# CHEMICAL STUDIES ON *RHIZOPUS JAPONICUS*

By

**Hoshik Lim**

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## INTRODUCTION

A most interesting piece of research is that on the utilization of microörganisms for nutrition. They can be cultured in quite confined spaces and they grow so rapidly that one may presume to employ them in artificial diets, particularly for some most important nutritive elements. From this standpoint, the author has made some chemical investigations on *Rhizopus japonicus,* hoping to gain some conception of its nutritive value.

*Rhizopus japonicus* is a saccharifying fungus, and was found by Boidin[1] in some Japanese "Koji". It was Vuillemin[2] who described it as a species of *Rhizopus.* Afterwards, numerous reports were published, but most of them are microbiological, and the chemical studies are very few: Cramer[3] Marschall[4] and others have reported on the ash and nitrogen contents of *Mucor* species: Nikolski[5], on the relation between the nitrogen content and the time of culture: Munz[6], Gerard[7] and Wisselingh[8], isolated trehalose, ergosterol and chitin respectively. Recently Nielsen[9] proved the existence of some materials which promote the growth of yeast and named them "Rhizopin". The author has studied the components such as the lipid, carbohydrates, proteins and vitamins of *Rhizopus japonicus,* from the view point of nutrition, the results of which studies will be described here.

## CHAPTER I

## PREPARATION OF SAMPLE

The species of fungus used in this experiment is *Rhizopus japonicus Vuillemin,* specimen kept in the Institute of Applied Mycology, College of Agriculture, Hokkaido Imperial University.

As a culture solution, Raulin's solution[10] was used of which the composition was as follows:—

| | | |
|---|---|---|
| 1. | Tartaric Acid ..... | 2.667% |
| | and magnesium Carbonate ..... | 0.267 " |
| 2. | Ammonium Nitrate ..... | 2.667 " |
| 3. | Ammonium Phosphate ..... | 0.400 " |
| 4. | Potassium Carbonate ..... | 0.400 " |
| 5. | Ammonium Sulphate ..... | 0.167 " |
| 6. | Zinc Sulphate ..... | 0.047 " |
| 7. | Ferric Sulphate ..... | 0.047 " |
| 8. | Sodium Silicate ..... | 0.047 " |
| 9. | Thirty five grams sucrose, purified with alcohol, was dissolved in 150 cc. of distilled water. | |

Twenty five cc. of each of 1, 2, 3, 4, 5, 6, 7 and 8 were mixed and to this mixture 50 cc. of the 9 solution was added.

As is well known, the culture medium has a great influence on the character of the fungus. Recently Yamamoto[11] studied the relation between the culture media, their temperature and the fermentation of the *Rhizopus* species.

The stock fungus used by the author was cultured in Koji agar. The organic substances in Koji agar would therefore probably influence the fungus. Attempts were made to eliminate these probable effects of the medium by successive transferrences into Raulin's solution for 20 generations. As Raulin's solution contains no organic substance whatever except sucrose and tartaric acid, any influence caused by the other organic substances was negatived.

The successive culturing was carried out every 2–3 days, and the fungus of the 20th generation was cultured once more in Raulin's solution as follows.

Two hundred and fifty cc. of Raulin's solution were transferred into 1-liter Erlenmeyer's flasks. Plugged with cotton, they were sterilized at 100°C. in Koch's sterilizer for three successive days, for 30 minutes

every day. The flasks were then placed in an incubator of 28°–30°C. overnight to ascertain their sterility; next inoculation was made with the least possible quantities from the above material and kept at 28°–30°C. for 5–7 days. Thus 0.5–1.2 grams fungous bodies were obtained per flask. By this method 1.5 kg. of the sample was prepared.

To separate the fungous bodies thus grown on the surface of the culture solution, the solution was decanted off, and, to wash off the culture solution completely, they were washed with distilled water several times. By means of a pincette the fungous bodies were taken out, pressed between filter papers, and dried in a sulphuric acid desiccator under negative pressure. When completely dried, they were crushed in a mortar, and kept in a bottle in a cool place.

To examine their general composition, 5 grams of the sample were treated successively with ether, 95% alcohol, and water: the soluble matters in each of these solvents were determined to be:

| | |
|---|---|
| Ether soluble matters | 9.720% |
| Ninty five percent alcohol soluble matters | 4.635 " |
| Water soluble matters | 23.133 " |
| Insoluble matters | 62.512 " |

The general analysis of the sample shows the following results.

TABLE I
Constituents of *Rhizopus japonicus*

| | In air dried matter % | In dry matter % |
|---|---|---|
| Water | 5.336 | — |
| Crude protein | 32.988 | 38.847 |
| Crude fat | 9.201 | 9.720 |
| Crude fiber | 7.285 | 7.696 |
| Nitrogen free extracts | 39.961 | 42.214 |
| Ash | 5.230 | 5.525 |
| Total nitrogen | 5.278 | 5.575 |
| Non-protein nitrogen | 3.431 | 3.624 |

Two hundred grams of the sample were ignited and ten grams of white coloured ash were obtained. Its composition was determined.

TABLE II
Mineral composition of *Rhizopus japonicus*

| | | | |
|---|---|---|---|
| $SiO_2$ | 25.556% | $P_2O_5$ | 37.233% |
| $Fe_2O_5$ | 1.482" | MgO | 1.326" |
| $SO_2$ | 0.314" | $K_2O$ | 18.575" |
| $Na_2O$ | 9.786" | ZnO | 0.128" |
| The rest | 5.600" | | |

## CHAPTER II
## ETHER SOLUBLE MATTERS

As already shown, the ether soluble matters of the fungous bodies amount to about 9.72%. To study their character, from 1 kg. of the dried sample all ether soluble matters were extracted in the following way.

One kg. of dried sample was extracted with ether in Soxhlet's apparatus for a week; completely to get the still remaining ether soluble matter in the residue, it was treated with 95% alcohol in a cool place for 5 days. It was extracted further 3 times with hot 85% alcohol at 85°–90°C. for 10 hours. All these alcohol extracts were cooled to room temperature, all the precipitates which appeared by cooling filtered off; after the removal of alcohol by evaporation at low temperature the remainder was dissolved in water and the solution was repeatedly shaken with ether. This ether extract and the ether extract in the apparatus were mixed together. After dehydration by the addition of anhydrous sodium sulphate, the ether was evaporated. Thus 98 grams of brown solid fats were obtained.

When these fats were treated with acetone 1.5 grams of insoluble substance were precipitated.

### (1) General characters of the fats

Through the fraction soluble in cool acetone, $CO_2$ gas was passed at a low temperature for quite a long time, and kept in a vacuum desiccator for a week. Thus when the acetone was completely removed, 96 grams of brown solid fats were obtained.

By ordinary analysis the following results were obtained for these fats.

Table III

General characters of the fats

| | |
|---|---|
| Specific gravity (at 40°C.) | 0.965 |
| Refractive index (at 40°C.) | 1.457 |
| Acid value | 95.40 |
| Iodine value | 84.65 |
| Reichert-Meissl value | 8.16 |
| Acetyl value | 38.00 |
| Saponification value | 164.00 |
| Hehner value | 72.75 |
| Polenske value | 0.82 |
| Ester value | 68.60 |
| Unsaponifying matters | 3.78% |
| Unsaturated fatty acids | 61.25% |
| Saturated fatty acids | 38.75% |

From the above results, it may be seen that these fats resemble the general fats. However the acid value is comparatively higher than the saponification value and also the Reichert-Meissl value is high when compared to the fats.

### (2) Unsaponifying matters

#### (a) *Isolation of ergosterol and biological test of it*

Seventy grams of these fats were saponified for an hour according to the Lewkowitsch method[12]. The seventy grams of fats were taken in a one liter flask, and, after the addition of 250 cc. alcohol, 35 grams potassium hydroxide and 140 cc. distilled water, they were saponified for an hour on a boiling water-bath. To the saponified solution, after the removal of alcohol by $CO_2$ gas admission, a large quantity of water was added and repeatedly shaken with ether. All these ether extracts were again saponified as stated, and, after the saponification was completed, again shaken with ether, and dehydrated with anhydrous sodium sulphate. The ether was then evaporated. After laying aside, lustrous, scale-like crystals were abundantly obtained with absolute alcohol and ether the yield being 3.48 grams.

The crystals thus obtained were positive for Liebermann and Salkowski's reaction, and precipitated by digitonin, characteristic of sterol. As Tanret[13] and Ellis[14] isolated fungisterol and ergosterol from fungous bodies, and Gerard[7], ergosterol from *Mucor mucedo,* the obtained substances were examined in this respect. Three and four-tenths grams of the crystals were dissolved in 20 cc. of anhydrous acetic acid, and small pieces of anhydrous sodium acetate were added. After 3 hours boiling, 150 cc. of hot water were poured in and stirred. The oily fraction was collected and dissolved in ether; the ether soluble fraction was then washed several times with water in a separatory funnel, dehydrated, and the obtained acetate, after the evaporation of ether, was treated with acetone according to Tanret's method[13]. The soluble part was removed, and by recrystallization with acetone 0.85 gram of scale-like crystals was yielded.

The melting point of the crystals was 168°–171°C., the refractive index and the elementary analysis were as follows.

Refractive index:

One per cent alcohol solution; tube length, 10 cm.; temperature, 17°C.; Ventzke sugar scale, −2.5; corrective coefficient, 0.3465.

$$[\alpha]_D^{17°} = \frac{-2.5 \times 0.3465 \times 100}{1 \times 1} = -86$$

Elementary analysis:

| Sample | $CO_2$ | $H_2O$ | C | H |
|---|---|---|---|---|
| g. | g. | g. | % | % |
| 5.824 | 17.53 | 5.51 | 82.09 | 10.51 |
| 5.645 | 16.99 | 5.33 | 82.08 | 10.49 |
| $C_{29}H_{44}O_2$ | — | — | 82.07 | 10.37 |

From these data, the crystal will be seen to resemble ergosteryl-acetate. To verify this the crystalline substance was again saponified and recrystallized with alcohol. Seventy eight-one hundredths gram acicular crystal was obtained.

The crystal thus obtained is positive for Liebermann and Salkowski's reaction; the melting point is 159°–162°C., and the refractive index is as follows;

One per cent chloroform solution; tube length, 10 cm.; temperature, 18°C.; Ventzke sugar scale, −3.5; corrective coefficient, 0.3465.

$$[\alpha]_D^{18°} = \frac{-3.5 \times 0.3465 \times 100}{1 \times 1} = -120$$

From the above data, the crystal is seen quite definitely to be ergosterol.

Ergosterol will be activated to vitamin D when irradiated by the ultra-violet ray, and even quite small quantities are capable of curing experimental rachitis in rats, as has already been proved by Windaus[15] and Rosenheim[16], and substantiated by many other investigators. With the obtained crystal, biological tests were therefore executed on albino rats.

## Biological Test

As the basal diet of albino rats, Steenbock's No. 2965[17] was employed; when the rats are fed on the basal diet alone, characteristic skeletonial changes due to rachitis may be seen, owing to the shortage of vitamin D. Ossified tissue is formed at the basal end of the tibia by the lack of precipitation of calcium salts; but when the D-factor is supplied to those suffering from rachitis, the disease will be completely cured within 3 weeks. Thus, noting the presence or the absence of this disease in those animals which were fed with basal diet only and in those which were fed with the test sample accompanying the basal diet

for 5 weeks one can know easily the vitamin D content of the test sample. For diagnosis the animals were examined with Röntgen-rays. It was also investigated if the tested sample would cure the attacked rats in 3 weeks or not. For the generation of ultra-violet rays, a Shimazu Mercury Lamp was used of 2 amp. at 90–100 volts. The sample dissolved in olive oil was irradiated for 40 minutes, at a distance of 40 cm. from the source. The sample was given *per os.*

The results thus obtained were as shown in Tables IV and V. Among the rats, No. 260, which was fed on the basal diet alone, No. 261, which took daily 1 cc. of irradiated olive oil in addition to the basal diet, and No. 263, for which 1 cc. of irradiated Raulin's solution was added to the basal diet every day, suffered equally seriously. Among the other rats, No. 265, which was fed on the basal diet and on 0.1 cc. of irradiated cod-liver oil, and No. 266, which took 1/500 mg. of irradiated sample and the basal diet every day, kept their health quite well after 2 months feeding. (see Table IV).

