SUMMARY

1. Preformed mycelial felts of *A. niger* treated with cyanide at four concentrations between 0.006 and 0.040M, utilized more sugar, synthesized more mycelial substance and formed a greater total of acids (citric, oxalic and gluconic) in a given period than did controls. The most effective concentration used was 0.020M.

2. In presence of 0.010 or 0.020M cyanide the yields of all products were not only actually greater than with the controls but were also greater when expressed as percentages on the weight of sugar consumed.

3. The breakdown of citric acid by *A. niger* is improved by cyanide.

4. Possible modes of action of the cyanide are discussed.

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Heat Transformation Products of Cottonseed Oil

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The high incidence of cancer of the stomach has indicated the possibility that heated foodstuffs contain a carcinogen. However, in this laboratory examination of fats heated to relatively high temperatures in cooking has not revealed the presence of agents of the aromatic hydrocarbon type, which show characteristic fluorescence. Biological tests of the carcinogenicity of heated cottonseed-oil products by feeding experiments on mice have been described by Peacock, Beck & Chalmers (1953) and additional tests made by subcutaneous injection into mice will be described later in this communication.

In the feeding experiments to which reference has been made, the following cottonseed-oil products have been used: (1) Cottonseed oil heated at the b.p. (350°) for 4 hr. (2) Cottonseed oil heated at 320° for 2.5 hr. in the presence of iron, which favours ketonization of the oil.

Considering all tests carried out in this biological investigation, in 268 animals surviving 400 days and over, there were eighteen papillomata and six carcinomata of the forestomach. Although this is a smaller percentage than that which might be expected to follow the administration of a carcinogenic hydrocarbon, it is similar to the percentage of deaths in the human species due to gastric cancer. The present work was undertaken in an attempt to determine the nature of the heated products used in

these biological tests. For a preliminary communication of this work see Chalmers (1952).

EXPERIMENTAL AND RESULTS

Apparatus. In the earlier biological experiments (Peacock *et al.* 1953), the oil was heated in a stream of N_2 in a flask fitted with an electrically heated column as described for use in the distillation of fatty esters (Hilditch, 1947). In the present experiments, owing to difficulties due to frothing, the heated column was replaced by a vacuum-jacketed column (3 ft. $\times$ 2 in. diam.). A small receiver was inserted immediately above the vacuum jacket, and water formed during the reaction together with a small quantity of liquor smelling strongly of acrolein were run off. The still-head carried a jet for the admission of N_2 in case of frothing: the main receiver was attached directly to the still-head. The path to the receiver and exhaust was about 1 in. wide.

Injection experiments. In addition to the feeding experiments to which reference has been made, several of the cottonseed-oil products were tested by subcutaneous injection, either alone or in solution in tricaprilyn, into mice which were 3 months old at the start of the experiment (Table 1). Some of these experiments are still in progress. A number of the animals died within the first year of the experiment, owing to an intercurrent infection. In Expts. 3 and 4 few of the mice lived much longer than 12 months and the results of the tests are therefore inconclusive. There has been no obvious reaction at the site of injection in any of these experiments, although in some of the older animals remote tumours were observed.

Table 1. *Biological tests with heated cottonseed-oil products*

(For experimental details see text.)

Expt. no.	Temperature and duration of heating	Product tested	Mice surviving at (months)				Remote tumours observed
			12	15	18	24	
1	350° for 4-6 hr.	Crude product and unsaponifiable fraction	14	14	14	7	Mammary adenoma after 18 months. Hepatoma after 24 months. Lung adenocarcinoma after 24 months
2	320° for 2-3 hr. (+ iron)	Crude product	13	10	10	4	Lung adenoma after 23 months. Lung adenoma and hepatoma after 27 months. Squamous carcinoma of skin and generalized lymphomatosis of lymph nodes and lungs after 25 months
3	300° for 22-25 hr.	Crude product	8	2	2	0	Lung adenoma after 18 months
4	Peroxidized at room temperature	Crude product	8	4	2	0	Lung adenoma after 22 months

Decarboxylation of cottonseed oil. When testing for the presence of CO_2 , the volatile products of pyrolysis carried over in the stream of N_2 were led from the still-head through a wash bottle containing aqueous $\text{Ca}(\text{OH})_2$.

