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# PURIFICATION STUDIES ON THE INFECTIOUS CANINE HEPATITIS VIRUS

## I. VIRAL PARTICLES IN TISSUE CULTURE FLUID REVEALED BY ONE PURIFICATION PROCEDURE

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### INTRODUCTION

Morphological studies on the infectious canine hepatitis (ICH) virus have been reported on ultra-thin-sections of the liver of infected dog<sup>12)</sup> and infected monolayer epithelial cells of dog kidney<sup>13,14)</sup>. There have been observed viral particles of about 65 m $\mu$  or 71 m $\mu$  (TAJIMA, 1958), 61 m $\mu$  (TOKUI, 1959), 54 m $\mu$  (TAJIMA, 1959) in the nucleus of infected cells. However, the morphology of purified virus particles remains untouched.

The present report describes one method of purification of the ICH virus from infectious tissue culture fluid for electron microscopy.

### MATERIALS AND METHODS

#### Virus:

Strain "MATSUDA"<sup>8)</sup> has been preserved in this laboratory since 1954 by passage through puppies and it was subsequently transferred serially 4 to 6 times in monolayer cultures of dog kidney epithelial cells. Monolayer cultures of dog kidney cells were prepared according to the method described by YOUNGNER. Cell growths usually became confluent after 4 to 7 days. At this stage they were washed one time with Hanks' salt solution and inoculated with a virus suspension. One to two hours were allowed for adsorption, the inoculum was removed, and 50 to 70 ml of the medium consisting of 1% horse serum in Earle's balanced salt solution containing 0.5% lactalbumin hydrolysate were added. Then the flasks were incubated at 37°C. Virus was harvested in the tissue culture fluid (TCF) when the cytopathic effect became nearly complete. This occurred at 4 to 5 days after inoculation. Immediately after harvesting, the infectious TCF was subjected to purification or stored at -20°C until it could be purified.

#### Virus purification:

The procedure for the purification and concentration of the virus is in part a modification of the methods used in the purification of poliomyelitis virus<sup>1,9,10,11)</sup> and foot-and-mouth disease virus<sup>2)</sup>.

Infectious TCF (1,000 to 2,000 ml) was centrifuged for 30 minutes at 2,500 rpm in the horizontal centrifuge to remove the cellular debris; the pH may be adjusted to 4 with 1 N HCl. Chilled methanol at  $-20^{\circ}\text{C}$  was then added slowly to bring the final concentration to 15% by volume. Super Cel suspension (50 g Hyflo-Super Cel in 1 liter of pH 4 saline) was added to one-twentieth volume of the original TCF. The mixture was emulsified for 3 hours by means of a magnetic stirrer. The precipitate was collected on a fritted glass filter. Approximately 200 ml of pH 4 saline (0.13 M NaCl in 0.01 M  $\text{KH}_2\text{PO}_4$  adjusted to pH 4 with 1 N HCl) was drawn through the precipitate on the filter to remove excess methanol. The precipitate was then resuspended to one-twentieth of the original volume in pH 9 saline (0.88 M NaCl in 0.04 M  $\text{Na}_2\text{HPO}_4$  adjusted to pH 9 with 1 N NaOH) and allowed to stand overnight in the refrigerator. To elute the virus, the suspended precipitate was warmed to room temperature, filtered off, and collected in an ice-chilled flask. Additional elution of 1 hour duration was made with the same quantity of pH 9 saline at  $37^{\circ}\text{C}$ . Two volumes of a 50:50 mixture of n-butanol and chloroform were introduced with stirring and the mixture was stirred for 1/2 hour. The organic aqueous emulsion which resulted was broken down by centrifugation at 2,800 rpm for 1/2 hour. Further purification and concentration were accomplished by one cycle of high-and-low-speed centrifugation in the Hitachi Model-40P ultracentrifuge. The conditions for sedimentation were centrifugation for 1 hour at 40,000 rpm (100,000 g) in a rotor No. 40. The resulting virus pellet was suspended by vigorous pipetting and gentle rocking in a small volume of pH 7.8 to 8.0 isotonic saline (0.11 M NaCl in 0.01 M  $\text{Na}_2\text{HPO}_4$ ). The suspended pellets were pooled and clarified by centrifugation at 10,000 rpm (7,000 g) for 10 minutes, and the supernatant fluid which contained the virus was recovered. The concentration of the above supernatant fluid varied between 300- to 400-fold depending upon the initial volume of infectious TCF. The temperature was maintained at  $0^{\circ}$  to  $4^{\circ}\text{C}$  except where otherwise stated.

#### Electron microscopic preparation:

The various fractions were prepared for electron microscopy by the agar pseudo-replica method<sup>3</sup>. The specimens were shadowed with palladium or unshadowed, and electron micrographs were made from random areas of the screens use being made of Model JEM-4CHD instrument.

#### Infectivity titrations:

Infectious culture fluids and purified viral fractions therefrom were determined by inoculation of 0.2 ml of decimal dilutions into sets of three roller tubes containing renal epithelium. Following reincubation of 7 days the 50% tissue culture infectious doses ( $\text{TCID}_{50}$ ) were calculated by the method of Behrens-Kärber<sup>6</sup>.

#### Nitrogen analyses:

A digestion and nesslerization procedure were used to determine the protein-nitrogen content of TCF and purified virus fractions by the micro-Kjeldahl method.

## RESULTS

Recovery and purification of virus infectivity in 3 experiments are summarized in table 1.

TABLE 1. *Recovery of Infectivity and Degree of Purification of "MATSUDA" Strain of the ICH Virus*

FRACTION	RECOVERY OF INFECTIVITY*			PN**/ml ( $\mu$ g)
	Exp. 1	Exp. 2	Exp. 3	
Original TCF	4.5	4.5	6.0	772
Soluble part of methanol ppt	4.5	6.0	6.5	128
Aqueous phase after extraction with organic solvent	3.5	4.8	6.0	56
Purified virus concentrates after differential centrifugation	3.5	4.5	5.5	28

\* TCID<sub>50</sub> per milliliter expressed as the  $-\log$  of the dilution.

