HISTOCHEMICAL STUDIES ON THE FEED TISSUES IN THE RUMINAL FLUIDS (REPORT I), ESPECIALLY ON THE ALTERATION OF THE CLOVER LEAVES PUT IN THE ARTIFICIAL RUMINAL FISTULA OF THE GOATS

HISTOCHEMICAL STUDIES ON THE FEED TISSUES IN THE RUMINAL FLUIDS (REPORT I), ESPECIALLY ON THE ALTERATION OF THE CLOVER LEAVES PUT IN THE ARTIFICIAL RUMINAL FISTULA OF THE GOATS

By

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Introduction

No discussion of cellular pathology, according to CAMERON (1), would be complete without some account of how plants respond to injury, though brief references have been made (NÄGELI and CRAMER 1855, HOFMEISTER 1867, von SACHS 1887, KÜSTER 1924~1937, BROOKS 1928, GUILLIERMOND et al 1933, HEALD 1947, MELHUS and KENT 1939, and CHESTER 1947). CAMERON described the cellular changes following the disturbances of the cell water, cell sap, cell plastids, and nucleus. He stated that the causes of plant disease fell into three main groups: (1) parasitic, such as bacteria, fungi and nematoda; (2) virus infections; (3) non-parasitic, such as nutritional disturbances or injurious mechanical influences. Local reactions were determined by the relations between the injurious agent and the host and these comprised a variety of responses from death to stimulated development.

Histochemical studies on the plant tissues in the ruminal fluids has been done on the digestive alteration of the leaves, grains and grasses put in the artificial ruminal fistula of the goat. Through these results, the morphology of the digestible feed was established from histochemistry and pathology.

By the present study there was found the onset of the autolytic phenomena with necrobiosis, foamy degeneration, discharges of the protoplasm from the cells such as plasmoptysis, coagulation and necrosis of the protoplasm, degeneration of the plastids such as lipophanerosis and hydrolysis, nuclear changes, and the invasion of bacteria into the intercellular spaces and cell bodies. No

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one has investigated the alteration of the digestible feed in the ruminal fluid from the histochemical view.

The mechanism of digestion of the feed in the rumen has been clarified. To the effect that the feed was considered as the lesions of autolysis and fermentation under the different medium-conditions, it might be important to observe the relationships between the solubility of the cytoplasms and nuclei and the multiplication of the microorganisms.

In the present study it was planned to examine the histochemical changes of the clovers, put in the artificial ruminal fistula, as the fresh protein-containing plants. Other feed, corns as a kind of the concentrates; grasses as a kind of the roughages and the dry fishes as a kind of the cocentrates, will be described on another occasion.

Materials and Methods for Studies

The clover-leaves were cut from the plants grown during October to November in the cultivated field of the school. They were immediately put in silk-bag-nets, and then kept in the artificial ruminal fistula of the goat in the Animal Physiological Laboratory. The sheep had been given various feed, so the ruminal fluids might contain portions of the various feed. The materials for the studies in the silk-bag-net, were pulled up in their original forms, and the ones broken beyond recognition were removed. At the time of 0, 1, 2, 4, 6, 8, 12, 16, and 24 hours after administration of the materials, these leaves were gathered from the silk-bag-net, and fixed with CARNOY's solution for DNA, starch, cellulose, pectin, lignin and protein, and with buffered formol for lipids and fat. The clover-leaves fixed in CARNOY's fluid, were embedded in paraffine and cut into 6 μ sections, and that in formol were embedded in formol-gelatin, frozen and cut with microtome for fat-staining. The stains employed were Hematoxylin-eosin stain, Azan stain for general histology, PAS-hematoxylin stain with or without saliva-digestion for the starch and other polysaccharides, Acrolein-SCHIFF reaction for the protein, pyronine-methyl green stain with or without ribonuclease digestion for the RNA, depolymerized DNA and polymerized DNA, and FEULGEN's reaction counterstained with light green for the DNA.

Results

I. Postmortem changes of the clover-leaves in the ruminal fluids

After the death of the individual there occur the death of the total cells. These processes played in the tissues is a kind of self-digestion or postmortem autolysis unless putrefaction happens. Postmortem self-digestion is the decomposition of the cells, tissues and organs of the dead body. This decomposition begins just after death, and then it developes to putrefaction by the participation of the microorganisms.

The fresh clover leaves were cut from the plant, and immediately put into the ruminal fluids of the ruminal fistula. Accordingly, the clover-leaves develope to the postmortem alterations followed by such disturbances which may affect (1) the cell water and cell sap, resulting, in plasmolysis, protoplasmic swelling or vascular degeneration, (2) the cytoplasm, as its displacement within the cell or protrusion plasmoptysis, with splitting of protoplasm into separate parts by the intercellular membranes or changes in the protoplasmic layers, or coagulation and necrosis, (3) the cell plastids and (4) the nucleus.

Postmortem changes with the progress of time are indicated in Table 1.

(a) At one hour after putting the leaves in the fistula, the papillary process in the epithelial tissue indicates protrusion and plasmoptysis, and produces injury in the cell membrane, and then destruction; the swelling in the cell membrane of the collenchyma cells and soft cells are shown.

(b) In the leaves after two hours in the fistula the desqumation and flattening of the epithelium, remarkable swelling of the collenchyma cells, palisade- and spongy- parenchym cells, and fragmentation of the thick-walled parenchym cells were found. At this time the plastid produced vaccuolisation and then disappears.

(c) In the leaves after four hours in the rumen the papillary process indicated shrinkage and destruction atrophy of the collenchym cells and parenchym cells, atrophy or swelling of the plastid, and enlargement of the intercellular spaces, and swollen cell-membrane of the soft cells and thick-walled parenchym cells.

(d) At six hours, there were shown the complete destruction of the papillary process, atrophy of the collenchym cells, assimilation tissue-cells and thick-walled parenceym cells, atrophy of the plastids, severe swelling of the cell-membrane of the thick-walled parenchym cells, and enlargement of the intercellular spaces in the cortex and medullary.

(e) After eight hours in the ruminal fluids there was found the disappearance of the papillary process, collenchym cells and thick-walled parenchym cells, loss of stainability in the plastids of the assimilation tissue, and fragmentation of the vessel bundles.

According to CAMERON, the protoplasm between the cell membrane and cell sap space become laminated, and the external portion (exoplasm) next to the membrane and the internal fragment (endoplasm) adjacent to the sap differs in physical and chemical qualities from the protoplasm in the middle (mesoplasm). BRINK (1) described that the protoplasm can also break up into discrete pieces within the cell by acids, ammonia and temperature variations. Severe disturbance of either layer produces the damaged hyalophasm fusing (PFEFFER) (1). Accordingly the alterations called the swelling of the cell membrane in Table 1 may contain the abnormal lamination, damaged lamination and fusing.

