AN ATTEMPT TO DETECT THREE NUTRIENTS AND MICROORGANISMS COINCIDENTALLY STAINED WITH HISTOCHEMICAL PROCEDURES UPON THE SAME FROZEN-SECTION OF SAUSAGE OR FISH PASTE

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By

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Introduction

No one has yet attempted histochemical detection of protein, polysaccharids (glycogen, starch, mucopolysaccharide, or glycoprotein), fat and bacteria upon the same section of sausage or fish-paste. This method might well contribute to food-histology from the point of qualitative and quantitative analysis. There were observed reddish starch grains or glycogen granules in periodic acid-Schiff's reaction (PAS), orange-colored fat in Sudan III-staining, blueish violet-colored microorganisms stained with carbol-thionine, and green-colored protein stained with acidic light green upon a frozen section of sausage or fish-paste. Accordingly, three or four different colors in a section indicated histochemically starch, glycogen, fat, protein or microorganisms. This method might be applied to show glycogen, fat and protein in a parenchymatous cell such as the heaptic cell, or to see starch, fat and protein in soft cells such as potato parenchym.

An attempt resembling this method was done by Affini (1) and Itikawa (2, 3). The prasent method upon the frozen section differed from Affin's concurrent demonstration of DNA and 1, 2-glycols, and Itikawa's coincidental staining of glycogen (or RNA and DNA), succinic dehydrogenase and lipid upon the same deparaffinized section.

This staining procedure might play an important role in solving histochemically, the problems of bacterial multiplication or chemical structure in fresh or processed foods from the oint of food-hygiene.

Materials and Methods for Studies

Meat-sausage and fish-paste were used for the study. These tissue samples were fixed in neutral formol for a month, washed in tap water, and cut with frozen

microtome at −10°C, 10～15μ thick. These frozen sections were kept in the distilled water for the removal of formol. For rapid completion, there was no necessity for formol fixation because of the heat coagulation in the processed food. Also it might be advisable to cut with a cryostat apparatus at −25°C and less for the preparation of thin sections for the observation of detailed structure.

The sections were stained starch, glycogen, mucopolysaccharides and glycoprotein with PAS, fat lipid and chromolipid with Sudan III staining, microorganisms with carbol-thionine staining, and protein with light-green staining. Light green staining for protein was based on the Michaelis's opinion (4) in which the basic dyes (such as methylene blue or thionine) indicated positive charge combined with the acidic substances (such as nucleic acid or mucopolysaccharides) with negative charge, but the acidic dyes (such as eosin or light green) indicated negative charge united with the basic substances (such as basic protein) with positive charge. These attempts to detect three nutrients (sach as polysaccharides, fat and protein) and microorganisms coincidently stained with histochemical procedures upon the same frozen-section of sausage or fish-paste might contribute to food-histology[5]. Sausage or fish-paste kept in the incubator for 1 to 7 days at 37°C. were used for studies on the detection of the bacterial maltiplciation in the denaturated substances.

Coincidental detection of polysaccharides, protein, fat and bacteria

This method consisted of three or four steps as follows:

Step one: *Staining for polysaccharide staining*: A frozen section was oxidized in 0.5% aqueous periodic acid solution for 10 minutes, washed in tap water for 10 minutes, and immersed in Schiff's reagent for 20 minutes at the room temperature. Further section was treated with a sulphite wash solution containing 0.5 cc concenttrated hydrochloric acid and 2cc. 10% potassium metadsulfite to 50 cc distilled water) for three minutes each three times. Histochemical procedures of polysaccharides used, either Hotchkiss-McManus's periodic acid Schiff reaction (8) or Lillie's Na_2IO_3 Shciff's one (9). As a result, carbohydrate-containing protein stained in various shades of purplish-red, and glycogen or starch stained deeply. According to Pearse (10) PAS-positive substances belonged to glycogen, starch, cellulose, mucoprotein (or glycoprotein), glycolipids and sphingolipids.

Step two : *Staining for fat*: A frozen section stained with PAS reaction, was put into the distilled water, and then treated into Sudan III solution (saturated dye in 70% alcoholic solution as a lipid soluble dye for 20 to 25 minutes at 37°C). The stained section was rinsed in distilled water for 5 minutes. As a result, fats are shown by the orange-colored tone.

Step three: *staining for microorganisms*: This step was used in the case of the decomposed food such as sausage or fish meat-paste experimentally kept in the incubator at 37°C for 1 to 7 days. The section stained with PAS and Sudan III -stain, was treated with 50% solution of carbolthionine (added 1 gm of thionine

and 5 gm of carbol to 100 cc of dstilled water) for 5 minutes, and then rinsed in distilled water for 3 minutes. As a result, microorganisms were indicated by the bluish or bluish red color, and existed in the decomposed starch, protein and the wall of cavities.