\*\* Protein-nitrogen value

The recovery of virus after methanol precipitation was very good, ranging 100% or more in 1/6 of the original protein-nitrogen. However, the infectivity in aqueous phase after extraction with n-butanol-chloroform mixture decreased to about 1/5 to 1/10 in 1/2 protein-nitrogen value of methanol precipitates.

Electron micrographs of agar pseudo-replica derived from purified virus concentrates revealed a uniform-sized, discrete particle in large number (Figs. 1, 2 & 3). Occasionally, these particles formed clusters (Fig. 7).

Measurement of 100 monodispersed particles revealed the diameters to be in the range of 75 to 100  $m\mu$  (average 85  $m\mu$ ) in unshadowed specimens, 88 to 102  $m\mu$  (average 96  $m\mu$ ) by shadow-casting method. It will be noted that in the unshadowed preparation the particles appear to be approximately spherical in shape and some of them have a central dense core surrounded by less dense material enclosed in a membrane (Fig. 4). The central objects continue to be 40 to 50  $m\mu$  across. In the shadowed preparation a central raised area is also apparent, the outline of particle is sharp and some particles contain a central protuberance (Fig. 5). It appears that the central core observed in unshadowed preparation is equivalent to this protuberance. It is interesting that the central dense core was observed in the purified viral particle as in sections of cells.

The morphology of the particles from soluble part of methanol precipitate was also similar to that of the final purified fraction, but the preparation contained a large amount of amorphous material (Fig. 6 a, b).

#### DISCUSSION

The application of methanol precipitation was excellent for partial purification and concentration of the ICH virus from TCF. However, after the extraction with n-butanol-chloroform mixture showed decrease of infectivity. The results in one experiment using several organic solvents are presented in table 2.

It was observed that the infectivity was lost to a considerable extent during the treatment with n-butanol, ethyl ether or n-butanol-chloroform mixture. Only

TABLE 2. Recovery of Infectivity\* after Extraction with Various Organic Solvents

ORIGINAL TCF	SOLUBLE PART OF METHANOL PPT	AQUEOUS PHASE AFTER EXTRACTION WITH			
		n-BuOH**	CHCl <sub>3</sub>	C <sub>2</sub> H <sub>5</sub> ·O·C <sub>2</sub> H <sub>5</sub>	n-BuOH-CHCl <sub>3</sub> *** (50 : 50)
> 7.5	> 7.5	6.0	> 7.5	4.8	6.8

\* TCID<sub>50</sub>/ml

\*\* n-butanol

\*\*\* 50 : 50 mixture of n-butanol and chloroform

chloroform treatment was satisfactory for the maintenance of infectivity. It seems that the profusion of 85 m $\mu$  or 96 m $\mu$  particles revealed by electron microscopy occurred in parallel with viral infectivity. The particles shown in nuclei of epithelial cells by TOKUI et al. and TAJIMA et al. (1959) were average 61 m $\mu$ , 54 m $\mu$  in size, respectively.

On the other hand, it has been reported that adenovirus particles were approximately 50 by 30 m $\mu$  in size and were separated from each other by a clear area measuring about 27 m $\mu$  in nuclei of HeLa cells (KJELLEN et al.), but the purified particle was 90 $\pm$ 4 m $\mu$  (HILLEMANN et al.). According to HILLEMANN, if one assumes that the particles observed were but the central portions of larger particles which touched each other, the full particle size may be estimated to be around 77 by 57 m $\mu$ , so that the size is not greatly different between nuclear and purified particles. It may be assumed to be the same on the ICH virus particle, too.

KAPSENBERG recently reported that the virus of infectious canine hepatitis shares the four main properties of the adenovirus group, including common complement-fixing antigen. Adding to these characters, morphological resemblance was clarified by the authors, viz., approximately the same size of this virus compared with the size of adenovirus group reported by HILLEMANN et al. On the other hand, TOKUI in this laboratory proved the crystalline arrangement of ICH virus in the infected dog kidney cell nuclei with tissue culture. These morphological finding will support the views of KAPSENBERG.

#### SUMMARY

Infectious canine hepatitis virus, strain "MATSUDA", from dog kidney tissue culture has been purified by methanol precipitation, extraction with organic solvents, and differential centrifugation. The ICH virus particle in the resulting purified virus concentrates has been identified by electron microscopy in agar pseudo-replica specimens as a uniform-sized particle 85 m $\mu$  (unshadow), 96 m $\mu$

(Pd-shadow) in diameter. These particles were found in parallel with viral infectivity. Some of the particles contain a central dense core surrounded by less dense material enclosed in a membrane when unshadowed and this central core when shadowed appears to be protuberant.

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## EXPLANATION OF PLATES

## PLATE I.

- Fig. 1. Unshadowed micrograph of a final preparation of ICH virus  $\times 27,000$   
 Fig. 2. The same preparation as fig. 1 after being shadowed with palladium  $\times 27,000$