Piper *et al.* (1931) described the preparation of certain aliphatic ketones by heating the corresponding fatty acids under reflux at temperatures in the region of 300° in the presence of an iron ring, and it was anticipated that heating cottonseed oil in the presence of iron would result in the formation of ketonic products. In the present experiments, it was found that in the presence of iron filings a vigorous reaction was reached after 0.5 hr. at 340°, after 1.25 hr. at 320° or after 4 hr. at 300°. Iron strips catalysed the decarboxylation reaction, although not quite so effectively as iron filings. The surface area of the iron strips used was equal to that of an iron vessel capable of holding the volume of oil heated. It was considered that the results of this experiment would be comparable with those of Grün, Ulbrich & Krezil (1926) who prepared aliphatic ketones by heating the corresponding fatty acids in an iron vessel and also with ordinary domestic cooking in iron utensils. On heating cottonseed oil alone under the conditions described above, CO_2 was first detected at 345°; in the presence of iron strips it was detected at 305° and in the presence of iron filings at 250°.

Polymerization of cottonseed oil. In the present experiments cottonseed oil was heated at 300° in an atmosphere of N_2 . There was a steady increase in viscosity on heating up to 20 hr., between 20 and 25 hr. there was a sharp rise and from 25 to 35 hr. the steady rate of increase was resumed. Fig. 1 shows changes in iodine and free fatty acid values during polymerization.

The polymerization of a number of highly unsaturated oils, including cottonseed oil, by heating at 280° for periods up to 20 hr. has been described by Lips, Promislow & Grace (1953). Under the conditions used by these workers, free fatty acid values remained low and the changes in viscosity and iodine value were lower than those found in the present experiments.

Peroxidation of cottonseed oil. This was done by passing air through a sample of cottonseed oil at room temperature until a high peroxide value was obtained. The sample used

Fig. 1. Iodine and free fatty acid values during polymerization of cottonseed oil.

for biological test had a peroxide value of 165 and a free fatty acid value of 4.3%.

Fractionation of heated cottonseed oil. The heated product (100 g.) was well stirred with hot acetone (1 l.) and allowed to stand overnight at room temperature. 2N-NaOH was used to separate the free fatty acid (FFA) from an ether solution of the acetone-soluble material. Saponification of the remainder of the acetone-soluble material gave acid (FA2) and unsaponifiable (U1) fractions. The acetone-insoluble fraction of the heated product was also saponified giving acid (FA3) and unsaponifiable (U2) fractions. An examination has been made in this way of the composition of the heated cottonseed-oil products used in the biological tests and a comparison made with cottonseed oil polymerized by heating at 300° for 22 hr. The results shown in Table 2 are typical, but there were variations in the results for samples heated on different occasions.

Polymerized cottonseed oil. An examination of the polymeric material in the acidic products has been made, using the method of Cowan, Falkenburg & Teeter (1944). The methyl ester of the acid was distilled in an alembic-type still under reduced pressure and the distillate collected in small fractions. The refractive index of the fractions was determined and plotted against the total percentage distilled up to the mid-point of the fraction. The acetone-insoluble fraction (FA3) of the oil heated at 350° for 4 hr., the free fatty acids (FFA) and the fatty acids of the neutral oil (FA2) were examined and found to contain polymeric

Table 2. *Comparison of heated cottonseed-oil products*

(There was a significant production of free fatty acid on polymerizing cottonseed oil at 300°. At 350°, less polymer (acetone-insoluble fraction) was produced and there was a corresponding increase in free fatty acid and unsaponifiable material (acetone-soluble fraction). Heating in the presence of iron filings resulted in the production of acetone-soluble and acetone-insoluble unsaponifiable material. Some of these fractions were ketonic but there was also a large amount of chemically inert material of a tarry nature.)

Temperature and duration of heating	Acetone-soluble fraction			Acetone-insoluble fraction	
	Free acids		Unsaponifiable (%)	Free acids,	Unsaponifiable (%)
	FFA (%)	FA 2 (%)		FA 2 (%)	
300° for 22 hr.	18	10	1.5	60	2
350° for 4 hr.	48	11	11	18	4
320° for 2.5 hr. (+ iron)	2	0	35	0	62

Table 3. *Analysis of methyl esters of heated cottonseed oil*

(The figures in parentheses are percentages of the total heated product. For other details see text.)

