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SOME PROBLEMS ABOUT PETROPROTEIN AS A NEW FEEDSTUFF1/

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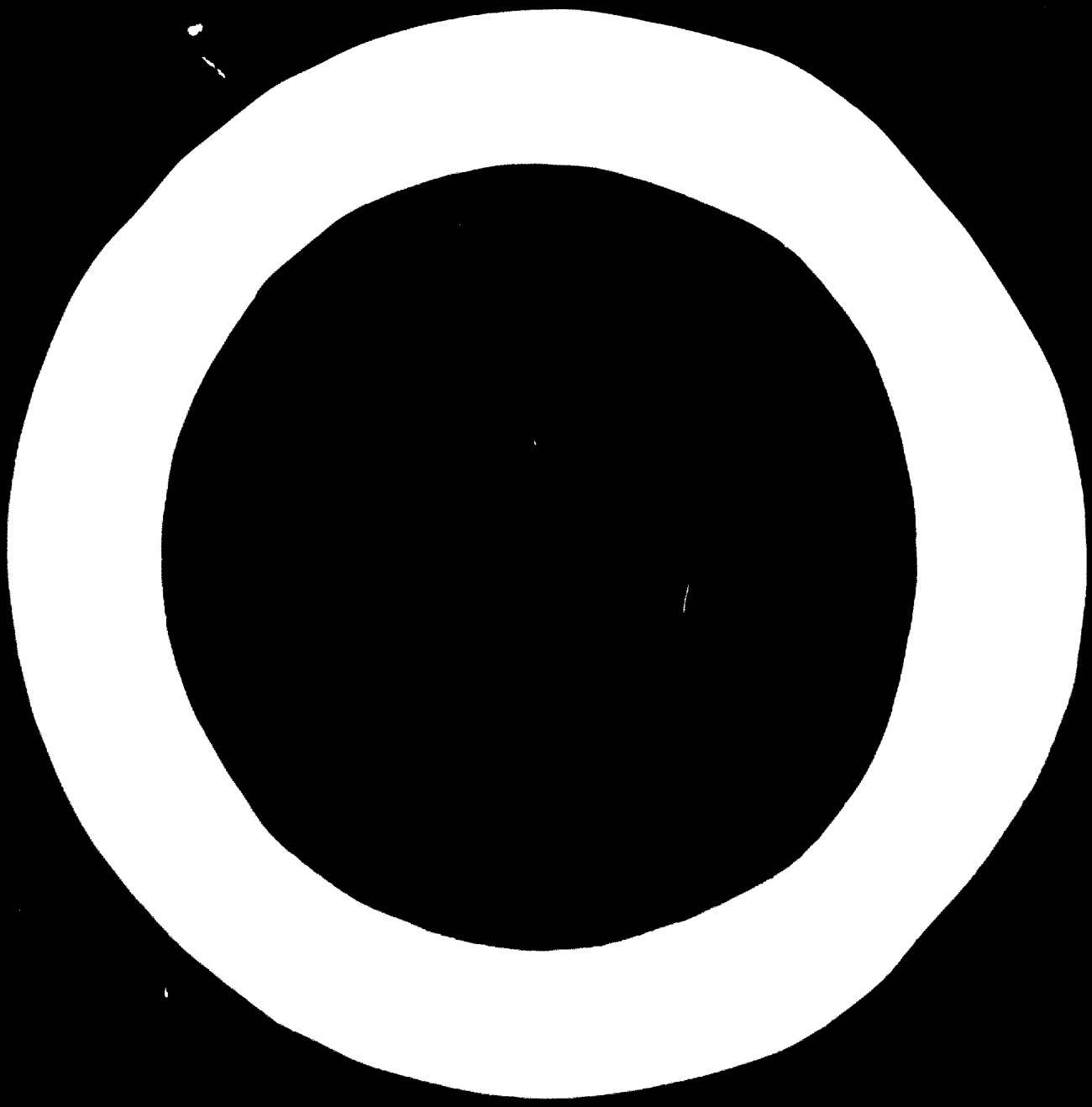