Table 1. Post-mortem Changes of the Clover-Leaves in the Ruminal Fluids

Plant tissue \ time			Progress of time after putting the leaves into the Fistula					
			0 hr.	1 hr.	2 hrs	4 hrs.	6 hrs.	8 hrs.
Epithelial tissue		Papillary process	Normal	Protrusion of cytoplasmic process, damage, destruction	Desquamation, flattening of epithelium, destruction	Shrinkage wavy membrane, severe destruction	Complete destruction	Disappearance
Cortex	Mechanical tissue	Collen-chyma cell	Normal	Swelling of homogeneous membrane	Swollen homogeneous membrane	Atrophy	Atrophy	Disappearance
		Inter-cellular space	Completely addhered	Somewhat enlarged	Enlarged	Enlarged	Enlarged	Disappearance
	Assimilation tissue	Palisade parenchyma	Normal	Globule-like arrangement around the peripheral part	Loss of stainability with basic dyes	Swollen plastid in the cell-atrophy, swollen membrane, irregular form	Cell-atrophy atrophy of plastid, thread-like, globoid-like plastid	Severe loss of stainability
		Inter-cellular space	Completely attached	Swelling of cell-membrane	Swelling of cell-membrane	Enlargement of cavity	Enlargement of cavity	
		Spongy parenchyma	Normal	Displacement of nucleus, atrophy of plastid	Vaccuolisation in the center of plastid. disappearance	Irregular form, shrinkage, atrophy of plastid	Cell atrophy, disappearance of plastid	Loss of stainability
Medullary	Vascular bundle tissue	Inter-cellular space	Somewhat with space	Swelling of cell-membrane	Enlarged	Enlargement of cavity	Enlargement of caivty, liquefaction	
		Vessel	Normal	Swelling of cell-membrane	Severe swelling of cell membrane, swollen C.M.	Destraction of vessel swollen C.M.	Swollen C.M.	Fragment of vessel chain
		Mechanical tissue: Thick-walled paren-chyma	Normal		Decrease of plastid fragmentation of C.M.	Severe swelling of C.M. atrophy of plastid	Severe swelling of C.M. atrophy of plastid, disappearance of plastid	
		Mechanical tissue: Inter-cellular space	Completely addhered	Somewhat release		Enlargement of cavity	Enlargement of cavity	

Remarks: C. M. indicated the abbreviation of the cell-membrane.

II. Histochemical changes of polysaccharides and fats in the clover-leaves put into the ruminal fluids

From the point of histochemistry, in the normal leaves there were found fat, pectin, cellulose and starch. With the progress of time after putting the leaves in the fistula, these substances appear or disappear within the cell membrane, cytoplasm and plastid by means of stomach digestion and self-digestion as shown in Table 2.

(a) **Pectin and cellulose in the cell membrane** (contained a part of cytoplasm)

According to KONOSHIMA (2), the 1st layer consists of pectin, and the 2nd and 3rd layers contain cellulose. The cells which are not developed in the 3rd layer, are called the parenchyma cells. But the cell membrane increases the width of the 3rd layer which is called lignification with the combination of cellulose and lignin. The cell membranes of the papillary process in the epithelial tissues, collenchyma cells and thick-walled parenchyma cells in the mechanical tissues, and that of the soft cells in the assimilation tissues seem to contain these pection and cellulose. These materials stained with PAS reaction, and the vessel system stained with PAS reaction seem to show the existence of the combination of cellulose and lignin.

Cellulose and pectin in the epithelial-assimilation and mechanical-tissues disappear and decrease gradually at four to eight hours after putting the leaves into the fistula, but no changes of cellulose and lignin in the vascular bundle system are found.

According to MUHLETHALER (3) (1961), the cell wall fibrils which are visible under the light microscope are called the macrofibrils, and are composed of many parallel microfibrils as an aggregate; the macrofibril may contain as many as 400 microfibrils. The capillary spaces between the microfibrils in the cell wall are filled by incrusting substances such as pectin, lignin, etc. Myofibrils consist of about 2000 cellulose chain molecules giving a diameter of the order of 100—250 Å (RANBY (4) 1949, FREY-WYSSLING (5) 1948, MUHLETHALER (6) 1949, PRESTON (7) 1951).

Pectic substances are generally localized in the middle lamella the adjacent primary wall, or the outermost layers of the secondary wall. The elementary structural unit of this polymer has been shown by EHRLICH (8) (1917) to be galacturronic acid. Additional constituent molecules are glucuronic acid and arabinose. Because of their hydrophily, pectic substances are usually in a highly swollen and water-saturated state. The cell walls contain pectic substances as incrustation or as in the middle lamella and corner thickenings of the collenchyma cells, and in layers between the cellulose lamellae.

Lignin is a condensation, or polymerization, product of coniferyl alcohol (SCHUBERT (9) 1954), and this compound is structurally closely related to the

Table 2. Histochemical Changes of Polysaccharides and Fats in the Clover-Leaves put into Ruminal Fluids.

tissues			Cells	Progress / Substances	Progress of Time after throwing Leaves in the Fistula: 0 hr.	1 hr.	2 hrs.	4 hrs.	6 hrs.	8 hrs.
Epithelial		Papillary process	Cell membr.	Pectin, Cellulose	+++	+++～++	+++～++	++～+	+～−	−
			Cytoplasm	Fat Pect., Cellulose	+++(3)	++(2)	−(2)	−(1)	−(0)	−(0)
Cortex	Mechanical tissue	Collenchyma cells	Cell membr.	Pectin, Cellulose	+++	+++～++	++	+	+～−	−
			Cytoplasm							
	Assimilation tissue: Palisade tissue	Soft cells	Cell membr.	Pectin, Cellulose	++	++	++～−	++～−	+～−	−
			Cytoplasm	Fat	−(4)	−(4)	+(3)	++(2)	+++(2～1)	+++(2～0)
			Plastids	Fat	−(4)	−(4)	+(3)	++(2)	+++(2～1)	+++(2～0)
				Starch	+++	+++	++～−	++, +, −	+	−
	Assimilation tissue: Spongy tissue	Soft cells	Cell membr.	Pectin Cellulose	++	++	++～−	++～−	+ −	−
			Cytoplasm	Fat	−(4)	−s(4)	−−(3)	++(2～1)	+++(2～1)	+++(2～0)
			Plastids	Fat	−(4)	+s(4)	++(3)	++(2～1)	+++(2～1)	+++(2～0)
				Starch	+++	+++	+	++～−	−	−
Medullary	Vascular bundle system: Vessel tissue	Vessel	Cell membr.	Cellulose, Lignin	+++	+++	+++	+++	+++	++
			Cytoplasm	Fat	−(2)	+(3)	+s(2)	+s(1)	+++(2)	++(2)
	Vascular bundle system: Mechanical tissue	Thick-walled	Cell membr.	Cellulose, Pectin,	+++	++	++	+++	++++	+
			Cytoplasm	Fat	−(4)	+s(3)	+s(3)	+(2)	+++(2 1)	+++(2)
		Parenchym cells	Plastids	Fat	−(4)	+s(3)	−s(3)	+(2)	+++(2 1)	+++(2)
				Starch	+++	++	+	−～+	−	−

In the column of fat-storage, +++, ++, +, $+_s$, − showed the intensity of Sudan III-staining, and 4, 3, 2, 1, 0 indicate the intensity of Sudan Black-B-staining.

derivatives of lignin. FREUDENBERGER's recent investigation (10) (1955) has shown that the tissues in the process of lignification contained a cell-bound β-glucosidases. This enzyme may split the glucoside d-coniferin which is present in glucose and coniferyl alcohol. Coniferyl alcohol is immediately converted into lignin by the reductases present. Lignin can be deteced with basic dyes such as gentian violet and chrysoidine. The yellow which color forms by treatment with aniline sulfate and the cherry red appearing upon staining with phloroglucinol and hydrochloric acid are characteristic for lignin. Chlor-zinc-iodine gives a yellow color to a lignified cell wall.