## PLATE II.

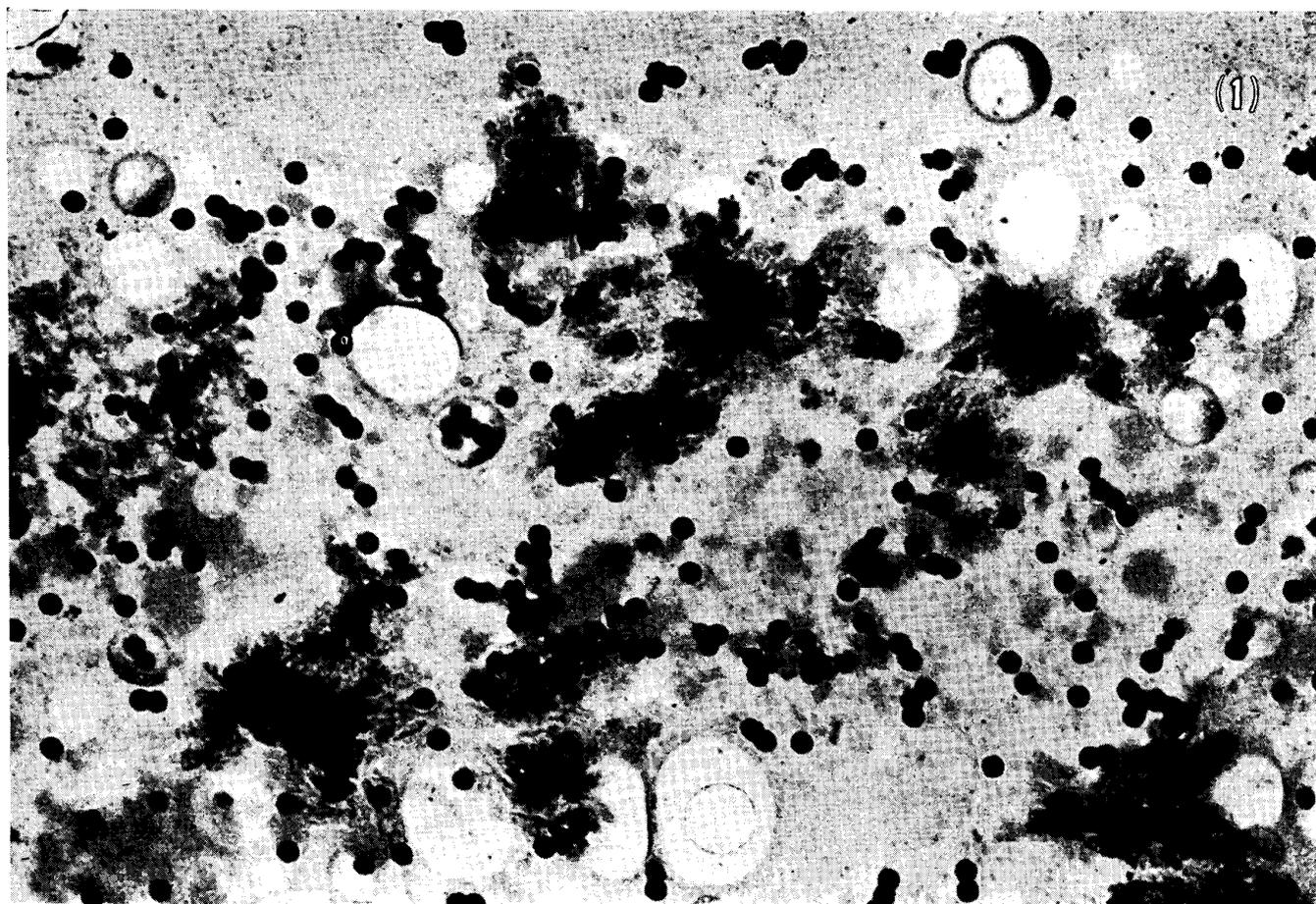
- Fig. 3. Electron micrograph of purified viral particles at higher magnification Pd-shadow  $\times 63,000$

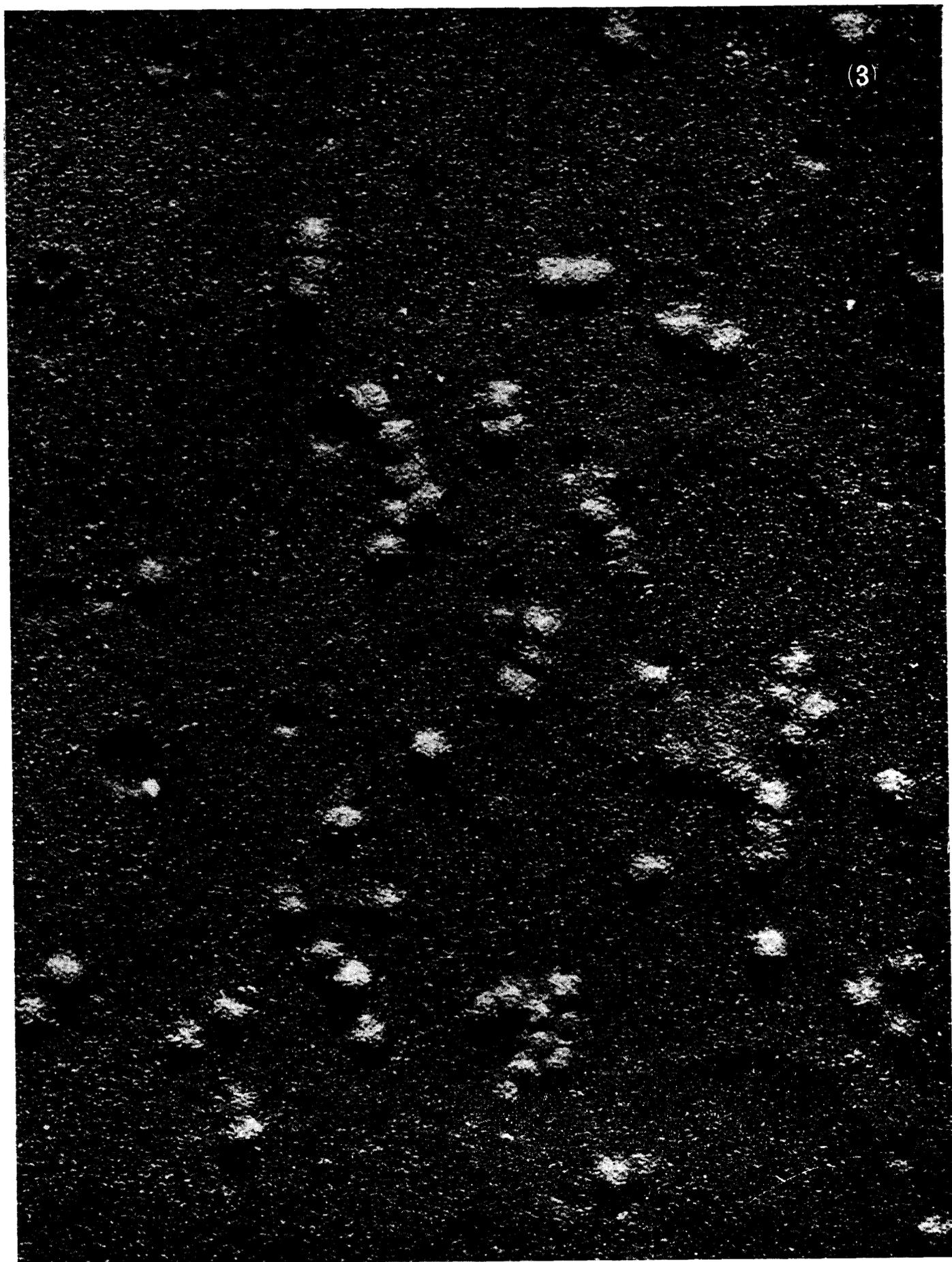
## PLATE III.

