CXXV. THE PRODUCTION OF FAT FROM CARBOHYDRATE AND SIMILAR MEDIA BY A SPECIES OF *PENICILLIUM*.

By HAROLD HAYDEN BARBER.

From the Department of Chemistry, University College, Nottingham.

(Received September 20th, 1929.)

WHILST it is generally considered that the origin of fats found in the vegetable kingdom is some form of carbohydrate, the mode of formation of the numerous fatty acids from relatively few carbohydrates is still a matter of speculation and the subject of considerable investigation.

The two principal views advanced to explain the occurrence of the various fatty acids in vegetable fats and oils are somewhat opposed, but both assume that the parent substance is glucose either as such or in the form of starch.

E. Fischer appears to have been the first to suggest that glucose or other hexose molecules are condensed, and, after oxidation and reduction, give higher fatty acids directly, thus accounting for the predominance of C_{18} fatty acids in natural fats and oils. From the C_{18} acids the formation in the animal body of palmitic acid and the lower members of the series has been explained by the β -oxidation theory of Knoop [1904], Dakin [1908, 1, 2, 3; 1909, 1, 2] and Embden *et al.* [1906]. This explanation accords with the fact of the almost universal occurrence in natural fats of acids containing an even number of carbon atoms. Later it was suggested by E. F. Armstrong and Allen [1924] that the C_{18} unit of starch rather than three molecules of glucose might be utilised by the plant to give C_{18} acids more directly, a change involving disruption of the oxygen linkage in the starch molecule followed by a direct union of the carbon atoms.

The other view of the mode of formation of vegetable fatty acids, and one which gives a more satisfactory explanation of the presence of the lower acids, is that glucose is broken down into acetaldehyde [Nencki, 1878; Hoppe-Seyler, 1879] and by aldol condensation the fatty acid skeleton is built up step by step by the addition of carbon atoms two at a time [Magnus Levy, 1902]. The experiments of Raper [1907] confirm this view so far as the lower fatty acids are concerned. A similar suggestion has been advanced by Smedley [1911] and by Smedley and Lubrzenska [1913], according to whom acetaldehyde—derived from pyruvic acid—condenses with a second molecule of pyruvic acid to give an unsaturated α -keto-acid, which on losing carbon dioxide gives

crotonaldehyde. The crotonaldehyde by similar condensations with pyruvic acid gives higher unsaturated aldehydes which are finally oxidised to acids of varying length of carbon chain and of varying degree of unsaturation.

A third view more recently advanced by Smedley-MacLean and Hoffert [1926] appears to reconcile the above two hypotheses by the suggestion, based on experiments with yeast, that acetaldehyde is first converted into hexose and the latter directly condensed to form higher fatty acids without passing through a fatty aldehyde stage, probably by a direct linking of the hexose molecules in a manner not yet known or suggested.

In the course of an investigation on the products of the growth of a pure culture of a species of *Penicillium* on various common carbohydrate media, the author [1927] considered the possibility of testing the general theory of direct fatty acid synthesis from hexose molecules by the method of cultivating the mould on solutions of individual sugars as the sole source of carbon and comparing the nature of the fatty products in relation to the carbohydrate employed. It follows that if the mechanism of fatty acid synthesis is direct combination of glucose molecules to give C_{18} fatty acids, normal growth on a pentose medium, if fatty acids are produced at all, should not yield C_{18} acids, although possibly C_{15} , C_{10} or C_5 acids might be produced.

The organism in question was found by preliminary experiments to grow on both xylose and arabinose as readily and as freely as on glucose or sucrose. Xylose was selected for the investigation because of the identity of its configuration with that of the α , β and γ carbon atoms of glucose. Mass culture of the mould on this medium yielded fat in amount similar to that produced from glucose or sucrose. The fatty acids present, whichever medium was employed, were found to consist of a mixture of palmitic, stearic, oleic and α - and β -linoleic acids—linolenic acid being definitely absent. Free acids were present to some extent and resembled those combined as glycerides. Small amounts of sterols were isolated, which will be the subject of a separate investigation. The saturated acids, as indicated by their mean molecular weights and melting points, were apparently a mixture of palmitic and stearic acids.