(b) Fats in the cell membranes, cytoplasms and plastids.

Acccording to GRANICK (11) (1961), the plant cell wall can be shown to consist of the substances stained with Sudan III. He described fatty acids as the cuticular substances. And also the lipid content of the chloroplasts is high, being from 20 to 40 percent of the dry weight, depending on the species. The runner bean leaves contain from 20 to 40 percent of the dry weight, depending on the species. The runner bean leaves contain from 20 to 30 percent of the dry weight and their components consist of 50 percent of fats, 20 percent of sterols, 16 percent of waxes and 2—7 percent of phosphatides (EBERHARDT (12), KATES 1957). GRANIK described that the mitochondria are also rich in lipoproteins, but their volume in the cytoplasm of the mature cell is negligible compared to the chloroplast volume, so their contribution to the lipid content of the cell is small.

The clover-leaves were stained with Sudan III or Sudan Black B stains. The results of stainability with Sudan III were different from that with Sudan Black B in the plastids. The plastids of the soft cells in the assimilation tissue and that of the thick-walled parenchym cells in the mechanical tissue were stained strongly with Sudan Black B in spite of the negative stainability with Sudan III. Generally in the plastids the fats stained with Sudan III appeared and increased by the progress of time after putting the leaves in the fistula, but that with Sudan Black B disappeared and decreased successively. From the point of lipophanerosis in the chloroplasts lipoids bound to the plastids were liberated from the proteins and appear in the stroma or at the surface of the plastids. It seemed to be very important to consider the difference of both stainability.

(c) Starch in the plastids.

According to GRANICK (1961), starch is formed in the stroma of the plastids, and two kinds of starch molecules are generally present as follows: amylase the minor component and amylopectin as the major component. The former stains with iodine in a blue color and later stains red with iodine.

From the results of Table 2. the starch in the plastids decreased gradually, and disappared at six to eight hours after putting the leaves in the ruminal

fluids.

III. Histochemical changes of nucleic acids and proteins in the clover-leaves, put into the ruminal fluids.

From the point of histochemistry, in the normal leaves were found DNA, RNA and protein. By the progress of time after throwing the leaves into the ruminal fistula, these substances disappeared within the cytoplasms and nuclei by means of the stomach digestion and self-digestion as shown in Table 3.

(a) Nucleic acids of the plastids

Proplastids in the meristematic leaf cells contain both RNA and DNA localized in the primary granum; in addition some RNA is detectable in the stroma (SPERKERMANN (13), 1957). In mature chloroplasts the evidence for the presence of RNA was perhaps positive, but it is negative for DNA according to the studies of LITTAU (14) (1958) on four species of monocotyledons (GRANIK, 1961). METZNER (15) (1952) could detect no DNA in *Agapanthus* with the FEULGEN reaction, but the methyl green-pyronine stain appears to indicate that DNA is present in the grana. CHIBA (16) (1951) reported a positive FEULGEN reaction for DNA in chloroplasts of *Selaginella*, *Tradescantia* and *Rheo*. Both METZNER and CHIBA reported RNA in the grana.

From the results of Table 3, there were found RNA in the plastids of the soft cells in the association tissues, but the RNA in the plastids disappeared on and after the time of two hours after throwing the leaves into the ruminal fluids. DNA was localized in the nuclei of epithelial cells, soft cells and thick-walled parenchyma cells on and before the time of 2 hours after throwing the leaves, but thereafter DNA decomposes and disappeares. Decomposition of DNA is detectable as the type of depolimerized DNA stained with pyronine.

(b) Protein of the plastids

GRANICK (17) described that the mature chloroplasts of tomato and tomato leaves (GRANICK (17), 1938) Sudangrass (HANSON (18), 1941), and oat leaves (GALSTON (19), 1943) make up 35—45 percent of the total protein-N of the cell; and 35—55 percent of dry weight of the chloroplasts is protein. And according to his discription about 80 percent of the total protein-N of the chloroplast is in an insoluble form, probably as lipoproteins and structurally built in enzymes; and a breakdown of the chloroplast structure is readily observed in the older leaves when bean plants are placed in the dark for several days.

From the results of Table 3, the protein in the plastids disappeared in and after four hours after throwing the leaves into the ruminal fluids. Nuclear protein disappeared completely at eight hours after throwing them in the fluids. Accordingly protein in the plastids and nuclear histon disappeared gradually in the digestive process.

IV. Histochemical changes of the plastids of clover-leaves in the

Table 3. Histochemical Changes of Nucleic Acids and Protein in the Clover-Leaves put into Ruminal Fluids.

Tissues			Cells	Progress	Progress of Time after Throwing Leaves in the Fistula					
					0 hr.	1 hr.	2 hrs.	4 hrs.	6 hrs.	8 hrs.
Epithelial tissue			Papillary process	Cytoplasm	–	–	–	–	–	–
				Nuclei	DNA Protein	DNA Protein	DNA Protein	–	–	–
Cortex	Mechanical		Collenchym cells	Cytoplasm	–	–	–	–	–	–
				Nuclei	DNA Protein	DNA Protein	DNA Protein	–	–	–
	Association tissue	Spongy t. Palisade t.	Soft cells	Cytoplasm	RNA	RNA	–	–	–	–
				Plastids	Protein + + +	Protein + + ~ +	Protein + + ~ –	Protein + ~ –	Protein – ~ $+_s$	Protein – $+_s$
				Nuclei	DNA Protein	DNA Protein	DNA Protein	dep. DNA Protein	dep. DNA Protein	–
			Soft cells	Cytoplasm	RNA Protein ?	RNA Protein ?	–	–	–	–
				Plastids	Protein + + +	Protein + + ~ +	Protein + + ~ –	Protein +	Protein – $+_s$	Protein – ~ $+_s$
				Nuclei	DNA Protein	DNA Protein	DNA, dep. DNA Protein	dep. DNA Protein	dep. DNA Protein	–
Medullary	Vascular bundle system	vessel	Vessel	Cytoplasm	–	–	–	–	–	–
				Nuclei	!	!	!	–	–	–
		Mechanical tissue	Thick-walled parenchym cells	Cytoplasm	–	–	–	–	–	–
				Plastids	Protein + + +	Protein + + +	Protein + +	Protein +	Protein – ~ +	Protein – ~ $+_s$
				Nuclei	DNA Protein	DNA Protein	dep. DNA Protein	–	–	–

Remarks : RNA stained with pyronine and DNA stained with methyl green and FEULGEN reaction, and the depolymerized DNA stained with pyronine. Protein stained with DUJUIN's acrolein-SCHIFF reaction. Accordingly the existence of RNA, DNA dep. DNA and protein are described in the table.

ruminal fluids.