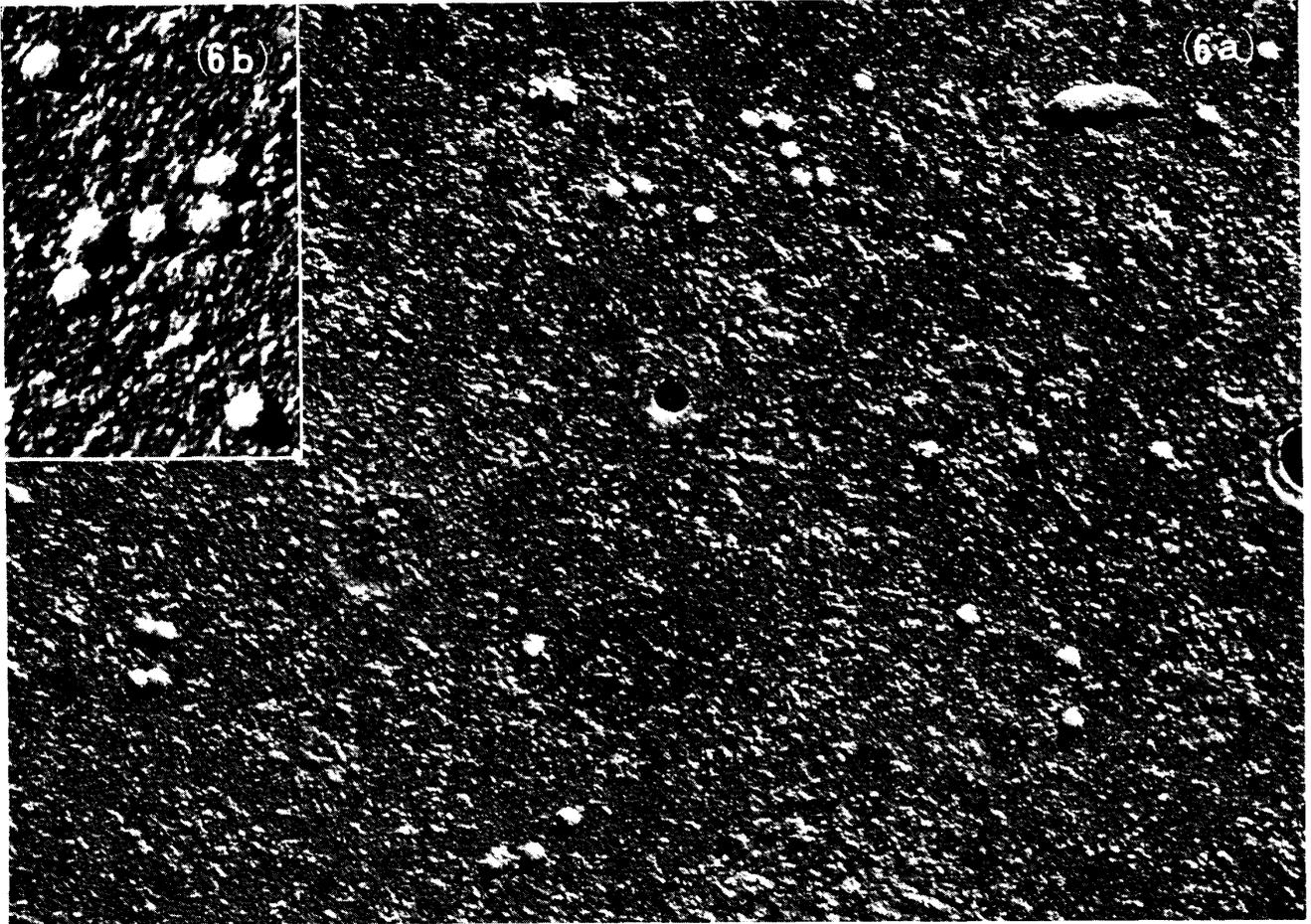
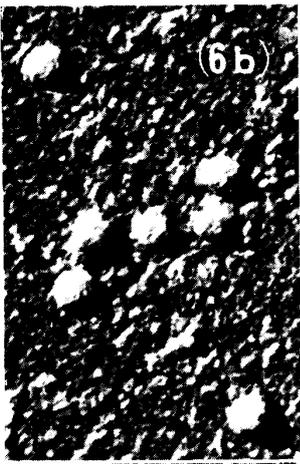
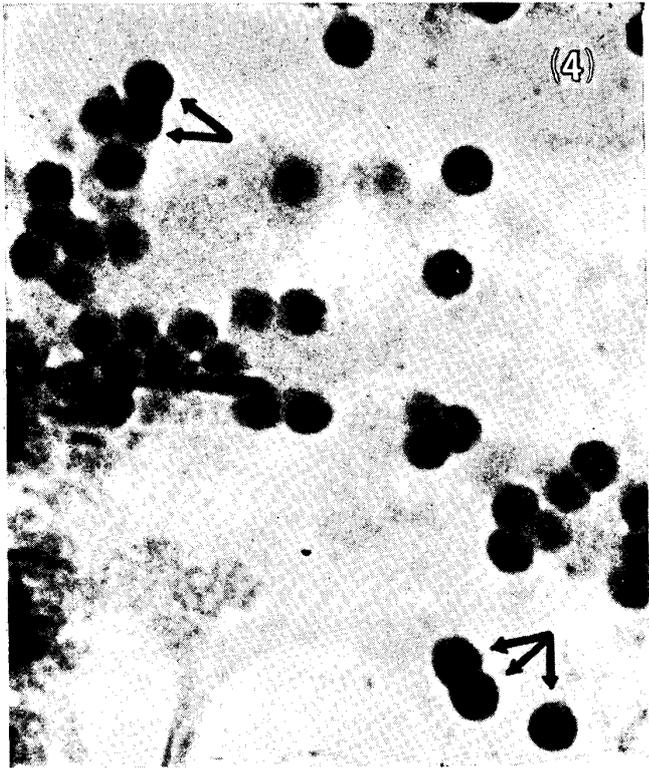
- Fig. 4. Part of fig. 1 at higher magnification Arrows indicate central dense core.  $\times 63,000$   
 Fig. 5. Part of fig. 2 at higher magnification Arrows indicate flattened and central protuberant particles.  $\times 63,000$   
 Fig. 6. Viral particles in methanol precipitate The preparation contains a large amount of amorphous material.  
 Pd-shadow a)  $\times 22,500$  b)  $\times 45,000$

