167. *Toxicological Studies on the Yellowed Rice by
P. islandicum* Sopp. II

*Isolation of the Two Toxic Substances from the Noxious Fungus,
and Their Chemical and Biological Properties*

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We have previously reported that acute liver injuries and liver cirrhosis occur as noticeable toxicological effects in mice and rats when they are fed with *P. islandicum* growing rice as well as the fungus mat. This time we announce the experimental data regarding the new toxic substances among the fungus metabolites,¹⁻⁴⁾ which were followed up since 1954 by our team. Although the main current of research activities lay in chemistry, there were many issues to be solved from the standpoints of mycology, toxicology, and pathology. Therefore chemical fractionations from the fungus mat have been carried out in parallel with screenings and testings, mycological, phytochemical, phytopathological, toxicological, and pathological.

I. *Preliminary examination on the methanol extract from the fungus mat—Presumption on presence of two kinds of toxic components.* According to the fatality test on mice, subcutaneously injected with camellia oil dissolved methanol extracts of the fungus mat, *P. islandicum* Sopp, Ub, Uc, Ud, and Ue strains cultivated in Czapeck solution, the fatality proved to depend upon the doses, without any remarkable difference from each other in toxicity per group. As to the life-time, a trend was noticed that fatal cases occurred frequently in the period before 15 hours and after 24 hours of injection. Judging from these data on fatality and autopsy as well as those⁵⁾ in hand on oral administration, the fungus mat must contain some toxic substances acting fatally to mice or rats and causing specific injury to liver, and the active agents are presumed not to be one kind but two at least. This presumption was ascertained by the BSP test;⁶⁾ the functional damage in liver by the methanol extract of Ea strain fungus mat is proved to be superimposed with two different kinds. Furthermore, considering the fact that the pathological findings are markedly observed with the extract suspended in water before 24th hour and

with the extract dissolved in camellia oil after 24th hour, it can be supposed that the relatively prompt acting substance is hydrophilic, while the slow acting one is lipophilic. We adopted these presumptions as our working hypothesis for the subsequent extraction researches.

II. *Systematic extraction from the fungus mat.* To isolate purely the desired substances, we have established the whole process of extractions with a scale systematized as shown in the figure.

Methods. The direction of further fractionation was determined by the toxicity testing and histopathological examination each time one fraction was obtained; ruling out the non- or less-toxic fractions to pursue the desired components. And in case a fraction of high toxicity was obtained, measurement was made on LD_{50} , and compounds in the fraction were examined further.

Results. 1) The important data regarding chemical fractionations and animal testings during the whole extraction process can be summarized as shown in the table.

2) Toxic reaction of mice. a) Luteoskyrin. In general, life-time to death after subcutaneous injection is so long that there is few under 24 hours regardless of dose and it is measurable in unit of day; 2 or 3 days for the most mice after a single injection with around the lethal dose. Although there appear large variations within doses, the life-time is certain to be shortened as the dose is increased.

Acute symptoms. Almost normal appearance of the mice following the injection lasts usually fairly long time until 10-odd hours or 7-8 hours preceding death symptoms appear evidently; the mice become inactive with ruffled fur and then crouch longer and longer in the corner of the cage with eyes closed and eat hardly their feed. Then they make round their back and soon take a particular posture, i.e. bending their dorsal spine in kyphosis and falling down their loin, and walk sinistrally and tottering, as if the lower half of their body are paretic. The body temperature