Of the four rats typically attacked by the disease after 5 weeks feeding with basal diet alone, two rats, Nos. 267 and 268, received an addition of 0.2 cc. of irradiated cod-liver oil to their daily diet. To the diet of the other two, Nos. 260 and 261, 1/1000 mg. of irradiated sample were added every day; after 3 weeks feeding, it was seen that all these rats had been completely cured. (see Table V).

TABLE IV

Effect of the sample upon the rachitis of rats

| Animal No. | Duration of experiment (days) | | Body weight | | Röntgen diagnosis |
|---|---|---|---|---|---|
| | | | Initial weight | After disease | |
| 260 | 35 | Steenbock No. 2965. | 38 | 68 | Serious |
| 261 | " | Steenbock No. 2965 1 cc. of irradiated olive oil. | 40 | 65 | " |
| 263 | " | Steenbock No. 2965 1 cc. of irradiated Raulin's solution. | 32 | 60 | " |
| 265 | " | Steenbock No. 2965 0.1 cc. of irradiated codliver oil. | 36 | 82 | Ordinary |
| 266 | " | Steenbock No. 2965 1/500 mg. of irradiated sample. | 41 | 95 | " |

TABLE V

Curative effect of the sample upon the rachitis of rats

| Animal No. | Basal diet | Duration of expt. (days) | Sample | Duration of expt. (days) | Body weight | | | Röntgen diagnosis | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Initial weight | After disease | After expt. | After disease | After expt. |
| | | | | | g. | g. | g. | | |
| 267 | Steenbock No. 2965. | 35 | 0.2 cc. of irradiated cod-liver oil. | 21 | 42 | 86 | 105 | Serious | Completly cured |
| 268 | " | " | " | " | 36 | 60 | 82 | " | " |
| 260 | " | " | 1/1000 mg. of irradiated sample | " | 38 | 68 | 93 | " | " |
| 261 | " | " | " | " | 40 | 65 | 85 | " | " |

From these results it may be inferred that the crystal is doubtlessly ergosterol.

### (b) *Isolation of fungisterol*

When kept at room temperature, crystalline substances appeared in the filtrate after the separation of ergosterylacetate. The melting point of these crystals was 158°C., which agrees with that of fungisterylacetate. The crystals were saponified, and recrystallized in alcohol; 1.85 grams of crystal were thus obtained.

The melting point of this recrystallized substance is 143–145°C. The refractive index and the elementary analysis are as follows:

#### Refractive index

Three per cent chloroform solution; tube length, 10 cm.; temperature, 18°C.; Ventzke sugar scale, −1.9; corrective coefficient, 0.3465.

$$[\alpha]_D^{18°} = \frac{-1.9 \times 0.3465 \times 100}{1 \times 3} = -23$$

Elementary analysis:

| Sample | $CO_2$ | $H_2O$ | C | H |
|---|---|---|---|---|
| mg. | mg. | mg. | % | % |
| 4.212 | 13.055 | 4.359 | 84.53 | 11.50 |
| 4.362 | 13.455 | 4.829 | 84.08 | 12.32 |
| $C_{25}H_{40}O$ | — | — | 84.27 | 11.24 |

From the results obtained, it is to be judged that the crystal is apparently fungisterol.

### (3) SAPONIFYING MATTERS

### (a) *Isolation of saturated fatty acids*

The saponified solution, after the removal of excessive ether by

warming on the water-bath, was cooled, mixed with a large quantity of water, and neutralized with dilute acetic acid. Ten per cent lead acetate solution was added under gentle agitation. Fine precipitates appeared. After having been put away overnight, the clear supernatant liquid was filtered off and the precipitates were successively washed with water until they became completely free from lead, a fact which was ascertained by testing with sodium carbonate. The precipitates were then shaken with ether, and stood overnight. The material was then centrifuged to separate the precipitates from ether. This procedure was repeated, and the precipitates thus obtained were dissolved by the addition of dilute hydrochloric acid and shaken with ether. The ether solution was then washed with water until it became quite free from chloride. Dehydrated by the addition of anhydrous sodium sulphate, the ether was evaporated off. Thus 31.2 grams of yellow, solid fatty acid were obtained.

General characteristics

The melting point and neutralization value of this fatty acid were determined in the ordinary way, and the iodine value by Wij's method.

| | |
|---|---|
| Melting point | 56°–58°C. |
| Neutralization value | 214.2 |
| Iodine value | 4.85 |

From the fact that the iodine value is 4.85, it is clear that the fatty acid certainly contains a small quantity of unsaturated fatty acids, but because the quantity is very small they were at once methylated according to Haller's method[18] without giving them much attention.

Fifteen grams of these saturated fatty acids were dissolved in 18.8 grams of anhydrous methyl alcohol and 26 grams of ether containing 2.5% hydrochloric acid, and heated for 12 hours with a reflex condenser. Cooled, the solution was neutralized with barium carbonate, and brought into a separatory funnel. Excessive methylalcohol was washed off with sodium chloride solution. The ethereal fraction was then dehydrated with anhydrous sodium sulphate and the ether was evaporated. Fourteen and four-tenths grams methylester of fatty acid were obtained.

The methylester thus obtained was distilled under a pressure of 12 mm. atm.; distillation began at 180°C. Fractional distillation was

therefore made at 180°–190°C., 190°–200°C. and 200°–210°C. The fatty acids thus obtained were as follows.

TABLE VI
Effects of fractional distillation of fatty acids at varying temperature

| Distillation Number | Temperature C. | Atm. pressure mm. | Saponification value | Melting point C. | Yield g. |
|---|---|---|---|---|---|
| I | 180–190 | 12 | 208.2 | 28 | 3.5 |
| II | 190–200 | „ | 205.7 | 29 | 6.2 |
| III | 200–210 | „ | 206.5 | 29 | 2.4 |

After this distillation 1.8 grams of dark brown residue were found in the flask.

From the above data, all methylesters of these fractions I, II and III seem to consist mostly of palmitic acid methylester. The three fractions were therefore mixed together, saponified with alcoholic potash solution, and then slightly acidified with hydrochloric acid. Free fatty acid was obtained. It was recrystallized in alcohol, the melting point of which was 60°C. After several recrystallizations in alcohol, 5.3 grams of white acicular crystal were obtained.

The melting point of this recrystallized white sample is 62°C. and the neutralization value 218.8 both of which figures agree with those of palmitic acid. Elementary analysis shows the following results.

| Sample | $CO_2$ | $H_2O$ | C | H |
|---|---|---|---|---|
| g. | g. | g. | % | % |
| 0.0562 | 0.1543 | 0.0638 | 74.88 | 12.61 |
| $C_{16}H_{32}O_2$ | — | — | 74.93 | 12.58 |

Therefore, the crystal is certainly palmitic acid.

The dark brown mass remaining in the distillation flask was saponified with alcoholic potash solution, and then acidified slightly with hydrochloric acid solution. The fatty acid thus freed was washed with ether in the separatory funnel. This ether solution was repeatedly washed with water to remove the hydrochloric acid, then dehydrated with anhydrous sodium sulphate; ether was evaporated off and fatty acid was obtained. On fractional crystallization, fatty acids with melting points of 68°C. and of 28°C. were obtained. When both these crystalline substances were recrystallized with alcohol, the former was obtained as a lustrous laminar crystal, and the latter as a white acicular

one. The melting points were 69°C. and 61.5°C. respectively, and the yields were 0.28 gram and 0.85 gram. The neutralization values were 197.88 and 216.85 respectively.

| | Yield | Melting point | Neutralization value |
|---|---|---|---|
| Laminar crystal | 0.28 g. | 69°C. | 197.88 |
| Acicular crystal | 0.85 g. | 61.5°C. | 216.85 |

From these results it is clear that the acicular crystal must be palmitic acid and the laminar crystal stearic acid. The latter was subjected to elementary analysis, the results of which were as follows;

| Sample | $CO_2$ | $H_2O$ | C | H |
|---|---|---|---|---|
| g. | g. | g. | % | % |
| 0.1254 | 0.3468 | 0.1447 | 75.42 | 12.83 |
| 0.0883 | 0.2449 | 0.1022 | 75.64 | 12.86 |
| $C_{18}H_{36}O_2$ | — | — | 75.98 | 12.76 |

These data agree quite well with those for stearic acid.

(b) *Isolation of unsaturated fatty acids*

The saturated fatty acids could be removed by the ether lead soap method.

The remaining ether solution of lead soap was transferred next into a separatory funnel, and acidified slightly with hydrochloric acid: fatty acids thus freed were washed with water several times, then dehydrated with anhydrous sodium sulphate. After the removal of ether, 45.8 grams of dark brown, oily, unsaturated fatty acids were obtained.

The general characteristics of these unsaturated fatty acids were as follows:

Specific gravity (at 15°C.) ..... 0.9675
Iodine value ..... 95.84
Neutralization value ..... 187.95

Twenty grams of unsaturated fatty acids thus obtained were dissolved in 170 cc. of ether and cooled to −5°C.; bromine water was then added, but no crystal was formed even after 20 hours. There was therefore evidently no octabromide. The solution was then poured into a separatory funnel, excessive bromine was removed by washing with dilute sodium thiosulphite solution. Sodium thiosulphite was then removed by washing with water. Then, the preparate having been dehydrated with anhydrous sodium sulphate, ether was finally driven

off. It was next treated with petroleum ether (B.P. 40–50°C.). Although nearly all of it was dissolved, a dark brown syrupy substance remained undissolved; the ether layer was then decanted off, and the undissolved material was washed several times and dried in a vacuum desiccator. The yield was 0.39 gram. The bromine content was determined by the Baubigny u. Chavanne method[19]. The results are as follows:

| Sample | AgBr | Br |
|---|---|---|
| g. | g. | % |
| 0.2243 | 0.2805 | 53.23 |
| 0.1675 | 0.2102 | 53.43 |
| $C_{18}H_{32}O_2Br_4$ | — | 53.33 |

These results agree perfectly with the contents of tetrabromstearic acid; linoleic acid must therefore exist in the sample.

From the petroleum ether soluble fraction, petroleum ether was evaporated almost to dryness and kept for 3 weeks at −7°C. Small quantities of white crystal resulted. On filtration of the solution and purification of the crystal, 0.62 gram was obtained.

This substance has a melting point of 112°–114°C., and on determination of its bromine content, the following results were obtained.

| Sample | AgBr | Br |
|---|---|---|
| g. | g. | % |
| 0.2553 | 0.3185 | 53.55 |
| $C_{18}H_{32}O_2Br_4$ | — | 53.33 |

The crystal agrees with tetrabromstearic acid.

After the separation of tetrabromide from the filtrate petroleum ether was completely evaporated and 29.4 grams of a brown, transparent oily substance were gained.