SUMMARY

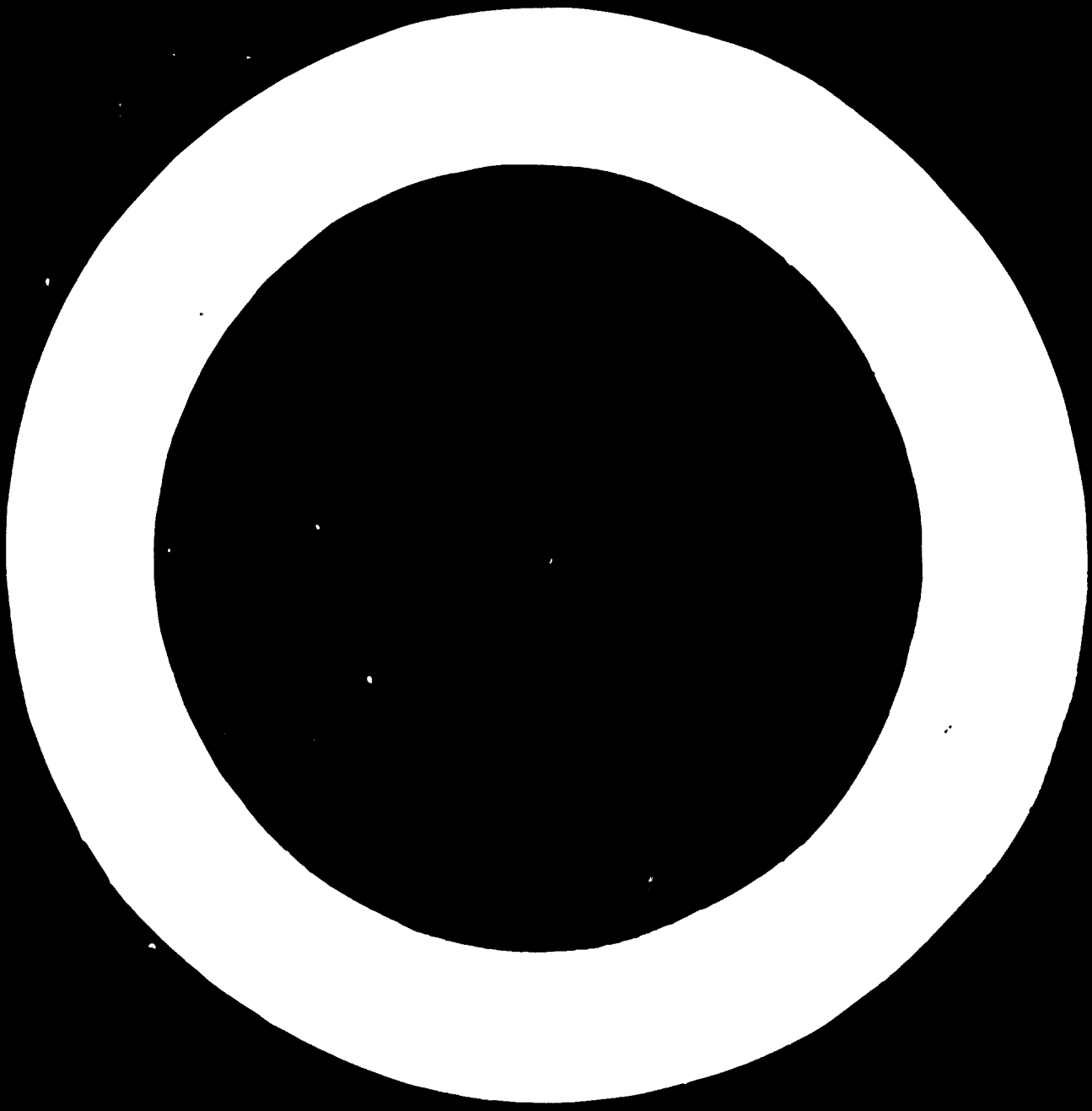
Two biocatalytic processes of the production of protein from petroleum oil have been studied in a pilot plant scale. *Candida lipolytica* as well as other yeasts have been used as the microorganisms for the fermentation. In one of the processes, the substrate used is n-paraffin, and, in the other, it is gas-oil.

In general, the continuous fermentation technique has been adopted. In some cases the petroyeasts were cultivated in batch processes. The percentage of conversion of n-alkanes into petroyeasts is about 70—80%.

Preliminary results from experiments on feeding pigs and chickens with n-paraffin yeasts at 10—25% levels show no specific pathological changes in the tested animals. The yeasts from gas-oil fermentation are toxic, but become non-toxic after the extractions with alcohol.

More extensive studies on petroyeasts as proteinous feedstuff are now being carried on.

Petroyeasts contain C_{16}- and C_{18}-fatty acids. The body fats of pigs fed on petroyeasts contain also C_{16}- and C_{18}-fatty acids which are negligible in the normal control pigs.



SOME PROBLEMS ABOUT PETROPROTEIN AS A NEW FEEDSTUFF

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The research on petroleum fermentation in China began in the sixty, when the Chinese people guided by the principle of "selfreliance and hard struggle" discovered and exploited the petroleum resources of Daching. Because Daching crude petroleum is highly rich in paraffin, it was then decided to make use of this material and to find ways by means of biocatalytic processes to convert paraffin into fats and proteins.

At present, the problem of petropotein production in China has been considered on the basis of her own concrete conditions. On account of different properties of crude oils from different petroleum resources and specific conditions of different regions of the country, two processes of petroprotein productions are being investigated in parallel. One of them is the production of the protein from the fermentation of *n*-paraffin, and the other, that from the fermentation of gas-oil, by *Candida lipolytica*, other yeasts being also employed in special cases. From the gas-oil fermentation dewaxed products may be obtained.

Some results from the studies on a pilot plant are summarized as follows:

1. PETROPOTEINS FROM *n*-PARAFFINS

The substrate used was *n*-paraffin (so-called heavy oil) prepared from the 220—320° distillate of a crude petroleum oil by urea molecular complex method. It is composed of C_{18}—C_{24} *n*-alkanes, mainly of C_{18}—C_{20} hydrocarbons (97.3%), the aromatic hydrocarbons being less than 0.5%. In the fermentation media ammonium sulphate (1%) is used as nitrogen source, and *n*-paraffin is added at 3—5% (by volume) levels together with adequate amounts of mineral salts (Table 1). *C. lipolytica* and *C. tropicalis* have been used, and the former has been employed in the most cases. The pH was maintained at 4—4.5 by adding ammonium hydroxide solution, and the temperature, kept at 30—32°C. For the sake of comparison as well as for other purposes, a continuous process with three-stage fermentators of 5000 *l* (with working volumes of 2400 *l*) and a batch process have been studied side by side.

In the case of the continuous fermentation, the volume ratio of aeration was around 1:2 to 1:2.5; the yeast concentration in the first stage fermentor in average, 5—7 g/*l*; the rate of flow, 400 *l*/hr; and the rate of dilution in average, 1/5—1/6. The fermentation has been continuued uninterruptedly for a month. The fermentation products were separated by a high speed centrifuge and the crude yeast was washed by water. Finally the product either directly or after subjection to subsequent treatments was drum-dried or spray-dried. The percentage of conversion of *n*-alkanes into dry yeasts was about 70—80%.