According to WILSON's cytology(20), plastids are generally classified on the basis of color, though such a classification is not neccessarily related to function. The noncolored plastids are refered to as the leucoplasts, while the colored one are called the chromoplasts. Leucoplasts often may function as the storage organelles by accumulating large amounts of starch (amyloplasts), oil (elaioplasts), or protein (aleurone-plasts) within their interior. Chromoplasts contain both chlorophyll and carotenoid pigments. The typical chloroplast is a more or less ovoid structure bounded by a double-layer membrane and containing relatively homogeneous matrix called the stroma in which are embedded the granules or lamellae referred to as the grana.

The results of alterations in the plastids are indicated in Table 4. Following the progress of time after throwing the leaves into the artificial fistula, there were found various alterations in the plastids as undermentioned:

(a) The plastids stained with Azan stains showed stainability of reddish orange, orange yellowish, orange yellow, yellowish blue to blue color. From this, while the Azan stains consist of azocarmin B, aniline blue and Gold-orange G; according to SEKI (21) if the dyes are arranged to the micro-melecule to macro-molecule, there would be found the following orders: azocarmin (molecularweight 433, 921) (red) <orange G (452, 370), yellow (red) <aniline blue (732, 718) (bluish). To use these dyes as the mixture, the loose structure with wide holes stains with the macromolecular dyes such as aniline blue and the fine structure with narrow holes by the micromolecular dyes such as orange. At first the plastids were stained with orange, and then with aniline blue because of the decomposition of plastids structure. Stainability of plastids with eosin decreased gradually by the dimished protein.

(b) The plastids consisted of starch, protein and lipids. The alteration of the plastids following the progress of time after putting the leaves in the artificial fistula, were indicated as the decrease of starch grains and protein, and the increase of lipoids. Accordingly there were found alterations such as the decomposition of the plastids, vaccuolisation of the plastids, and lipophanerosis.

(c) The nuclear alterations of plastids were shown as the decomposition of the nuclei such as karyorrhexis, karyolysis and disappearance of the nuclei. In and after two hours after throwing the leaves into the artificial fistula there were indicated the depolymerization of DNA and disappearance of RNA.

V. Modus of bacterial invasion into the clover-leaves in the ruminal fluids.

What is the difference between the infection and fermentation? Infection is the invasion in the body by bacteria and other harmful organisms. An infection can be restricted to a single part of the body (local infection) or be scattered throughout the entire living system (infection). Infectious organisms

Table 4. Histochemical Changes of the Plastids of Clover-Leaves in the Ruminal Fluids.

		Progress of Time after Throwing Leaves in the Artificial Fistula					
		0 hr	1 hr	2 hrs.	4 hrs.	6 hrs.	8 hrs.
Stainability	Azan-staining	Reddish orange	Orange	Yellowish orange	Yellow, light orange	Yellow, blue	Blue
	H.E. stains	Eosin	Pink, red	Pink, red	Pink	Light yellow	Light yellow
Swelling or Disappearance of Plastid	Starch in PAS-R	Masses of fine granules ++	Masses of fine granules ++	Slight decrease of fine granules ++～−	Decrease of granules ++, +, −	Disappearance of granules +～−	Complete disappearance of starch −
	Protein in Acrolein-Schiff-R.	Wide ring zone near cell wall +++	Wide ring zone near cell wall	Slight narrow ring zone near cell wall	Narrow ring zone near cell wall +～−	Isolated globule- or island-like zone near cell wall −s	Decrease of zone or disappearance −～+$_s$
	Unstained cavity formation	−	−	++	++	++	+++
Nuclear changes	RNA pyronino-philia	Clear	Clear	−	−	−	−
	DNA methyl-philia	+++	+++	++ (Mg) + (Py)	+ (Mg) ++(Pyr) Violet	+ (Mg) ++(Py) Violet	−
Lipo-phanerosis	Sudan III	−	−～+$_s$	+～++	+～++	+++	+++
	Sunan Black B	++++	++++～+++	+++	++	++	++

Remarks : Mg. or Py. showed methyl green-philic or pyroninophylic substances stained with pyronine methyl-green staining.

may gain entrance to the body by a number of ways (through the mouth, injected into the blood stream, by insects and other animal carriers, and through open wounds, in animals; through open wounds in the plant). On the other hand, fermentation is a chemical change of organic matter caused by the action of enzymes which are catalytic materials to change the speed of chemical reaction and to be produced by living cells. Many fermentation processes are undesirable but some are necessary to the process of natural life. In the living body the breakdown of starch to sugars is a form of fermentation. In this meaning the alterations of modus of bacterial invasion into the clover-leaves in the ruminal fluids are of interest to the authors. These results are indicated in Table 5.

The penetration of bacteria into the clover-leaves was shown in Table 5 to be as follows

(a) adhesion: attached to the surface of the papillary process

(b) penetration into the cell membrane of the papillary process with destruction of the cell membrane and protrusion of the cytoplasmic process.

(c) proliferation of bacteria within the swollen spaces between the papillary process and collenchyma- and parenchyma-cells.

(d) bacteria around the parenchymatous cells.

(e) penetration through the pits of the cell membranes. The cell membrane generally consists of three layers such as the first layer with pectin (middle lamella), the second layer (its inner side called the first membrane) with cellulose and the third layer (furthermore inner surface called the second membrane) with cellulose. When the cell membranes thicken and grow, there occurred a pit through the middle lamella. The pits correspond to the neighbor pits of the connected cells. Bacteria may penetrate into the cells through this pits. If the cell wall should remarkably thicken, the pits form long grooves as a bordered pit in the clover-leaves. These formations of the bordered pits were obscure in the present observation of the clover leaves.

(f) nodule formation as if an abscess in the diseased tissues of the animal within the intercellular spaces and soft cells.

(g) bacterial multiplication within the cells with the destructed plastids.

(h) The arts of microorganisms consisted of bacillus and coccus, and there existed bacillus at first, and then appeared bacillus and coccus, and finally coccus principally. Bacterial colonies contained one kind of microorganisms, and then mixed types such as bacillus and coccus appeared.

Discussion and Summary

Histochemical studies on the plant tissues such as the clover-leaves put in the artificial fistula of the goat has been investigated. In a meaning this

Table 5. Modus of Bacterial Invasion into the Clover-Leaves into the Ruminal Fluids.