Step four: *Staining for protein*: This step was used in the section after finishing the first, second and third steps or the first and second steps. After staining through two or three steps, the section was stained with 2% light green solution within a few seconds for the detection of protein. Light green as an acidic dye was a valuable plasma stain often used for staining animal tissues in contrast to nuclear dyes (11). Light green as an acidic dye combined with basic protein. The stained section was put into the color-fixing solution (added few drops of amigen as a fatty acid esters such as nonsurfaced activator in distilled water). After finishing all steps of staining, the section was mounted in glycerol-jelly.

Results

1) *Morphological analysis of the sausage and fish paste indicated by planimeter*

The section stained coincidently three nutrients and microorganisms with various staining procedures, was drawn by Abbe's Zeichen-apparatus, and calculated the ratio of the areas indicated protein, fat and starch in a constant area by the planimeter. These results showed the interesting values of the morphological analysis in the same thin section as Table 1.

Table 1. Morphological analytical values in the section stained histochemically with protein, starch, fat and unstained cavity (with air bubble and without substances).

Cases		Area of substances	Morphological analytical values				
			Protein muscles and other	Starch grains	Fat droplets extramuscular	Air bubbles in cavities	Total area
Sausage	No.1	area $\times 10^4\mu^2$	23.6	11.5	10.4	1.9	47.4
		%	50	24	22	4	100
	No.2	area $\times 10^4\mu^2$	22.3	8.7	11.2	5.2	47.4
		%	47	18	24	11	100
Fish paste	No.1	area $\times 10^4\mu^2$	28.0	16.5	0.1	2.8	47.4
		%	59	35	0	6	100
	No.2	area $\times 10^4\mu^2$	28.0	16.5	0.1	2.8	47.4
		%	59	35	0	6	100

In a comparison of sausage and fish paste, more fat and less starch were found in sausage than in fish paste (see Table 1).

2) *Morphological observation of the composition and varieties of the Vienna sausage.*

It is convenient and useful in food-histology to show the composition of various tissues and annexes, and to know a variety of proteinrich tissue such as pork-meat, glutein, gelatin, blood-vessels, kidney, thymus, lymphatic nodes, stomach, intestine, esophagus, skin and hair in the Veinna-sausage. These chemical structure and variety in the sausage might be differed from the determination by the chemical analysis. Accordingly the present authors called them morphological-analysis in the fresh and processed foods. Materials used for studies consisted of ten Vienna sausages of good or inferior quality. The composition and variety of the tissues and annexes in the sausage are indicated in Table 2.

According to Table 2, there were found 1) the admixture of tissuehomogenates such as blood vessels, kidney, thymus, salivary gland, stomach, intestine, esophagus, skin and hair. 2) sa usage of the substitute products such as glutein, gelatin, cheese, starch in substitution of porkemat, horsemeat, and fish meat, 3) the mxiture of fat and species as a seasoning, and 4) the addition of air-bubbles in the staffer before boiling and pasteurization.

There existed fat-rich muscles (No. 41), glycogen-rich horsemuscles (No. 37), glycogen-rich Tunna-muscles (No. 37), fat dropletcontained glutein (Nos. 35, 36, 38, 39, 40, 42 and 43), gelatin (Nos. 35,36,37, and 38), cheese (No.36) α-starch grains (all cases, No. 35 to 43 and Tokuyo), fatty tisue (all cases, No. 35 to 43 and Tokuyo), species (all cases, No. 35 to 43 and Tokuyo), blood vessels with artery and vein (Tokuyo, Nos. 38, 39 and 43), kidney, thymus, lymphatic nodes, salivary gland stomach, intestine, esophagus, skin and hair (Tokuyo alone).

Table 2. Various tissues and

Tissue / Name	Muscles				Added substances						
	Glycogen-rich	Fat-rich	Protein	Tuna glycogen	Glutein	Glutein, fat-rich	Gelatin	Cheese	Starch	Fat	Spices
T	−	−	++	−	−	−	−	−	+++	+++	++
35	−	−	++	−	++	++	+++	−	+++	++	++
36	−	−	++	−	++	++	++	++	+++	+	+
37	−	−	+++	+++	−	−	+	−	+++	++	++
38	−	−	+	−	+++	+++	+	−	++++	+S	++
39	−	−	++	−	+++	+++	−	−	++++	+	++
40	−	−	++	−	+	+	−	−	++++	++	++
41	−	+++	+++	−	−	−	−	−	++	+++	+
42	−	+	+++	−	−	+	−	−	+++	++	+++
43	−	−	++	−	++++	+++	−	−	+++	+++	+++