Fraction	Compound	Chemical identification	LD ₅₀	Remarks
[2]	a) Ergosterol b) Palmitic acid	a) Lieberman-Burchard reaction, mp. 165°C UVλ max 262, 270, 281, 293 mμ b) Esterification bp ₃ 158-160°C, hydrazide mp. 108°C	Non fatal	Not related to toxic substance. Fatty metamorphosis of liver cells, diffuse, or centrolobular
[3]	a) Isl. b) Cat. c) Irido. d) Erythro. e) Fig. X (luteo.) f) Sky. g) Rubro.	Paper chromatography	LD ₅₀ /10 g subc. 8.8 mg (8th day) and 3.0mg (21st)	Slow acting component approved. Centrolobular necrosis, marked.
[4]	a) Luteo. b) Rubro. c) Erythro., trace	Paper chromatography	Fatal	All mice fatal 12-48 hrs. after 6, 2 and 1 mg/10 g subc. Major component in [3] is presumably approved. Centrolobular necrosis, marked.
[5]	a) Isl. b) Cat. c) Irido. d) Erythro.	Paper chromatography		Fatality test omitted since all pigments in [3] and [4] apparent, with a small quantity
[6]	a) Isl. b) Irido.	Paper chromatography	Non fatal	Survived with 3 mg/10 g subc.
[7]	Luteo. (Fig. X)	Paper chromatography mp. 278°C (decomp.) UVλ max 245, 275, 430 mμ IR 1623, 3378 cm ⁻¹	LD ₅₀ /10 g 1.47 mg subc., 2.21 mg oral	Principal toxic pigment isolated Centrolobular necrosis, marked.
[8]	a) Nitrogen cont. fraction b) d-Mannitol	a) Qualitative test N (+), S (-), P (-) b) Mp. 166°C (from ethanol) No depression on admixture with standard one	LD ₅₀ /10 g 3.9 mg subc.	All died 2-17 hrs. after 5.3 mg/10 g subc. Prompt acting hydrophilic component comes here. Hydropic degeneration of liver cells, peripheral, etc.*
[9]	Nitrogen cont. fraction	Qualitative test N (+), S (-), P (-)	Fatal	All died 3-15hrs. after 4mg and 3mg/10g subc. The same substance as in [8] transferred.
[10]	a) Nitrogen cont. fraction b) Organic acid, trace	a) Qualitative test N (+), S (-), P (-) b) Precipitates with basic lead carbonate	Fatal	All died 2-3 hrs. after 5.0-0.5mg/10 g subc. and some died 3-15 hrs. after 0.25 mg/10 g Hydropic degeneration of liver cells, peripheral, etc.*
[11]	Chlorine cont. peptide	Pauli (+), Burett (+) mp. 245°C; mp. 251-252°C (recryst. from methanol), qualitative test Cl (+) UVλ 257 mμ IR 3400, 1670, 1640 cm ⁻¹ , etc.	LD ₅₀ /10 g 4.75 γ subc., 3.38 γ i. v. 65.5 γ oral	Prompt acting hydrophilic substance obtained in crystal. Peripheral necrosis, marked, and hydropic degeneration of liver cells, etc.*
Note:	Isl. Islandicin Cat. Catenarin Irid. Iridoskyrin Erythro. ... Erythroskyrine	Luteo. Luteoskyrin Sky. Skyrin Rubro. Rubroskyrin	* Hydropic degeneration with hyalin droplets in liver cells, peripheral necrosis of liver cells, bleeding in liver cell plates and dilatation of sinusoids	

gradually falls down and the muscles and skins are deprived of normal tension as time passes. Respiration is more disturbed. Most of mice have a slight agony, but not any convulsion. In a small number of the mice is noticed some jaundice-like colour on their auricles. The final symptoms give us the impression of the hepatic coma of human being.

b) Chlorine containing peptide. Life-time after subcutaneous injection is so short that it is measurable in unit of hour; 2 or 3 hours for the most of mice and over 24 hours for a few of them.

Acute symptoms. After a transient auricular congestion, mice begin to crouch in the corner of the cage respirating more frequently. Their posture gradually comes down, lying down flat with their mouth on the floor; walking and moving with a poor coordination and their auricles turn pale with a disturbing respiration. After all these mice fell in dyspnoea, ending in death with a convulsion or without it. As for the mice with a comparatively long life-time, the same symptoms develop more slowly, and in these cases surviving over 24 hours, jaundice is often present on the auricles and extremities. Carefully observing these cases, it is disclosed that, though the disturbance in consciousness is unknown, their final symptoms give us a strong impression of resembling to the hepatic coma of human patient.

