μg/ml., Caffeic acid 12·5 μg/ml., Adamantanamine hydrochloride 50 μg/ml., 5-iodo-deoxyuridine 30 μg/ml. and 5-bromo-deoxyuridine 30 μg/ml.

In vitro *tests* were carried out by mixing equal volumes (0·2 ml.) of serial 10-fold dilutions of RH 10 liver suspension with suspensions of the drugs listed below and incubating them for 1 hr in a 37° water bath. Each dilution was then inoculated into guinea-pigs, a control titration in BBS being included for comparison. The drugs used were: Tylosan, Marboran, γ-dithiosemicarbazone, Ledermycin, Sulphamezathine and Gantrisin.

In vivo *tests*.—Guinea-pigs infected with approximately 1000 guinea-pig LD_{50} of RH 10 liver suspension were treated daily with the following drugs in the dosages shown: Erythromycin 50 mg., Chloramphenicol 37·5 mg., Terramycin 12·5 mg., Ledermycin 10 mg., Sulphamezathine 100 mg., Gantrisin 200 mg., Midicel 30 mg., Gantanol 30 mg., Bidizole 20 mg., γ-ethoxyethylglyoxal-dithiosemicabazone (356C61) 5 mg., Primaquine 5 mg., Chloroquine 5 mg., Fanasil 130 mg., Trimethoprim 5 mg., Pyrimethamine 13 mg., Gantanol (30 mg.) in conjunction with trimethoprim (5 mg.), Fanasil (100 mg.) in conjunction with pyrimethamine (10 mg.) and Sulphamezathine (100 mg.) in conjunction with chloroquine (5 mg.).

Untreated infected control guinea-pigs were included in all the experiments and uninfected control guinea-pigs were given equivalent dosages of each drug.

Electron microscopic examination

Monkey blood, which appeared to be one of the richest sources of agent in body fluids with titres of approximately 10^6 guinea-pig i.p. LD_{50}/ml. was used for electron microscope examination.

Six days after infection blood was obtained from monkeys under anaesthesia, by cardiac puncture using heparin as an anticoagulant. The blood was immediately subjected to light centrifugation (2000 rpm for 5 min.). Plasma was collected and centrifuged at 30,000 rpm in a Spinco centrifuge for 3 hr. at 2°. The pellet was reconstituted in Michaelis buffer pH 7·2 to give a 10-fold concentration of the agent compared to the original concentration in the blood. Subsequently it was filtered through a 1250 mμ Millipore cellulose filter. The filtrate was mixed with an equal volume of 2 per cent osmium tetroxide in Michaelis buffer pH 7·2 and left for 24 hr.

The fixed plasma was mixed with an equal volume of 2 per cent ammonium molybdate at pH 7·0 and small drops were placed on electron microscope specimen grids coated with Formvar, the excess fluid being removed with filter paper. The grids were then stabilized with a film of evaporated carbon and examined in a Philips EM 300 electron microscope using double condenser illumination and an accelerating voltage of 80 kV.

RESULTS

Filtration studies

Having examined electron micrographs of this agent and seen structures measuring up to 1500 mμ in length by about 100 mμ in diameter one can appreciate the difficulty in trying to estimate its size by filtration. We have attempted in these experiments to give some idea not of the size of the agent but the average porosity size of filter that will produce a non infectious filtrate. The results of 3 experiments are presented in Table I. Although the majority of infectivity is lost after filtration through a 300 mμ filter, this and the 220 mμ filter does allow a small percentage of the agent to pass into the filtrate. It was only when we used

TABLE 1.—*Infectivity Log Titre**

Average filter porosity size	1250 mμ	450 mμ	300 mμ	220 mμ	100 mμ	50 mμ	Control
Exp. I	5	4	2	2	0	0	5
Exp. II	5	4·5	2·5	0·5	0	0	5
Exp. III	5	5	2	2	0	0	5

* Guinea-pig i.p. LD_{50}/0·1 ml.

filters with an average porosity of 100 mμ or less that we were able to produce non-infectious filtrates.