		Monomer	Intermediate fraction	Dimer	Non-volatile residue
Fraction of heated product (350° for 4 hr.)					
Free fatty acid, FFA (48%)	Yield (%)	40	10	15	30
	Mol.wt.	308		531	1205
	n_D^{30}	1.4260–		1.4775	1.4925
		1.4435			
Neutral oil, FA 2 (11%)	Yield (%)	50	15	10	25
	Mol.wt.	329		466	1263
	n_D^{30}	1.4325–		1.4805	1.5020
		1.4445			
Acetone-insoluble polymer, FA 3 (18%)	Yield (%)	15	10	7	67
	Mol.wt.	309		544	934
	n_D^{30}	1.4330–		1.4780	1.4970
		1.4470			
Fraction of heated product (300° for 22 hr.)					
Acetone-insoluble polymer, FA 3 (60%)	Yield (%)	35	15	15	32
	Mol.wt.			440	932
	n_D^{30}	1.4355–		1.4750	1.4900
		1.4450			

material. The results of these distillations and, for comparative purposes, of the distillation of the FA 3 fraction of cottonseed oil polymerized by heating at 300° for 22 hr., are shown in Table 3. The acetone-insoluble polymeric fraction of cottonseed oil heated at 350° contained less monomer, less dimer, and more non-volatile polymeric material than the product heated at 300°. The free fatty acid fraction of the oil heated at 350° contained a significant amount of polymeric material and the importance of this is increased by the fact that almost half of this heated product was free fatty acid. The polymerization of the free fatty acid from soy-bean oil has been described by Croston, Tubb, Cowan & Teeter (1952) and presumably in the present case the fatty acid liberated on heating was subsequently polymerized. The neutral-oil fraction was also shown to contain polymeric material.

Cooking tests. Chang, Tchen & Watts (1952) examined the effect of deep-fat frying on the component fatty acids of lard and corn oil. They found that the iodine value of the corn oil dropped from 126.1 to 122.1 after 4 hr. at 180° and that the percentage of conjugated diene showed a slight increase. Changes in fatty acid values were slight, the most marked being shown by linoleic acid, which dropped from 55.0 to 52.2%.

In this laboratory a sample of cottonseed oil which had been used in a local factory in frying potato crisps has been examined by the methods which have been described. In the factory the fat was heated in a deep iron pan to a temperature of about 250°. The sample did not contain any acetone-insoluble polymeric material and the methyl ester of the fatty acids from the neutral oil behaved similarly to the unheated cottonseed oil on distillation in the alembic still, consisting almost entirely of monomer.

In a further experiment potatoes were fried in cottonseed oil in the laboratory. In view of the catalytic effect of iron referred to earlier in this paper, an iron and an aluminium pan were used in parallel experiments. One litre of cottonseed oil was used for each experiment and when the temperature reached 190° the potatoes were added. The temperature dropped to 120° and remained low for about 15 min. Subsequently the temperature rose steadily to 140° at which point the chip potatoes were cooked brown. The time of heating was approx. 30 min. The operation was repeated using the same oil until the total time of heating was 7 hr. An analysis of the oil remaining and of the oil used commercially gave the results shown in Table 4. In the laboratory experiments, the reduction in the iodine value of the used oil was greater than the corresponding increase in free

Table 4. *Effect of potato-frying temperatures on cottonseed oil*

(For details see text.)

Test	Unused cottonseed oil	Cottonseed oil used in frying potatoes		
		Commercially (iron pan)	Laboratory (iron pan)	Laboratory (aluminium pan)
Solubility in acetone	Soluble	Soluble	Soluble	Soluble
Iodine value	104.4	100.1	91.4	89.2
Free fatty acid (%)	None	0.85	0.38	0.33
Peroxide value	1.2	4.7	13.3	9.9
Refractive index (n_D^{20})	1.4680	1.4685	1.4710	1.4720

fatty acid content when compared with oil heated at 300°. In this respect the commercially heated oil occupied an intermediate position. The laboratory heated oil was also oxidized to a greater extent than the commercially heated oil but this was possibly due to the exposure of a relatively greater surface to air. It was noted that the refractive index values varied with the iodine value. Distillation of the fatty acid methyl esters from the neutral fraction of cottonseed oil which had been used commercially in frying potatoes gave no evidence of polymer formation.

DISCUSSION

There is evidence that distillation of the glycerides of saturated fatty acids in the presence of iron converts the fatty acids into the corresponding ketones. Holleman & Koolhaas (1939) distilled coconut oil in the presence of iron and isolated an aliphatic ketone from the distillate. They concluded that small quantities of higher aliphatic ketones are generally formed when food is fried.

There is also evidence that on heating cottonseed oil at 320–340° in the presence of iron the palmitic acid fraction of the oil is converted into palmitone (Chalmers, 1951). In previous experiments, cottonseed oil was distilled in the presence of iron and the solid distillate fractionated by treatment with ethanol. In the higher melting fraction palmitic acid was identified. It was presumed that the fatty acid was first split from the fat and under suitable conditions, e.g. in the presence of iron at about 320°, converted into the corresponding ketone.