					Progress of Time after the throwing Leaves into the Fistula					
					0 hr	1 hr	2 hrs.	4 hrs.	6 hrs.	8 hrs.
Outside of epithelium					−	++ Bac. monococc.	+++ Bac. Coccus.	+~+++ Bac. red-Coccus	+++ Bac. Coccus.	++++ Colonial coccus Bac.
Epithelial tissue			Papillary process		−	+	++ Bac. Coccus mixed abscess	+~+++ Bac. Coccus	+++ Coccus.	×
			Intercellular space		−	+	++ Bac.	++ Bac.	+++ Bac.	×
Cortex	Mechanical tissue		Collenchyma cell		−	−	−	+ Bac.	+++ Bac. Nodule Coccus	×
			Intercellular space		−	−	−	+	++ Bac. Coccus	×
	Assimilation tissue	Palisade tissue	Soft cells	Cytopl.	−	−	+ Bac.	+ Coccus Bac.	+++ coccus	++++ Coccus
				Plastid	−	−	+ Bac.	++ Bac. Coccus	·Bac. +++ Coccus	++++ Coccus
			intercellular space		−	−	+ Bac.	++ Bac.	+++ Bac. Nodules Coccus	×
		Spongy tissue	Soft cells	Cytopl.	−	−	+ Bac.	++ Bac.	++ Bac. red Coccus	++++ Coccus
				Plastid	−	−	+ Bac.	++ Bac.	Bac. ++ ·Coccus	++++ Coccus
			intercellular space		−	−	++ Bac. nodule	++ Bac.	++++ Bac. mixed Coccus	×
Medullary	Vascular bundle system	Vessel t.	Space of vessel		−	−	−	−	++ Bac.	×
			vessel		−	−	−	+ red coccus	+ Bac.	·Bac. +++ ·Coccus
			intercellular space		−	−	−	+ Bac.	+ red−Coccus	+++ ·Coccus
		Mechanical t.	thick-walled parenchym		−	−	+ Bac.	++ Bac. Coccus	++ ·Bac. nodules·Coccus	·Bac. ++++ Coccus
			intercelluar space		−	−	−	++ Bac.	++ Bac. nodules Coccus	++++ ·Coccts

Remarks: ++++, +++, ++, +, − indicate the degree of parasitic microorganisms. Bac. abreviation of bacillus, × means obscure for the sake of fragmentation.

investigation seemed to be a study on the decomposition of plant tissues by the digestion of domestic animals. The present study described the histopathological postmortem alterations; histochemical alterations such as pectin and cellulose in the cell membranes, fats in the epithelium, fats, protein, nucleic acids and starch within the plastids; the relationships between the decomposition of protein and lipophanerosis in the plastids; and modus of bacterial invasion into the leaves.

The results of the investigaion are summerized as follows:

1) Histopathologically following on the progress of time after putting the leaves in the ruminal fistula revealed the following alterations:

In the leaves after one to two hours:

Protrusion of the cytoplasmic process, destruction and desquamation of the epithelial tissue; flattening of the epithelium; swelling of the cell membrans of the collenchyma cells, parisade- and spongy-tissues; enlargement of the intercellular spaces in the mechanical and assimilation tissues, displacement of the nucleus, vaccuolisation in the plastids, and decrease of the plastids.

In the leaves after four to six hours:

Shrinkage and severe destruction of the epithelium; atrophy of the collenchyma cells, parisade- and spongy-tissues; atrophy of the plastids, and disappearance of the plastids; enlargement of the intercellular spaces in the mechanical and assimilation tissues.

In the leaves after eight hours:

Disappearance of the epithelium and collenchyma cells; loss of stainability of the plastids: fragmentation of the vascular bundle system.

2) Histochemically following on the progress of time after throwing the leaves into the ruminal fluids showed the following alterations:

In the leaves after one to two hours:

Slight decrease of pectin and cellulose in the cell membranes of the epithelium, collenchyma cells, soft cells and thick-walled parenchyma cells; slight decrease of fat in the epithelium; slight lipophanerosis of the plastids in the soft cells; slight decrease of starch in the plastids of the soft cells and thick-walled parenchyma cells; slight decrease of RNA in the soft cells; slight decrease of protein in the plastids of the soft cells; slight depolymerization of DNA in the soft cells.

In the leaves after four to six hours:

Slight decrease of pectin and cellulose of the epithelium and soft cells; disappearance of fat in the epithelium; lipophanerosis of the plastids in the soft cells and thick-walled parenchyma cells; disappearance of starch in the plastids of the soft cells and thick-walled parenchyma cells; disappearance of RNA in the papillary process, collenchyma cells and soft cells; disappearance of DNA in the nucleus of the papillary process, collenchyma cells and

thick-walled parenchyma cells; depolymerization of DNA in the nucleus of the soft cells in the assimilation tissues; decrease and disappearance of protein in the plastids.

In the leaves after eight hours:

Disappearance of pectin and cellulose of the epithelium, collenchyma cells and soft cells; decrease of fat in the epithelium; severe lipophanerosis in the plastids of the soft cells and thick-walled parenchyma cells; disappearance of starch in the plastids of the soft cells and thick-walled parenchyma cells; severe decrease of protein in the plastids of the soft cells and thick-walled parenchyma cells.

3) Bacteriologically following on the progress of tmie after throwing the leaves into the ruminal fluids there were found the following multiplication:

In the leaves after one hour:

Bacterial-penetration into the epithelium.

In the leaves after two hours:

Nodule-like multiplication-of the bacteria in the papillary process; slight invasion to the soft cells and thick-walled parenchyma cells; slight invasion in the intercellular spaces of the epithelial-assimilation- and vascular-tissues.

At six hours:

Severe nodule-like multiplication of the bacteria in the papillary process, intercellular spaces of the mechanical tissue, assimilation tissue and vascular tissue, and that in the thick-walled parenchyma cells: severe multiplication in the plastids.

At eight hours:

Severe multiplication of the bacteria in the fragments of the soft cells and thick-walled parenchyma cells; severe multiplication in the vessel.

The relationships between pathological, histochemical and bacteriological alteration of the leaves in the ruminal fluids are shown in Table 6.

To summarize the results as shown in Table, it seemed to be quite clear that the relations between the three alterations were associated with each other.

1) The leaves after one hour in the ruminal fluids showed slightly the alteration such as destruction of the epithelium, enlargement of the intercellular spaces and atrophy of the plastids, no changes of histochemical substances in the various types of cells, and slight penetration of the bacterium in the epithelium and slight bacterial invasion to the interepithelial spaces. Accordingly the first bacterial invasion began at the lession such as epithelial damage.

2) The leaves after two hours in the ruminal fluids showed the epithelial alterations such as destruction, desquamation and flattening, swelling of the cell-membrane; enlargement of the intercellular spaces; decrease, fragmentation and vaccuolisation of the plastids; swelling of vessels; histochemical changes such as disappearance of RNA, slight decrease of starch and protein in the plastids;

Table 6. Relationships between Pathohistological, Histochemical and Bacteriological Alterations in the Ruminal Fluids.