II. Petroproteins From Gas-oil (Or From Other Petroleum Distillates)

The essetial features of the fermentation process for gas-oil are the same as the above-mentioned ones, with the exception that gas-oil containing ca. 15% n-alkanes (or other distillates) is used as the substrate. The percentage of conversion was in average 75%. Besides the petroyeast as the main product, there is also obtained dewaxed oil as a byproduct. After centrifugation and subsequent treatments, the product yeast was dried.

III. Subsequent Treatments After Separation

The yeast obtained from n-paraffin was either directly dried after water washing or first subjected to alcoholic extraction with or without pretreatment with alkali and then dried.

The yeast from gas-oil was subsequently treated in the following ways:

(1) The yeast concentrate from the centrifugation was stirred up in dilute sodium hydroxide solution (10% of dry yeast) at 40°C for 1 hr. to remove nucleic acids, centrifuged and dried.

(2) The alkali-treated yeast was further extracted with petroleum ether chiefly to remove the residue of non-fermentable hydrocarbons and then dried.

(3) The alkali-treated yeast was further extracted with alcohol to remove oils and fats until the fat content was below 2% and then dried.

The defatted yeast thus obtained consists of 50% crude protein, 1.1% fat, 5.0% ash and ≤10% water (Table 2). The essential amino acid contents of the petroyeasts are given in Table 3.

IV. Toxicity Tests

Preliminary tests show that *C. lipolytica* and other yeasts employed are non-pyrogenic and produce no toxic substances. Toxicity tests have been performed on pigs, chickens and albino rats.

In a number of experiments with pigs, groups of five animals of 3 months old (except one experiment with pigs of 7 months of age) of the same litters were employed. Control groups were put on a ration consisting of wheat bran, rice bran, barley meal, corn meal, soybean cake, stone powder and table salt, vegetables being given daily in extra. In test groups, petroyeasts with different subsequent treatments were fed at two different levels (10 and 25%) in substitution for soy bean cake, and in case of the 25% group the yeast also partially displaced the other feedstuffs (Table 4). During the experimental periods of four months, the average daily consumption of the rations and the gain in body weights have been noted. At the end of the experiments the animals of both the control and test groups were sacrificed and examined for gross pathological changes and histological lesions of internal organs (liver, heart, stomach, kidney *etc.*).

In case of tests for n-paraffin yeast treated with alkaline and alcoholic extractions, the average daily consumptions of the fodders of the test and control groups were practically the same, 1.6—2.0 kg per pig (Table 5). In average, each test pig had

taken 200 and 500g petroyeasts respectively in the 10% and 25% level groups. At the end of the tests, the average body weight increases per pig of the two test groups, were greater than those of the controls by 7 and 11% respectively, corresponding to 0.4 and 0.49 kg/day/pig in the test groups, in comparison with 0.38 and 0.44 kg/day/pig in the control group. (Table 6). Experiments with defatted petroyeasts gave similar results. Composition analysis of the pork from both the control and test groups shown no significant differences (Table 7). The appearance of the growth as well as the results from gross autopsy and histological examination of the internal organs of all test groups did not indicate any specific pathological lesions due to petroyeast-feeding.

In chicken tests, groups of 80 chickens of Brock race, 10 days after hatching, were employed. One group was put on a control ration, consisting of corn, broken rices, barley meal, wheat bran, soybean cake, fish meal, table salt, stone powder, phosphates, trace elements, vitamins A, B$_{12}$ and D and also green vegetables. The test groups were put on rations similarly composed but with the exception that 10% of soy bean cake was substituted by petroyeast from *n*-paraffin (Table 8). The duration of experiment was two months. In the second month, more corn was given at the expense of broken rices, barley meal, wheat bran and fish meal. (Table 8). During the period of two months, the average daily consumption of the fodder (77 g) and the average weight increase (1670 g) per chicken were practically the same for both the control and the test groups (Tables 9 and 10). The feather appearance and the results of composition analysis of the breast muscles of the chickens in the test and control groups show no significant differences (Table 11). A number of the chickens in each group were killed for autopsy. Gross examinations as well as histological studies of the internal organs of both control and test chickens did not reveal any specific pathological changes ascribable to the toxicity of petroyeasts. The rest of the chickens are being kept alive on the same rations. The number of eggs laid and the percentages of the successful hatching of the fertilized eggs of the test groups did not differ significantly from those of the control groups. Experiments have been carried on for three generations. The general growth of the descendants of the test group and the laying and hatching of their eggs were all normal in comparison with those of the controls, and gave no sign of specific symptoms of petroyeast toxicity.

In case of tests for petroyeasts obtained from gas-oil and only freed from nucleic acids and residual petroleum, feeding experiments (at 10, 20 and 50% levels) show that all the test pigs, chickens and albinorats gained less bodyweights than the control ones during the four months period. Furthermore, the stomachs and livers of the test pigs showed significant hypertrophy and pathological lesions. The changes occurring to the 20% petroyeast group was more severe than the 10% group, and the 50% level group shown the severest lesions together with accumulation of ascitic fluid. In the chicken tests, so far only the separation of horny layer from the wall of muscular stomach could be observed in the test groups. But there were no pathological changes found in the internal organs of the test rats fed on gas-oil yeasts. On the other hand, the pigs fed on petroyeasts defatted by alcohol show that their increases in body weights (Table 12) and their autopsy findings indicate no specific differences from those of the control animals, while the pigs fed on an alcoholic extract from the gas-oil yeasts presented clear pathological lesions in their livers and stomachs similar to

those of the test pigs fed on untreated gas-oil-yeast. Therefore, the toxic substance could be removed by alcoholic extraction.