On determining the bromine content, the following resulted:

| Sample | AgBr | Br |
|---|---|---|
| g. | g. | % |
| 0.2635 | 0.225 | 36.32 |
| $C_{18}H_{34}O_2Br_2$ | — | 36.18 |

The substance agrees with dibromstearic acid. The existence of oleic acid is consequently obvious. Twenty nine and four-tenths grams of dibromstearic acid corresponds, when calculated as oleic acid, to 19.48 grams; the content of oleic acid in the unsaturated fatty acid therefore amounts to 94.98%.

### (4) Isolation of phosphatids

In 98 grams of the ether soluble matters there were 1.5 grams of

acetone insoluble matter. This was again dissolved in a little ether and precipitated with acetone, then filtered and treated with absolute alcohol. Thus two fractions were separated, the one soluble in alcohol, and the other insoluble.

The fraction soluble in absolute alcohol was then precipitated by the addition of a large quantity of acetone, and the precipitate dissolved in ether. Then, repeating the procedure several times, precipitation by acetone and dissolution by alcohol, 0.78 gram of a white waxy powder was finally obtained.

This substance contains phosphorus and nitrogen, and its water solution is colloidal. In alcoholic solution it will be precipitated by a chloride solution of cadomium or platinum. The nitrogen and the phosphorus were determined by the micro-Kjeldahl method and by Neumann's method respectively. The results are:

Phosphorus 1.90% Nitrogen 1.81% P : N = 1.0 : 2.03

From these data it is apparent that this substance must be a diamino-monophosphatid.

The fraction insoluble in absolute alcohol was again dissolved in a small quantity of ether and a large quantity of alcohol was added. Forty one-one hundredths gram of a white powder was obtained.

The substance thus obtained has the general characteristics of phosphatid. The content of phosphorus and nitrogen was:

Phosphorus 3.25% Nitrogen 1.51% P : N = 1.0 : 1.04

This substance is therefore a monoamino-monophosphatid.

### (5) Summary

By chemical analysis of the *Rhizopus japonicus* cultivated in Raulin's solution, 1.08 grams of ergosterol, 2.55 grams of fungisterol, 7.6 grams of palmitic acid, 1.16 grams of stearic acid and 1.19 grams of phosphatids were found per 1 kg. of dried fungous bodies. The unsaturated fatty acids are mainly oleic acid; the existence of a small quantity of linoleic acid was proved.

## CHAPTER III

## ALCOHOL SOLUBLE MATTERS

The content of the alcohol soluble matters in the fungous bodies is 4.635%.

## (A) Alcohol and saccharides

### (1) *Isolation of mannit*

Nine hundred grams of the fat free fungous body were extracted for 5 days with 2.5 liters 95% alcohol and filtered. (1/5 volume of this filtrate was used for the detection of vitamin C). The residue was then extracted three times with two liters 85% alcohol at 85–90°C. for 10 hours. Both of these filtrates and wash alcohol were mixed and cooled. Abundant crystallized precipitates appeared. Recrystallized in methyl alcohol several times, and washed with alcohol and ether, 16.3 grams of white acicular crystals were obtained.

This crystal is very soluble in water, but with difficulty soluble in alcohol and quite insoluble in ether; its taste is sweet, and the melting point lies at 166°C. By elementary analysis and the determination of the refractive index, the following results were obtained.

Elementary analysis

| Sample g. | $CO_2$ g. | $H_2O$ g. | C % | H % |
|---|---|---|---|---|
| 0.1254 | 0.0356 | 0.4482 | 7.74 | 39.80 |
| 0.1422 | 0.0400 | 0.5120 | 7.67 | 40.00 |
| $C_6H_{14}O_6$ | — | — | 7.72 | 39.54 |

Refractive index

Five per cent boric acid solution; tube length, 10 cm.; temperature, 25°C.; Ventzke sugar scale, 3.95; corrective coefficient, 0.3465.

$$[\alpha]_D^{25°} = \frac{3.95 \times 0.3465 \times 100}{1 \times 5} = 27.3$$

From these results it is clear that this acicular crystal is mannit.

After the separation of the mannit, alcohol was removed from this alcohol extract and it was dissolved in water. After the removal of all ether soluble matters in this solution by shaking it several times with ether, it was concentrated at 40°C. Then neutral and basic lead acetate solutions were added; the precipitates thus resulting were filtered off. The lead in the filtrate was removed by hydrogen sulphide and the acetic acid by ether and calcium carbonate. The solution was then kept in a cool place for several days. Sulphuric acid was added to the concentration of 5% and saturated phosphotungstic acid solution was poured in. The precipitates thus resulting were separated. The filtrate was treated as usual, and several times decolorized with animal charcoal,

condensing to a syrupy state. One hundred and thirty five grams of syrup were obtained.

(2) *Characteristics of the syrup*

1. Water: 53.25%. 2. Solid matters: 46.75%. 3. Reducing sugar (as glucose): 6.72% (14.38% for solid matter). 4. Reducing sugar, after the syrup was hydrolized (as glucose): 28.25% (61.08% for solid matter. 5. Molisch's reaction for the carbohydrates: positive. 6. Seliwanoff's reaction and Pinoff's reaction for ketose: positive. 7. Iodine reaction for starch and dextrine: negative. 8. Aniline acetate reaction for furfurol: negative. 9. Mucic acid was not formed by keeping 1 g. of syrup dissolved in 20 cc. of nitric acid of sp. gr. 1.15 and concentrated on a water-bath with occasional stirring for several days in a cool place. 10. The above solution, after the removal of nitric acid by heating on the water-bath until it was almost dried up, was cooled. Potassium carbonate was added to saturate, then acidified by adding acetic acid drop by drop, and kept in a cool place. Abundant white crystals were obtained. On examination through the microscope the crystal seems to be potassium saccarate. 11. By excessive addition of acidic solution of phenylhydrazin to the 5% aqueous solution of the syrup a precipitate was made after a day in a cool place. The precipitate was washed with a mixed solution of ether and alcohol, recrystallized from 80% alcohol. The crystal is identical with that of mannose phenylhydrazone, its melting point being 196°C. After the removal of mannose phenylhydrazone, the filtrate was heated for 30 minutes at 100°C. on the water-bath. Abundant yellowish acicular crystals resulted. After recrystalization in alcohol, the melting point was estimated. It was 204°C., which quite agrees with that of glucosazone.

(3) *Isolation of trehalose*

Munz[6] proved the presence of trehalose in *Mucor,* and, as stated, the reducing power of the syrup after hydrolysis is very different from that before hydrolysis. One may therefore suppose the presence of trehalose in the syrup. Its isolation was consequently attempted.

One hundred grams of the syrup were dissolved in a small quantity of 95% alcohol, and kept cool (at about 10°C.) for 3 weeks. Some crystals appeared. Recrystallized several times from alcohol, 0.26 gram of white crystals was obtained.

This white, rhombic crystal melts at 203°C., reducing Fehling's

solution as sucrose. However, when the crystals are heated in 5% sulphuric acid solution for 6 hours, they do not reduce Fehling's solution; the refractive index is;

One per cent aqueous solution, tube length, 10 cm.; temperature, 20°C.; Ventzke sugar scale, 5.7; corrective coefficient, 0.3465.

$$[\alpha]_D^{20°}=\frac{5.7\times 0.3465\times 100}{1\times 1}=197$$

From these results, the crystal is probably trehalose.

(4) *Detection and isolation of saccharose*

After the removal of trehalose, alcohol was evaporated from the solution under diminished pressure at low temperature. As its reducing power is changed by hydrolysis, there must exist an appreciable quantity of unreduced sugar; an attempt was therefore made to isolate saccharose by Schultze's method[20]. Mixing with saturated strontium hydroxide solution, strontium bisaccharate was obtained. Suspended in water, strontium was removed by $CO_2$ gas; then when sufficiently concentrated, tiny crystal particles of saccharose were added. The crystal particles soon grew larger. The solution was filtered after several days, washed several times with absolute alcohol, and dried. Eight and six-tenths grams of white crystal were thus obtained.

This white, very sweet crystal melts at 183°C. It does not reduce Fehling's solution directly, but it reduces the reagent after hydrolysis. Seliwanoff's reaction is positive. The refractive index is as follows:

Two per cent aqueous solution; tube length, 10 cm.; temperature, 20°C.; Ventzke sugar scale, 3.8; corrective coefficient, 0.3465.

$$[\alpha]_D^{20°}=\frac{3.8\times 0.3465\times 100}{1\times 2}=65.8$$

Therefore the crystal is quite certainly saccharose. To verify this, its hydrolysis products were examined. Five grams of the crystal were dissolved in 50 cc. of water, 5 cc. of 0.279% hydrochloric acid solution were added, and the solution heated for 30 minutes on a water-bath. It was then neutralized and made up to 100 cc. with water. This hydrolyzed solution forms a brownish, black ring at the contact surface when poured gently on to concentrated sulphuric acid. Seliwanoff's reaction was also notably positive, the refractive index being—

Five per cent aqueous solution; tube length, 10 cm.; temperature, 19°C.; Ventzke sugar scale, −2.9; corrective coefficient, 0.3465.

$$[\alpha]_D^{19°} = \frac{-2.9 \times 0.3465 \times 100}{1 \times 5} = -20$$

Two grams of phenylhydrazine hydrochloride and 3 grams of sodium acetate were added to 20 cc. of the above hydrolyzed solution and heated for 30 minutes on a water-bath. The abundant acicular crystalline precipitates thus obtained were recrystallized from alcohol. They melt at 205°C., and when put under the microscope, the characteristic acicular crystals of glucosazone were observed.

From these results it is clear that the substance is certainly saccharose, since it consists of ketose and aldose as shown. The refractive index on hydrolization shows invertose. Furthermore, glucosazone was obtained.

(5) *Detection of fructose, glucose and mannose*

After the separation of trehalose from the filtrate, excess of strontium was removed and condensed to about 70 grams. The syrup thus obtained yields both Seliwanoff's and Pinoff's reactions. The presence of fructose being obvious, the methylphenylosazone of fructose was found by Neuberg's method[21]. Ten grams of the syrup were dissolved in small quantities of water, 6 grams of methylphenylhydrazine and alcohol were added, and, when the mixture was cooled after heating, reddish orange coloured crystals were obtained. Recrystallized several times in 10% alcohol containing several drops of pyridine, 0.35 gram of the crystal, which melts at 156–158°C., was obtained. Since Neuberg showed that the melting point of the methylphenylosazone of *d*-fructose when crystallized in 10% alcohol, is 158°C., it is to be inferred that the crystal is probably methylphenylosazone of *d*-fructose.

If it is assumed that the fructose was completely osazonized, the fungous bodies contain 0.154% of fructose.

From the qualitative tests above mentioned, glucose and mannose seem to exist in the syrup. But other sugars may also exist. The diphenylhydrazone of glucose was therefore made according to Fischer's[22] and Stahel's method[23].

Twenty grams of the syrup were dissolved in a small quantity of water and 20 cc. of alcoholic solution of diphenylhydrazine were added. As the solution showed some turbidity, it was filtered and kept at room temperature for several days. A colourless prismatic crystal appeared. Recrystallized from warm water several times, washed with ether, and

dried, 2.8 grams were obtained. It melts at 161°–162°C. corresponding perfectly with diphenylhydrazone of glucose.

Moreover, by excessive addition of acidic solution of phenylhydrazine to 20 cc. of 5% aqueous solution of the syrup a precipitate was made after a day in a cool place. The precipitate was washed with a mixed solution of ether and alcohol, recrystallized from 80% alcohol. Thus 0.223 gram was obtained. The crystal is identical with that of mannose phenylhydrazone, its melting point being 196°C. Hence the content of mannose in the fungous bodies must be 1.403%.