Cell-Form	Methods	Progress / Change	Progress of Time after the throwing Leaves into the Fistula				
			0 hr.	1 hr.	2 hrs.	4 hrs.	6~8 hrs.
Epithelium	Pathol.		Normal	Destruction, swelling	Desquamation, flattening, destruction	Shrinkage destruction	Destruction disappearance
	Histo-chem	Pectin, cellulose, Fat	+++ +++	+++~++ ++	+++~++ −	++~+ −	+~−
	Bact.	Penetration, nodlle multipl.	−	⊕	⊕⊕	⊕⊕⊕	⊕⊕⊕
Cell membrane	Path.	Pectin, cellulose	Normal	Swelling	Swelling	Cell-atrophy	Cell-atrophy
	Histo-chem.	Pectin, cellulose	++	++	++~−	++~−	+~−
	Bact.	Multiplication	⊖	⊖	⊖	⊖	⊖
Inter-cellular space	Path.		Normal	Enlargement	Enlargement	Enlargement	Disappearance
	Histo-chem						
	Bact.	Nodule-like multiplication	0, 0, 0, 0, 0, 0	1, 0, 0, 0, 0, 0 only epithelium	2, 0, 1, 2, 0, 0	2, 1, 2, 2, 1, 2	3, 2, 3, 3, 3, 3
Plastids of Soft cells, Collenchym, thick-walled cells	Path.		Normal	Atrophy	Vacuolization, decrease, fragmentation	Aatrophy irregular form. shrinkage	Atrophy fragmentation, loss of stainability
	Histo-chem.	Fat Starch RNA DNA Protein Azan-staining	− +++ RNA DNA +++ Reddish orange	− +++ RNA DNA ++~+ Orange	+ ++~− − DNA ++~− Yellowish orange	++ ++~− − Depolym. DNA − Light orange	+++ +~− − Depolym. DNA, − +s~− blue
	Bact.	Nodule-like multiplication	−	−	⊕	⊕⊕	⊕⊕⊕
Vessels	Path.		Normal	Slight swelling	Swelling	Destruction swelling	Fragmentation
	Histo-chem	Cellulose, Lignin	+++	+++	+++	+++	+++~++
	Bact.	Multiplication.	−	−	−	⊕	⊕⊕

Remarks : In the colum of intercellular space, there were indicated six places such as around the epithelium, collenchyme cells, soft cells of parade-or spongy tissues, thick walled parenchyma cells and vessels. In the colum of bacteriology the degrees showed the intensity (3, 2, 1, 0) of bacterial multiplication.

decrease of fat in the epithelium; slight decrease of pectin and cellulose in the cell membrane; nodule multiplication of bacteria in the epithelium and intercellular spaces, and slight multiplication in the plastids of the soft cells. Accordingly, the bacteria penetrated from the epithelium to the interior places, and bacterial multiplication within the intercellular spaces from the epithelium to the soft cells followed on the degenerative alterations in the epithelium and plastids of the soft cells.

3) The leaves after four hours in the ruminal luids showed the epithelial lesions such as destruction and shrinkage, cell atrophy, enlargement of the intercellular spaces, alterations of the plastids such as atrophy, shrinkage and disappearance and remarkable multiplication of bacteria in the epithelium, intercellular spaces and plastids; histochemical changes such as decrease of pectin and cellulose of the epithelium and cell-membrane, decrease or disappearance of starch and protein, depolymerization of DNA, increase of fat in the plastids, and disappearance of fat in the epithelium. Accordingly the remarkable multiplications of bacteria in the various cells of the leaves based on the destruction of epithelium and histochemical degeneration of the plastids such as the depolymerization of DNA, decomposition of protein and starch, and lipophanerosis.

4) The leaves after six to eight hours in the ruminal fluids were similar to the ones at four hour's case. However, at eight hours, the leaves fractionated and became isolates as the cellular groups, and also bacterial multiplication in or around the vessels became severe in degrees.

The bacterial multiplication and the histochemical changes in the leaves within the ruminal fluids may be called a part of digestion and fermentation of the leaves in the rumen. What is the difference between the infection and fermentation from the points of morphology? As a mater of fact, infection was the invasion of the body by bacteria and other harmful organisms, and fermentation was the chemical change caused by the digestive ferments as the catalyser produced by the living cells. The phenomenon called a digestion-symbiosis (Verdaungssymbiose) is the relation of symbiosis that some microorganisms normally parasitised in the alimental canal render valuable services to digestive function. And the ruminants utilized positively these functions of the microorganisms such as the enzymatic activity to the protein and carbohydrate. In

	Infection in animal	Fermentation in plant
Modus	Perosr, intravascular, insect, animal carriers of injuries	Cellular injuries, through intercellular spaces
Reaction	The reaction of living tissue to injuries such as bacterial invasion: inflammation, immunity, abscess, and putrefaction	Negative

Morphological changes by the micro-organisms	a. Adhesion of bacteria over the damaged epithelium b. Pynocytosis and phagocytosis c. Swelling in the connective tissue d. Enter into the blood vessels. e. Embolus of bacterial masses in the blood vessels. f. Phagocytosis by the leukocytes g. Decompositon and death of phagocytized leukocytes as an abscess formation. h. Necrosis and necrobiosis of cells in the neighbor of abscess i. Liquefaction and cavity	a. Adhesion of bacteria over the damaged epithelium b. Penetration into swollen epithelium. c. Enlargement of intercellular space and bacterial invasion in this place. e. Bacterial multiplication in the intercellular spaces. f. None. h. Decomposition of cells. breakdown of cell membrane. i. Isolation of cells.
Morphological changes by autolytic process.	a. Hydropic degeneration b. Decrease of protein c. Decrease of glycogen d. Loss of nuclei and nuclear fragmentation e. Fat deposition f. Mucin production g. Liquefaction and cell-destruction	a. Hydropic degeneration b. Decrease of protein c. Decrease of starch. d. Loss of nuclei and nuclear fragmentation. e. Fat lipophanerosis. f. Swelling and atrophy of cell walls. g. Liquefaccion and cell-destruction.

the rumen itself there were found the digestive enzymes secreted from the cells of the digestive glands as digestible ability of the hosts. In comparison between the animal infection and plant fermentation the following table shows the distinction in the points of modus, reaction, and changes;

According to CAMERON, the onset of the autolytic phenomena in the natural death of the plant cells, generally lead to increasing vesiculation of the mitochondria and plastids, so that an alveolar or foamy structure resulted. Also described that the vacuolar membrane was destroyed and the vacuole disappeared, leading to a contraction of the cytoplasm with detaching of itself from the cellulose wall as if plasmolysed and coagulated. According to BALBACH (1), in some cases the plastids became granular and stained with osmium tetroxide, swollen and then appeared as enormous vesicles.

Lipophanerosis is a condition occurring in chloro-, achromo- and leuko-plasts, in which the lipoids bound to the plastids are liberated from the proteins and appeared in the stroma or at the surface of the plastids. According to GUILLIERMON *et al*, it occurs normally during ageing, but other writers stressed that it can be produced by the osmotic disturbances.

In the present study it was described that the onset of autolytic phenomena with necrobiosis, coagulation and necrosis of the cell walls, degeneration of the plastids such as lipophanerosis, decrease of protein and starch, nuclear changes, and the multiplication of microorganisms in the intercellular spaces and cells existed in the digestible clover-leaves used as a feed in the rumen, from the side of histochemistry.

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Plate 1

Explanation of Figures

Fig. 1. Desoxyribose nucleic acids localized in the nuclei of the epithelial cells, soft cells and thick-walled parenchyma cells of the clover-leaves. This was a fresh leave cut from the plants grown in the cultivated field. FEULGEN nuclear reaction counterstained with light green.
Microphoto ×200.

Fig. 2. Disappearance and decomposion of desoxyribose nuclei acids in the nuclei of the epithelium, soft cells and thick-walled parenchyma cells of the clover-leaves for eight hours after throwing them in the rumen. FEULGEN nuclear reaction counterstained with light green.
Microphoto ×200.