From time to time several workers have claimed the isolation of daturic acid from various natural sources, and in view of the fact that Dieterle, Diester and Thimann [1927] report the presence of daturic acid in the oil of *Secale cornutum* it was thought desirable that the saturated acids should be further examined. Since the usual methods of differentiation are difficult to apply in any case and are obviously out of the question where the amount of material is so small, recourse was had to X-ray analysis, and through the kindness of Mr Piper of Bristol University, who examined three samples of acids by this means, it was found (*a*) that the combined saturated fatty acids from glucose, xylose and glycerol were all identical, and (*b*) that all these mixtures showed distinct evidence of the presence not of a C_{15} acid but of one containing 19 carbon atoms, together with stearic acid.

Examination of the spent or partially spent medium yielded very little except unchanged carbohydrate and traces of a yellow solid obtained by prolonged extraction of the filtered medium with ether. This substance consisted of a neutral ester and an acid of mean molecular weight (by titration) of 160 to 200; both acid and ester were insoluble in light petroleum but soluble in ether and readily soluble in alcohol. Oxalic acid and volatile acids were entirely absent.

In the case of glycerol growth proceeded much more slowly than on either the pentose or hexose.

EXPERIMENTAL.

The particular species of *Penicillium* used in this investigation was isolated by the author from various moulds found growing on maize-starch pastes used in the finishing of cotton textile fabrics.

A single spore culture was obtained in the usual manner by means of successive dilutions in sterile water and plating on to a 5 % sucrose—2 % agar medium containing Czapek's solution, the same medium in tubes being used for preserving the stock cultures. During the course of the work the stock culture was plated out from time to time in order to ascertain and ensure its continued purity.

Pure cultures of the *Penicillium* were grown in round flat-bottomed litre flasks each containing 500 cc. of liquid. The medium, containing 3, 5, 10 or 20 % of the carbohydrate with the addition of Czapek's solution was sterilised in moist steam at 95–100° on three successive days and the flasks kept for at least 10 days before inoculation. The xylose used was Difco brand and had m.p. 149–149.5°.

The temperature generally employed for cultivation was 21° since it was found that at temperatures only a few degrees lower, *e.g.* 17°, growth was much retarded in every case.

After several weeks, when a thick surface mat had been formed, this was removed, washed in water and partially dried between several layers of filter paper and finally *in vacuo* over sulphuric acid. Meanwhile, the filtered culture medium, which was of a deep orange-red colour, was extracted six times with ether and the ethereal solution washed twice with water. This ether extract gave on evaporation in a stream of dry carbon dioxide a brown solid substance insoluble in light petroleum (40–50°). The total yield was about 0.1–0.8 per litre of culture medium. The substance when treated with dilute sodium hydroxide solution and ether gave a neutral ester and an acid. The ester, which was insoluble in light petroleum, on saponification yielded acids also insoluble in petroleum and resembling the free acid originally present. These acids gave dark-coloured blue-black solutions in dilute sodium carbonate solution from which the acids were precipitated as a yellow solid on adding dilute sulphuric acid. By extraction with ether they were obtained as a dark brown fatty solid, melting above 100°. Determination of mean molecular weight by titration

with $N/10$ sodium hydroxide solution in neutral alcohol with phenolphthalein gave the following values:

0.165 g. acids from 5 % glucose medium. Mean molecular weight 172.

0.032 g. acids from 10 % glucose medium. Mean molecular weight 160.

0.447 g. acids from 5, 10 and 20 % sucrose medium. Mean molecular weight 198.

On oxidation with moderately concentrated nitric acid the principal product was oxalic acid.

The yields of surface mat etc. are summarised in Table I.

Table I. *Yield of surface growth, etc.*

No. of flasks each con- taining 500 cc. medium	Concen- tration of sugar (%)	Temp. ° C.	Duration of growth (days)	Total ether extract from medium g.	Dry surface mat	
					g.	g. per flask
Glucose:						
4	5	17	98	0.09	9.0	2.25
4	5	17	34	0.03	9.0	2.25
8	10	17	34	0.14	15.8	2.00
4	5	21	21	0.36	15.0	3.75
5	5	21	21	0.36	15.0	3.75
8	5	21	35	0.59	35.0	4.38
Sucrose:						
7	5	21	28	—	26.0	3.70
4	5	21	34	0.26	13.0	3.25
4	10	21	34	0.27	10.0	2.50
4	20	21	34	0.33	12.0	3.00
8	5	21	35	—	30.0	3.75
Glycerol:						
8	10	21	80	—	21.0	2.63
3	10	21	87	1.17	9.0	3.00
8	10	21	96	2.40	22.0	2.75
8	10	21	201	—	50.0	6.25
Xylose:						
4	3	21	33	0.16	13.0	3.25
6	3	21	60	0.32	21.0	3.50

The dry surface mat, finely powdered and extracted with dry ether in a Soxhlet apparatus yielded a pale yellow viscous oil. This was treated with dilute sodium hydroxide solution and ether to remove free acids as soaps, the latter were then extracted with ether after acidifying their aqueous solutions with dilute sulphuric acid. The free acids were pale yellow solids and on treatment with light petroleum yielded a very small proportion of darker insoluble acids presumably hydroxy-acids. The free fatty acids taken up by the light petroleum were examined as regards their melting points, mean molecular weights and iodine values in the manner described below for the examination of the combined fatty acids.