3) Histopathological findings. a) Luteoskyrin. The livers of mice dying after 24 hours showed microscopically centrilobular necrosis with diffuse fatty metamorphosis of liver cells^{7,8)} and if heavily damaged, interconnection between necrotic areas. In the centrilobular areas, the liver cells were in coagulation necrosis, having showed the so-called Mallory's body-like appearance. The cytoplasm became round and eosinophilic. The nuclei were either pyknotic or lost. In these necrotic areas, proliferation of endothelial cells and infiltration of leucocytes and mononuclear cells were present together with debris possibly originated from the nuclei of damaged liver cell. The liver cells just adjacent to the centrilobular necrotic areas showed such nuclear degenerations as hyperchromatosis of nucleic membrane, caryorrhexis and caryolysis. b) Chlorine containing peptide. The livers showed, microscopically within relatively short time, presence of vacuolation and fibrin positive hyalin droplets in liver cells, and swelling and cloudification of mitochondria.^{7,8)} And were noticed dilatation of sinusoids, hemorrhage in liver cells and trabeculae. The liver lobules, if extensively damaged, looked like a blood lake save in centrilobular regions. However, the structure of the sinusoids were well maintained, while the damaged liver cells and endothelial cells showed caryorrhexis in their nuclei. The animals surviving more than 48 hours showed markedly diffuse fatty metamorphosis but little vacuolation of liver cells.

As for rats were obtained almost similar findings in livers and moreover a noticeable change in pancreas: hemorrhage in the head of pancreas and in the peritoneal cavity around liver hilum in cases of surviving more than 10 hours, and microscopically, acute necrosis of pancreas along with acute liver injuries.

By the way, liver cirrhosis was demonstrated after more than 100 days of the repeated administration of each toxic substance, either luteoskyrin or peptide.

III. *Purification of the toxic components, and their physical and chemical properties.* The mass purification of the two toxic substances was conducted with the under-mentioned methods and results.

A) *Luteoskyrin.* a) Purification. Dried fungus mat of Ud or Ea strain was treated with normal hexane to exclude the component of the fraction [2] on the table, and then treated with ether to extract the fraction [3]. The latter fraction of crude pigments was subjected to column chromatography (active carbon-sodium sulfate in ratio of 1 to 10-20). Since erythroskyrin, islandicin, skyrin, and iridoskyrin were tightly adsorbed on the carbon and not eluted easily, at first was eluted luteoskyrin and then rubroskyrin was completely separated by repeated chromatography on the mixed elutes. The over-all yield of luteoskyrin is 2% of the weight of dried fungus mat.

b) Physical and chemical properties⁴⁾

- i) Crystal: Yellow rectangular (recryst. from 95% acetone), mp. 278°C (decomp.)
- ii) Optical rotation: $[\alpha]_D^{25} - 880^\circ$ (0.1% in acetone)
- iii) Ultraviolet absorption: λ max (m μ) 245, 275, 430,
log ϵ 4.32, 4.33, 4.46
- iv) Infrared absorption spectrum: cm⁻¹ 1623, 3378

v) Chemical degradations:		
Luteoskyrin	pyrolysis	Islandicin and catenarin
„	$\xrightarrow{\text{Na}_2\text{S}_2\text{O}_4}$	Islandicin
„	$\xrightarrow{\text{H}\cdot\text{COOH}}$	Iridoskyrin
„	$\xrightarrow{60\% \text{ H}_2\text{SO}_4\text{aq}}$	Iridoskyrin and islandicin
„	$\xrightarrow{\text{NaOHaq}}$	Decomposed
vi) Derivatives:		
Luteoskyrin	$\xrightarrow{\text{CH}_3\text{COCl}}$	Luteoskyrin-hexa-acetate, mp. 188°C
„	pyridine	
„	$\xrightarrow{h\nu}$	Irrad. luteoskyrin, purple rhomboid pillars
„	acetone	mp. > 360°C
Irrad. luteo.	pyrolysis	Islandicin and catenarin

B) *Chlorine containing peptide*. a) Purification. Filtrate of 20 l of *P. islandicum* culture media was concentrated to about one-tenth in volume, added with acetone to exclude inorganic salts and polysaccharides of high molecule, and then, evaporating the acetone, an aqueous solution thus obtained contained considerable amount of pigments. Excluding these pigments by extraction with ethyl acetate, the solution was added with normal butanol to extract the toxic substance. *n*-Butanol solution was washed with pH 7 phosphate buffer solution to remove the contaminates, polysaccharides of low molecular weight and organic acids, disturbing precipitation of the desired toxic substance. Removing completely the normal butanol, the residue was gum, which was treated with absolute methanol to give chlorine containing peptide, white needles of m.p. 248° and 50–30 mg.