Various tissue-homogenates located in the Vienna-sausage of inferior uality were distinguished from polysaccharide-containing membranous glomerulus in the kidney; PAS-positive internal elastic lamina of the blood vessels, thicker tunica interna et media (protein) of the arteries; thinner tunica interna et media (protein) of the veins; dark cortex with rich DNA and light medulla of the thymus -lobules, existence of the Hassal's corpuscles in the medullar area of the thymus; subcapsular and perifollicular sinuses with DNA-rich follicles in the centrum of the lymph-ndoes; protein-rich serous alveoli and interstitial abundant fat cells in the parotid gland; prtein-rich serous alveoli principally and PAS-positive mucous alveoli with serous cresent in the submaxillary gland; PAS-positive mucous alveoli predominantly and mucous alveoli with a few proteinic serous cresent in the sub-lingual gland; distinction of the parotid, submaxillary and sublingual glands as the salivary glands; protein-rich and polysaccharidic mucosa with deep gastric pits and thick tunica muscularis with protein-rich smooth muscles; mucoprotein rich mucosal villi with crypts and relatively thick tunica muscularis with protein-rich smooth muscles in the small and large intestine; thick stratified squamous epithelium with rich protein and some keratohyalin granules and protein rich striated or smooth muscles in the esophagus; protein-rich epidermis with keratinization and desquamation, DNA-rich four strata, fat-rich gland and tissue, protein-rich coll-agenous dermis in the skin; and hair shaft, outer sheath, inner sheath, bulb, papilla in the hair.

In the annexes in the Vienna-sausage there were distinguished the dispersed fat droplets within the homogenesus protein-rich glutein from no fat droplets in the homogeneous gelatin with protein and polysaccharide. It is easy to find cheese with fat and protein gloubles, starch grain (wave-like or thread-like α-starch), and characteristic appearances of the leaves and seeds with polysaccharide and fat within the Vienna sausage.

added substances in the sausage.

Cavities	Added tissue homogenates								
	Blood vessels	Kidney	Thymus lymph. n.	Salivary gland	Stomach	Intestine	Esophagus	Hair	Skin
++	++	++	+++	+++	++	++	++	++	++
+++	−	−	−	−	−	−	−	−	−
+++	−	−	−	−	−	−	−	−	−
+++	−	−	−	−	−	−	−	−	−
++	++	−	−	−	−	−	−	−	−
+++	++	−	−	−	−	−	−	−	−
++	−	−	−	−	−	−	−	−	−
++	−	−	−	−	−	−	−	−	−
+++	−	−	−	−	−	−	−	−	−
+++	+	−	−	−	−	−	−	−	−

Table 3. The locus of bacterial multiplication

Name \ Tissue		Muscles				Added substances						
		Glycogen-rich	Fat-rich	Protein	Tuna glycogen	Glutein	Glutein, fat-rich	Gelatin	Cheese	Starch	Fat	Spices
Fresh sausage	T	−	−	−	−	−	−	−	−	−	−	−
	35	−	−	−	−	−	−	−	−	−	−	−
	36	−	−	−	−	−	−	−	−	−	−	−
	37	−	−	−	−	−	−	−	−	−	−	−
	38	−	−	−	−	−	−	−	−	−	−	−
	39	−	−	−	−	−	−	−	−	−	−	−
	40	−	−	−	−	−	−	−	−	−	−	−
	41	−	−	−	−	−	−	−	−	−	−	−
	42	−	−	−	−	−	−	−	−	−	−	−
	43	−	−	−	−	−	−	−	−	−	−	−
Decomposed sausage (for 7 dys, 37°C)	T					unexamined						
	35	−	−	+	−	+	−	−	−	++	+	−
	36	−	−	++	−	−	−	−	−	++	+	−
	37	−	−	+	−	−	−	−	−	+++	++	−
	38	−	−	+++	−	+++	−	−	−	+++	+	−
	39	−	−	++	−	−	++	−	−	+++	++	−
	40	−	−	−	−	++	−	−	−	++	−	−
	41	−	+	+++	−	−	−	−	−	++	−	−
	42	−	−	−	−	−	−	−	−	+	−	−
	43	−	−	−	−	−	−	−	−	++	+	−

3) *The locus of bacterial multiplication in the decomposed susage.*

For the detection of the putrefied or decomposed foods the materials used for study were sausage sold on the market and then kept in the incubator of 1 to 7 days at 37°C. Frozen sections of sausage were stained with PAS-Fat-Carbolthionine-light green staining, and observed the locus of bacterial multiplication in the sausage. The results are shown in Table 3.