The neutral fat after removal of free acids was quantitatively saponified with $N/2$ alcoholic potassium hydroxide solution and after removal of the alcohol in a stream of carbon dioxide on the water-bath the solution was made alkaline with dilute sodium hydroxide solution and the sterols etc. removed by extraction three times with ether. The extracted material appeared to be sparingly soluble in ether and separated as a fine white powder at the interface

of the two liquids. The total ether extract was finally washed with water till free from alkali. The ethereal solution and suspension were then run into a weighed beaker, evaporated and the weight of the residue taken as "crude sterols." From the aqueous soap solution the combined acids were obtained by acidifying with dilute sulphuric acid and extracting with ether. The ethereal solution after being washed was evaporated and the residue of combined acids was taken up in light petroleum leaving traces of insoluble hydroxy-acids. The combined fatty acids after determination of their melting point were titrated with $N/10$ sodium hydroxide solution and phenolphthalein in the presence of at least 50 % by volume of neutral alcohol and their mean molecular weight thus obtained. The iodine value (Wijs) was then determined on a portion of the acids and another portion was brominated in ethereal solution at 0° . No insoluble hexabromides were ever obtained, but on the removal of the ether and the addition of light petroleum ($40-50^\circ$), boiling and filtering, the tetrabromide of α -linoleic acid was deposited from the cooled filtrate and after recrystallisation from alcohol melted at $114-115^\circ$. The fraction of the brominated acids which was soluble in cold light petroleum was weighed, titrated with $N/10$ sodium hydroxide solution and found to have a molecular weight indicative of the presence of soluble tetrabromides of β -linoleic acid.

These results are summarised in Table II.

Table II. *Fat from surface mat grown at 21° .*

Concentration of sugar (%)	Weight of mat extracted (g.)	Free fatty acids				Neutral fat and sterols		Combined fatty acids				Crude sterols % in neutral fat
		Weight (g.)	M.P.	Mean molecular weight	Iodine value (Wijs)	Weight (g.)	Sapn. value	M.P.	Mean molecular weight	Iodine value (Wijs)	α -Linoleic acid (%)	
Glucose:												
5	30	0.33	33	285	96	2.02	186	34	280	90	9.4	—
10	20	0.14	35	295	91	1.08	188	34	288	93	12.0	—
5	31	0.83	37.5	288	81	4.92	191	37	286	88	—	2.5
Sucrose:												
5	26	0.18	36	282	70	2.03	195	36	291	80	—	13.0
5	33	0.15	37	301	84	1.85	186	37	281	90	13.0	—
10	10	0.05	37	298	78	0.54	201	37	282	78	7.0	13.6
Glycerol:												
10	30	0.22	35	306	78	3.21	185	35	284	86	8.4	4.5
10	22	0.09	37	—	88	1.53	176	37-39	283	101	14.5	9.9
10	50	0.33	35-36	281*	—	5.45	184	32	289	98	7.5	7.9
Xylose:												
3	13	0.22	36	288	—	0.86	183	35	279	91	10.0	9.7
		0.12	37	279	—	0.77	189	38	280	—	—	5.8
3	21	0.31	36	292	76	1.15	166	35-36	296	80	—	22.1

* Solid fraction only.

The combined fatty acids were further examined by quantitative separation into solid and liquid fractions by the lead salt-alcohol method of Twitchell [1921]. The melting points and mean molecular weights of the saturated acids thus obtained were then determined. The results, summarised in Table III, suggest the presence of palmitic and stearic acids, but, whatever may be the ultimate composition of the mixture, there is no evidence of the formation of lower acids when xylose is employed.