b) Physical and chemical properties⁴⁾

- i) Crystal: White needle (recryst. from abs. methanol), mp. 251–252°C (decomp.)
- ii) Optical rotation: $[\alpha]_D^{16} -92^\circ.9$ (in $\text{C}_2\text{H}_5\text{OH}$), $-100^\circ.5$ (in $\text{C}_2\text{H}_5\text{OH}$)
- iii) Ultraviolet absorption: max $m\mu$ 257, $\log \epsilon$ 2.75
- iv) Infrared absorption: cm^{-1} 3400, 1670, 1640, 1535, 1510, 1495, 1460, 695
- v) Qualitative tests:

Buret reaction	positive,	Neubauer-Rhode reaction	negative
Sakaguchi reaction	negative,	Ninhydrin reaction	negative
Pauli reaction	positive,	Millon reaction	negative
- vi) Elementary analysis: Observed %
 C. 51.0, H. 6.1, N. 11.0, Cl. 11.3, C. 50.8, H. 6.05, N. 11.9, Cl. 11.9
 Proposed formula $\text{C}_{25}\text{H}_{36}\text{N}_5\text{O}_8\text{Cl}_2$
- vii) Chemical degradations:

Chlorine cont. peptide	$\xrightarrow{6\text{N}\cdot\text{HCl aq.}}$	Serine 3-2, α -aminobutyric acid 1, β -phenyl- β -aminopropionic acid 1, and unknown substance (Ehrlich reaction positive)
„	$\xrightarrow{0.5\% \text{ or } 28\% \text{ NH}_4\text{OH aq.}}$	white needle, mp. 230–231°C, (Halogen test is negative.)
- viii) pKa: Not observed between pH 1–14.

Viewing from the above properties, the chemical constitutions in both luteoskyrin and chlorine containing peptide have been revealed to a great extent. As to luteoskyrin the proposed structural formula was presented by Shibata⁹⁾ in connection with rugulosin and rubroskyrin. As we think, as he says, the formula is not decisive, we are engaging in further study as well. As to chlorine containing peptide, the report by Sumiki and Marumo¹⁰⁾ has some points different from the outcome of our study, though the experimental materials of both sides have not been yet identified. Besides the above data, we have in hand those of partial hydrolysis, according to which the combining order of

amino acids constructing the molecule has been almost disclosed as reported later. Although the chlorine contained in the compound proved to have a close relation with the toxicity, how this halogen is located in the molecule remains to be examined.

Summary. Since 1954, our team has been engaging in the joint approach to the toxic substance in the metabolites of *P. islandicum* Sopp, with the following results obtained. 1. The preliminary examinations on animals gave us a presumption that there must be at least two kinds of toxic components in the methanol extracts of the fungus mat; one is relatively prompt acting and hydrophilic and the other is slow acting and lipophilic. This presumption was proved as working hypothesis of great use to bring our whole fractionations to the fruitful end. 2. Isolation of the desired toxic substances was carried out along the whole process of chemical extraction, in tight combination with toxicity testings and pathological examinations. In consequence were successfully isolated a yellow pigment named luteoskyrin and a chlorine containing peptide, both unknown hitherto. Then, the mass purifying methods thereof have been established to disclose their physical and chemical properties including structural formula. 3. Luteoskyrin is lipophilic and the relatively slow acting. LD₅₀ is subcutaneously 1.47 mg/10 g and orally 2.21, when dissolved in water, and expected to be much less if being possible to make it soluble in water. Chlorine containing peptide is hydrophilic and the relatively prompt acting. LD₅₀ is subcutaneously 4.75 microgram/10g, and orally 65.5. The two substances appear different from each other not only in life-time but also in acute symptoms of poisoning. Nevertheless, they cause final symptoms in acute cases surviving rather longer, which give us an impression of resembling to the so-called hepatic coma. 4. Pathologically the two toxic substances cause acute liver injuries. In cases of luteoskyrin, most of mice survived over 24 hours, microscopically demonstrating centrolobular necrosis and diffuse fatty metamorphosis of liver cells, and if heavily damaged, interconnection between necrotic areas. In cases of chlorine containing peptide, were demonstrable vacuolation in liver cells, dilatation of sinusoids, and hemorrhage within a relatively short time. In the animals surviving more than 48 hours was observed diffuse fatty metamorphosis. Which of both the purified toxic substances was administered repeatedly, liver cirrhosis was demonstrable.

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