### (6) *Summary*

*Rhizopus japonicus Vuillemin* cultivated pure in Raulin's solution contains 16.3 grams of mannit, 0.26 gram of trehalose and 8.6 grams of saccharose in 1 kilogram of dried bodies; in addition to this, it contains 0.154% of fructose, 1.403% of mannose, and about as much glucose. A great deal of sucrose separated from the sample seems to have been transported from the culture solution.

## (B) Separation of organic bases

The precipitate formed by phosphotungstic acid was made sludgy with a little water, mixed with excess of barium hydroxide, and distilled under negative pressure; the distillate was introduced into a dilute hydrochloric acid solution, the volatile base being thus absorbed in it. The solution was then concentrated and afterwards dried in the vacuum desiccator. Some crystalline substance was recognized which does not dissolve in absolute alcohol. It was therefore recrystallized with water, and 0.15 gram of a white crystal was obtained.

The nitrogen content of this crystal was determined. It resembles that of ammonium chloride.

| Sample g. | Nitrogen g. | Nitrogen % |
|---|---|---|
| 0.012 | 0.0031 | 26.08 |
| 0.015 | 0.0039 | 26.07 |
| $NH_4Cl$ | — | 26.05 |

### (1) *Adenine*

The residue after the removal of the volatile base, was treated as usual; the free base solution was concentrated, acidified with nitric acid, and mixed with silver nitrate solution. A small quantity of a brownish precipitate was formed. This precipitate was washed several times with dilute nitric acid solution, and suspended in water; a slow

current of $H_2S$ gas was passed, thus removing the silver. Then, with phosphotungstic acid, precipitation was caused as usual. From this precipitate, a free base solution was prepared after the usual treatment, and concentrated under negative pressure till almost dry; treated with 10% ammonia solution, the greater part was dissolved. The solution was filtered and the ammonia removed by acidifying with dilute hydrochloric acid and mixed with a saturated solution of picric acid, by which a small amount of picrate was obtained.

This picrate is a beautiful yellowish acicular crystal, quite characteristic of adenine picrate. Its yield was 0.13 gram, its melting point 285°C. The picric acid content was determined, the result of which coincides with that for adenine picrate, as the next table shows.

| Sample | Picric acid | Picric acid |
|---|---|---|
| g. | g. | % |
| 0.045 | 0.0280 | 62.22 |
| 0.050 | 0.0312 | 62.40 |
| $C_5H_5N_5 . C_6H_3N_3O_7$ | — | 62.34 |

(2) *Hypoxanthine*

The filtrate, after the separation of adenine picrate, was shaken several times with ether, to remove the picric acid contained therein. It was then concentrated, dissolved in a small quantity of water, and made alkaline with ammonia. Precipitation was caused by the addition of ammonia silver nitrate solution. The precipitate was collected on filter paper, washed several times with ammonia water, and boiled with nitric acid of specific gravity 1.1, filtered and cooled. The filtrate was mixed with some hot water. By passing an $H_2S$ current through, the silver in this solution was removed. The filtrate was again slightly alkalized by the addition of ammonia, concentrated, and kept at room temperature. A white precipitate was formed.

The substance thus obtained is a white amorphous powder which has no definite melting point, but carbonizes easily. The yield was 0.076 gram; the nitrogen content in it agrees quite well with that of hypoxanthine.

| Sample | Nitrogen | Nitrogen |
|---|---|---|
| g. | g. | % |
| 0.035 | 0.0144 | 41.17 |
| 0.040 | 0.0165 | 41.14 |
| $C_5H_4N_4O$ | — | 41.25 |

(3) *Histidine*

Of the above mentioned filtrate from ammonia silver nitrate, after

the removal of the precipitate caused by silver nitrate, one part was used for the detection of vitamin B. To the other part an excess of silver nitrate solution was added. It was saturated with barium hydroxide, and a considerable quantity of dark brownish precipitate was formed. The precipitate was washed with barium hydroxide solution, and decomposed by nitric acid and hydrochloric acid. A brown coloured solution was thus obtained; a free basic solution was then made in the usual way. With mercurous chloride solution some precipitate was obtained. Washed with mercurous chloride solution, and suspended in water, mercury was removed by $H_2S$. By treating as usual, a free base solution was obtained; then, neutralized with hydrochloric acid, the solution was concentrated, and kept in a vacuum desiccator. A white crystal was obtained. It showed the diazo-reaction after Pauli's method. The strongly yellowish, acicular crystal melts at 86°C. It is therefore apparently histidine picrate. The yield was 0.027 gram and the estimation of picric acid content gives the following result.

| Sample | Picric acid | Picric acid |
|---|---|---|
| g. | g. | % |
| 0.022 | 0.0131 | 59.55 |
| $C_6H_9N_3O_2 \cdot C_6H_3N_3O_7$ | — | 59.64 |

The filtrate obtained by filtering the precipitate formed by the addition of mercurous chloride was treated with $H_2S$ gas to remove the mercury. The free base solution was then made by the usual method, neutralized with nitric acid, concentrated, and kept in a vacuum desiccator. No crystallization was observed, however. The substance was then dissolved in water and sodium picrate added, but no arginine picrate was obtained.

The filtrate obtained by filtering the brown precipitate resulting from the addition of silver nitrate and barium hydroxide, and used for the detection of histidine, was treated with hydrochloric and sulphuric acid to remove the excess of silver and baryta. The free base solution was concentrated at a low temperature and acidified with hydrochloric acid, kept in a vacuum desiccator; then a considerable amount of prismatic crystaline mass was obtained with some adhesive matter. By treating these substances with cold absolute alcohol, soluble and insoluble parts were separated. From the insoluble part betain was obtained and from the soluble part stachydrin, as shown below.

### (4) *Betain*

The chloride which does not dissolve in cold absolute alcohol was extracted with pure methyl alcohol. After evaporation some crystals were found. Recrystallization was carried out several times with methyl alcohol. Fourteen-one hundredths gram of colourless tabular crystals was obtained.

The crystal melts at 228°C., and forms with picric acid a yellowish prismatic crystal of picrate. This is decided to be betain picrate since it melts at 183°C. The content of picric acid was as follows.

| Sample | Picric acid | Picric acid |
|---|---|---|
| g. | g. | % |
| 0.180 | 0.1190 | 66.11 |
| 0.220 | 0.1455 | 66.16 |
| $C_5H_{11}NO_5 \cdot C_6H_3N_3O_7$ | — | 66.18 |

### (5) *Stachydrin*

To the fraction soluble in cold absolute alcohol, a saturated alcoholic solution of mercurous chloride was added. Precipitation occurred. After it was kept overnight at room temperature the precipitate was separated. It was filtered, concentrated after being acidified with hydrochloric acid, and kept in a vacuum desiccator. No crystal resulted, although a small quantity of adhesive material was recognized. These materials were therefore dissolved in alcohol from which the double salt of gold chloride was prepared; 0.012 gram of prismatic crystal was obtained.

This salt melts at 224°C. and by the estimation of its gold content it is recognized as the double salt of stachydrin gold chloride.

| Sample | Gold | Gold |
|---|---|---|
| g. | g. | % |
| 0.011 | 0.0045 | 40.82 |
| $C_7H_{14}NO_2 \cdot AuCl_4$ | — | 40.79 |

### (6) *Summary*

By the chemical analysis of *Rhizopus japonicus Vuillemin* cultivated with Raulin solution, the following compounds were isolated from 1 kg. dried fungous bodies: ammonium chloride, 0.15 gram; adenine picrate, 0.13 gram; hypoxanthine 0.076 gram; histidine picrate, 0.027 gram; betain picrate, 0.14 gram; stachydrin gold chloride, 0.012 gram.

## (C) Vitamins

### (1) *Vitamin A*

To determine the presence of vitamin A, the following experiments

were made:

### Methods and samples employed

As the subject for the experiments, healthy albino rats each weighing 30–60 grams, were employed. All these rats were separately bred in a cage of wire netting. The cages were always kept clean and much attention was paid to ventilation and sunlight. As the basal diet, Euler's vitamin A free diet[24] modified a little by the author, was used. The composition is as follows:

| | |
|---|---|
| Casein | 18 grams |
| Lard | 10 " |
| Starch | 60 " |
| McCollum's salts (185) | 5 " |
| Oryzanin | 5 " |
| Lemon juice | 2 " |
| Irradiated ergosterol | 0.002 mg per day. |

### Purification of casein

To the casein of pharmacopoeia, three times the amount of 95% alcohol were added, and boiled in a flask equipped with a reflux condenser at 88–95°C. for 10 hours every day for 5 successive days. After filtration, the casein was shaken with ether, dried in a thermostat of 80°C., and pulverized.

### Purification of starch

Starch bought in the open market was repeatedly washed with water and mixed with three times its volume of 95% alcohol, shaken for 10 hours, left overnight, and decanted. This procedure was repeated five times. The starch was then extracted with ether several times and dried in a thermostat at 80°C.

### Purification of lard

To the market lard, three times the volume of 95% alcohol was added, and heated in a flask equipped with a reflex condenser at 80–85°C. for 10 hours. When cooled, the upper liquid and floating sediments were rejected. These procedures were repeated several times until the solution became colourless. Keeping it in a thermostat of 80°C., the alcohol was completely evaporated.

## Vitamins

As vitamin B, oryzanin was used; as vitamin D, ergosterol isolated from *Aspergillus oryzae;* and as vitamin C, lemon juice.

Casein, starch, lard and salts were mixed together in the proportion shown above. The same volume of water was added to it, and boiled on a water-bath. Then, mixed with vitamins B, C and D the preparation was given to the animals in dumpling form. The weight of the animals was examined at the same hour every day. The feeding samples to be examined for their vitamin contents were given *per os.*

## Sample

The residual solution obtained by the removal of phosphatids and sterols from the ether soluble matters was used for the test of vitamin A.

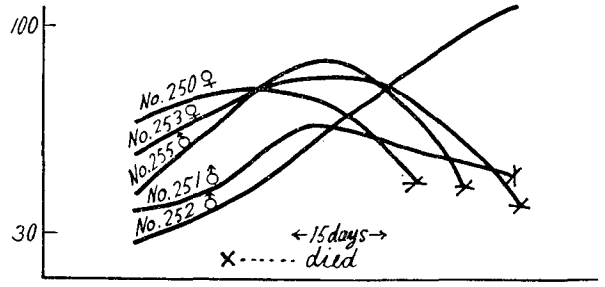


Fig. 1.
The results of feeding *Rhizopus japonicus* extract to rats

The results are shown in Fig. 1. While Albino rat No. 252, which was fed on the basal diet and carrots grew very healthily even after 2 months, two rats No. 250 and No. 255, which were fed on the basal diet and water, gained weight only at the beginning. After 3 weeks they ceased growing. Finally they began to lose weight every day, and both died before 65 days had elapsed. No. 253 was fed on the basal diet, water, and 0.1 cc. of the sample daily, which corresponds to 5 grams of the original dried fungous bodies. No. 251 was fed quite similarly, only the given quantity of the sample being different, 0.2 cc. every day, that is, corresponding to 10 grams of the original dried fungous bodies. These two rats showed quite the same results as rats Nos. 250 and 255, which were fed on the basal diet only. They died after 66 days.

From these results, it is to be concluded that *Rhizopus japonicus* cannot synthesize vitamin A.

### (2) *Vitamin B*

A part of the residual solution from which adenine had been separated, was employed as the material in examining for the presence of vitamin B.

Nielsen[9] has reported recently that *Rhizopus suinus* synthesizes a substance "Rhizopin," which promotes the growth and the fermentative activities of yeast. Therefore in *Rhizopus japonicus* also the same substance may be synthesized. To examine this point, the following experiments were carried out.

Effect of the sample upon the multiplication of yeasts.

