The First Interim Report by

The Analytical Sub-Committee

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I. On constant(s) of the oil

1) Acid value (by Sugano)

Analysis was done in the standard analysis method for oils and fats.

Kanemi's rice oil owned by one of the oil disease patients, 0.75. The control oil considered not related with the oil disease, 0.57 - 0.70.

2) Peroxides (by Inagami and Sugano)

Done in the standard methods.

Pt's Kanemi oil	Iodine method mag/kg	Lodin method OD500/pmg
	13.4 - 39.4	0.431 - 0.79
Control oil	1.2 - 12.1	0.044 - 0.458

II. Metals (Inorganic matters)

1) Arsenic

i) Arsenic in oil

a) Gutzeit Method (by Yoshimura, Kuratsune, Yamaguchi)

The rice oil samples of six patients and four control oil samples were prepared in the moist method and the combustion method and analized in Gutzeit method. The results were all under 1 ppm as arsenious acid, with no significant difference between the patients' oil and the control oil.

b) Xray-fluorescence analysis and radiation analysis (by Yamada)

The result of the former on one patient's oil was 0 ± 5 ppm. The result of the latter on another patient's oil was below 0.1 ppm.

ii) Urine before and after BAL.

BAL was given to three patients of one family and discharge of arsenic in urine before and after the administration was measured. No significant figure was obtained in any of the cases.

III. Organic Chlorines

1) P C P

i) Color reaction (by Inagami)

Alkali extract from patients' oil sample and its acid vapor distillation fluid were placed to color reaction tests in 4-amino-antiphyrine method, and results were negative.

2) Other metals

i) Chemical analysis (by Yoshimura, Pref. Sanitation Lab.)
Rice oil samples used by six patients were prepared in the moist method and in the conbustion method for analysis of Pb and Ni, and the results were all negative.

ii) Xray-fluorescence analysis and radiation analysis (by Yamada)
The patients' oil and the control oil (Nisshoku Salad Oil) showed no marked difference in the fluouscence spectre of heavy metal elements.
By radiation analysis one patient's oil (Kanemi Rice Oil) showed the following results.

Na	7 ppm
Mn	0.3 ppm
Ni	2 ppm or less
Cu	1 ppm or less
2n	2 ppm or less
Hg	1 ppm or less

3) Discussion
It was concluded that, in view of the above results, the oil used by patients did not contain arsenic or any other heavy metal in quantity enough to cause the disease.

3) Carbonyl (by Sugano)

In 24-dinitrophenyl-hydrazine method

	(Total carbonyl mag/kg)
The pt's rice oil	16.5
Control oil	9.6 - 11.0

4) Unsaponificated material

By Prefectural Sanitary Research Lab. in the Sanitation Test Method.

Two samples of pt's oil	3.01%, 3.51%
Control oil (2 samples)	2.73 3.08

5) Fatty acid composition (by Nagai)

Both the patients' rice oil and the control oil mostly consisted of palmitic acid, oleic acid, linoleic acid, and the gas-chro patterns were also very much alike.

6) Discussion

As mentioned in the above, the difference of the oil used by the patients and the control oil was in peroxide and carbonyl values, higher in the patients' oil. However, the patients' oil by the time of the examination had passed more time than the control, and these differences may be due to natural denature of the oil.

ii) Gas chromatography (by Yoshimura)

Patients' Kanemi Rice Oil samples and a sample of a recent lot Kanemi Rice Oil, as well as samples of two other brands were separated into acid, alkali and neutral parts at each, and PCP was sought at the acid part using the Shimazu GL-2C Type apparatus (detector ECD column : 1.5% SE-30 Auakron AS 4mm x 0.75). In all samples, the peaks were close to that of the standard PCP RT, with no significant difference between the patients' oil and others.

iii) Thin layer gas chromatography (by Inagami and Yoshimura)

The above i) and ii) samples were used for search of PCP in this method also. No evidence of its existence was recognized.

iv) Discussion

The above is sufficient to prove that PCP cannot be considered a cause of the disease.

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2) Heat catalyst diphenyl chloride (Kane Chlor)

i) Gas chromatography (by Inagami, Makizumi, Yoshimura, & Prefectural Sanitation Lab.)

a) Preparation of samples

Kanemi Rice Oil samples of four patients were prepared in *saponification method or in **AoAC method to get concentrated organic chlorine contents as samples for gas-chromatography. (*The oil is heated in 15% alcoholic NaOH for 60 minutes at 100°C, and the unsaponificated matter is extracted, using ethyl ether).

(** The oil solution in hexane or petroleum ether is put to an agitation extraction process with acetonitrile saturated with the same solvent, and the extract is thrown in 2% NaCl or mirabilite aqueous solution and extracted again with petroleum ether and the extract is put through a florisil column to get the ethyl ether or the hexane content in the extract solve out).

