Downloaded from rstb.royalsocietypublishing.org

On the Production from Glucose by Penicillium spiculisporum Lehman of a New Polybasic Fatty Acid, C<latex>$_{17}$</latex>H<latex>$_{28}$</latex>O<latex>$_{6}$</latex> (The Lactone of <latex>γ</latex>-hydroxy-<latex>$\beta \delta$</latex>-dicarboxypentadecoic Acid)

Percival Walter Clutterbuck, Harold Raistrick and Margaret Lesley Rintoul

Phil. Trans. R. Soc. Lond. B 1931 **220**, 301-330
doi: 10.1098/rstb.1931.0027

Email alerting service Receive free email alerts when new articles cite this article - sign up in the box at the top right-hand corner of the article or click **here**

To subscribe to *Phil. Trans. R. Soc. Lond. B* go to: **http://rstb.royalsocietypublishing.org/subscriptions**

This journal is © 1931 The Royal Society

Downloaded from rstb.royalsocietypublishing.org

Studies in the Biochemistry of Micro-organisms.

PART XVI.—*On the production from Glucose by* Penicillium Spiculisporum LEHMAN *of a new polybasic fatty acid, $C_{17}H_{28}O_6$ (the lactone of γ-hydroxy-βδ-dicarboxypentadecoic acid).*

By PERCIVAL WALTER CLUTTERBUCK, HAROLD RAISTRICK *and* MARGARET LESLEY RINTOUL.

The acid described in this paper is a metabolic product of *P. spiculisporum* LEHMAN. This species was isolated by S. G. LEHMAN (1919) from the rootlets of apparently healthy cotton plants taken from a field in Anson County, N.C., U.S.A. It derives its name from the fact that "the walls of the ascospores bear minute spines." The culture used was obtained from the American Type Culture Collection and bears their Catalogue No. 1136, THOM and CHURCH Collection 4391.

It was noticed that, when cultures of *P. spiculisporum*, grown in test tubes on the usual CZAPEK-DOX medium, were filtered and acidified, an amorphous insoluble white precipitate was produced. This precipitate may be readily crystallised from boiling water and constitutes the product, the preparation, properties and constitution of which will now be described in detail. This product was shown to be the lactone of γ-hydroxy-βδ-dicarboxypentadecoic acid and for the sake of brevity *will be referred to throughout as Substance "A."*

It is worthy of mention here, as a further example of the extraordinary specificity of the lower fungi, that Substance "A" is produced only by *P. spiculisporum* and not by any other species either of *Penicillium* or of any genus in our collection.

Preparation of Substance "A."

Thirty litres of the modified CZAPEK-DOX glucose medium containing twenty times the amount of ferrous sulphate given in Part I, p. 7, were made up.

350 c.c. of this medium were distributed in each of a number of 1-litre conical flasks which were plugged with cotton wool, and sterilized by steaming for half an hour on each of three consecutive days. The flasks were then sown with a suspension in distilled water of the spores of *P. spiculisporum* grown in ROUX bottles on beer wort agar (or CZAPEK-DOX agar). The flasks were incubated at 27°–28° C. (and in one case at 37° C.) until the sugar content as determined by the polarimeter was less than 1 per cent. The following table is a typical example of the progress of the growth of the mould.

The Royal Society is collaborating with JSTOR to digitize, preserve, and extend access to
Philosophical Transactions of the Royal Society of London. Series B, Containing Papers of a Biological Character.
www.jstor.org

Downloaded from rstb.royalsocietypublishing.org

TABLE I.—Batch of flasks sown 31st March, 1928. Taken off 27th April, 1928.

Date of Testing.	Incubation Period in Days.	Percentage Glucose by Polarimeter.	Titration in c.c. N/1 NaOH per 250 c.c. Medium.
11.4.28	11	3·62	4·5
13.4.28	13	3·41	4·6
16.4.28	16	2·86	4·83
19.4.28	19	2·86	5·4
23.4.28	23	2·25	6·23
26.4.28	26	1·36	7·0
27.4.28	27	0·99	6·35

The metabolism solution was then filtered and acidified with HCl. This gave rise to a voluminous, light, white, amorphous precipitate which was filtered off on a large Buchner funnel and dried. The amount of material obtained from the metabolism solution depends largely on its final acidity and the temperature of incubation. If the final acidity is high and the temperature of incubation low a considerable proportion of Substance " A " is precipitated in the metabolism solution and hence is filtered off with the mycelium, from which it may be readily extracted by means of boiling water. Extraction is repeated two or three times and the boiling water extract slightly acidified with HCl. If the flasks are incubated at 37° C., however, and filtration is rapid a portion of the Substance " A " crystallises out from the filtered metabolism solution on cooling.