Two species of yeasts, *Saccharomyces sake* and *Saccharomyces cerevisiae* were inoculated in the smallest possible quantities in the Raulin solution. In the same medium containing 1/100 cc. of the sample per 10 cc., these were incubated in a thermostat at 33°C. for 24 hours. The number of the cells in the solutions was counted every 3 hours as graphically shown in Fig. 2. While the number of the cells in 1 cc. of Raulin's solution inoculated with *Saccharomyces cerevisiae* and *S. sake* was 4,000,000 and 18,000,000 respectively, at the end of 24 hours in the medium with the tested sample there were 76,000,000 of the former and 114,000,000 of the latter. Both of these yeast species grew far more vigorously in Raulin's solution with the tested sample.

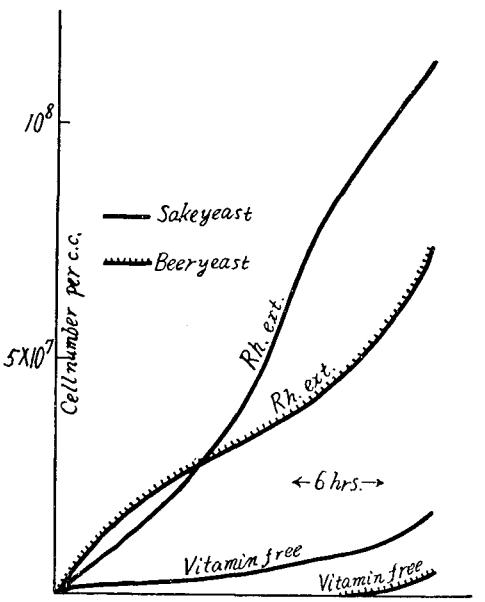


Fig. 2.
The effect of *Rhizopus japonicus* extract on the growth of yeasts

From these results, one may conclude that there is some substance in the sample which vigorously promotes the growth of the yeast.

Effect of the sample upon the growth of rats

Vitamin B is divided into three fractions from its function; the growth promoting factor for yeast, the antineuritic, and the growth accelerant. These three, however, exist, in most cases combined; as the factor which promotes the growth of the yeast has been proved, the other two factors must certainly be contained in the sample. Therefore, for the presence of growth promoting factor in the tested sample healthy white rats weighing 30–50 grams were experimented with. For the antineuritic factor, five cocks of 1300–1500 grams were employed.

The basal diet employed for the rats was of the following composition, each of the complements having been purified as already stated;

| | |
|---|---|
| Casein | 18 grams |
| Lard | 10 " |
| Cod-liver oil | 2 " |
| Starch | 65 " |
| McCollum's salts (185) | 5 " |
| Lemon juice | 2 cc. per day |

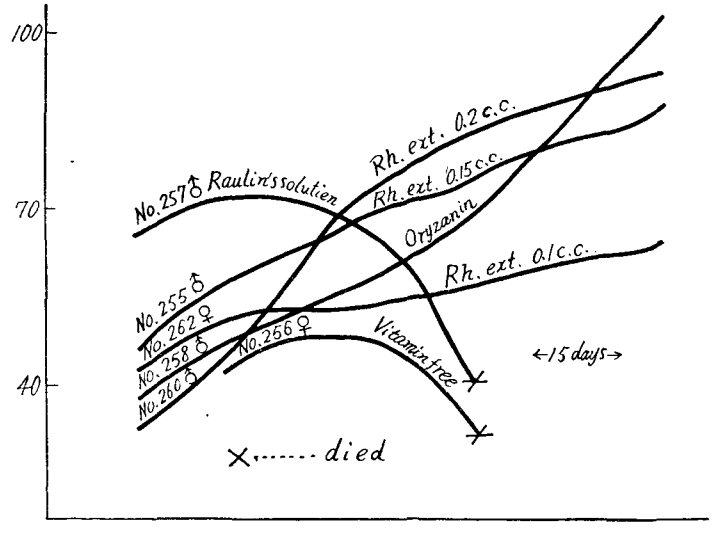


Fig. 3.
The effect of *Rhizopus japonicus* extract on the growth of young rats

The results are shown in Fig. 3. Animal No. 256, which was fed on the basal diet alone, did not grow at all and died after 43 days. No. 257, which was fed on the basal diet and 10 cc. of Raulin's solution, did not

grow well and died after 57 days, just as in the former case. But No's. 262, 255 and 260, which were raised with the basal diet and 0.1, 0.15 and 0.2 cc. of the sample for 3 months, grew excellently, even up to the end of 3 months. Compared with rat No. 258, which was fed on the basal diet plus 0.1 gram oryzanin, no great inferiority was observed.

Efficiency in curing neuritis in domestic fowls

The basal diet in the above experiment contains no vitamin B. Still, those rats which took the solution prepared from fungous bodies were able to grow well without suffering from any neuritic disease. This fact shows that the solution contains two factors of vitamin B, the growth promoting and the antineuritic. To ascertain the presence of the antineuritic factor and to determine its content, the following experiment was made with some cocks.

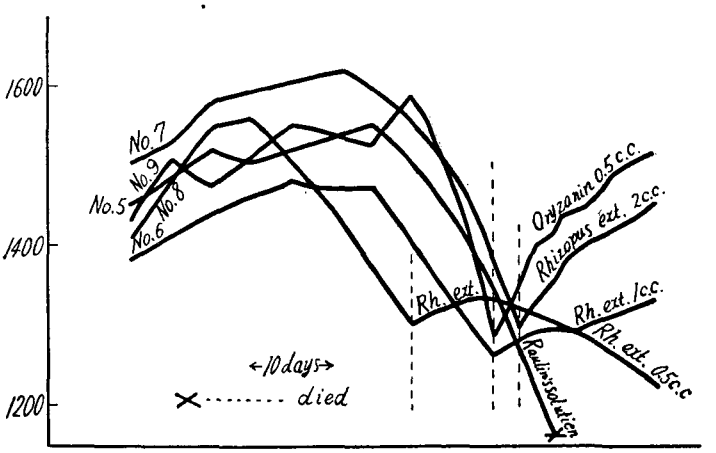


Fig. 4.
*The curative effect of Rhizopus japonicus extract upon* polyneutritis of Cocks.

Five cocks, No's. 5–9, weighing 1300–1500 grams, were fed with polished rice and water. To one of these five, No. 5, 10 cc. of Raulin's solution was compulsorily given daily. In seven weeks all these five showed typical neuritic symptoms. Now to cock No. 5, the Raulin soltion given was doubled, but without any effect whatever. It died after 8 days. But Nos. 6 and 7, to which 1 or 2 cc. of the sample were given daily, increased their weight even from the next day, and began to walk almost normally. Day after day their weight increased rapidly,

and they walked about quite freely. No. 8, however, which took only 0.5 cc. of the sample was not able to recover its health completely and after showing a slight increase of weight for one week, it began to decrease in weight day by day. Compared with No. 9, which took daily 0.5 cc. of oryzanin, No. 7 to which 2 cc. of the sample was given, showed the same effect. Clearly, in the tested solution there exists an antineuritic vitamin about one fourth of that of oryzanin.

### (3) *Vitamin C*

Guinea pigs were fed daily on the following basal diet: 10–15 grams of oats, and the same quantity of wheat bran and 40–50 grams milk autoclaved for one hour at 120°C.

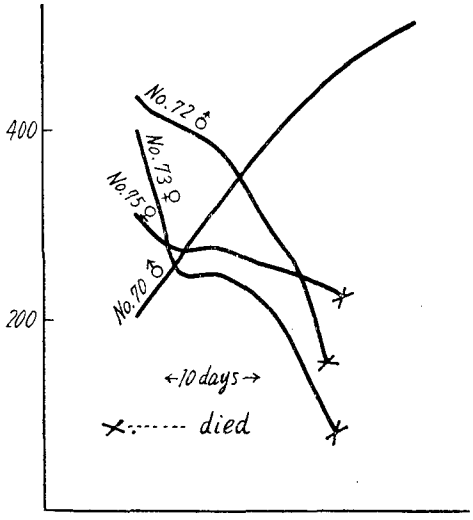


Fig. 5.
The results of feeding *Rhizopus japonicus* extract to guinea pigs.

Guinea pigs, thus nourished will suffer from scurvy in two weeks and generally die in three weeks, as already reported[25]. The substance to be tested for the existence of vitamin C was therefore added to the basal diet from the beginning of the experiment. As this material 95% alcohol extract of the fungous bodies was used.

Of four normal healthy guinea pigs, No. 72, which was fed on the basal diet only, and No's. 73 and 75 to the diet of which 2 cc. or 5 cc. of the sample were given daily, decreased in body weight every day and died in 3 weeks just as did No. 72, which had been raised on the basal diet alone, all showing the symptoms of scurvy.

From this experiment it is proved that vitamin C is not present in the fungous body.

### (4) *Discussion*

From the above experiments, it is evident that vitamins A and C are not present in the fungous bodies, but that vitamin B is. It stimulates the growth of yeast, accelerates the growth of the animal,

and acts as an antineuritic agent.

Now the fungous bodies were obtained from the *Rhizopus japonicus*, in the vitamin-free Raulin's solution. Attention must be called to the fact that 20 generations had been successively cultured in the vitamin-free Raulin's solution. *Rhizopus japonicus* must therefore have the power of synthesizing vitamin B in Raulin's solution. It cannot, however, synthesize either vitamin A or C.

### (5) *Summary*

*Rhizopus japonicus Vuillemin* synthesizes in Raulin's solution neither vitamin A nor C. Vitamin B, however, can be synthesized very well.

## (D) *Conclusion*

One kg. dried fungous bodies of *Rhizopus japonicus Vuillemin* cultivated with Raulin's solution was extracted after the removal of all ether soluble matter with 95% alcohol. From this alcohol extract the following substances were isolated:

| | |
|---|---|
| Mannit | 16.3 grams. |
| Sucrose | 8.6 " |
| Trehalose | 0.26 " |
| Mannose | 14.03% |
| Fructose | 1.54 " |
| Glucose | an appreciable amount. |
| Ammonium chloride | 0.15 grams. |
| Adenine (picrate) | 0.13 " |
| Hypoxanthine | 0.076 " |
| Histidine (picrate) | 0.027 " |
| Betain (hydrochloride) | 0.14 " |
| Stachydrin (gold chloride) | 0.012 " |

Vitamin A in the residual solution was not obtained after separating phosphatids and sterols from ether extract. Vitamin B was found in sufficient quantity in the residual solution after filtering off the adenine. Vitamin C was not found in the extract by 95% alcohol of fungous bodies formerly treated with ether.

# CHAPTER IV
# PROTEINS

## (1) Determination of soluble proteins in water, sodium chloride, alcohol and alkali solution

To examine the distribution of nitrogen, ten grams of the dry fungous body were extracted successively with 1) water, 2) 10% sodium chloride solution, 3) 70% alcohol, 4) 0.2% NaOH solution. The nitrogen in each fraction was determined by the Kjeldahl method.

Table VII
The distribution of nitrogen

| Fraction | % in the sample | % in dry matter | % of total nitrogen |
|---|---|---|---|
| Water soluble nitrogen ..... | 1.447 | 1.560 | 27.984 |
| 10% NaCl soluble nitrogen ..... | 0.544 | 0.575 | 10.307 |
| 70% alcohol soluble nitrogen .... | 0.058 | 0.061 | 1.098 |
| 0.2% NaOH soluble nitrogen .... | 0.915 | 0.967 | 17.336 |
| Residue ..... | 2.284 | 2.413 | 43.274 |

As shown by the above table, the amount of nitrogenous compounds soluble in 70% alcohol is extremely small compared with the others. The amount is increased in the order of 10% NaCl solution soluble, 0.2% NaOH solution soluble and the water soluble.