Thermal inactivation

Fig. 1 shows the inactivation curve at 56°. Two components were produced, (i) a linear component between 0–20 min. when the majority of the agent is

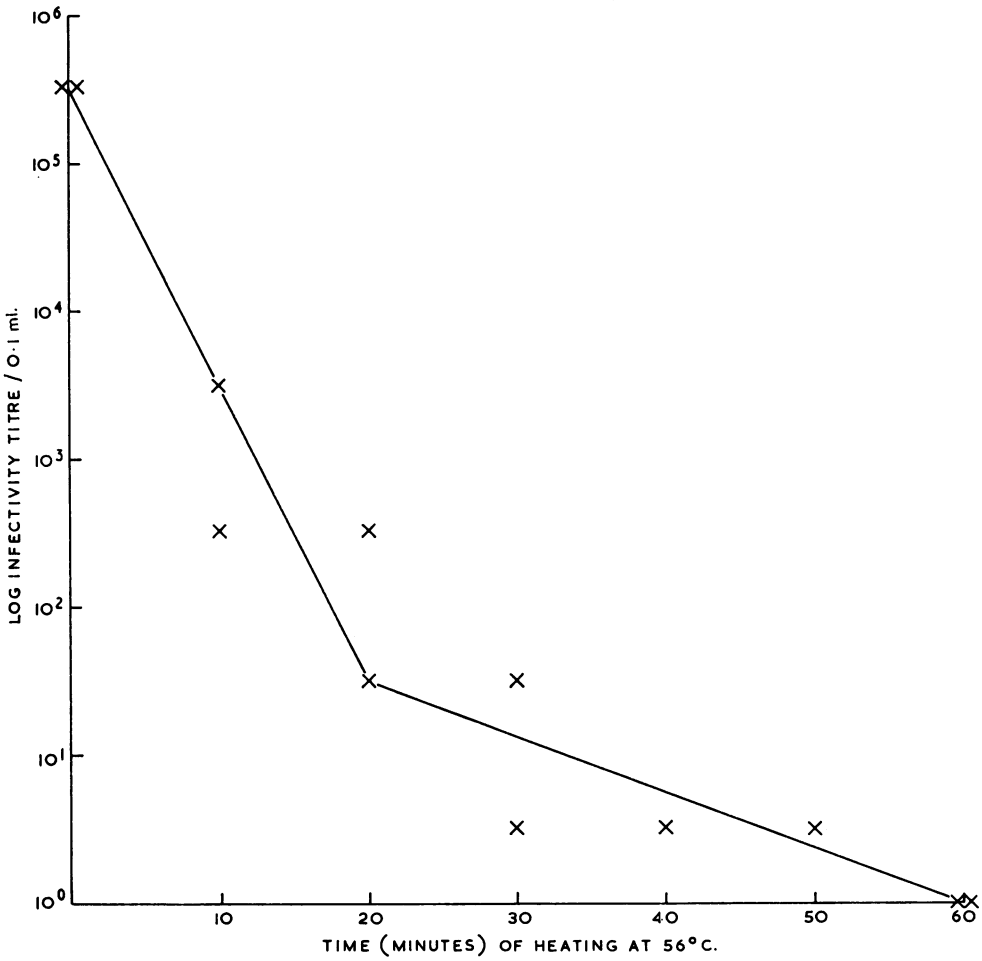


FIG. 1. Heat inactivation curve of the vervet monkey agent.

inactivated and (ii) a second component which contains a small residual fraction that is relatively heat resistant, inactivation taking place at a much slower rate. Slopes of best fitting lines were computed together with their standard errors. These were significantly different at the 1 per cent level.

Heating at 60° for 1 hr completely destroyed all infectivity in three separate experiments.

The stability experiments at — 70°, 4° and room temperature are presented in Table II. There appears to be very little fall in infectivity on storage at these temperatures for periods of up to 5 weeks. At 8 weeks the infectivity titre of the room temperature sample was reduced to 0·5 logs and the 4° sample to 2·5 logs. The — 70° sample showed no drop in titre and has in fact been fairly stable over a period of 12 months.

TABLE II.—*Effect of Storage at Room Temperature, 4° and — 70° on Virus Infectivity*

Days	Infectivity log titre* −70°	4°	Room temp.
0	5·5	5·5	5·5
2		5·5	5·5
5		5·5	5·5
9		5·0	4·5
14		5·5	5·5
19	5·5	5·5	4·0
28		5·0	4·5
35		5·0	5·0
56	5·5	2·5	0·5

* Guinea-pig i.p. LD_{50}/0·1 ml.

Ultraviolet light inactivation

Ultraviolet light reduced the infectivity titre from 5·5–1·5 log LD_{50} in 30 sec. and this was reduced further to 0·5 log LD_{50} in 1 min., complete inactivation being achieved by 2 min.