Subsequently it was suggested by Hendry, Homer, Rose & Walpole (1951) that ketonization of heated fat might take place with formation of glycidyl esters. Although heated fat products have been tested in this laboratory for the presence of glycidyl esters, with negative results, it is possible that the glycidyl esters are unstable intermediates.

In addition to palmitic acid, cottonseed oil contains oleic and linoleic acids as major components. Isomerization of these acids has been described by Bauer & Krallis (1931) who heated oleic acid in nitrogen at 250° and were able to identify an isomeric acid in the heated product. In the course of a study of the synthesis of triolein, Desnuelle & Naudet (1945) found that 'isoleic acid' was formed

in amounts varying from 5.1 to 11.8% when oleic acid or triolein was heated at 135–180°.

In a recent paper, Barker, Crawford & Hilditch (1951a) have described the interchange of acyl groups in triglycerides containing saturated and unsaturated acids. They heated oleodistearin at 300° in glass or silica vessels and after 10 hr. at this temperature the composition of the mixture was tristearin 25, oleodistearin 54.2, steardiolein 20.2 and triolein 0.6%. The production of free fatty acid was the only side reaction detected. Acyl migration in the presence of catalysts has been demonstrated by other workers in the case of the glycerides of soy-bean oil or cottonseed oil and similar results were obtained with mixtures of tripalmitin and triolein in the presence of stannous hydroxide at 225° (Norris & Mattil, 1946, 1947) or of tristearin and triolein at 205° in the presence of sodium methoxide (Naudet & Desnuelle, 1947).

The primary change which takes place on heating an oil containing polyethenoid acids is a rearrangement of unconjugated pentadienes to the corresponding conjugated dienes. This change, which thermally takes place at about 300°, can take place at a lower temperature in the presence of catalysts. Following the conjugation stage, polymerization of the oil takes place. The mechanism of polymerization is not as yet completely understood but is a subject that has received considerable study. Thus the kinetics of thermal polymerization of isomers of methyl linoleate have been investigated by Paschke, Jackson & Wheeler (1952) and chemical changes taking place on thermal polymerization of sunflower-seed and linseed oils have been described by Barker, Crawford & Hilditch (1951b). Sunflower-seed oil, like cottonseed oil, contains oleic and linoleic acids as major component unsaturated acids, and Hilditch and his co-workers consider that dimerization is effected by a single cross-linking between two carbon atoms in different linoleic acid radicals, intramolecularly in the earlier and intermolecularly in the later stages.

Polymerization increases the flavour stability of oils containing unsaturated acids but has been found to have a deleterious effect on the nutritional value. Lassen, Bacon & Dunn (1949) investigated

the digestibility of polymerized sardine oil incorporated in the diet of rats and found that the polymerized portion of the oil was not absorbed by the gastro-intestinal tract. Further work from a nutritional standpoint has been carried out by Crampton, Common, Farmer, Berryhill & Wiseblatt (1951) and Crampton, Common, Farmer, Wells & Crawford (1953), who fractionated polymerized linseed oil by distillation at low pressure and then by the capacity of the distillate to form urea adducts. When fed to rats, the non-distillable polymeric fraction was largely unabsorbed and the portion of the distillate which did not form urea adducts was found to be toxic. Chemical aspects of this thermal effect on the nutritive value of the polymerized oil have been examined by Wiseblatt, Wells & Common (1953) and by Wells & Common (1953). In the first paper the authors give analytical data, including changes in the fatty acid composition, for linseed oil polymerized by heating at 275° in an atmosphere of CO₂ for various times up to 17 hr. Analytical data are also presented for soy-bean oil heated at various time intervals up to 30 hr. It is suggested that the relationship between the values for polymeric acyl radicals and mean molecular weights of the heated oils indicates the presence of intrapolymers. In passing, it may be mentioned that, unlike in the present experiments with cottonseed oil, little free fatty acid was formed (0.69 % after 30 hr. heating of soy-bean oil). The second paper describes the fractionation of linseed oil polymerized as before by heating in CO₂ at 275° for 12 hr. Distillation of the ethyl esters at low pressure gave a monomeric distillate of which one-fifth was incapable of forming urea adducts. It is suggested, on the basis of fatty acid composition, that this fraction contains cyclic acids. Estimations of acetyl values and of the proportion of terminal groups excluded the presence of hydroxy acids and branched fatty acids.