### (a) *Nitrogenous compound soluble in water*

The amount of nitrogen soluble in water is the largest. To study its chemical nature, it was examined with the water extract, with the following results.

1. Acetic acid does not produce a precipitate.
2. Neutralized with NaOH solution and heated there is no coagulation whatever.
3. One percent $CuSO_4$ solution does not produce a precipitate.
4. Trichloracetic acid does not produce a precipitate.
5. Tungstic acid produces a precipitate, the nitrogen content of which is only 0.552%.
6. Nessler's reagent gives a brownish precipitate.

These results show that there is no albumin in the water extract, and the nitrogen in it is all inorganic. It must have come to the fungous bodies from the culture solution.

### (b) *Ten percent NaCl solution soluble protein*

Of the total nitrogen 10.307% is contained in the fraction extracted

with 10% NaCl solution; this may be globulin. But, as shown in the next table, dilution with distilled water causes no precipitation at all, a fact which shows that either there is no globulin whatever or that it is in very small quantity.

TABLE VIII

The degree of turbidity of sodium chloride solution of protein for different quantities of water

| 10% NaCl solution extract (cc.) | Water (cc.) | Turbidity |
|---|---|---|
| 1 | 1 | — |
| 1 | 2 | — |
| 1 | 4 | — |
| 1 | 6 | — |
| 1 | 8 | — |
| 1 | 10 | — |
| 1 | 12 | — |
| 1 | 16 | — |
| 1 | 20 | — |
| 1 | 24 | — |
| 1 | 28 | — |
| 1 | 30 | — |

Now, to ascertain whether a small quantity of glubulin existed, a similar extract of 1 kilogram of the sample was dialysed in a bladder membrane for two weeks. A precipitate appeared, which showed all the colour reactions of protein and contained 15.68% nitrogen of dried matter. This substance may be considered as a globulin. Hence a small quantity of globulin seems to exist in the sample.

### (c) *Seventy percent alcohol soluble protein*

As described above, the nitrogen contained in the fraction extracted with 70% alcohol was very small in quantity and comprised only 1.098% of the total nitrogen. Further studies were consequently not made.

### (d) *NaOH solution soluble protein*

The nitrogen content in the extract with 0.2% NaOH solution is very high. To determine the best concentration of NaOH for extracting nitrogenous compounds from the fungous bodies and to get the largest amount of precipitate from it with acetic acid, the following experiments were tried. In four bottles containing 20 cc. of 0.1, 0.15, 0.2 and 0.25% NaOH solution respectively 2 grams of the fungous bodies were shaken for an hour with a shaking machine. The nitrogen content in each

extract was determined, and then the remainder was acidified with acetic acid. The resulting precipitates were filtered, dried and weighed. The nitrogen contents of these filtrates were also determined.

The results are shown in the following tables.

TABLE IX

Solubility of the sample in NaOH solution of different concentrations

| | % in air dried matter | % in anhydrous matter | % in total nitrogen |
|---|---|---|---|
| 0.1% NaOH soluble nitrogen | 2.661 | 2.809 | 50.417 |
| 0.15% " " " | 2.697 | 2.849 | 51.099 |
| 0.2% " " " | 2.742 | 2.897 | 51.951 |
| 0.25% " " " | 2.585 | 2.731 | 50.872 |

TABLE X.

Nitrogen precipitated by acetic acid of the sample in NaOH solution of different concentrations.

| | % of nitrogen precipitated by acetic acid | % of total nitrogen | Precipitate g. |
|---|---|---|---|
| 0.1% NaOH solution | 1.206 | 22.849 | 0.080 |
| 0.15% " " | 1.613 | 30.561 | 0.082 |
| 0.2% " " | 1.983 | 37.609 | 0.110 |
| 0.25% " " | 1.899 | 35.999 | 0.933 |

From the above results, it can be seen that 0.2% NaOH solution extracts the nitrogenous compound best and gives larger quantities of precipitate by acetic acid. The nitrogen content in this precipitate, moreover, is highest.

### (2) SEPARATION AND PURIFICATION OF THE PROTEIN

To 750 grams of the residue obtained after extracting all ether and alcohol soluble matters, 2250 cc. of distilled water were added and shaken vigorously. The mixture was left overnight at room temperature. The upper liquid part was decanted off and the residue filtered. With this residue the same treatment was repeated twice.

To the residue thus obtained 2250 cc. 10% NaCl solution were added and shaken well. The mixture was left overnight at room temperature. The upper liquid was decanted, and the same treatment was repeated. The residue was then thoroughly washed with water until it became quite free from chlorine. Two tenths percent NaOH solution was added until the whole was made up to 3000 cc. The mixture was shaken well and left overnight at room temperature. It was decanted and filtered. With this residue the same treatment was repeated three

times. All these NaOH solutions were collected and filtered several times to make quite clear. To this clear filtrate dilute acetic acid was added. The protein was precipitated as a brownish amorphous mass. It was washed with water, alcohol and ether, dried in a sulphuric acid desiccator at diminished pressure, and when dried, well crushed to a fine powder. Twenty nine and seven tenths grams of brownish powder were obtained.

### (3) Chemical properties of the protein

The brownish powder thus prepared is insoluble in water, 10% NaCl solution and alcohol, while it dissolves very easily in alkaline solution, yielding a quite brown solution which may be precipitated with acids.

This substance shows Biuret, Xanthoprotein, Millon's and Molisch's reactions, but is negative for lead sulphite reaction. It is certainly, therefore, a kind of protein.

The water and ash contents of this protein matter were determined by the ordinary method, the nitrogen content by the usual micro-method, the sulphur by Denis-Benedict's, and the phosphorus by Neumann's micro method. The results obtained were as follows:

Table XI
Composition of the protein

| | Water | Ash | Nitrogen | Phosphorus | Sulphur |
|---|---|---|---|---|---|
| % in air dry | 10.714 | 0.187 | 13.592 | 1.148 | 0.192 |
| % in water and ash free subst. | — | — | 15.255 | 1.288 | 0.215 |

### (4) Determination of amino acids

As the nutritive value of proteins is influenced by the kinds and the amounts of amino acids contained therein, the quantities of various amino acids contained in this protein must be determined. Van Slyke's method[31] was employed for this purpose but Okuda's method[32] was used for cystine estimation.

The sample was boiled for 12 hours on a sand-bath under a reflux condenser with 20 times the amount of 20% HCl. Hydrolysis completed, almost all HCl in the solution was driven off under diminished pressure at 40°C. or lower. The residue was neutralized and made slightly alkaline by the addition of 10% calcium hydroxide solution. The ammonia nitrogen liberated was distilled into a standard sulphuric acid under diminished pressure at below 40°C. The remaining fluid

was then filtered and separated from melanine nitrogen. The melanine nitrogen was determined by Kjeldahl's method. The filtrate was then acidified with HCl and evaporated under negative pressure at 40°C. or below. To the concentrated filtrate 18 cc. of concentrated hydrochloric acid and 15 grams of phosphotungstic acid were added and the diamino acids were precipitated. After 48 hours undisturbed standing, the precipitate was filtered by suction and treated with a mixture of ether and amylalcohol according to Van Slyke's modified method. The results obtained were as follows:

TABLE XII
The kinds of nitrogen found in protein

| | | |
|---|---|---|
| Total nitrogen ..... | 15.255% | 100.00 % |
| Amide nitrogen ..... | 2.524" | 16.545" |
| Humin nitrogen ..... | 0.623" | 4.084" |
| Diamino nitrogen ..... | 3.965" | 25.991" |
| Arginine nitrogen ..... | 2.375" | 15.568" |
| Histidine nitrogen ..... | 1.114" | 7.302" |
| Lysine nitrogen ..... | 0.471" | 3.088" |
| Cystine nitrogen ..... | 0.005" | 0.033" |
| Monoamino nitrogen ..... | 7.498" | 49.092" |
| Free amino nitrogen ..... | 7.072" | 46.359" |
| Non-amino nitrogen ..... | 0.426" | 2.733" |
| Loss nitrogen ..... | 0.645" | 4.288" |

In the last column are shown the values calculated when the total nitrogen is taken as 100.

### (5) DETERMINATION OF TYROSINE AND TRYPTOPHANE

For the determination of tyrosine and tryptophane contained in the protein, Folin and Loony's method[33] was employed. One gram of the protein was hydrolyzed with 3.5 grams of barium hydroxide and 25 cc. of distilled water under a reflux condenser for 48 hours. To the solution 30 cc. of 20% $H_2SO_4$ solution were added and heated on the water-bath for an hour. The precipitate was filtered after cooling and was then diluted with distilled water until this volume reached 100 cc.

By means of a pipette, 2 cc. of the filtrate were measured into a 15 cc. centrifuging tube graduated at a volume of 10 cc. Two cc. of 10 per cent mercuric sulphate solution were added, and the mixture was at once diluted with 5% sulphuric acid to the 10 cc. mark. A rubber stopper was inserted, and the solution was shaken vigorously to ensure thorough mixing. The tube was allowed to stand for two hours and was

then centrifuged.

The clear supernatant liquid containing tyrosine was poured into a clean dry test tube and set aside for tyrosine determination. The centrifuging tube containing the mercuric tryptophane sediment was then filled to the 10 cc. mark with 5% sulphuric acid, its own rubber stopper was again inserted, and the mixture shaken. After removing the stopper, the liquid in the container was drained for half a minute.

Tyrosine.—Five cc. of the liquid containing tyrosine were transferred into a 100 cc. volumetric flask. Into another similar flask, 1 cc. of the standard sulphuric acid tyrosine solution containing 1 mg. was introduced. To the latter, 1 cc. of the acidic mercuric sulphate solution and 3 cc. of 5 per cent sulphuric acid solution was added. Then, to each flask about 30 cc. of water, 20 cc. of saturated sodium carbonate solution, and 4 cc. of 5 per cent sodium cyanide solution were added, in the order described. Two cc. of phenol reagent were added and the whole was mixed and stood for 30 minutes. Colour comparison was made in the usual manner. Setting the standard at 20 mm., the reading of the sample was 13.8 mm.

Tryptophane.—To the unknown mercury precipitate and to a similarly precipitated and centrifuged standard which contains 1 mg. tryptophane, 6 cc. of water were added, and shaken so as to secure a uniform suspension. Without any unnecessary delay, 4 cc. of 5 per cent sodium cyanide were added to each, and again shaken. A complete solution was at once obtained. The standard and the unknown solution substance were each rinsed into a 100 cc. volumetric flask, keeping approximately equal volumes. Twenty cc. of saturated sodium carbonate solution were added and, finally, shaking the while, 2 cc. of the phenol reagent were added, and stood for 30 minutes. It was then diluted to the volume and colour comparison was carried out, setting the standard at 20 mm. The reading was found to be 72.7 mm.

The amounts of tyrosine and tryptophane contained in the sample are accordingly as follows:

Tyrosine ..... 7.245% Tryptophane .... 1.435%

The above results show that although the amount of diamino nitrogen is smaller than that of monoamino, it occupies 25.991% of the total nitrogen in the isolated protein. It is also rich in arginine, and histidine, and contains suitable amounts of lysine and also a small quantity of cystine. Tyrosine and tryptophane are also contained in considerable amounts. These suggest that this protein has undoubtedly

a high nutritive value.

### (6) Ratio of cystine sulphur to the total sulphur

As already described, the total sulphur is 0.216 per cent and the cystine sulphur is 0.005 per cent: therefore, the ratio of cystine sulphur to the total sulphur is 1:18.

### (7) The formation of iodine-protein

As iodine combines with the tyrosine group of protein, each protein must show a different degree of combination with iodine, as shown by Blum and Strauss[37] and by Tadokoro and Nakamura[38]. The writer tried next the preparation of the iodine-protein with the isolated protein.