Chemical effects

Both diethyl ether and sodium desoxycholate completely destroyed the infectivity of the agent as tested in guinea-pigs, indicating the presence of an essential lipid. Formalin, acetone, Chloros, methyl alcohol and Tego MGH completely inactivated the agent after 1 hr exposure. Fixation in osmium tetroxide for 24 hr was also capable of destroying infectivity. Phenol, Cetramide and trypsin did not completely destroy but only reduced the infectivity titre. These results are summarized in Table III.

TABLE III.—*The Effect of Various Chemicals on Virus Infectivity*

Temperature tested	Infectivity log titre* after 1 hr exposure 4°	Room temp.	37°
Control	6	6	6
Trypsin	–	–	5
Phenol	–	3	–
Cetramide	–	2	–
Ether	0	–	–
Sodium desoxycholate	–	0	–
Formalin	–	0	–
Acetone	–	0	–
Chloros	–	0	–
Methyl alcohol	–	0	–
Tego MHG	–	0	–

* Guinea-pig i.p. LD_{50}/0·1 ml.

In order to ascertain, whether the above treatments which were capable of inactivating the agent, would confer any form of immunity on the animals,

surviving guinea-pigs were challenged with a 1000 guinea-pig i.p. LD_{50} 3 weeks following the initial inoculum. All the guinea-pigs succumbed to infection, indicating that after this type of treatment the agent may also lose its capacity to act as an immunogen.

Chemotherapeutic studies

In general none of the drugs used to treat infected guinea-pigs had any effect on the course of illness which was always fatal. Occasionally the illness was prolonged for a further 1–2 days. In the experiments carried out in BHK cell cultures 5-iodo-deoxyuridine (IUDR) and 5-bromo-deoxyuridine (BUDR) had no effect on the agent's infectivity as judged by the appearance of intracytoplasmic inclusions. This would suggest that the agent contained RNA rather than DNA. None of the other drugs used affected the infectivity.

The *in vitro* studies with the longer acting sulphonamides—Sulphamezathine and Gantrisin—were very interesting in that there was a marked fall in infectivity after incubating the RH 10 liver suspensions with these drugs. The fall in titre was 3 log LD_{50} with Sulphamezathine and 2 log LD_{50} with Gantrisin. No corresponding effect was produced in the *in vivo* studies when these drugs were used to treat infected guinea-pigs.

Electron microscope studies

Particles of a definite morphology could be distinguished under the electron microscope. The particles were elongate and often twisted. Some particles were short, rod-shaped, semi-circular or hook-like (Fig. 2 and 3), some particles ended in bulbous structures of 250–300 mμ diameter (Fig. 6). It was difficult to ascertain whether the short particles were fractions of larger particles. The particle diameter was approximately 100 mμ and the length of the particle ranged from 900 mμ for the short to approximately 1500 mμ for the longest observed. It is worth pointing out that whereas the short particles had no side branches or bulbous ends (Fig. 2 and 3) the longer ones were either twisted in various directions or folded over or showed evidence of branching (Fig. 4, 5, 6 and 7). A small number of particles showed either complex twisting of one end (Fig. 6) or a bulbous structure with small side branches (Fig. 7).

The inner structure of the particle consisted of an inner electron dense cylinder surrounded by a light cylinder and finally an outer very light zone. Cross striations could be discerned in the middle layer.

DISCUSSION

Laboratories involved in serological studies have relied a great deal on heat inactivation at 56° for 30 min. as a means of rendering potentially infected serum non-infectious. Several workers have demonstrated heat resistant fractions at this temperature with a number of viruses and studies reported here indicate that this type of treatment is also insufficient to bring about the complete inactivation of Marburg vervet monkey disease agent. There appears to be a relatively heat resistant fraction which requires longer treatment at 56° to render samples safe for handling. The results of our heat inactivation at 60° for 60 min. suggest this might be a safer procedure for routine use.

Filtration experiments by other workers have shown the size of this agent to be 450–1000 mμ (May and Knothe, 1968) and structures seen in electron micrographs measure anything between 900 mμ–1500 mμ in length by 100 mμ in diameter. Our studies indicate that small amounts (approx. 100 guinea-pig i.p. LD_{50}) of infective agent are capable of passing through filters with an average porosity of 220 mμ diameter. Filtration methods are universally used as a means of sterilizing body fluids such as serum, where heat inactivation needs to be avoided. These results should be borne in mind, particularly in the filtration of body fluids from monkeys.