A further small amount of Substance " A " may be recovered from the filtered, acidified, metabolism solution either by evaporation *in vacuo* and extraction with ether, or by freezing in the cold room and filtering immediately the frozen solution has been thawed. The amount recovered by either of these methods is small, however, averaging about 5–10 per cent. of the total amount.

Identification of succinic acid as a metabolic product of P. spiculisporum.

The whole of the titratable acidity of the metabolism solution is not accounted for by Substance " A," the greater part being accounted for by the presence of succinic acid. This was proved as follows :—

A quantity of metabolism solution from which Substance " A " had been separated by acidifying with HCl and filtration was evaporated *in vacuo* and extracted with ether. On evaporation of the ether extract a quantity of white crystals remained. A portion of this was sublimed in a high vacuum and had then a melting point of 189° C. which was not changed on mixing with a sample of succinic acid. 0·0484 gm. of the same sublimed material required 8·32 c.c. of N/10 NaOH for neutralisation corresponding

Downloaded from rstb.royalsocietypublishing.org

to an equivalent weight of 58·2. (Theoretical for succinic acid = 59·0.) A further amount gave the following results on combustion :—

TABLE II.

Weight of Material Analysed.	Weight of CO_2.	Weight of H_2O.	Percentage Carbon.	Percentage Hydrogen.
Gm. 0·1585	Gm. 0·2366	Gm. 0·0744	40·70	5·25
Theoretical for $C_4H_6O_4$ ...	...	...	40·66	5·11

The yield of Substance "A" obtained under the conditions described is not very large. Details of the various batches prepared are given in the following tables :—

TABLE III.

Date of Sowing.	Incubation Period in Days.	Temperature of Incubation.	Apparatus.	Volume of CZAPEK-Dox 5 per cent. Glucose Solution used.	Weight of Substance "A" obtained.
				Litres.	Gm.
3.2.28	18	28° C.	44 flasks (750 c.c.)	11	10·0
10.3.28	17	28° C.	121 flasks	30	15·3
31.3.28	27	28° C.	169 flasks	60	23·7 (A further 3·2 gm. separated on freezing)
23.4.28	35	Room temp.	6 enamelled trays in aluminium incubator	30	6·4
	36	Room temp.	6 silica trays in aluminium incubator	30	29·5
7.5.28	22	27–28° C. (3 flasks were incubated at 37° C.)	86 flasks	30	13·6
5.6.28	17	37° C.	108 flasks	37	13·6
9.6.28	17	37° C.	110 flasks	35	12·6
10.7.28	17	37° C.	101 flasks	35	6·2
10.7.28	20	37° C.	108 flasks	35	6·8

Downloaded from rstb.royalsocietypublishing.org

TABLE IV.—Analysis of average sample from different batches.

Date of Sowing.	Percentage Glucose by Polarimeter.	Acidity in c.c. N/1 NaOH per 250 c.c. Medium.	Weight of Substance " A " precipitated from 250 c.c. of Acidified Medium Filtered through a Gooch Crucible and dried at 100° C.
3.2.28	0·782	4·3 c.c.	—
10.3.28	0·660	2·65 c.c.	—
31.3.28	0·994	6·35 c.c.	—
23.4.28 (enamelled trays)	0·218	5·28 c.c.	—
23.4.28 (silica trays)	0·935	5·53 c.c.	—
7.5.28	1·298	6·58 c.c. (Average of flasks incubated at 28° C.)	—
	0·715	9·23 c.c. 1 flask incubated at 37° C.	—
	0·875	8·8 c.c. 1 flask incubated at 37° C.	—
5.6.28	1·024	8·53 c.c.	0·0043 gm. after 6 days. 0·0823 gm. after 13 days. 0·0860 gm. after 15 days. 0·0604 gm. after 17 days.
9.6.28	1·051	8·12 c.c.	0·0352 gm. after 9 days. 0·0683 gm. after 17 days.
10.7.28	1·576	7·4 c.c.	0·0239 gm. after 15 days. 0·0389 gm. after 17 days.
10.7.28	0·825	7·5 c.c.	0·0415 gm. after 20 days.
9.3.29	1·009	5·59 c.c.	—

Purification of Substance " A."

The Substance " A " obtained by acidification of the metabolism solution is almost pure and homogeneous. A quite pure product may be obtained by recrystallising from boiling water with the addition of a little animal charcoal. Alternatively Substance " A " may be purified by recrystallising from boiling ethyl ether to which is added boiling light petroleum (boiling point 40°–45° C.) until crystallisation commences. It may also be recrystallised from alcohol, but is so readily soluble in this solvent that this method is not recommended.

Isolation of γ-ketopentadecoic acid from the mother liquors remaining after crystallisation of Substance " A."