## PLATE IV.

- Fig. 7. Cluster of viral particles Pd-shadow  $\times 50,000$









## INFORMATION

The Hokkaido University has granted the degrees of Doctor and of Master of Veterinary Medicine to the following nine graduates on March 25, 1960. The authors' summaries of the doctor's and master's courses are as follows:

1) Theses for the doctor's course

ABE, Mitsuo (*Department of Veterinary Anatomy*) : A STUDY ON THE INTERVERTEBRAL DISC OF THE MINK

All intervertebral discs of 43 minks aged 30 days to 2 years were investigated with special attention to normal structures and pathological features. According to the findings of the author himself the characteristics of the normal structures and abnormal features found in his specimens were discussed; they are briefly summarized as follows:

- 1) Out of the regional differences the specific character of the disc-like structure at the base of the processus dentis was discussed at first, and the differences in the structures of the nucleus pulposus (Np) of the cervical, thoracic and lumbar vertebrae were pointed out.
- 2) The postnatal changes of the Np were traced comparing 30 days, 50 days, 60 days, and over 1 year old specimens. Also attention was given to changes of the annulus fibrosus (Af).
- 3) The presence of the costal conjugal ligaments on the dorsal region of the Af of the thoracic disc (1-10) was noted. The delayed development of the cartilage of the Np of the thoracic disc, and the very thin and most degenerated state of the dorsal annulus fibrosus (Afd) of the thoracic disc were discussed.
- 4) As the abnormal features of the intervertebral discs there were noted: a) the splitting and desquamation of the Afd with rupture of the bone epiphysis, b) the outflow of the Np into the epiphyseal bone marrow cavity, c) the proliferation of the cartilage and the hyaline degeneration of the cartilaginous plate (Cp), d) the fragmentation of the dorsal longitudinal ligament and e) the abnormal thickness of the disc.
- 5) Out of very frequent splittings and bone ruptures only a few cases were seen to have occurred in vivo; the majority of them seemed to be artefacts resulting from the pelting processes.
- 6) Some extrusions of the Np seemed to be artefacts, but some seemed to be abnormal features which had occurred in vivo.
- 7) The proliferous activity and the hyaline degeneration in the region of the Cp seemed to be a sort of reaction to some mechanical stresses exerted upon the disc in vivo.
- 8) Ruptures of the dorsal longitudinal ligament are also pathological features which suggest the effect of some strong stress brought to bear upon these regions.
- 9) By the rates of the thickness of the Afd and the ventral annulus fibrosus (Afv) it is easy to define the top region of the so-called lordosis of the thoracic vertebrae. These regions show such topographic anatomical characteristics as sudden disappearance of the costal conjugal ligaments and *m. rotatores* and unusual features of the Afd.
- 10) As the dominating feature of the lordosis there are shown, a) sudden elevation of the ratio Afv/Afd, b) hemorrhage and some sort of degeneration of the Np. But these are not only specific in lordosis but also are seen in other regions.
- 11) The above mentioned abnormal features of the disc such as the splitting and rupture of the Afd, the prolapse of the Np into the bone marrow, the proliferous activity and degeneration of the Cp and the fragmentation of the dorsal longitudinal ligament do not

correspond to the lordosis topographically but there are some intimate agreements between their occurrence and the lordosis.

12) In view of these facts, the so-called lordosis seems to be one of the appearances of some unknown disturbance of a certain fundamental phenomenon such as general cartilagopoesy or calcium metabolism.

13) Concerning the direct cause of the abnormal features at the lumbar regions consideration is given to the mode of copulation of the mink and the behavior of the mink in resistance to handling by man.

AMADA, Akio (*Department of Physiology*) : A STUDY ON THE ELECTROCARDIOGRAM OF THE CAT

Since there have been few publications about the electrocardiogram of the cat, no reliable data with respect to the normal value of the electrocardiogram have been gathered and the excitation process in the heart of the cat has not been described distinctly. In the present experiment, standard limb, unipolar limb and unipolar leads from 70 positions on the body surface were employed. In addition, the intracavity leads of right auricle and ventricle were also used. The results obtained are summarized as follows:

The normal value of the standard limb lead, viz., amplitudes and intervals were measured. The results of the unipolar limb and unipolar lead from the 70 positions both on the ventral and dorsal face of the chest warrant the following generalization in respect to the P wave and QRS complex. a) Unipolar leads that face over the cranial surface of the auricle show a negative P wave whilst those that face over the caudal surface of the auricle show a positive P wave. b) Unipolar leads that face over the base of the heart show a QS pattern. c) Unipolar leads that face over the epicardial surface of the left ventricle show a qR pattern. d) Unipolar leads that face over the epicardial surface of the right ventricle show an rS pattern. A general rule with respect to T wave, as seen in the QRS complex, was not obtained. Concerning the intracavity lead, when the different electrodes were situated in the *vena cava cranialis* and *caudalis* in the neighbourhood of the auricle, a negative P wave and QS or rS patterns were obtained. When the electrode was in the cavity of the right auricle, diphasic P wave and QS pattern were recorded. When the electrode was situated in the cavity of the right ventricle, a small positive P wave and QS pattern were obtained. The distribution of the instantaneous potential on the chest was analogous to the results reported in the human being and dog. The above described results show that the excitation process in the heart of the cat bears resemblance to that in the human being, dog and rabbit.

SATÔ, Yûji (*Department of Biochemistry*) : STUDIES ON THE TOXIN PRODUCTION OF *CLOSTRIDIUM BOTULINUM* TYPE E (IWANAI STRAIN)

Recently, some researchers have demonstrated that a large amount of botulinum toxin is found closely associated with the cells and can be obtained directly from washed cells.