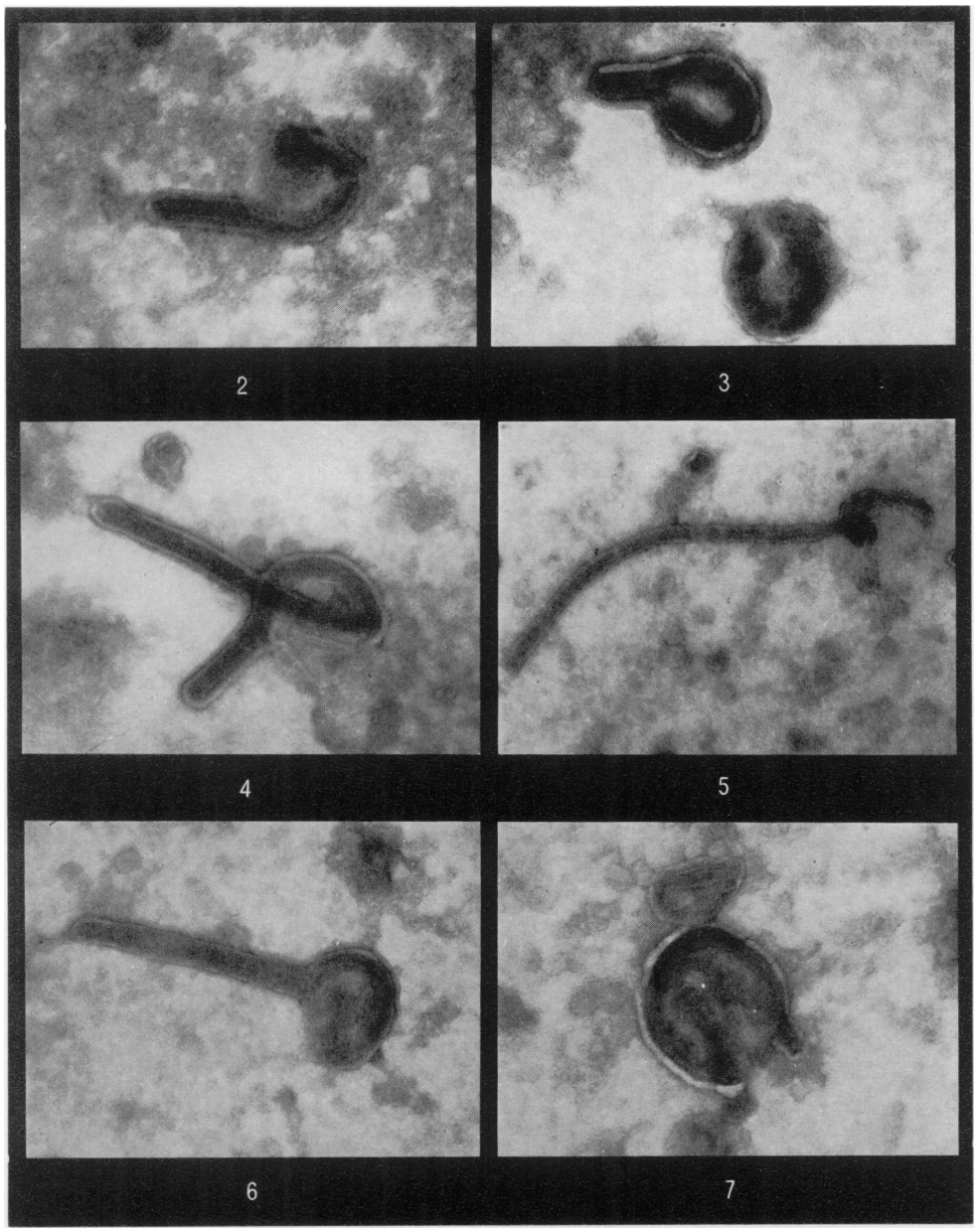
Hennessen, Bonin and Mauler (1968) showed that 4 of the German laboratory workers probably became infected through handling cell suspensions prepared from the kidneys of incriminated monkeys. This is in accord with our findings in that the agent withstands treatment with trypsin for the period normally used in the preparation of tissue culture material. There is also very little, if any, loss of infectivity on storage at room temperature over periods of up to 5 weeks. Findings of this kind must once again emphasize the need for some attention to be directed towards carefully designed procedures for handling tissues and body fluids removed from monkeys.

The only interesting results to emerge from the chemotherapeutic studies were the non-inhibitory effects of IUDR and BUDR which suggests a nucleic acid composed of RNA. This is consistent with the results of Kissling *et al.* (1968). The poor response of infected guinea-pigs to the variety of drugs used is disappointing but not surprising in view of the fact that the agent resembles certain viruses.