Fig. 3. Proteins localized in the nuclei of the epithelium, in both nuclei and cytoplasms of the soft cells of the parisade- and spongy tissue, and in the nuclei of the thick-walled parenchyma cells of the vascular systesm, within the fresh clover-leaves cut from the plants grown in the cultivated field. JUIJN's acrolein SCHIFF reaction for the demonstration of protein.
Microphoto. ×200.

Fig. 4. Proteins localized in the cytoplasms of the soft cells of the parisade- and spongy-tissue, and decrease of protein within the cells. These cells of the clover leaves were put in the rumen for eight hours, JUIJN's acrolein-SCHIFF reaction for the demonstration of protein.
Microphoto. ×200.

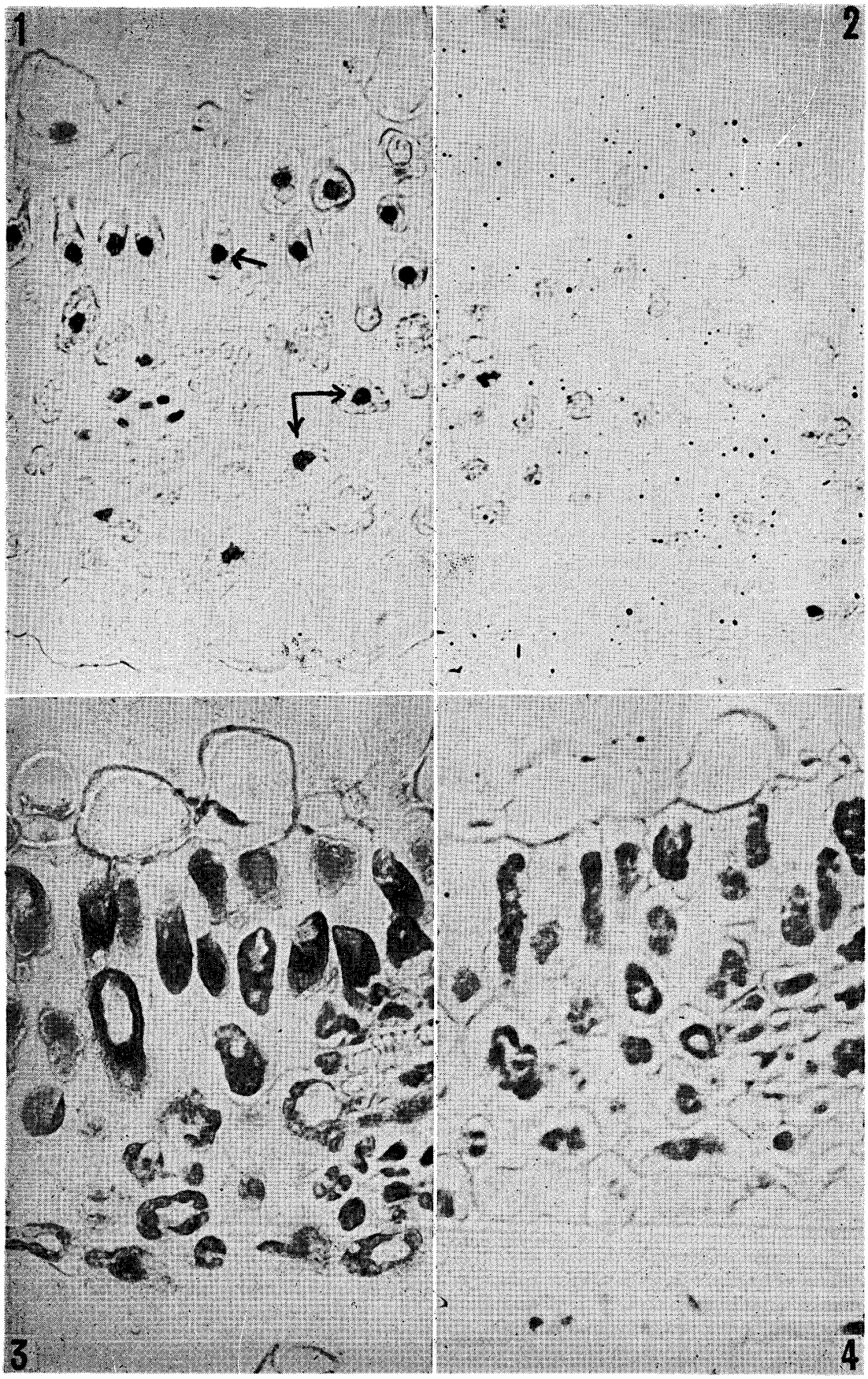
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Plate 2

Explanation of Figures

Fig. 5. Starch(Remark : St.) in the plastids of the palisade- and spongy-tissue of the fresh clover leaves cut from the plants grown in the cultivated field, stained with McMANUS's PAS reaction.
Microphoto. ×1000.

Fig. 6. Disappearance and decrease of starch in the plastids of the palisade- and spongy-tissue of the clover leaves for eight hours after putting in the rumen, stained with McMANUS PAS reaction.
Microphoto. ×1000.

Fig. 7. No deposition of fat in the epithelium, palisade- and spongy-tissue of the fresh clover leaves cut from the plants grown in the cultivated field, stained with Sudan III stain.
Microphoto. ×1000.

Fig. 8. Lipophanerosis (Remark : F) in the cells of the palisade- and spongy-tissue of the clover-leaves put in the rumen for eight hours, stained with Sudan III stain.
Microphoto. ×1000.