In this paper, the mechanisms of toxin production in *Clostridium botulinum* type E (Iwanai strain), especially in relation to autolysis have been studied and the results are as follows:

- 1) *Clostridium botulinum* type E (Iwanai strain) was readily autolysed in the culture, after the maximum phase of its growth. The autolysis of the cell was closely parallel to the increase in toxicity of the culture supernatant.
- 2) A large amount of toxin was obtained from the cells by mechanical treatment or by exposure to hypertonic solution.
- 3) The toxin exists within the cells growing in the culture medium before it appears in

that medium. At the early period of cultivation, the amount of toxin existing within the cells was much greater than that in the culture supernatant.

4) The amount of toxin obtained from the cells at the maximum phase of cell growth by sonic oscillation was equal to the total amount in the supernatant cultured by the same method, while only 1/3 to 1/5 of the amount was liberated by treatment with hypertonic solution.

5) A great increase in toxicity was observed by treatment with trypsin against the supernatant of the culture, the suspension or extract of the cells, and against the supernatant prepared from the cells ruptured mechanically.

6) In the case of cell extract, optimum increase in toxicity caused by incubation with trypsin at 30°C was observed at pH 6.0 to 8.0 for 15 to 30 minutes. This reaction was inhibited completely by the addition of crystalline soy bean trypsin inhibitor. The greatest increase in toxicity at pH 8.0 occurred for approximately 15 minutes, and the toxicity was decreased rapidly by further incubation.

7) No proteolytic enzyme, such as proteinase or peptidase was detected in the cell extract or in the culture supernatant tested. Furthermore, no mechanism concerning the conversion of "protoxin into toxin" in the cell free filtrate, in the culture supernatant or in the extract of the cells examined was demonstrated.

From the results described above, the author concluded that the toxin, as well as the protoxin, is produced within the cell and exists in it.

## 2) Theses for the master's course

### ITAKURA, Chitoshi (*Department of Veterinary Pathology*): A PATHOLOGICAL INVESTIGATION ON SOME PERIPHERAL NERVES OF SLAUGHTERED HORSES—CENTERING AROUND THE COELIAC AND ANTERIOR MESENTERIC GANGLIA—

Histo-pathological examination was made of some peripheral nerves principally the coeliac and anterior mesenteric ganglia, thoracic part of sympathetic trunk and femoral nerve from 152 slaughtered horses (1 to 15 years of age) gathered indiscriminately. Various remarkable alterations of the nerve cells and nerve fibers, being quantitatively rather a few, were distinctly recognized in very many cases: vacuolization in cytoplasm, chromatolysis, profound cell changes, liquefaction, dissolution, loss, etc. in the nerve cells, and desolation (degeneration and loss) in the nerve fibers and bundles; it appears likely that the latter displays a picture of multicentral appearance. Almost all of these changes were regarded to be pathological; they were substantiated by many photographs taken of numerous cases.

With regard to interpretation of the lesions, the author has offered several histopathogenetical considerations, and furthermore, "Koshifura disease", "Yanagawa disease", both of them B<sub>1</sub>-avitaminosis in horses, and "Grass disease" of horses were referred to from the patho-morphological viewpoint.

### ITO, Akiharu (*Department of Epizootiology*): STUDIES ON HEMOLYTIC *ESCHERICHIA COLI* STRAINS ISOLATED FROM EDEMA DISEASE OF SWINE IN JAPAN

1) In Japan, MIURA et al. (1958) reported the isolation of hemolytic *E. coli* strains of 2 different O-groups from pigs which suffered from edema disease. The present writer made it clear that the strains isolated most frequently by MIURA et al. belonged to O139:K82(B):H1, whilst the strains of the other O-group were O2:K?:H1.

- 2) In 1958 and 1959, 19 *E. coli* strains, 18 hemolytic and 1 non-hemolytic, were isolated from 17 piglets suffering from the disease which occurred in 4 districts of Hokkaido. Of the hemolytic strains, 12 belonged to O139:K82(B):H1, whilst 4 hemolytic and 1 non-hemolytic strains belonged to O2:K?:H1. The remaining 2 strains did not belong to O-1~140 groups (this O-antigen was assumed as O-E4).
- 3) Duodenal and colonic contents and mesenteric lymph nodes obtained from carcasses of the diseased pigs indicated frequently pure or almost pure growths of the hemolytic *E. coli*.
- 4) From 2 out of 7 piglets which recovered from the disease, 2 *E. coli* strains were obtained. Of the two strains, a hemolytic one belonged to O139:K82(B):H1 and the remaining non-hemolytic one to O-2 (K- and H-antigens were not investigated).
- 5) From feces of 12 piglets in contact with those affected with the disease, 4 hemolytic and 2 non-hemolytic *E. coli* strains were isolated. Among the hemolytic strains, 3 were O139:K82(B):H1 and one was non-typable (this had no antigens common to the above described O-groups).
- 6) From feces of 25 apparently healthy piglets obtained from 10 unaffected herds in 3 out of the above-mentioned 4 districts, 8 hemolytic *E. coli* strains were isolated. Out of the 8 strains, 3 belonged to O139:K82(B):H1, 2 to O2:K?:H?, 2 in common with O-E4 group and the remainder was non-typable.
- 7) Intestinal contents, tonsils and other materials of 185 pigs and of 110 cattle and a number of materials from horses and sheep in an abattoir were investigated for hemolytic *E. coli*. A total of 46 isolated strains did not belong to any of the O-groups such as O-139, O-138, O-2 and O-E4.
- 8) Broth cultures of hemolytic strains of O-139 and O-2 groups and those of hemolytic strains isolated from healthy cattle and pigs killed mice more rapidly than non-hemolytic strains of pig origin, but the filtrates of these cultures did not kill the animals.

Some of the hemolytic and non-hemolytic strains isolated from the piglets affected with edema disease, and hemolytic and non-hemolytic strains from healthy pigs were examined by DE's test. The duodena of rabbits inoculated with the strains from edema disease and the hemolytic one from healthy pig showed severe pathological changes such as *enteritis diphtherica haemorrhagica acuta et peritonitis purulenta acuta* but, in case of the non-hemolytic strain, the above described changes were not observed.