The ether-light petroleum mother liquors from a long series of purifications of over 100 gm. of crude Substance " A " were combined and the solvent removed. 4·1 gm. of a semi-solid material were obtained. This was boiled with light petroleum several times and the petroleum decanted off, whilst boiling, through a Buchner funnel. 0·5 gm. of crystalline material separated from the petroleum on cooling and melted at 87°–89° C.

Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

After recrystallising twice from boiling petroleum it melted at 92·6° C. and the melting point was unchanged on mixing with the keto-acid prepared by oxidation of Substance "A" with permanganate in acetone solution (see p. 325). It also had the same equivalent, 0·1170 gm. requiring 4·65 c.c. of N/10 NaOH corresponding to an equivalent of 251·6. (Theoretical for $C_{15}H_{28}O_3 = 256$.)

It appears probable, therefore, that, in addition to Substance "A" and succinic acid, γ-ketopentadecoic acid is also a metabolic product of *P. spiculisporum* LEHMAN. The possibility cannot be ruled out, however, that γ-ketopentadecoic acid is merely an artefact arising from Substance "A" during the purification of the latter substance.

The residue insoluble in light petroleum was dissolved in a little ether and light petroleum added. A small amount (0·3 gm.) of Substance "A" separated but the bulk of the material remained in solution and on removal of solvent proved to be a yellow oil which did not crystallise on standing for a long time in the cold room.

General properties of Substance "A."

When crystallised from boiling water Substance "A" separates in large, shining, white plates which in the liquid appear like fine silky needles. It separates from a mixture of ether and light petroleum in small plates. It is readily soluble in boiling water but almost insoluble in cold water. It is also readily soluble in alcohol and acetone, fairly soluble in ethyl ether, less soluble in chloroform, and almost insoluble in light petroleum. It melts at 145°–146° C. without decomposition.

Analysis of Substance "A."

A sample recrystallised from water gave on combustion the results given in Table V.

TABLE V.

Experiment No.	Weight of Substance Analysed.	Weight of CO_2.	Weight of H_2O.	Percentage Carbon.	Percentage Hydrogen.
	Gm.	Gm.	Gm.		
1	0·1319	0·3010	0·1004	62·25	8·52
2	0·1246	0·2823	0·0970	62·02	8·71
	Theoretical for $C_{17}H_{28}O_6$ ...	...	... ...	62·15	8·59

Molecular weight of Substance "A."

(*a*) *By titration.*—0·4200 gm. was dissolved in alcohol and titrated to phenolphthalein with N/10 NaOH. 25·68 c.c. were required corresponding to a combining weight of 163·6. (Theory for $C_{17}H_{28}O_6$ assuming that this titrates as a dibasic acid = 164.)

Downloaded from rstb.royalsocietypublishing.org

(*b*) *By analysis of silver salt.*—The silver salt was prepared by precipitation of a solution of the sodium salt with 5 per cent. $AgNO_3$ solution. A white flocculent precipitate was obtained which was filtered off in the dark, well washed and dried to constant weight at 100° C. 0·2105 gm. of this left 0·0841 gm. of silver on ignition, equivalent to 39·95 per cent. Ag. (Theoretical percentage of Ag in $C_{17}H_{26}O_6Ag_2$ = 39·83.) This corresponds to a molecular weight of 326·3. (Theoretical for $C_{17}H_{28}O_6$, = 328.)

(*c*) *By depression of freezing point of camphor.*—(Modified RAST's method. See SMITH and YOUNG, 1927.)

0·00211 gm. of Substance " A " depressed the melting point of 0·02904 gm. of camphor 17·02° C. corresponding to a molecular weight of 169·5. (Theoretical for $C_{17}H_{28}O_6$ = 328.) No explanation is at present forthcoming for the discrepancy between these figures, but it is interesting to note that among the substances which give incorrect molecular weight values by this method are certain well-known polybasic acids.

Optical activity of Substance "A."

The molecule of Substance " A " must contain an asymmetric carbon atom since its solution in alcohol is optically lævorotatory, though curiously enough the solution of its sodium salt in water is dextrorotatory.

(1) *Optical rotation in alcohol.*—1·0335 gm. of Substance " A " dried at 100° C. were dissolved in 25·07 c.c. of absolute alcohol and polarised in a 40 cm. tube using the mercury green light. The average of ten readings = $-2\cdot440°$ corresponding to $[\alpha]_{\text{Hg. green}} = -14\cdot76°$.

(2) *Optical rotation of sodium salt in water.*—0·5011 gm. of Substance " A " was dissolved in the theoretical amount of N/1 NaOH, the solution made up to 25 c.c. and polarised in the mercury green light. In a 40 cm. tube the mean rotation from a number of observations was $+1\cdot201°$ corresponding to $[\alpha]_{\text{Hg. green}} = +14\cdot98°$.