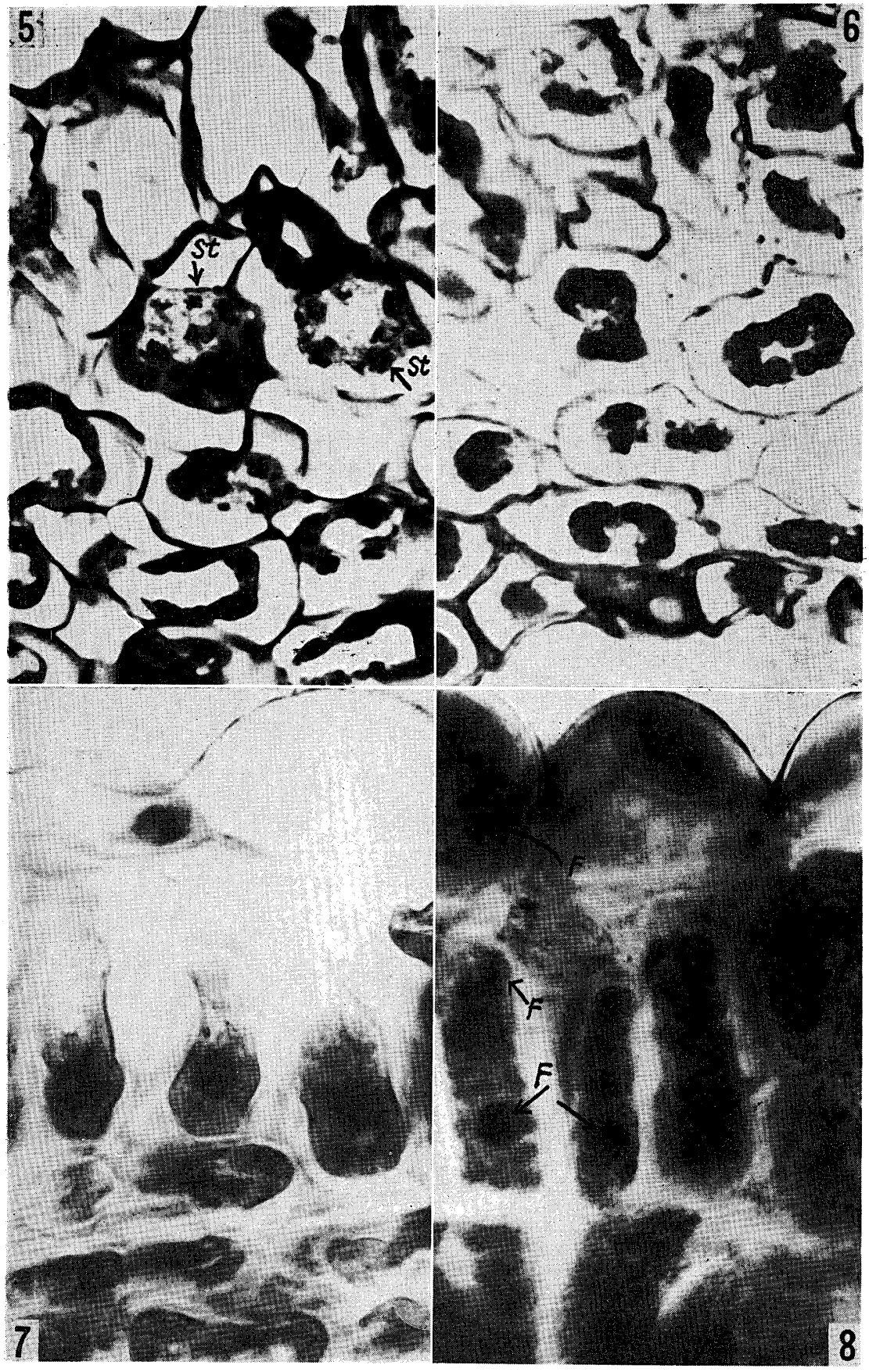
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Plate 3

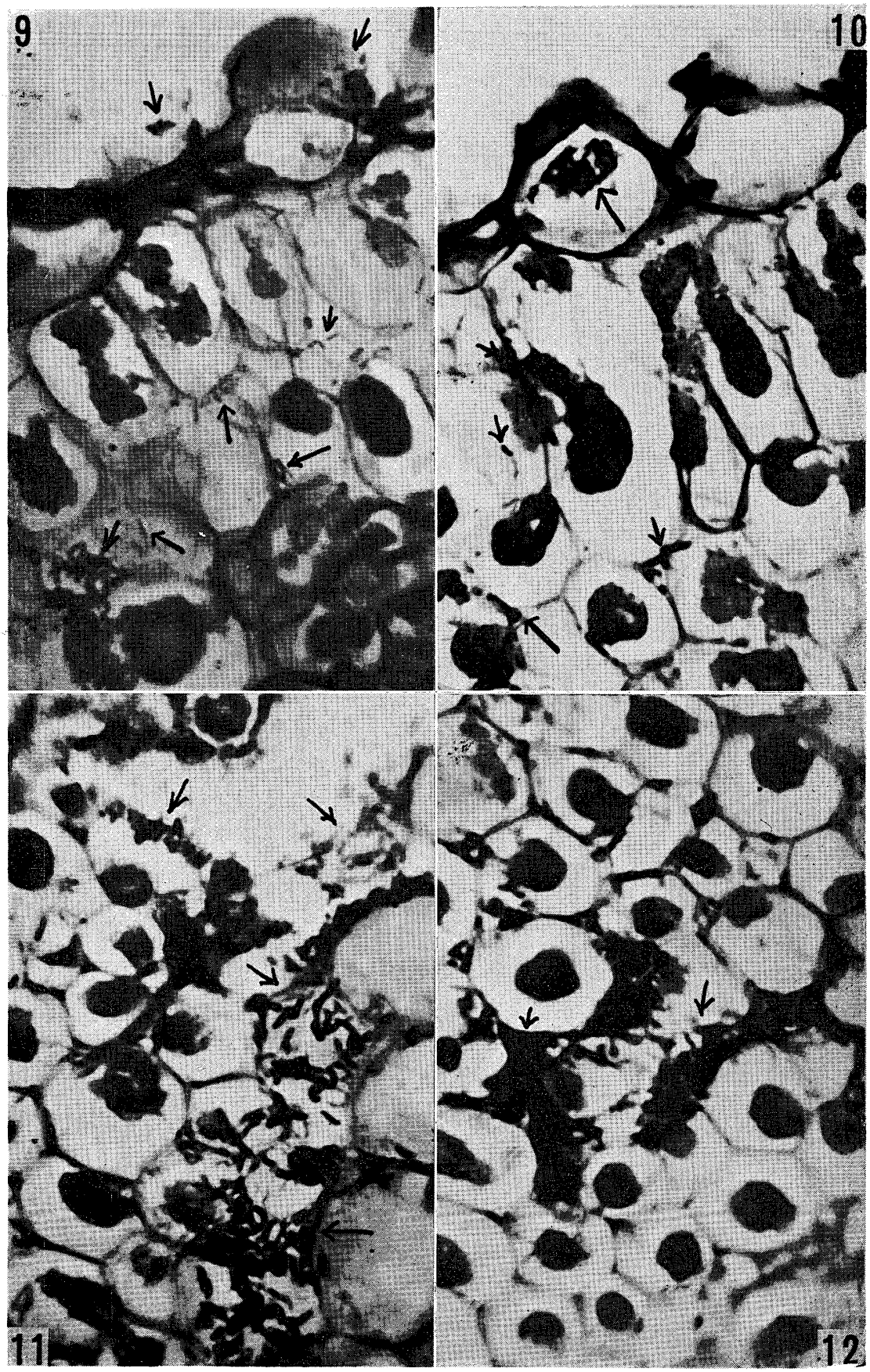
Explanation of Figuers

Fig. 9. Bacterial invasion to the papillary process of the epithelium, adhesion to the epithelium, imigration in the intracellular space of the palisade tissue, and the bacterial nodules in the intracellular space of the spongy tissue, and penetration to the cell membrane of the soft cells. Stained with carbol-thionine stain and enlarged ×1000.

Fig. 10. Bacterial multiplication in the papillary process and migration into the intracellular spaces of the soft cells in both palisade- and spongy tissue of the clover leave put in the rumen for eight hours. Stained with carbol-thionine, and enlarged ×1000.

Fig. 11. Bacterial migration to the intercellular spaces of the soft cells, bacterial multiplication within the anuleated soft-cells of the clover leave put in the rumen for eight hours. Stained with PAS-hematoxylin stain and enlarged ×1000.

Fig. 12. Bacterial multiplication within the soft cells of the spongy tissue of the clover leave put in the rumen for eight hours. Stained with PAS-hematoxylin stain and enlarged ×1000.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