KANAGAWA, Hiroshi (*Department of Veterinary Obstetrics*): ANATOMICAL AND PATHOLOGICAL STUDIES ON THE SEX ORGANS FROM SLAUGHTERED BULLS IN HOKKAIDO

Anatomical and histo-pathological investigations were made on the genital organs from 15 bulls slaughtered at Sapporo from 1958 to 1959. They comprised 14 Holstein bulls and one Guernsey bull, ranging from 3 to 11 years of age. Their previous histories informed from the owners including the causes for disposal were discussed on the basis of post-mortem examinations.

The observations made were as follows:

- 1) Clinically, several of the cases seemed to be suffering from *impotencia coeundi* or *impotencia generandi*. Three cases suffering from the former disease showed inability for copulation caused by lameness, and another similar case was diagnosed as having psychic impotence, probably due to the fear originated in the failure at a previous service; although its libido was satisfactory at the following services, its penis could not protrude enough out of the preputial sheath which showed no adhesion. Two cases suffering from the latter disease had an abnormality in the semen picture showing low fertility, and

another similar case was suspected as a carrier of some hereditary prolonged gestation factor.

2) The anatomical positions of attachment of the ampullae to the seminal vesicle were classified into 4 types; dorsal type (5 cases), ventral type (1), intermediate type (7) and mixed type (2).

3) Among the testis, epididymis and tunica vaginalis, fine fibrous adhesions were observed in 11 cases most of which were bilateral. These adhesions were not considered as having any adverse effect on the fertility problems.

4) Remnants of the Müllerian duct were observed in 5 cases whose conditions were distinguished into two forms: one had a tubular construction beneath the prostate with a narrow passageway connecting to the urogenital canal, and the other had one or more firm vesicles located in the anterior or medial part of the urogenital fold without connection to the urogenital canal. All these Müllerian remnants were filled with translucent gelatinous mucus. Histologically, they had a narrow central cavity covered with single or stratified cubic, columnar and ciliated epithelium, and several smaller accessory cavities surrounding the central cavity.

5) Urinary calculi were found in 2 cases. In both of them, most of the calculi were light gray or white in color, of the size of a grain of sand or of rice and of the consistency of hard stones. The urinary tract and bladder were affected by chronic catarrhal inflammation. The chemical analysis of the urinary calculi showed mainly silicic acid.

6) Testicular degeneration was observed in 3 cases and orchitis in 2 cases. The former were similar to partial hypoplasia, showing degeneration in several seminiferous tubules with a single layer of markedly vacuolized spermatogonia, proliferated cells of Sertoli, moderately thickened basal membrane and almost empty cavities. The histological findings of the latter were accordant with those of chronic local interstitial orchitis, showing atrophy and vacuolation in the seminiferous tubules with moderately thickened basal membrane. In the interstitial tissue, there existed marked edema, proliferation of histiocytic cells, fibrosis and cellular infiltration or cellular foci. One of the orchitis cases had been treated with hormone preparations (1,000 mg of testosterone propionate and 10,000 IU of PMSG in total). During administration of these preparations, the number of spermatozoa increased about 2~3 times as compared with the finding before the therapy.

7) In the prepuce, chronic catarrhal posthitis was observed in 9 cases. In the corium intense cellular infiltration and foci were noted. The lymphatic nodules of the preputial mucosa were enlarged with marked hemorrhages in most of the cases.

SATÔ, Nanao (*Department of Hygiene and Microbiology*): STUDIES ON THE DISTRIBUTION OF DYE-TEST ANTIBODIES AMONG ANIMALS IN HOKKAIDO AND ON THE COMPLEMENT FIXING ANTIGEN FOR TOXOPLASMOSIS

The author investigated the status of latent infection among the several animal species (7 spp., 1053 serum samples) making use of the dye test.

On the other hand, as to the complement fixing antigen, comparative studies have been conducted from standpoints such as its antigenicity, anticomplementary activity and specificity, with the antigens extracted from chorio-allantoic membrane (AMA), mouse peritoneal fluid (MPA) and tissue culture fluid (TCA). The results obtained may be summarized as follows:

Distribution of dye-test antibodies

1) The author found dye-test antibodies (titers of 1:16 or more) for *Toxoplasma* in

high percentages such as in 44.2 per cent of 52 sheep and in the following decreasing order: 27.5 of 69 dogs, 25.2 of 580 cattle, 17.6 of 165 swine, 15.0 of 40 guinea-pigs, and 1.9 of 105 horses. All 42 domestic fowls tested were negative.

2) Some cases in sheep (1.9%), swine (3.5%) and dogs (3.6%) indicated high titers of 1:1024 or more. These data seem to suggest the existence of active infection of toxoplasmosis among these animals.

3) The relation between dye-test antibodies and several animal diseases was also investigated, however, there was no distinct relation found between them.

#### Comparative studies of complement fixing antigens

1) Antigenicity was superior in MPA and AMA to that in TCA. But in MPA, anticomplementary activity was observed in 1:4 dilutions, and also in TCA in 1:1, however in AMA, such activity was unrecognized absolutely.

2) To test the specificity of these 3 kinds of antigens, complement fixation test was conducted use being made of experimentally infected rabbit sera, dog sera and human sera. The results were almost similar to each other, and were quite parallel with those obtained by use of dye-test.

Accordingly, the author confirmed that these 3 antigens in any case had specific antigenicity and especially, TCA was superior in its reactivity.

#### TAKAGAKI, Yoshio (*Department of Hygiene and Microbiology*) : STUDIES ON BACTERIOPHAGE TYPING OF STAPHYLOCOCCI ISOLATED FROM BOVINE MILK

In 1959, NAKAGAWA proposed a new method of classification of the types of the coagulase-positive staphylococci from bovine milk samples. The author attempted to determine whether the same method was available as a phage typing method for the bovine staphylococci.

A total of 67 strains of coagulase-positive staphylococci were isolated from cow milk in 2 different areas (Sapporo & Nayoro) in Hokkaido.

The phages of NAKAGAWA which were isolated from bovine staphylococci by FISK's cross-culture method and 20 phages from N.C.T.C. (the National Collection of Type Cultures) were used for bacteriophage typing of the isolated strains.