The particles of the agent found in the blood of monkeys resemble those described previously by Siegert *et al.* (1967) for guinea-pig blood, by Zlotnik *et al.* (1968*b*) for guinea-pig and monkey livers and BHK cells, by Kissling *et al.* (1968) in guinea-pig liver and BHK cells, by Peters and Muller (1968) in guinea-pig liver and by Haas, Maass, Muller and Oehlert (1968) for monkey liver. A consensus of opinion exists as to the actual recognition of the agent. There is however a very important point of difference in morphology of the particles as seen in tissue cells from that seen in body fluids. The large membranaceous bulbous heads of the agent as described by Zlotnik *et al.* (1968*b*) measuring up to 750 mμ in diameter and frequently seen in tissue cells could not be found in the blood plasma. At the same time some branching of the particle or special twisting of one end could be observed. Some of the long particles appear to have a small bulbous ending measuring 250–300 mμ in diameter which could not be explained by branching or twisting. The cross striation and cylindrical morphology is again present in each case and no basic difference could be found in the structure irrespective of the source, whether it be guinea-pig or monkey liver, guinea-pig or monkey blood or BHK cells. Recently tubular structures associated with leptospires have been described which bear a morphological resemblance to the agent of vervet monkey diseases (Almeida, Waterson, Berry and Turner, 1969).

EXPLANATION OF PLATES

FIG. 2–7.—Electron microscope pictures of various particles of vervet monkey disease agent in vervet monkey plasma ($\times$ 48,000).



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The actual characterization and classification of the agent is still premature until further properties have been elucidated.

Opinion has been expressed of similarities between the agent of vervet monkey disease and viruses of the Stomatoviridae or rhabdovirus group. Although admitting the general resemblance, the fact that it is the first virus of such morphology that can form agglomerations visible under the light microscope in guinea-pig tissue (Smith *et al.*, 1967; Simpson *et al.*, 1968) and in hamster tissue (Zlotnik, 1969; Zlotnik and Simpson, 1969) points to some special properties which require further investigation.

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REFERENCES

ALMEIDA, JUNE, WATERSON, A. P., BERRY, D. M. AND TURNER, L. H.—(1969) *Lancet*, i, 235.

CAPSTICK, P. B., TELLING, R. C., CHAPMAN, W. G. AND STEWART, D. L.—(1962) *Nature, Lond.*, **195**, 1163.

HAAS, R., MAASS, G., MULLER, J. AND OEHLERT, W.—(1968) *Z. med. Mikrobiol. Immun.*, **154**, 210.

HENNESSEN, W., BONIN, O. AND MAULER, R.—(1968) *Dt. med. Wschr.*, **93**, 582.

KISSLING, R. E., ROBINSON, R. Q., MURPHY, F. A. AND WHITFIELD, S. G.—(1968) *Science*, **160**, 888.

MAY, G. AND KNOTHE, H.—(1968) *Dt. med. Wschr.*, **93**, 620.

PETERS, D. AND MULLER, G.—(1968) *Arzneimittelforsch.*, **65**, 1831.

SIEGERT, R. V., SHU, H.-L., SLENCZKA, W., PETERS, D. AND MULLER, G.—(1967) *Dt. med. Wschr.*, **92**, 2341.

SIMPSON, D. I. H., BOWEN, E. T. W. AND BRIGHT, W. F.—(1968) *Lab. Anim.*, **2**, 75.

SIMPSON, D. I. H., ZLOTNIK, I. AND RUTTER, D. A.—(1968) *Br. J. exp. Path.*, **49**, 458.

SMITH, C. E. G., SIMPSON, D. I. H., BOWEN, E. T. W., AND ZLOTNIK, I.—(1967) *Lancet*, ii, 1119.

ZLOTNIK, I.—(1953) *Nature, Lond.*, **172**, 962.—(1969) *Trans. R. Soc. trop. Med. Hyg.*, in the press.

ZLOTNIK, I. AND SIMPSON, D. I. H.—(1969) *Br. J. exp. Path.*, **50**, 393.

ZLOTNIK, I., SIMPSON, D. I. H., BRIGHT, W. F., BOWEN, E. T. W. AND BATTER-HATTON, D.—(1968*a*) *Br. J. exp. Path.*, **49**, 311.

ZLOTNIK, I., SIMPSON, D. I. H. AND HOWARD, D. M. R.—(1968*b*) *Lancet*, 26.