Analysis of 3,4-benzopyrene contents in the adipose tissue and lean meat of pigs and in the breast muscles and eggs of chickens of the control and test groups, fed on n-paraffin yeasts, indicates that 3,4-benzopyrene is present in an amount around 1 or less than 1 ppb (Table 13).

V. Discussion

1. *About the problem of the toxicity.* The preliminary results from the present feeding experiments with yeasts from n-paraffin fermentation show that petroyeasts when taken at levels 10 to 25% do not cause specific pathological changes in tested pigs and chickens, and seem to be quite hopeful to be used as a new proteinous feedstuff. Of course, further extensive feeding investigations about chronic effects and nutritive values of the n-paraffin yeasts must be carried out and extended to longer periods and more generations of test animals. The crude yeast from gas-oil fermentation is toxic, and it becomes non-toxic after alcoholic extraction. The toxic substance seems to be diffieultly soluble in petroleum ether and easily soluble in alcohol. Other series of experiments are now being carried out to study the nature of the toxic substance.

Results from toxicity experiments indicate that albino rats, chicks and pigs show perceptible differences in the response to the toxicity of the preparations of petroyeasts. The sensitiveness to the toxicity is in the following order: Pig>chicken>albino rat.

2. *About the problem of the technology of petroyeast production.* Although the technology of the production of petroyeasts from n-paraffin seems simpler and more economic in spite of the production of n-paraffin itself, the gas-oil fermentation can, however, yield not only protein-rich petroyeasts, but also dewaxed oils and other valuable byproducts such as ergosterol, fats *etc.*, which may be isolated easily from the alcoholic extracts. Thus the extra costs caused by the additional defatting process may be well balanced by the benefits of comprehensive utilization.

Therefore, which one of the two processes of petroyeast productions is preferred is better decided according to the concrete conditions of the countries or the regions concerned. At present, in China, the policy of "walk on two legs" is adopted.

3. *About the fatty acids of the test animals.* It is worth while to point out, that, in an experiment with pigs fed on *C. tropicalis* from n-paraffin there were found in the adipose tissue of the test animals less liquid unsaturated fatty acids than the control ones (Table 14), and, moreover, in the solid fatty acids, unmistakable amounts of C_{16}- and especially C_{17}-fatty acids, which were negligible in the controls. The n-paraffin yeasts contain C_{16}- and C_{17}-fatty acids in its fats. The yeast from molasses is also low in liquid unsaturated fatty acids. Pigs fed on a ration containing 25% of yeasts, cultivated in a molasses medium, yielded fatty acids also low in liquid unsaturated fatty acids, but with no abnormal C_{16}- and C_{17}-saturated fatty acids. (Table 14, Figs. 1, 2 and 3). In one test group, in which the animals were fed first on untreated petroyeasts, and afterwards shifted to the control ration during the last two months, the C_{16}- and C_{17}-fatty acids of the adipose tissues of these animals decreased in amount remarkedly. These results agree well with those of some earlier classical nutrition experiments

with animals which show, besides the portion of species-specific glycerides of the animal, the composition of the remaining body fats is influenced by the enviromental conditions and ingested fodders. In general the change is reversible.

The question of the effects of prologed feeding of petroyeasts on animals, however, remains to be answered by further studies.

Table 1

Compositions of the media for yeast fermentations of petroleum

Constituents	Propotions	Constituents	Propotions
$(NH_4)_2SO_4$	2 Kg	$ZnSO_4$	0.002 Kg
KCl	3 Kg	H_3PO_4	2 Kg
NaCl	0.2 Kg	H_2O	2000 l
$MnSO_4$	0.04 Kg	n-Paraffin	60-160 l
$MgSO_4$	1 Kg	(or Gas-Oil)	(or 400 l)
$FeSO_4$	0.002 Kg		

pH was maintained at 4.0–4.5 by adding NH_4OH solution.

Table 2

A comparison of the protein contents (on dry basis) of petroyeasts with those of some other feedstuffs

Feedstuffs	Protein (%)	Fat (%)	Ash (%)
Petroyeast*	65.84	2.44	4.43
Petroyeast**	48.18	0.16	4.30
Petroyeast***	53.67	1.12	5.66
Fish meal	70.09	4.80	16.48
Soybean cake	49.04	5.49	5.08
Peanut cake	48.23	5.01	5.17

* *C. lipolytica* cultivated on gas-oil and defatted.
** *C. lipolytica* of a different strain cultivated on n-paraffin and defatted.
*** *C. tropicalis* cultivated on n-paraffin and defatted.

Table 3

Distributions of essential amino acids in the proteins of petroyeast (*C. lipolytica*) and other feedstuffs

Amino acids*	Petroyeast protein	Fish meal	Soybean cake	Cotton seed cake
Arginine	5.05	5.03	6.61	9.38
Histidine	2.08	1.90	2.84	2.62
Isoleucine	4.84	4.43	4.60	3.17
Leucine	6.84	6.70	8.20	5.86
Lysine	6.48	5.70	5.81	4.40
Methionine	1.17	--	--	--
Phenylalanine	6.17	4.10	5.60	5.65
Threonine	5.83	4.25	4.60	3.75
Valine	6.45	5.29	4.98	4.22

* Tryptophane had not been determined.