The possible carcinogenicity of a mixed fat (equal parts of cottonseed oil and whale oil) which had been repeatedly heated at 200–220° was examined by Beck & Peacock (1941). The heated fat was added to an adequate basal diet containing 0.5 % cod liver oil and fed to rats and mice. Within a year signs of avitaminosis-A appeared among the rats and this was followed by gastropapillomatosis in the forestomach. The papillomatosis, however, could be avoided or cured by the addition of raw carrot to the heated fat diet. Beck & Peacock also showed by experiments in which rats were fed a diet supplemented by milk, carrot and heated lard that 'the vitamin-A content of the liver was reduced in proportion to the degree and duration of heating to which the lard was subjected'. They concluded that 'there is present in repeatedly heated fats a factor which interferes in some way with the absorption or metabolism of vitamin A'. It is therefore possible

that this condition was caused by the inefficient absorption of vitamin A from the diet due to its retention in the polymeric material of the heated lard which was, to a greater or less extent, unabsorbed.

In the present work consideration was given to the nature of the agent inducing tumours of the forestomach of mice fed a diet containing heated cottonseed oil (Peacock *et al.* 1953). The oil tested was heated (a) at 350° for 4 hr. and (b) at 320° in the presence of iron for 2.5 hr. These products were fractionated by treatment with acetone and by distillation of the fatty acid after methanolysis in an alembic still. For comparative purposes a sample of cottonseed oil polymerized by heating at 300° for 22 hr. was also examined. In the case of the oil heated at 350°, a high percentage of free fatty acid was formed and this fraction, as well as the acetone-soluble (monomeric acid) and acetone-insoluble (polymeric acid) fractions, contained appreciable amounts of undistillable polymeric material. While the figures given for the oil heated at 320° in the presence of iron indicate that this product is mainly unsaponifiable, some of the samples used in the biological tests contained polymers. Moreover, the unsaponifiable fractions contained, in addition to ketonic material, chemically inert substances of a tarry nature which presumably were decomposition products of polymeric material. In a preliminary account of this work (Chalmers, 1952) the presence of polymers in heated cottonseed-oil products was stressed.

No tumours occurred at the site of injection in the present experiments with cottonseed oil polymerized at 300° or oil heated at 350° for 4 hr. and at 320° for 2.5 hr. in the presence of iron. The experiments with oil polymerized at 300° were inconclusive since many of the animals under test died at a comparatively early age due to an infection. However, in the test with oil heated at 350°, which also contained polymeric material, more animals were used and there was a good survival rate. In this experiment, also, no tumours were found at the site of injection. In the feeding tests, where the route of administration is more satisfactory (Peacock *et al.* 1953), cottonseed oil heated at 350° gave fewer tumours of the forestomach of mice than the product heated at 320° in the presence of iron. It therefore appears that the substance responsible for the induction of tumours of the forestomach of mice fed a heated fat diet is a weak carcinogenic agent. Although there is at present no conclusive evidence concerning the nature of this carcinogen, it is possible that it may be a polymer.

In considering the abnormal products in heated cottonseed oil from the point of view of possible carcinogenicity, reference may be made to the sterol fraction of the oil which, although small in

amount, may be important in view of the carcinogenic activity of cholesterol-rich extracts from biological sources (Hieger, 1947). It is also possible that small quantities of polymeric acyl radicals are formed during potato frying as carried out in the laboratory, although the heated fat did not contain demonstrable amounts of polymeric material. Another possibility would be the formation in heated fats of compounds of a 'cyclic' type, the toxicity of which was described by Crampton *et al.* (1953) in the case of heated linseed oil.

Although there is no evidence associating stearoylethyleneimine with heated fat products, it is interesting that it has been suggested that polymerization plays a part in the action of this synthetic carcinogen in the cell. It has been postulated by Hendry *et al.* (1951) that cytotoxic agents condense intermolecularly within the cell with the formation of polymer units with chemically reactive side chains, which then combine with the protein or nucleoprotein components of the cell. These authors suggested that certain carcinogens acted in the same way and prepared stearoylethyleneimine, which was regarded as a micelle-forming substance capable of reacting with cell components, and showed it to be carcinogenic.

SUMMARY

1. An analysis has been made of cottonseed oil heated (a) at 350° for 4 hr. and (b) at 320° for 2.5 hr. in the presence of iron. These products, which have been reported to induce tumours of the forestomach of mice, were fractionated by treatment with acetone and by distillation in an alembic-type still. Product (a) contained polymeric material in both the acetone-soluble and acetone-insoluble fractions. The major component of (b) was unsaponifiable material.

2. Subcutaneous injections into mice of these products and also of polymerized and peroxidized cottonseed oil have not so far, after more than a year, induced any tumours at the site of injection.

3. The changes observed in cottonseed oil used in frying potatoes were comparatively slight and polymerized products were not detected.

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