No bacteria stained with carbol-thionine were found in the fresh sausage on the market, but there existed a large amount of microorganisms as colonies of coccus or bacteria within the decomposed starch grains and cavity-lumen with air-bubbles. In the decomposed sausage with severe damage the unstained zone with dematuration of starch grains and proteinic muscles in the cortex (peripheral portion at 1/4 in width transverse section) and stained zone with starch grains and proteinic muscles in the medulla (central portion at 3/4 in width of transverse section) are shown. Also the unstained decomposed zone contained a markedly large amount of microorganisms in the cavity with air-bubbles, decomposed starch grains and degenerative protein-contained muscles.

in the sausage kept in the incubator at 37°C for 7 days.

Cavities	Added tissue homogenates								
	Blood vessles	Kidney	Thymus lymph. n.	Salivary gland	Stomach	Intestine	Esophagus	Hair	Skin
−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−
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Summary and Conclusion

The present authors have attempted the detection of three nutrients (protein, carbohydrates and fat) and microorganisms coincidently stained with histochemical procedures upon the same frozen section of sausage or fish paste. This method is convenient and useful to show a morphological analysis of the sausage and fish-paste by means of the measurement of areas of stained histochemically protein, starch and fat by the plannimeter, and to observe the composition and varieties of the sausage, and to find the locus of bacterial multiplication in the decomposed sausage.

In shrot this method might contribute to study on the morphological analysis of the food and food hygiene.

Acknowledgement

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Plate 1.