Several other brands of food oil considered not related with the disease were also gas chromatographed as control samples.

b) The type of the equipment and the experiment conditions

i) Shimazu GC-IC Type(Makizumi)

Detector:

Pulse type electron-capture detector (ECD) and hydrogen flame ionization detector (HFID) were connected for simaltaneous recording.

Column:

1.5% SE-30 on Chronosorb W (60 - 80 mesh)

Glass column 4mm x 2625m

Column temp.: 200°, Detector temp.: 200°

Carrier Gas: N_2, (1.6kg/cm^2)

E C D: Sensitivity 10^2, Range 0.8 or 1.6

HFID: Sensitivity 10^3, Range 0.8 or 1.6

ii) Apparatus:

Shimazu GC-IC-Type Gas Chromatography(Yoshimura)

Detector:

Hydrogen flame ignition detector(HFID)

Column: 1.5% OV-17 on Shimalite W

Glass Column 4mm x 2,625m

Column temp.: 200°, Detector temp.: 200°

Carrier gas: N_2(30ml/min, 3.2kg/cm^2) : H_2 40ml/min.

Sensitivity 10^3, Range 3.2

c) Conclusion

The most typical chromotograms of the samples of patients' oil prepared in AoAC process, tested by ECD under conditions of the above b) i) ~~are shown attached~~ and that of Kane-Chlor(10^{-8}g), are the attached Figs. 1. and 2, in which extreme peaks are frequent and the patterns are very similar, clearly showing that the patients' oil contains much Kane-Chlor. (The sample of Kane Chlor was obtained from Kanemi Warehouse KK Production Dept.) The control oil samples do not show such a pattern

The Figs. 3 and 4 are another typical chromatogram of a patient's oil sample and that of Kane-Chlor (10^{-6}g) done in AoAC process, tested by HIFD under conditions of b)ii). The two figures are alike again.

ii) Infra red spectrum(Yoshimura & Takeshita)

The acetone extraction of the above patients' Kanemi Rice Oil sample was done and its organic chlorine part was separated and purified by florisyl column chromatograph, which, in chloroform solution, was found to show almost the same spectrum as that of Kane-Chlor, as the figures enclosed.

iii) Organic chlorine contents in oil

a) By chemical analysis(Yoshimura, Kuratsune, Inagami, and Prefectural Sanitation Lab.)

Done in the "General Quantitative Determination Method of Organic Chlorines" of the "Sanitation Test Methods" on samples of six patients' Kanemi Rice Oil samples and control rice oil samples. The method was to dissolve the oil into benzene, wash it with water to remove inorganic chlorine, and then to reduce it by adding isopyl-alcohol, metal sodium to it and the resulted NaCl was titrated. The results were,

Sample	Cl content (ppm)
A	1,070
B	1,080
C	1,020
D	1,080
E	1,170
F	1,500
Control	Trace or none

b) Radiation analysis (Yamada)

Three patients' Kanemi Rice Oil samples and control oil samples were analized using Kyoto University Atomic Pile Laboratory's pile KUR (non-breaking).

Neutron ray group, 5 x 10^{12} n/cm^2, sec; radiation time 10 minutes, 1 hour; measurements, RCL 512 and 1024; channel analyzer Ge(Li); detector 2cm^3 and 22cm^3.

Sample	Cl content (ppm)
A	1,170
B	1,105
C	1,300
Control A brand	2
" B "	8
" C "	18

When the above A sample was washed twice with 70°C ANO_3(1 : 4), twenty times in quantity of the sample, almost none of Na remained in the oil, while almost all Cl remained. From this, it was estimated that the chlorine in the oil was organic chlorine.

c) Discussion

a) From the above a) and b) results, the patients' oil was confirmed to contain 1000 - 1500 ppm (as Cl) organic chlorine.

If the composition of organic chlorine in the patients' oil will be found to be same as that of Kane-Chlor, it will be concluded that the patients oil contains 2000 - 3000 ppm Kane-Chlor as Kane-Chlor Cl content is about 48% as will be reported in the next iv). Other Kanemi Rice Oil samples than the above are still under study.

iv) Chlorine content in Kane-Chlor

a) Burning in oxygen flask(Ueno)

According to Schoniger's method, the sample specimen 10 - 20 mg was capsuled and burned in an oxygen-filled and sealed flask, and the gas obtained from burning it was absorbed by the absorption fluid in the flask, consisting of 0.1 N NaOH 10 ml and 30% H_2O_2 2 ml, and titrated with 0.01N Hg $(NO_3)_2$, and the unused Kane-Chlor(obtained from Kanemi Warehouse KK), was found to contain 47.5% chlorine.

b) Sodium biphenyl analysis (Ueno)

20mg of sample was dissolved in toluene, added with 1N sodium biphenyl solution 10gms, shaken for 30 minutes, and organic halogenated material was taken out, at which point the designated test chemical is added in excessive quantity so that the chemical's blue-green color will remain , neutralized with nitric acid, extracted more than twice with 0.1 N HNO_3, and titrated with $Hg(NO3)_2$. The chlorine content of the Kane-Chlor sample examined in this method was 48.5%.

c) Reduction method, one of the Sanitation Test Methods (Yoshimura and Pref. Sanitation Lab.)