Forty-one (61.2%) of 67 coagulase-positive strains were phage-typed by one or more of the 13 phages of NAKAGAWA, when used at tenfold dilutions for determining the R.T.D.: 32 (47.8%) by R.T.D. and 47 (70.1%) by 100×R.T.D. Furthermore, 21 of these 41 typable strains could be classified into the phages of group A and the other 20 into B group.

On the other hand, only 24 (35.8%) were phage-typed by the 20 phages of N.C.T.C. while 43 (64.2%) were untypable; 14 of these 24 typable strains were susceptible to the phage of group IV and the remainder could be classified into 4 different phage groups.

It appears from these results that the method reported by NAKAGAWA could be applied as a suitable test for phage types of the staphylococci from bovine milk samples.

The author attempted to classify the group A strains into 3 types (A<sub>1</sub>, A<sub>2</sub>, A<sub>3</sub>) on the basis of phage patterns with the 3 phage strains in addition to NAKAGAWA's phages. It is possible to speculate that this new phage set may be available as a phage typing method for udder staphylococci.

All of 363 coagulase-negative strains were insusceptible to the phages of NAKAGAWA.

Phage typing of coagulase-positive staphylococci was attempted from 4 species of domestic animals (horse, sheep, swine and cow) other than cow milk and *Homo sapiens*; 6 (8.1%) out of 74 staphylococci strains which were isolated from horses, 6 (23.1%) out of

26 strains from swine, 4 out of 10 from sheep, 3 out of 8 from cows and 14 (16.9%) out of 83 from *Homo sapiens* proved to be susceptible to NAKAGAWA's phages. The majority of these strains with the exception of sheep's and cow's were non-susceptible to NAKAGAWA's phages. Similarly, 12.2% (9/74 horses), 42.3% (11/26 swine), 50% (5/10 sheep) and 50% (4/8 cows) of the strains isolated from each animal were typed by the phages of N.C.T.C.

These results indicate that the strains isolated from each animal differ in respect to sensitivity of the phages. The approach to a detailed typing of staphylococci will probably need more phages from many origins.

On the other hand, study was made of antibiotic resistance and its relation to phages. In the study of 191 coagulase-positive staphylococci from bovine milk, it was found that 19.3% were resistant to penicillin. However, most strains which were lysed by group III phages were resistant to penicillin. On the other hand, group IV and group A<sub>1</sub>+A<sub>2</sub> strains were entirely sensitive. The incidence of resistance to streptomycin was 12.5% and no significance was noticed in relation to phage types.

Ninety-nine per cent strains were sensitive to tetracycline or oxytetracycline and 100% were to erythromycine or chloramphenicol. It would seem, therefore, that the relation between antibiotic resistance and the phage types can not be determined.

#### TAMURA, Tatsudo (*Department of Veterinary Anatomy*) : ABOUT VASCULAR STRUCTURES IN THE SUPRARENAL BODY OF THE CAT

The vascular structure in the suprarenal body of 47 cats was observed three-dimensionally. Neoprene latex 601 A was diluted with tap water 2~4 times; in that condition or colored to black with 10~15% black ink (PILOT Co.), it was used as the main injection material.

Such black latex can be recommended as a suitable injection material for stereoscopic observations of detailed arrangements of fine blood vessels in organs, because the transparent slices prepared are transformable themselves into stereoscopic specimens.

In this observation, the obtained results are briefly stated as follows:

- 1) The principal suprarenal arteries nourishing the suprarenal body, approximately 15 in number in each side, originate from the abdominal aorta, the renal and phrenicoabdominal arteries in all cases, and also from the cranial mesenteric, coeliac and ovarian arteries in some cases. Each artery divides into many branches prior to reaching the capsule. The extra-capsular arteries show conspicuous snaking or coiling features adjacent to the capsule.
- 2) The present writer found that the capsular arteries, the existence of which was denied in cats by BENNETT, branch from the juxta-capsular arteries and supply comparatively poor capillary nets in the capsule.
- 3) After penetrating into the capsule, the intra-capsular arteries divide into a number of arterial branches in which they show remarkable snaking and coiling features in many cases. The branches of these arteries extend to the cortical and medullary arteries as defined by FLINT. The cortical arteries give rise to capillaries entering into the cortical parenchyma. In neoprene cast, the capillaries of the zona fasciculata show sinusoid features, especially in the external part.
- 4) The recurrent arteries were categorized into four types (R.I~R.IV) according to the appearance of their courses and branches. Type R.I shows an incomplete recurrent form; the recurrent loop does not reach as far as the capsule, but diverges into the capillaries in the internal half portion of the cortex. Types R. II and R. III exhibit a complete recurrent form similar to those described by BENNETT; in type R. III, the artery divides into two or three branches within the cortex on its course, while in types

R. II, there are no branches. Type R. IV is a mixed form of both types R. I and R. II or types R. I and R. III.

5) At the corticomedullary boundary, the cortical capillaries unite into the medullary vein, independently of the medullary capillary nets. In addition to the above general finding, the present writer observed that, in cats, some proportion of the cortical capillaries communicate directly with those of the medulla.

6) The medullary arteries, nourishing the medullary parenchyma, were classified into four types (M. I~M. IV) according to appearance of their courses and branches. Type M. I is the most simple form. Types M. II, M. III and M. IV show progressively more and more complex structures. In many cases, the arteries show a conspicuous dilatation, snaking and coiling at the corticomedullary boundary and in the medulla.

7) The writer found newly that there are arteries which supply capillaries which extend into both the cortical and medullary parenchyma, in addition to the ordinary recurrent and medullary arteries. Such arteries were provisionally called "the cortico-medullary arteries" and classified into six types. Type CM. I is a variant of the recurrent artery (R. II); from the curve of the recurrent loop, some capillaries dip down into the parenchyma of the medulla. Type CM. II appears as a variant of the medullary artery; from the arteries on their courses, capillaries diverge into the parenchyma of the cortex. Types CM. III, CM. IV, CM. V and CM. VI are combined forms made up of both medullary arteries and types R. I, R. II, R. III or R. IV of the recurrent arteries. There has been no description in previous publications regarding the writer's types CM. I, CM. V and CM. VI.

3) Conspicuous snaking, coiling and dilatation of the extra- and intra-capsular arteries and the medullary arteries seem to be less conspicuous in juvenile cats than in adult.