Table III. *Saturated fatty acids.*

Source		M.P.	Mean molecular weight	% of saturated acids in fatty acids
Glucose	Combined acids	55	264	27
Sucrose	Combined acids	54	271	29
Glycerol	Combined acids	55	275	16
Glycerol	Free acids	55	281	22
Xylose	Combined acids	54	293	21

The X-ray measurements obtained by Mr Piper, indicating the presence of a C_{19} acid are as follows:

	How mounted	Spacings
Acids from glucose	Pressed	Two spacings show in third order not accurately measurable. One spacing 40-45 Å units.
	Melted	
glycerol	Pressed	Two spacings in third order one too faint to measure, the other 43-9 Å units. One spacing 41-15 Å units.
	Melted	
An equimolar mixture of C ₁₈ and C ₁₉ acids gives 43-5 pressed, 41-6 melted, suggesting that the lines photographed are due to these two acids with a high proportion of C ₁₈ .		
xylose	Pressed	Two spacings in third order too diffuse to measure. One spacing 43-5 Å units. (43-7 for equimolar C ₁₉ and C ₂₀ melted.)
	Melted	

DISCUSSION.

The remarkable constancy of the analytical values obtained for the fats produced by the organism from both pentose and hexose would appear to indicate that the mechanism of fatty acid synthesis is more complex than the mere condensation of monosaccharides.

Since the final products are identical it seems safe to assume that there is a common intermediate in each case and that the hexose and pentose are both broken down with approximately equal readiness to this substance before the process of actual synthesis begins, the actual synthesis then following a common course.

A similar relationship between pentoses and hexoses in biochemical synthesis by fungi is that shown by the recent work of Challenger, Klein and Walker [1929] on the production of kojic acid by *Aspergillus oryzae*. Whilst it was previously known that kojic acid was produced from glucose by various species of *Aspergillus* it was stated that none was obtained from pentoses. These workers show, however, that *A. oryzae* grows readily on xylose or arabinose,

giving, not pyromeconic acid, as might be expected, but kojic acid. This non-formation of pyromeconic acid, they point out, is remarkable, especially as the formation of kojic acid involves a breakdown of the pentose molecule and the synthesis of a 6-carbon compound.

What the intermediate compound may be, whether it is the same in fatty acid synthesis as in the formation of kojic acid, and whether it is a 3-carbon compound as suggested by the work of Peterson, Fred and Schmidt [1924] or the acetaldehyde of alcoholic fermentation is a problem still awaiting solution.

SUMMARY.

- (1) The species of *Penicillium* under investigation grows equally well on solutions of glucose, sucrose or xylose but less freely on glycerol.
- (2) The same fat or mixture of fats is produced in every case.
- (3) This fat contains palmitic, stearic, oleic and α - and β -linoleic acids both free and as glycerides together with some sterols.

The author wishes to express his thanks to the Chemical Society for a grant towards the cost of material, to the Council of University College, Nottingham, for a maintenance grant during a part of the period, to Mr Piper for his kindness in carrying out the X-ray examination, and to Professor F. S. Kipping, F.R.S., for his kind interest throughout this work.

REFERENCES.

- Armstrong and Allen (1924). *J. Soc. Chem. Ind.* **43**, 208 T.
Barber (1927). *J. Soc. Chem. Ind.* **46**, 200 T.
Challenger, Klein and Walker (1929). *J. Chem. Soc.* 1498.
Dakin (1908, 1). *J. Biol. Chem.* **4**, 419.
— (1908, 2). *J. Biol. Chem.* **5**, 173.
— (1908, 3). *J. Biol. Chem.* **5**, 303.
— (1909, 1). *J. Biol. Chem.* **6**, 203.
— (1909, 2). *J. Biol. Chem.* **6**, 221.
Dieterle, Diester and Thimann (1927). *Arch. Pharm.* **265**, 171.
Embden, Saloman and Schmidt (1906). *Beitr. chem. Physiol. Path.* **8**, 129.
Hoppe-Seyler (1879). *Z. physiol. Chem.* **3**, 351.
Knoop (1904). *Beitr. chem. Physiol. Path.* **6**, 150.
Magnus Levy (1902). *Arch. Anat. Physiol., physiol. Abth.* 365.
Nencki (1878). *J. prakt. Chem.* **17**, 105.
Peterson, Fred and Schmidt (1924). *J. Biol. Chem.* **60**, 627.
Raper (1907). *J. Chem. Soc.* **91**, 1831.
Smedley (1911). *J. Chem. Soc.* **99**, 1627.
— and Lubrzynska (1913). *Biochem. J.* **7**, 364.
Smedley-MacLean and Hoffert (1926). *Biochem. J.* **20**, 343.
Twitchell (1921). *J. Ind. Eng. Chem.* **13**, 806.