To 0.2 gram of the protein 150 cc. of 3.5% ammonium hydroxide solution were added. The mixture was then stirred well and stood overnight, 50 cc. of alcoholic 1/10 normal iodine solution were added and laid aside overnight. The clear yellowish solution was filtered by suction, the filtrate acidified with dilute sulphuric acid. A precipitate of iodine protein was formed. After a while, the upper solution was decanted off, the precipitate was suspended in water, dissolved by the addition of sodium hydroxide solution, and again precipitated with sulphuric acid. These treatments were repeated twice so as to remove the excess of iodine from the protein. The precipitate was then washed with water, using a centrifugal machine until no more ammonia was detected in the washing. It was then washed with alcohol, absolute alcohol and ether. The iodine-protein thus obtained was dried over sulphuric acid in a desiccator of diminished pressure. A fine, slightly yellowish powder was obtained.

By means of Baubigny and Chavanne's method[39], the iodine content of the powder was determined. Forty cc. conc. $H_2SO_4$, 0.1 g. powdered silver nitrate and potassium bichromate were taken into a 300 cc.-Erlenmeyer's flask, and shaken well. To the mixture was added the iodine-protein, and the whole was heated to 150–170°C. until no more oxygen was evolved. After cooling, 150 cc. of water and 10 cc. of alcohol were added, and by reduction with a saturated solution of sodium sulphite, silver iodide precipitates were obtained. These were gathered, washed and weighed in a Gooch's crucible in the usual way.

| Sample | Silver iodine | Iodine | Iodine |
|---|---|---|---|
| g. | g. | mg. | % |
| 0.113 | 0.034 | 18.369 | 16.167 |

The iodine value of serum-albumin is 8.6% according to Blum[37] and that of an oryzenin isolated by Tadokoro[38] from glutinous rice is 11.764%. Comparing the present results with these values protein shows a much higher value, 16.167%, coinciding with the fact that tyrosine is contained in the protein to as great a quantity as 7.245%.

## (8) Partial hydrolysis

The degree of condensation of proteins seems to be observed depending upon their partial hydrolysis.

Two grams of the sample were hydrolyzed on a water bath in a 100 cc. flask with 20 cc. of hydrochloric acid of different concentrations, for varying periods of from 5 to 25 hours. At the end of the required period, the contents in these flasks were filtered, and the filtrates made up to a definite volume. Ammonia nitrogen, free amino nitrogen and the substance which is precipitated by sodium tungstate were determined in the usual way. The results were as follows:

Table XIII
Results of partial hydrolysis

| Concentration | Ammonia nitrogen | | | | |
|---|---|---|---|---|---|
| | Time (hrs.) | | | | |
| | 5 | 10 | 15 | 20 | 25 |
| 5% | 0.069 | 0.716 | 0.341 | 0.915 | 1.255 |
| 10% | 0.075 | 0.717 | 0.759 | 1.120 | 1.388 |
| 20% | 0.085 | 0.963 | 1.156 | 1.393 | 1.403 |

| Concentration | Free amino nitrogen | | | | |
|---|---|---|---|---|---|
| | Time (hrs.) | | | | |
| | 5 | 10 | 15 | 20 | 25 |
| 5% | 0.028 | 0.059 | 0.065 | 0.075 | 0.102 |
| 10% | 0.081 | 0.120 | 0.137 | 0.149 | 0.162 |
| 20% | 0.114 | 0.125 | 0.165 | 0.175 | 0.223 |

| Concentration | Nitrogen precipitated by sodium tungstate | | | | |
|---|---|---|---|---|---|
| | Time (hrs.) | | | | |
| | 5 | 10 | 15 | 20 | 25 |
| 5% | 3.159 | 2.984 | 2.591 | 2.550 | 3.928 |
| 10% | 2.409 | 2.353 | 2.599 | 1.984 | 1.579 |
| 20% | 1.646 | 1.822 | 3.793 | 1.700 | 1.241 |

If the total nitrogen is taken as 100, the results are as follows;

TABLE XIV
Results of partial hydrolysis

| Concentration | Ammonia nitrogen | | | | |
|---|---|---|---|---|---|
| | Time (hrs.) | | | | |
| | 5 | 10 | 15 | 20 | 25 |
| 5% | 1.307 | 13.566 | 14.040 | 17.336 | 23.779 |
| 10% | 1.427 | 13.567 | 14.380 | 21.220 | 26.297 |
| 20% | 1.610 | 18.246 | 21.921 | 26.395 | 26.581 |

| Concentration | Free amino nitrogen | | | | |
|---|---|---|---|---|---|
| | Time (hrs.) | | | | |
| | 5 | 10 | 15 | 20 | 25 |
| 5% | 0.531 | 1.118 | 1.220 | 1.421 | 1.952 |
| 10% | 1.530 | 2.274 | 2.596 | 2.823 | 3.067 |
| 20% | 2.160 | 2.372 | 3.126 | 3.316 | 4.225 |

| Concentration | Nitrogen precipitated by sodium tungstate | | | | |
|---|---|---|---|---|---|
| | Time (hrs.) | | | | |
| | 5 | 10 | 15 | 20 | 25 |
| 5% | 59.663 | 56.343 | 49.091 | 48.314 | 74.422 |
| 10% | 45.623 | 44.579 | 48.485 | 37.588 | 29.917 |
| 20% | 31.185 | 34.519 | 71.864 | 32.209 | 23.511 |

From the above results it can be seen that the protein is condensed just as ordinary proteins.

### (9) ISOELECTRIC POINT OF THE ISOLATED PROTEIN

Pauli and Matula[34], Loeb[35] and Michaelis[36] showed that all physical properties of proteins are maximum or minimum at their isoelectric point: minimum for osmotic pressure, viscosity, amount of alcohol required for their precipitation, electric conductivity and swelling, and maximum for turbidity and surface tension. The isoelectric point of the protein may consequently be easily determined. For the determination of the isoelectric point of the isolated protein surface tension, viscosity and turbidity were measured by the ordinary method. The results of this investigation were as follows:

TABLE XV
The degree of surface tension, turbidity and viscosity of alkali solution of protein for different quantities of HCl

| pH value | $N/100$ HCl(cc.) | Surface tension (degree) | Turbidity | Viscosity (minutes) |
|---|---|---|---|---|
| — | 1.0 | 74.0 | — | 0.49 |
| — | 1.5 | 72.5 | — | — |
| — | 2.0 | 70.0 | — | 0.56 |

| | | | | |
|---|---|---|---|---|
| — | 2.5 | 67.5 | − | — |
| — | 3.0 | 65.5 | − | 1.17 |
| — | 3.5 | 63.0 | − | — |
| — | 4.0 | 64.0 | + | 1.46 |
| — | 4.5 | 66.0 | ++ | — |
| — | 5.0 | 67.0 | +++ | 0.52 |
| — | 5.1 | 67.2 | ++,++ | — |
| — | 5.2 | 67.5 | +++++ | 0.50 |
| — | 5.3 | 67.8 | ++++++ | — |
| 3.02 | 5.4 | 68.0 | ++++++++++ | 0.48 |
| 2.95 | 5.5 | 68.0 | +++++++++ | 0.45 |
| — | 5.6 | 67.8 | +++++++++ | 0.49 |
| — | 5.8 | 67.4 | +++++++++ | — |
| — | 6.0 | 67.0 | +++++++++ | 0.59 |

The data show that the isoelectric point of the protein lies between pH 2.95–3.02.

### (10) On the protein

It must now be determined to what class of protein this substance belongs.

Very carefully prepared and purified substance was still very brown. It was therefore supposed to be one of the chromoproteids. It was examined by Kylin's method[26]. Two grams of it were hydrolyzed in 20 cc. of 0.5% HCl for an hour at 60°C., and vigorously shaken with ether; but the ether remained colourless. When treated with alkali and pepsin, and shaken with ether and amylalcohol, the solvents also remained quite colourless. From these results it seems that this protein is not a chromoproteid.

Molisch's reaction is strongly positive,—which indicates the existence of carbohydrate. But when vegetable proteins are isolated, carbohydrate is almost inevitably admixed. Therefore this carbohydrate may be seen as only some impurity admixed. It may be thought to be due, however, to the carbohydrate radical existing in the protein molecule itself as glucoproteid.

In general, glucoproteid is easily decomposed and produces carbohydrates; therefore, if hydrolyzed by weak mineral acids, Fehling's solution can be reduced. Such reducing sugars are glucosamine, glucose, etc., and they exist sometimes directly combined with protein, but mostly as one component of some compounds which combine with protein molecules. Anyway, in the decomposition products of glucoproteid or related substances, glucosamine will always be found and

detected by Oswald's method[27], as follows.

Five grams of the protein were hydrolyzed in 150 cc. of 3% hydrochloric acid for five hours. The material was cooled and filtered. The filtrate was concentrated under negative pressure at low temperature, decolorized with animal charcoal, phosphotungstic acid solution was added, and filtered after a day. The filtrate was washed several times with a mixed solution of equal parts of amylalcohol and ether, and concentrated at 40°C. under negative pressure into a syrupy state. Some tiny crystals of glucosamine hydrochloride were inoculated in it and kept in a vacuum desiccator for several weeks. But no crystallization of glucosamine was observed. Hence this protein does not seem to belong to the glucoproteids.

The phosphorus content of this protein matter is too high for that of an ordinary protein. Hence the substance is perhaps a phosphoprotein or nucleoproteid. To determine this, the content of purine nitrogen was estimated by the precipitation method according to Krüger and Wulff[28] with copper sulphate and sodium bisulphite. One hundred cc. of 0.5% solution of this protein were boiled, 10 cc. of 50% sodium bisulphite solution and 10 cc. of 13% copper sulphate solution were added, and heated. Five cc. of 10% barium chloride solution were poured in, and left for two hours. The precipitate was combusted together with filter paper by Gunning's method, and titrated with *N*/100 oxalic acid solution. Four and five-tenths cc. of *N*/100 oxalic acid were consumed. As 1 cc. of the oxalic acid equals 0.00014 g. N, 0.5 g. of this protein contains 0.63 mg. purine base nitrogen, this is, 1.26 mg. purine base nitrogen is contained in 1 gram of the protein, or the content of the nucleic acid nitrogen in this protein is only 1.26%.

As many investigators,[29] [30] insist that this method leads generally to a rather higher value and some quantities of purine base nitrogen are always found even in nucleic acid free proteins, this protein probably contains almost no nucleic acid, and is therefore a kind of phosphoprotein.

## Elementary composition of the studied protein

Elementary analysis shows the following results:

| Sample g. | Carbon dioxide g. | Water g. | Carbon % | Hydrogen % |
|---|---|---|---|---|
| 0.0772 | 0.1506 | 0.0611 | 53.203 | 8.769 |
| 0.0851 | 0.1662 | 0.0669 | 53.263 | 8.735 |
| Average ..... | | | 53.233 | 8.752 |

Therefore the elementary composition of the protein, is:

| Nitrogen | Hydrogen | Carbon | Sulphur | Phosphorus | Oxygen |
|---|---|---|---|---|---|
| % | % | % | % | % | % |
| 15.255 | 8.752 | 53.233 | 0.216 | 1.288 | 21.256 |

## (11) Discussion

As stated above, from 1 kg. of dried fungous bodies of *Rhizopus japonicus Vuillemin* cultivated in Raulin's solution, 29.7 grams of a certain kind of protein were isolated. This protein, which is nearly insoluble in water, sodium chloride solution and alcohol, dissolves easily in sodium hydroxide solution, is precipitated by acid, and was recognized as a kind of phosphoprotein.