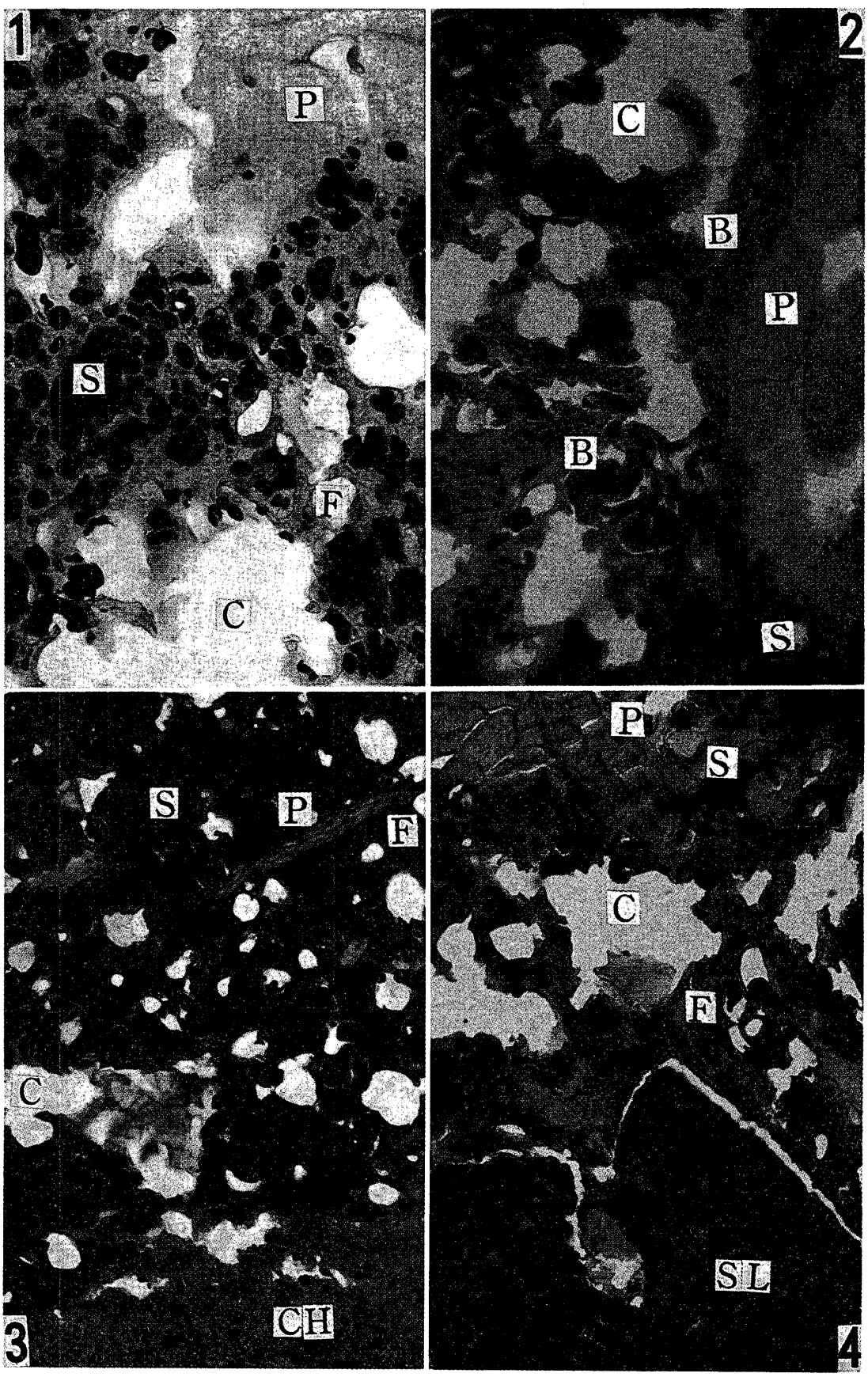
Explanation of the Figures

Fig. 1. Protein, starch and fat in the Vienna-sausage. There were shown greenish protein of the muscles (P), reddish violet starch (S), orange-colored fat (F) and colorless cavity with the air-bubbles (C) in the frozen section stained with coincident PAS-Sudan III-light green staining, and at 200×

Fig. 2. Protein, starch, fat and bacterial masses in the Vienna-sausage kept at the incubator at 37°C for 7 days. The locus of the bacterial multiplication was found in the decomposed portions of the starch grains (B_1) and cavity (B_2) and muscular protein (B_3). There were indicated reddish violet starch (S), orange-colored fat(F), coloress cavity (C), purple protein (P) and bluish bacteria (B–1, 2, 3) in the frozen section stained with coincident PAS-Sudan III-carbol thionine-light green staining, and enlarged at 200×.

Fig. 3. Protein, starch and fat in the cheese-contained Vienna-Sausage. There were showed bluish green protein of the muscles (P), orange-red-blue-colorn cheese (CH), orange-colored fat (F) and colorless cavity (C) in the frozen section stained with coincident PAS-Sudan III-light green staining and enlarged at 200×.

Fig. 4. Protein, starch and fat in the sailvary-gland mixed Vienna-sausage. There were shown bluish green protein (P) of the muscles, orange-coloredf at(F), red-orange-blue-colored salivary gland (SL) and colorless cavity (C) in the frozen section stained with coincident PAS-Sudann III-light green staining, and enlarged at 200×.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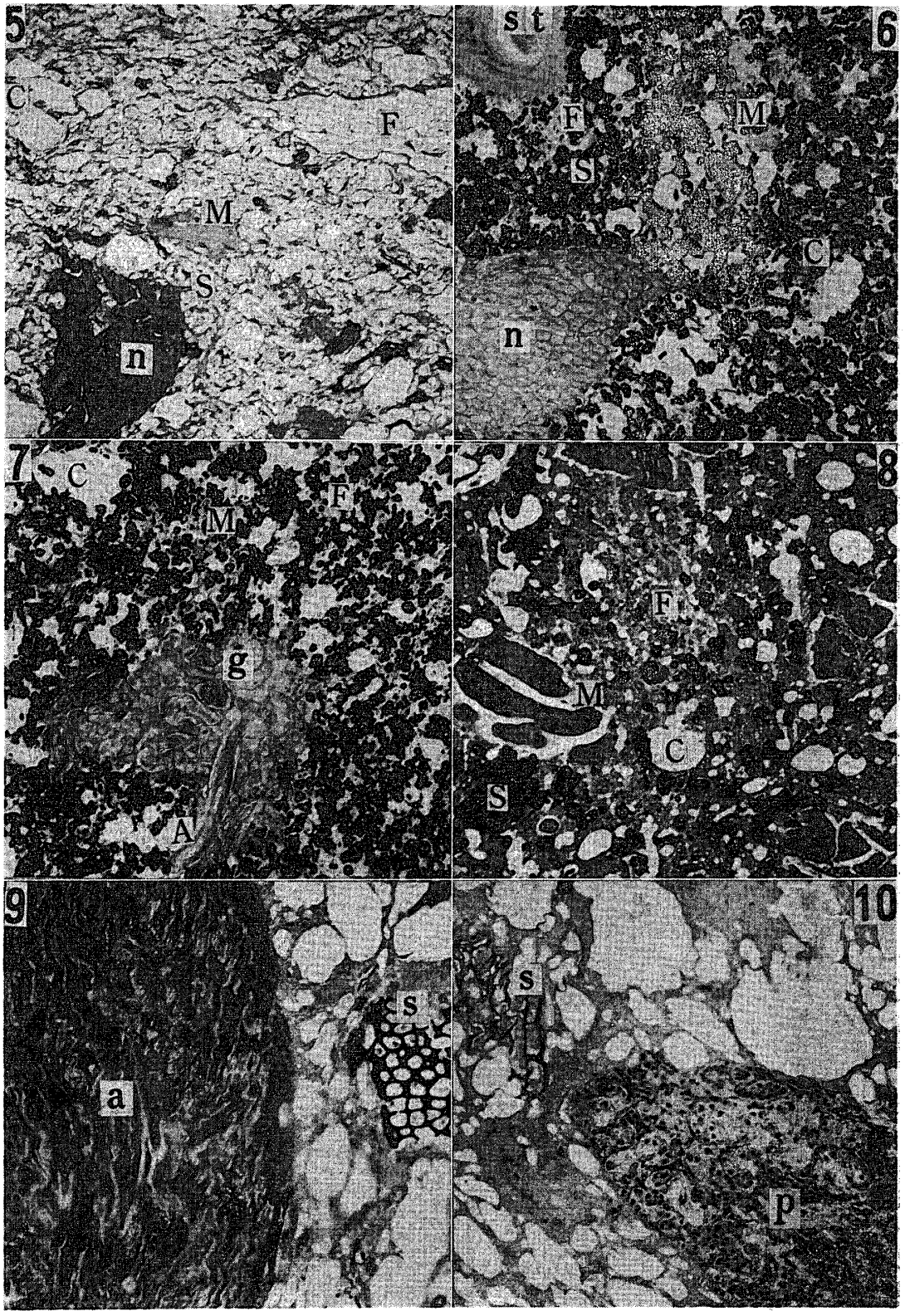
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Plate 2.