The method is same as the above iii) a). The result was 47.3%.

d) Discussion

The above three results 47.5, 48.5 and 47.3% were almost equal with the 48% as specified in Kane-Chlor specifications. The samples used in the above three analyses were all from unused lots.

v) Kane-Chlor composition (Ueno)

Kane-Chlor, first having its chlorine removed by using Raney alloy and NaOH aqueous solution, was gas-chromatographed, and the result pattern showed that two new peaks appeared in the

small RT area in addition to the pattern of the unreacted Kane-Chlor, of which, the one of large RT was identical with RT of biphenyl (Kane-Chlor material) and the other, with that of phenyl ticrohexan standard product.

The Kane-Chlor raw material, that is, Yawata Chemical's biphenyl, showed a single peak at gas-chromatography, which was identical with biphenyl standard product RT. From these results, it is not considered likely that other aromatic compound than biphenyl is in the Kane-Chlor composition.

IV. Search for other foreign materials

Patients' oil samples were separated into acid, alkali, and neutral parts or distilled by vaporization and compared with control oil samples at each of these with efforts for seach of any agricultural chemical, food additive, etc. in methods such as thin layer gas-chromatography, ultra-violet absorption spectrum and all sorts of color reactions, but no specific foreign material was found mixed in.

V. Search for Kane-Chlor component contents in living tissues

(Yoshimura, Makizumi, Inagami)

Skin biopsies specimen of a few of the oil disease patients prepared at Kyushu U. Dermatological Dept. were gas-chromatographed for finding Kane-Chlor component contents in them.

1) Skin fat

i) Preparation of specimen

Two patients' abdominal skin fat specimen, one pt's cheek skin fat specimen and three normal people's abdominal, neck and breast skin fat specimen(control) were chosen for the examination. The tissue was cut off by scissors and put through ethanol extraction three times for 10 - 15 minutes each time at 50 - 60°C. Then it was infiltrated in the same manner, using ethyl ether and hexane. All of the extracted fluid was placed on a hot plate for solvent removal and the residual matter was extracted with ethyl ether: petroleum ether = 1.5 : 8.5, and filtered, and the solvent was removed from the filtrate, which was dissolved in a small quantity of petroleum ether, poured in a florisil column to dissolve the ethyl-ether and petroleum ether out, to be made ready for gas chromatograph.

ii) Gas chromatograph apparatus and conditions

a) Shimazu GC-IC Type gas chromatograph(Yoshimura)
Detector: Election-Capture Detector(ECD)
Other details are the same as stated in Page b) (2).

b) Shimazu GC-IC Type Gas-chromatograph (Makizumi)
Same as stated in Page b) (1).

iii) Results

The typical gas chromatogram of the above ii) a) is the attached Fig. 6(Extracts of a pt's and Kane-Chlor). Fig 7 is of a control case and Fig 8 is of another pt's, co-chromatographed with Kane-Chlor.
The Fig 6 shows several peaks which are equal-patterned as those of Fig 7 in the small RT area, but there are peaks in the large RT area also, such not being seen in Fig 7, which are very similar to Kane-Chlor components' latter half peaks, and the identification was confirmed by co-chromatography(Fig. 8).

Extracts of two other patients, though there were minor differences of height, showed very similar patterns as of Fig. 6, while chromatograms of three control cases were very similar to Fig. 7. The same samples were analyzed on ii) b) equipment also, whose conditions are different, and showed data to lead to the same conclusion.

From the above results, it is suspected that some of the Kane-Chlor components remain in pts' fat composition for a fair length of time after the rice oil intake as some Kane-Chlor component peaks were confirmed in the pts' extract chromatograms.

2) Other ~~biopsies~~ specimen

Besides skin fat, perspiration, nails, mother's milk, fetus, placenta, etc. were suspected to contain Kane-Chlor. Analyses are underway.

VI. Conclusion

1) The constant measurements of the pts' Kanemi Rice Oil showed abnormal values in peroxides and carbonyl, but these were natural in view of the production dates of the oil. This fact does not necessarily relate with the disease as a cause.

2) Arsenic and other inorganic metals cannot be considered to have caused the disease.

3) PCP could not be the cause either.

4) Patients' oil was confirmed to contain much Kane-Chlor, a heat catalyst. The chlorine contents of the pts' oil samples were 1000 - 1300 ppm.

5) The chlorine content of unused Kane-Chlor obtained from Kanemi Warehouse KK was about 48%.

6) The major component of Kane-Chlor is biphenyl.

7) The oil disease patients' skin fat gas-chromatograms show that a few of Kane-Chlor components remain there for a fair length of time after the rice oil intake.