Table 4

The compositions of rations for experimental pigs on toxicity tests

Feedstuffs		Ration for test group		Ration for Control group
		10%	25%	
Wheat bran		30	25	30
Rice bran		25	21	25
Barley meal		20	16.5	20
Corn meal		15	12.5	15
Petroyeast		10	25	0
Soybean cake		0	0	10
Total		100	100	100
In addition	Stone powder	2	2	2
	Table salt	0.5	0.5	0.5

Vegetables were given extra.

Table 5

A comparison of average daily consumptions of the rations by pigs during the experimental period

Exp. No.		I		II		
Groups		Petroyeast* 25%	Control	Petroyeast**		Control
				10%	25%	
Ration consumption	Quantity (kg/day/pig)	1.55	1.40	2.04	2.02	1.97
	Percentage	96.8	100	104	102	100

* *C. lipolytica*, cultivated on *n*-paraffin and extracted with alcohol.
** *C. lipolytica*, treated with alkaline and alcoholic extractions.

Table 6

The average daily increases of the body weight of experimental pigs on toxicity tests for petroyeasts

Exp. No.	Groups	Number of pigs	Age (in months)		Average body weight per pig at start (kg)	Petroyeast added	Daily weight increase per pig	
			Start	End			Kg	Percentage
I	Test	5	3	7	19.65	25*	0.42	111
	Control	5	3	7	19.25	0	0.38	100
II	Test	5	7	11	45.15	10**	0.49	111
		5	7	11	44.20	25**	0.47	107
	Control	5	7	11	44.00	0	0.44	100

* *C. lipolytica* from *n*-paraffin defatted by alcoholic extraction.
** *C. lipolytica* from *n*-paraffin treated with alkaline and alcoholic extractions.

Table 7

Compositions of the back muscle between the sixth and seventh ribs of experimental pigs employed for toxicity tests on n-paraffin yeast (*C. lipolytica*), preliminarily treated with alkaline and alcoholic extractions

Groups		Moisture (%)	Protein (%)	Fat (%)	Ash (%)
Test	Petroyeast (10%)	74.67	21.92	1.77	1.17
	Petroyeast (25%)	74.66	22.04	1.42	1.13
Control		74.52	22.20	1.68	1.21

Table 8

Compositions of rations for experimental chickens

Feedstuffs	Early period 10~45 days old			Later period 46~73 days old		
	Test ration		Control ration	Test ration		Control ration
	1	2		1	2	
Corn	25	25	25	40	40	40
Broken rice	25	25	25	20	20	20
Barley meal	15	15	15	10	10	10
wheat bran	7	7	7	5	5	5
Soybean cake	20	0	20	10	0	20
Fish meal	8	8	8	5	5	5
Petroyeast	10	20	0	10	20	0
Total*	100	100	100	100	100	100

* In addition to 100kg of each of the above rations were mixed 0.5 kg table salt, 1.5 kg $CaCO_3$, 0.5 kg $CaHPO_4$, 0.25 kg trace elements and sufficient amounts of vitamins A, B, D and also vegetables.

Table 9

Average daily Consumptions of the rations by chickens during the experimental period

Groups	Number of chickens	Duration (days)	Petroyeast* added (%)	Average consumption (g/day/chicken)
Test	80	65	10	77
control	80	65	0	77

* *C. lipolytica* obtained from n-paraffin and treated by alkaline and alcoholic extractions.

Table 10

Average daily weight increase of experimental chickens on toxicity tests at different intervals (in days) of the experimental period

Groups	Number of chickens		Average start weight (g./chicken)	Average final weight (g./chicken)	Average weight increase per chicken							
					1st–75 days		1st–30 days		31st–60 days		61st–75 days	
	Start	End			Increase (g.)	%	Increase (g.)	%	Increase (g.)	%	Increase (g.)	%
Test group (On 10% petroyeast*)	90	80	101	1772	1671	100	255	115	867	96	549	101
Control	80	88	103	1772	1667	100	222	100	901	100	546	100

* *C. lipolytica* from n-paraffin treated by alkaline and alcoholic extractions.

Table 11

Compositions of the breast muscle of experimental chickens employed for toxicity tests on n-paraffin yeast (*C. lipolytica*) preliminarily treated with alkaline and alcoholic extractions

Group of chickens	Moisture (%)	Protein (%)	Fat (%)	Ash (%)
Petropeast (10%)	74.55	23.37	0.39	1.11
Control	74.93	23.27	0.34	1.13

Table 12

Effect of different preliminary treatments of petroyeasts on experimental pigs in respect of ration consumption and daily weight increase

Groups	Average daily weight increase per pig				Average daily consumption per pig	
	Start weight (kg)	Final weight (kg)	Daily increase (kg)	%	Quantity (kg)	%
n Paraffin yeast* (25%)	24.1	71	0.4	99	1.35	100
Gas-oil yeast** (25%)	24	58.8	0.29	70	1.26	92
Gas-oil yeast*** (25%)	23.6	76	0.14	107	1.23	100
Control	23.8	78.3	0.41	100	1.33	100

* *C. rugosa* from n-paraffin.
** *C. rugosa* from gas-oil, treated by alkaline and petroleum ether extractions.
*** *C. rugosa* from gas-oil, treated by alkaline and alcoholic extractions.