Explanation of the Figures.

Fig. 5. Mixture of the fragment of the kidney (N) in the Vienna-sausage of poor quality. There were indicated poor muscle (grey tone, (M), rich starch grain (irregular small vesicle, unstainable, colorless,(S) and rich fat (round large vacuole, unstainable,(F) in the section stained with acrolein-Schiff reaction for protein. 200×.

Fig. 6. Mixture of the fragment of the stomach (St) and kideny (N) in the Vienna-sausage of poor quality. There were indicated poor muscle (grey tone, M), rich starch grain (blackish, S), and rich fat (round large vacuole, unstainable, F) in the section stained with PAS reaction. enlarged at 200×.

Fig. 7. Mixture of the fragmented kindey with the glomerulus (G) and arcuate artery (A) in the Vienna Sausage of the poor quality. There were indicated poor muscle (grey tone, M.), rich starch grain (blackish, S), and rich fat (round large vacuole, unstainalbe, F) in the section stained with PAS reaction. enlarged at 200×.

Fig. 8. The arrangment of muscle (blackish,M), starch (blackish, St), and fat (colorless, vesicles, F) in the fish-paste stained with Haidenhein's iron-hematoxylin staining. enlarged at 200×.

Fig. 9. Mixture of the fragmented arterial wall (a) and spcie (s) in the Vienna sausage of the poor quality. The section was stained with PAS reaction and enlarged at 200×.

Fig. 10. Mixture of the fragmented parotid gland (P) and spice (S) in the Vienna sausage of the poor quality. The section was stained with pyronine-methyl green staining. 200×.

Plate 3.

Explanation of the Figures.

Fig. 11. Mixture of the fragmented thymus (h) in the Vienna suasage of the poor quality. The section was stained with pyronine-methyl green staining. DNA in the thymus stained greenish with methyl green and polysaccharide in the spices (S) stained reddish with pyronine 200×.