The elementary composition of this protein is: nitrogen, 15.25%; hydrogen, 8.752%; carbon, 53.233%; sulphur, 0.211%; phosphorus, 1.288%; oxygen, 21.256%. It contains tyrosine, 7.245%; tryptophane, 1.435%; arginine, 2.375%; histidine, 1.114%; lysine, 0.471% and cystine, 0.005%. From the results of partial hydrolysis, the substance seems to be condensed to the degree of ordinary proteins. The ratio of cystine sulphur to total sulphur is 1:18; iodine value, 16.167%. The isoelectric point lies between pH 2.95–3.02.

Isolation of proteins from *Rhizopus*-species, is very rare. Thomas[40] separated phosphoprotein from *Aspergillus niger*, and Takada[41] recently obtained a phosphoglucoproteid from *Aspergillus oryzae*. Compared with these, the protein isolated by the author shows the following properties.

Table XVI

Comparison of characters of fungus protein by different authors

| | Author (*Rh. japonicus*) | Takada (*Asp. oryzae*) | Thomas (*Asp. niger*) |
|---|---|---|---|
| Solubility in water | Insoluble | Insoluble | Insoluble |
| Solubility in NaCl solution | Almost insoluble | Slightly sol. | Partly sol. |
| Colour reaction for protein | All positive except PbS reaction | " | " |
| Carbohydrates | No. glucosamine | Glucosamine present | glucosamine present |
| Nitrogen content | 15.255% | 14.78% | 12.9% |
| Phosphorus content | 1.288% | 1.77% | 0.7% |
| Sulphur content | 0.216% | Trace | 0.5% |
| Humin nitrogen | 4.084% | 3.01% | 4.0% |
| Basic nitrogen | 25.991% | 29.11% | 15.6% |
| Monoamino nitrogen | 45.092% | 54.21% | 73.1% |
| Total Sulphur : Cystine Sulphur | 1 : 18 | — | — |
| Iodine-value | 16.167 | — | — |
| Isoelectric point | 2.95–3.02 | — | — |

These three sets of figures resemble each other in respect of solubility, colour reactions and amino acid content, but the protein isolated by the author contains no glucosamin and is fundamentally different in regard to its elementary composition, iodine value, ratio of cystine sulphur to total sulphur, and isoelectric point. And as no report has been previously made on the isolation of this kind of protein from the *Rhizopus* species, the author has named this protein "Rhizopenin."

### (12) Summary

From 1 kg. of dried fungous bodies of *Rhizopus japonicus Vuillemin* cultured in Raulin's solution, 29.7 grams of protein were isolated.

This protein is nearly insoluble in water, NaCl solution and alcohol, while soluble easily in NaOH solution and is precipitated by acid. It is a kind of phosphoprotein, and it is proposed by the author to name it "Rhizopenin."

## CHAPTER V

## DIGESTIVE EXAMINATION

As described, *Rhizopus japonicus* in Raulin's solution can nutritively synthesize valuable proteins, lipid, carbohydrates and vitamins in a very short time. In order to determine the digestibility of these components, the following digestive examination was carried out. There is a large number of methods for the examination of digestibility, but recently Bergeim[42] discovered a comparatively simple method: when a diet containing ferric oxide is given to experimental animals, the ferric oxide, without being absorbed in the intestinal canals, is excreted quantitatively in the faeces; therefore if the ratio of ferric oxide to the tested components in both diet and faeces is determined, the digestive coefficient of the sample being tested is to be calculated by the following equation:

$$\left(\frac{\%\text{ of tested component in diet}}{\%\text{ of ferric oxide in diet}} - \frac{\%\text{ of tested component in diet}}{\%\text{ of ferric oxide in faeces}}\right) \times \frac{\%\text{ of ferric oxide in diet}}{\%\text{ of tested component in faeces}} \times 100.$$

By this method the digestive coefficient of the components contained in *Rhizopus japonicus* was determined.

White rats were fed with diets No. 1 (15 g. casein, 65 g. starch, 10 g. lard, 2 g. cod-liver oil, 3 g. oryzanin, 5 g. McCollum's salts, 0.5 g.

ferric oxide) and No. 2 (100 g. *Rhizopus japonicus,* 0.5 g. ferric oxide), respectively, for a week. The components of the diet and the faeces were the determined, the results being as follows:

TABLE XVII

The composition of diets and faeces

| | Diet No. 1. | Faeces No. 1. | Diet No. 2. | Faeces No. 2. |
|---|---|---|---|---|
| | % | % | % | % |
| Water | 8.65 | 5.22 | 5.34 | 8.35 |
| Nitrogen | 2.84 | 3.51 | 5.28 | 5.62 |
| Carbohydrates | 57.22 | 53.20 | 27.50 | 22.32 |
| Ashes ($Fe_2O_3$ not included) | 3.18 | 8.58 | 4.53 | 5.22 |
| $Fe_2O_3$ | 0.25 | 3.43 | 0.32 | 1.21 |

The digestive coefficients of the tested components in diets Nos. 1 and 2, calculated by Bergeim's formula, are:

TABLE XVIII

Digestibility of the fungus bodies

| | Solid matters | Nitrogenous matters | Carbohydrates | Ash |
|---|---|---|---|---|
| Diet No. 1. | 92.4 | 91.5 | 93.2 | 80.2 |
| Diet No. 2. | 74.1 | 72.1 | 78.6 | 74.2 |

Thus the digestive coefficients of the components contained in *Rhizopus japonicus* are, compared with those of the synthetic diet, rather lower, but all components are digested and absorbed to a considerable extent.

## CONCLUSION

After 20 generations of culture in order to eliminate the effect caused by culture, the following substances were isolated from 1 kg. dried fungous bodies of *Rhizopus japonicus* cultivated for 5–7 days in Raulin's solution, which contains no organic substance other than sucrose and tartaric acid.

| | |
|---|---|
| Ergosterol | 1.08 g. |
| Fungisterol | 2.55 g. |
| Palmitic acid | 7.60 g. |
| Stearic acid | 1.16 g. |
| Phosphatids | 1.19 g. |
| Mannose | 14.03% |
| Mannit | 16.30 g. |
| Trehalose | 0.26 g. |
| Fructose | 1.54% |

| | |
|---|---|
| Glucose | an appreciable amount. |
| Ammonium chloride | 0.15 g. |
| Hypoxanthine | 0.076 g. |
| Betainhydrochloride | 0.14 g. |
| Adenine picrate | 0.13 g. |
| Histidine picrate | 0.027 g. |
| Stachydrin gold chloride | 0.012 g. |
| Vitamin B | |
| 0.2% NaOH solution soluble Protein | 29.7 g. |

From the above data, it is known that important nutritive materials are synthesized by *Rhizopus japonicus* in Raulin's solution in such a short time, and from its digestive coefficient of 72.1 in nitrogenous substances, 78.6 in carbohydrates and 74.2 in ashes, that these components are digested quite well.

The protein is synthesized by *Rhizopus japonicus,* which is soluble easily in sodium hydroxide solution and is precipitated by acid. It is a kind of phosphoprotein with the following elementary composition: nitrogen, 15.255%; hydrogen, 8.752%; carbon, 53.233%; sulphur, 0.216%; phosphorus, 1.288% and oxygen, 21.256%. Rich in basic amino acids, it is a valuable nutrient; it contains also appreciable amounts of tyrosine and tryptophane. The ratio of cystine sulphur to the total sulphur is 1:18, the iodine value, 16.167%. Its isoelectric point lies between pH 2.95 and 3.02.

As described above, the protein differs completely from those isolated from *Asp. niger* by Thomas, and from *Asp. oryzae* by Takada, though they resemble each other in respect to solubility, colour reaction and amino acid contents. The chief difference consists in our proteins lack of glucosamin content, its elementary composition, its iodine value, the ratio between cystine sulphur and total sulphur, and its isoelectric point. Consequently, the author proposes to name the studied protein "Rhizopenin," as no report concerning this protein has hitherto been published.

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## BIBLIOGRAPHY


1) BOIDIN—Revue gener' des sciences purer et app., 689 (1901).
2) VUILLEMIN, P.—Revue mycologique, Bd. 24, Nr. 93 (1902).
3) CRAMER, E.—Arch. Hyg., 28, 16 (1897).
4) MARSCHALL—ibid.
5) NIKOLSK, KRUSE—Allgemeine Mikrobiologie. (1910).
6) MUNZ—Boussingant agron., 6, 216 (1876).
7) GERARD, E.—Compt. rend., 114, 1544 (1895).
8) WISSELINGH, C.—Pringsheim'sche Jahrbucher, 31, 619 (1898).
9) NIELSEN, N.—Zentralbl. Bakt. usw. II. Bd. 82, 70 (1930).
10) BERTRAND, G. et THOMAS, P.—Guide pour les Manipulations de Chimie Biologique, 437 (1919).
11) YAMAMOTO, Y.—J. Faculty of Agr., Hokkaido Imp. Univ., 28 (1930).
12) LEWKOWITSCH—Chem. Teck. & Anal. Oils, Fats & Waxes, 103 (1914).
13) TANRET, C.—Compt. rend., 147, 75 (1908).
14) ELLIS, M. T.—Biochem. J., 12, 173 (1918).
15) WINDAUS, A. und HESS, A. F.—Proc. Soc. Expt. Biol. Med., 24, 269 (1927).
16) ROSENHEIM, O. & WEBSTER, A.—Biochem. J., 21, 389 (1927).
17) STEENBOCK, H. and BLACK, A.—J. Am. Chem. Soc., 64, 263 (1925).
18) HALLER, A.—Compt. rend., 143, 657 (1906).
19) BAUBIGNY u. CHAVANNE—Compt. rend., 138, 85 (1904).
20) BROWN—Handbook of Sugar Analysis, 647 (1912).
21) NEUBERG, C.—Ber., 35, 959 (1902).
22) FISCHER, E.—Ber., 23, 805 (1890).
23) STAHEL—Ann., 258, 242 (1890).
24) EULER, B. u. HELLSTORM, H.—Biochem. Z., 203, 370 (1928).
25) TAKAHASHI, E. and LIM, H.—J. Agr. Chem. Soc. Japan, 5, 262 (1929).
26) KYLIN, H.—Zeits. physiol. Chem., 69, 169 (1911).
27) OSWALD, A.—ibid., 68, 173 (1910).
28) KRÜGER, M. u. WULFF, C.—ibid., 20, 176 (1895).
29) BURIAN, R. u. SCHUR, H.—ibid., 23, 55 (1897).
30) ALDERS, N.—Biochem. Zeits., 181, 400 (1927).
31) VAN SLYKE, A.—J. Biol. Chem., 10, 15 (1911–1912).
32) OKUDA, Y.—J. Chem. Soc. Japan, 45, 19 (1924).
33) FOLIN, T. and LOONY, J. M.—J. Biol. Chem., 51, 421 (1922).
34) PAULI u. MATULA—Kolloid Zeit., 12, 222 (1913).
35) LOEB—J. General Physiol., 1, 363 (1918–19).
36) MICHAELIS u. RONA—Biochem. Zeits., 28, 193 (1910).
37) BLUM, F. u. STRAUSS, E.—Zeits. physiol. Chem., 127, 199 (1923).
38) TADOKORO, T. and NAKAMURA, S.—J. Sapporo agr. fores. Soc., 15, 121 (1924).
39) BAUBIGNY u. CHAVANNE—Compt. rend., 136, 1198 (1903).
40) THOMAS, P. et MORAN, R. C.—ibid., 159, 125 (1914).
41) TAKADA, R.—J. Soc. Chem. Industry, Japan, 379, 831 (1929).
42) BERGEIM, O.—J. Biol. Chem., 70, 29 (1926).