Table 13

3,4-Benzopyrene contents of experimental chickens and pigs employed for n-paraffin yeast (*C. lipolytica*) tests

Groups	3,4-Benzopyrene contents (ppb)*			
	Chickens		Pork	
	Fat	Breast muscle	Lean	Subcutaneous fatty tissue
Test group (on 10% petroyeast)	0.125	1.25	0.25	1.0
Control group	0.25	0.75	0.50	0.5

* 3,4-Benzopyrene was determined according to the method given in J. A. O. A. C., 1958, 194.51, p. 888

Table 14

Effect of yeast-feeding on the liquid unsaturated fatty acid contents of the adipose tissue of experimental pigs

Groups		Number of pigs	Start age (months)	Duration of experiment (months)	Yeast added (%)	$\frac{\text{Liquid unsat. acids}}{\text{Total fatty acids}} \times 100$ (%)
Test Groups	1	5	3	4	Petroyeast* (25)	57.4
	2	5	3	4△	Petroyeast* (25)	57.4
	3	5	3	4	Molasses yeast** (25)	54.2
	4	5	3	4	Molasses yeast*** (25)	54.9
Control group		5	3	4	0	55.8

* *C. tropicalis* from n-paraffin.
** *C. tropicalis* from molasses.
*** *Mac. cerevisiae* from molasses.
△ During the second half period, the test pigs were shifted to the control ration.

Fig. 1 The gas chromatography of the methyl fatty acids of *C. hyaginoides* from a plankton

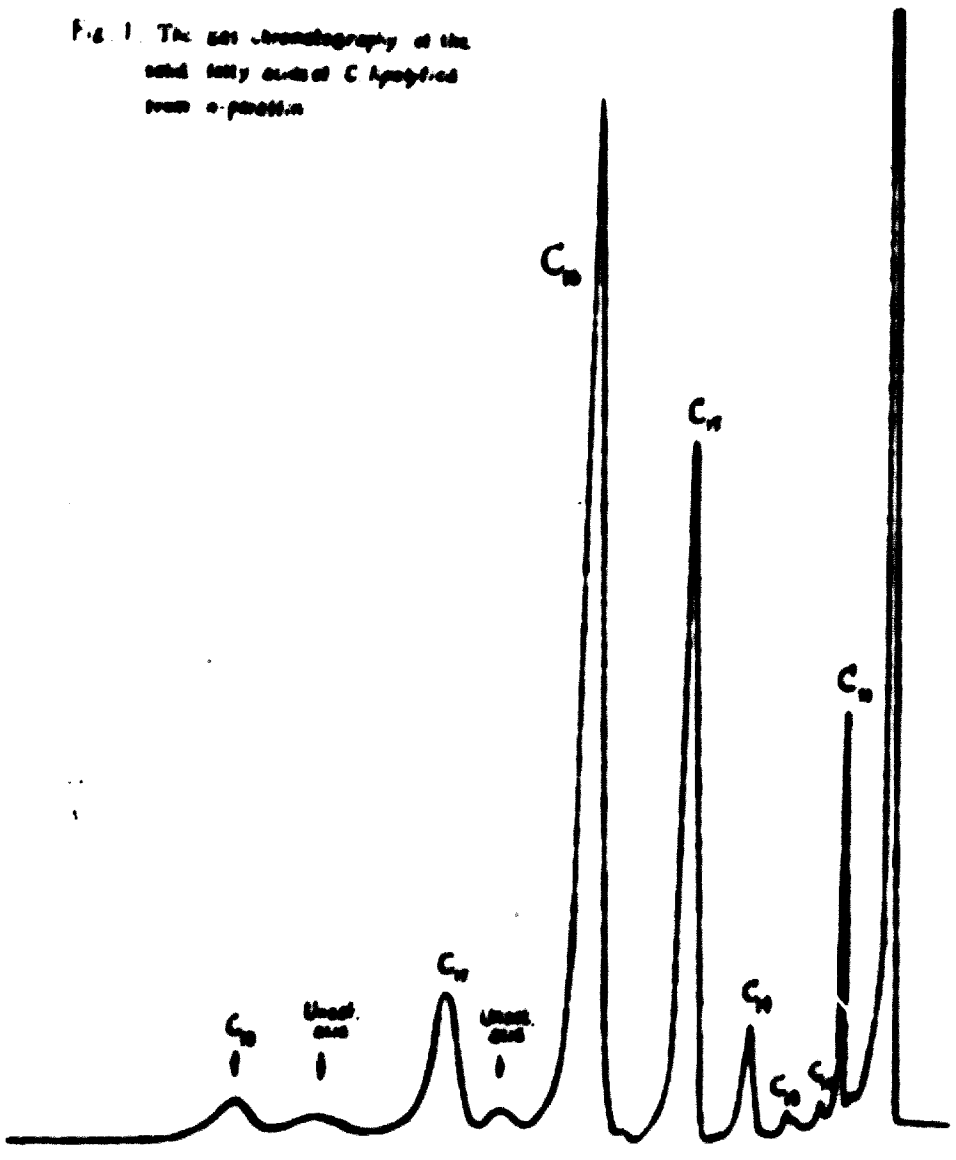


Fig. 2. The gas chromatography of the solid fatty acids of the adipose tissue of control pigs.