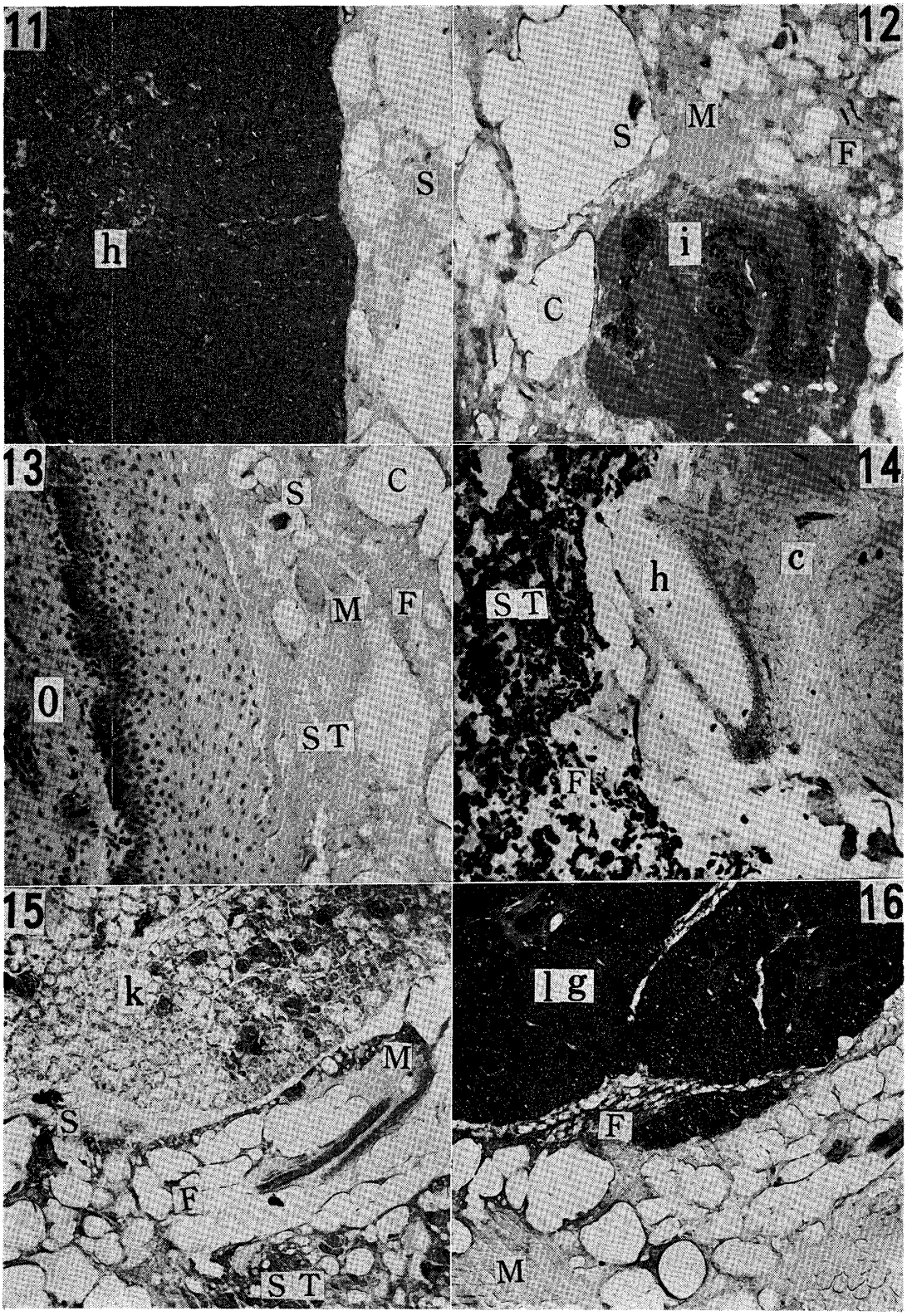
Fig. 12. Mixture of the fragmented small intestine (i), spices (S), muscle (M) and fat (F) in the Vienna sausage of the poor quality. The section was stained with PAS and enlarged at 200×.

Fig. 13. Mixture of the fragmented esophagus (O), muscle (M), spice (S), starch (small vesicle, unstainable, St), and fat (F, large vacuole, unstainable) in the Vienna sausage of poor quality. The section was stained with hematoxylin-eosin staining and enlarged at 200×.

Fig. 14. Mxiture of the fragmented skin (corneum, C) and hair (h), starch (small vesicle, blackish, St), and fat (F, large vacuole, unstainable) in the Vienna Sausage of poor quality. The section was stained with PAS reaction and enlarged at 200×.

Fig. 15. Mixture of the fragmented submaxillary gland (k) and fatty tissue (F), muscle (grey tone, M), starch (small vesicle, unstainable, St) and spice (S, blackish) in the Vienna sausage of the poor quality. The section was stained with hematoxylin-eosin staining and enlarged at 200×.

Fig. 16. Mixture of the fragmented sublingual gland (lg, blackish), muscle (m) and fatty tissue (F, unstainable) in the Vienna sausage of poor quality. The section was stained with PAS reaction and enlarged at 200×.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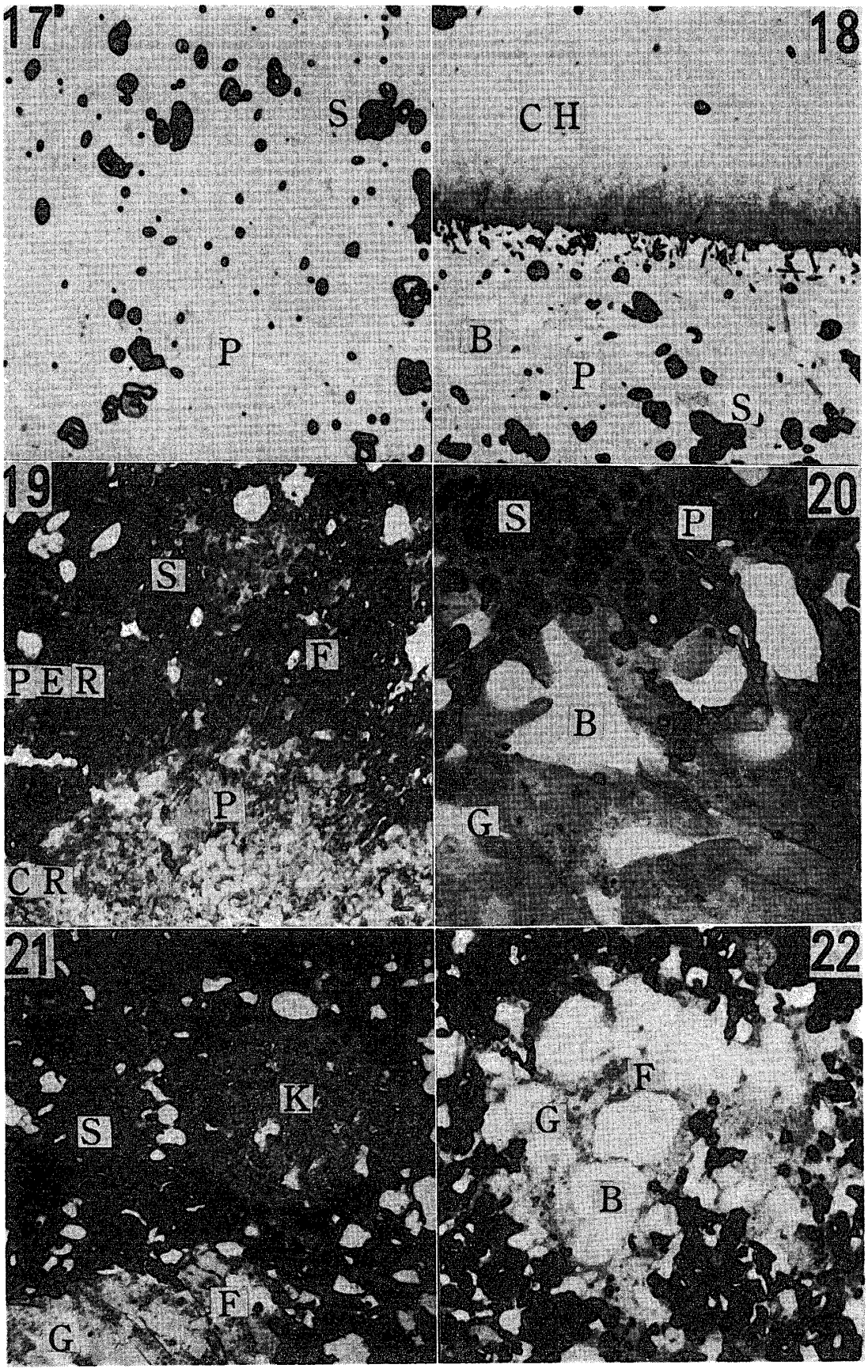
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Plate 4.

Explanation of the Figures.

Fig. 17. Protein (greyish tone, P), starch (blackish, St) and air-bubbles (coloress, B) in the fish-paste of good quality stained with PAS reaction and enlarged at 200×.

Fig. 18. Protein (greyish tone, P), starch (blackish, St),champinion (blackish hyphae, C) and air bubbles (coloress, B) in the champinion-contained fish-paste of good quality stained with PAS reaction and enlarged at 200×.

Fig. 19. Decomposed starch (St) and protein (P) in the peripheral portion (greyish tone, PER) and starch (blackish)-protein (greyish) in the centrum (blackish tone CR) of the Vienna-sausage kept in the incubator at 37°C for 7 days. This section was stained with PAS-Sudan III-light green staining and enlarged at 200×.

Fig. 20. Fat (F) rich glutein (G) greyish fat gloubles in the light grey glutein, starch (blackish, St), muscle protein (grey, P), fat (orange colored, F) and air-bubbles (coloress, B) in the Vienna sausage of poor quality stained with PAS-Sudan III-light green staining and enlarged at 200×.

Fig. 21. Fat (F)-rich glutein (G) within the Vienna sausage stained with PAS-Sudan III-light green staining and enlarged at 200×.

Fig. 22. Fat(F)-rich glutein (G) within the Vienna sausage stained with PAS-Sudan III-light green staining and enlarged at 200×.