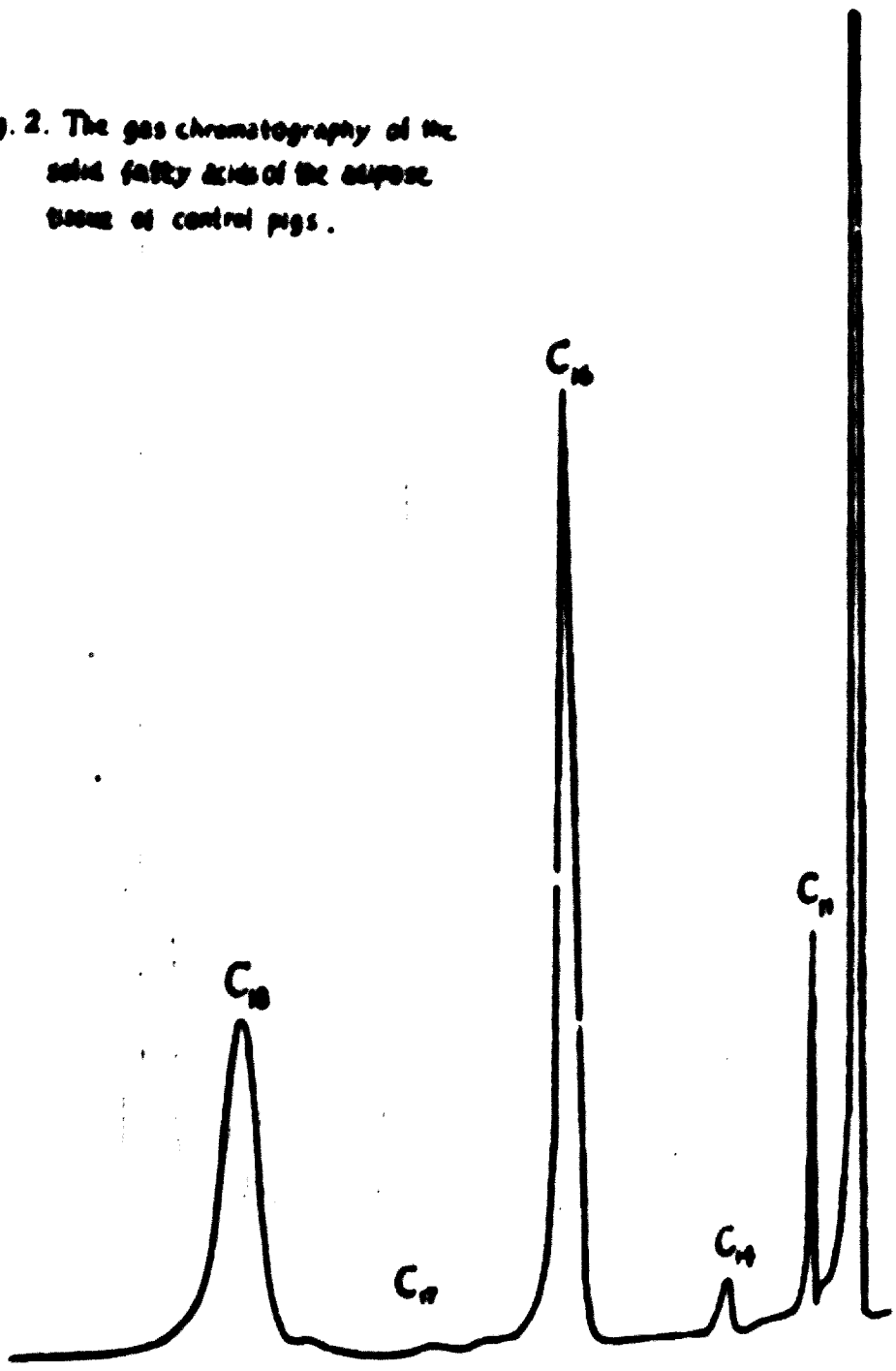
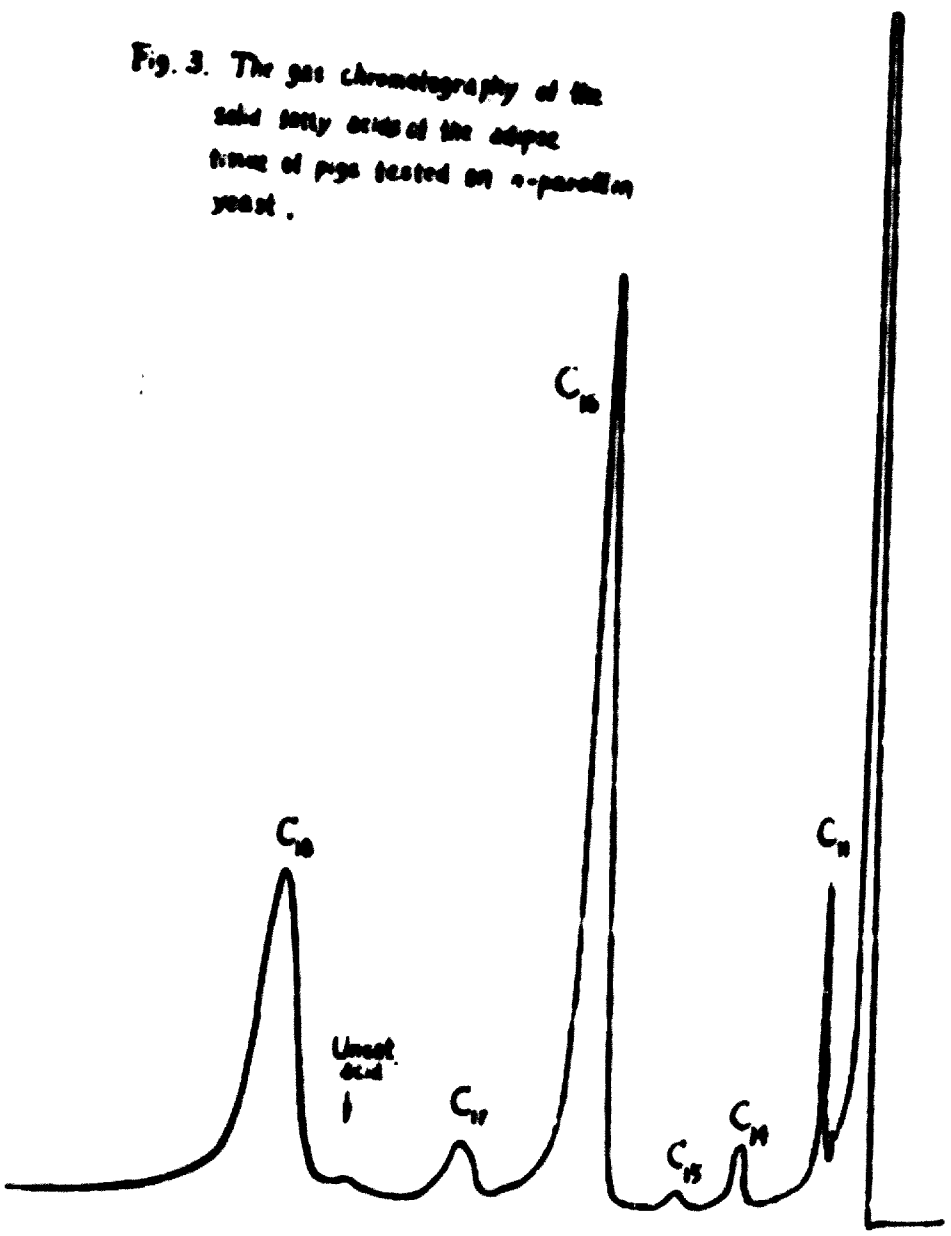
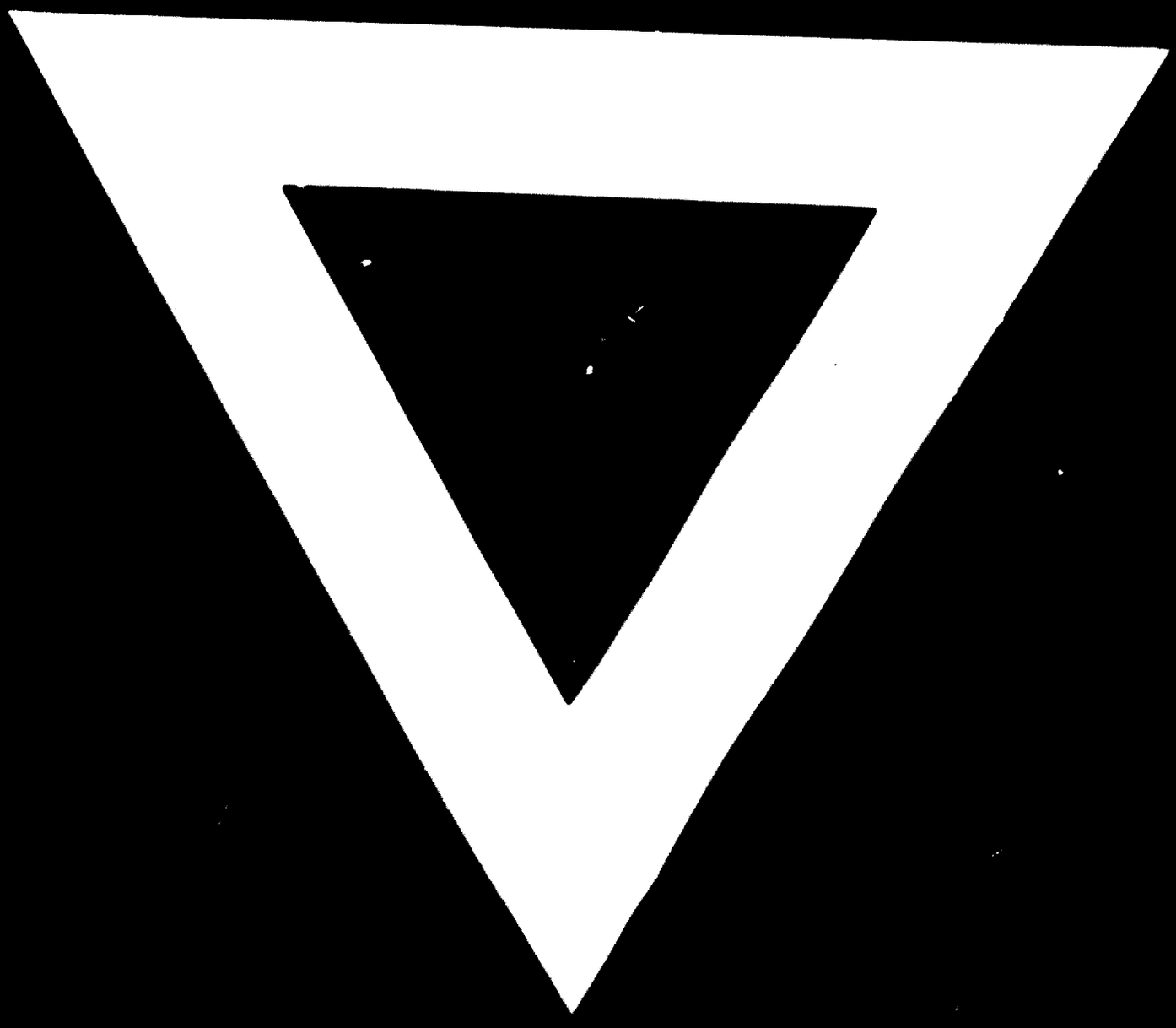


Fig. 3. The gas chromatography of the total fatty acids of the adipose tissue of pigs fed on n-paraffin yeast.





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