Reactions of an aqueous solution of the sodium salt of Substance "A."

A neutral aqueous solution of the sodium salt of Substance " A " has the following properties :—

(*a*) With calcium acetate solution it gives a white precipitate insoluble in warm water.

(*b*) With silver nitrate it gives a white precipitate turning red on standing.

(*c*) With neutral lead acetate solution it gives a white gelatinous precipitate which when dried is insoluble in ether.

(*d*) With copper sulphate solution it gives a blue precipitate.

(*e*) It does not reduce BENEDICT's solution on boiling.

(*f*) The aqueous solution froths on shaking, in a manner reminiscent of a soap solution.

Downloaded from rstb.royalsocietypublishing.org

Action of acids on Substance "A."

Substance " A " is unaffected by boiling with dilute sulphuric acid, or concentrated nitric acid, from which solvents it may be readily crystallised. Concentrated sulphuric acid dissolves it and the solution does not darken in colour until nearly boiling.

Action of alkalis on Substance "A."

On boiling Substance " A " with dilute sodium hydroxide solution (N/10 or N/1) hydrolysis takes place and this change is dealt with in more detail on p. 308.

Action of halogens on Substance "A."

Substance " A " is not acted on by aqueous alkaline iodine solution, by bromine in glacial acetic acid, or by iodine in carbon tetrachloride (Wijs' solution), and hence does not appear to contain a double bond.

Action of alcoholic potassium acetate solution on Substance "A."

When a solution of anhydrous potassium acetate in absolute alcohol is added to a solution of Substance " A " in the same solvent there is no separation of any material, even on standing, indicating that if a potassium salt of Substance " A " is formed under these conditions it must be readily soluble in absolute alcohol.

Acetylation of Substance "A."

(*a*) *With acetic anhydride and anhydrous sodium acetate.*—A quantity of Substance " A " was heated with acetic anhydride and anhydrous sodium acetate in an oil bath in the usual way. There was immediate darkening in colour, and after five minutes' heating the mixture was dark brown-black in colour, and on dilution with water gave rise to a small amount of a brownish-black oil which could not be purified. The filtrate from this oil was almost colourless but acidification of it gave no precipitate nor did ether extraction give rise to any product. It is somewhat remarkable, in view of the general stability of Substance " A " towards most reagents, that heating with acetic anhydride and anhydrous sodium acetate should decompose it so readily.

(*b*) *With acetyl chloride.*—On boiling with acetyl chloride Substance " A " gradually dissolved. The excess of acetyl chloride was removed on the water bath and finally *in vacuo* when a colourless oil remained. This oil is readily soluble in absolute alcohol and separates also as an oil from aqueous alcohol. It resisted all attempts to crystallise it.

(*c*) *With acetic anhydride and pyridine.*—A quantitative acetylation of Substance " A " was carried out by the method described by Peterson and West (1927). About 0·5 gm. of Substance "A" was dissolved in 10 c.c. of a mixture of 8 c.c. of acetic anhydride and 40 c.c. of pure pyridine and incubated at 37° C. for five days in a tube fitted with

Downloaded from rstb.royalsocietypublishing.org

a ground glass stopper. Blanks containing 10 c.c. of the pyridine-acetic anhydride mixture were incubated alongside. After five days the contents of each tube were washed out with 200 c.c. of chilled water and titrated with N/1 NaOH.

TABLE VI.

Weight of Substance Acetylated.	c.c. N/1 NaOH required to Neutralise.	c.c. N/1 NaOH required to Neutralise Blank.	c.c. N/1 NaOH excess of estimation over Blank.	c.c. N/1 NaOH due to original Acidity of Substance "A."	c.c. N/1 NaOH equivalent to Acetic Anhydride used up.	Number of Acetyl groups in one Molecule of Substance "A."
Gm. 0·5051	36·23	33·99	2·24	3·08	(3·08–2·24) 0·84	0·55
0·5036	36·16	33·96	2·20	3·07	(3·07–2·20) 0·87	0·57

HYDROLYSED SUBSTANCE "A."

When Substance "A" is boiled for a short time with an excess of N/1 NaOH, although no apparent change takes place, the product recovered on acidifying the alkaline solution is different in appearance and has a different melting point from Substance "A." This reaction has therefore been examined in some detail.

0·5015 gm. of Substance "A" was boiled with 100 c.c. of N/10 NaOH for eight hours but no CO_2 or volatile acid was produced during the hydrolysis.

Subsequent investigation has shown that on boiling Substance "A" with dilute alkali, the original acidity of Substance "A" is increased by approximately 50 per cent., and that if this substance with the increased acidity is now boiled with dilute acid it reverts to the original Substance "A." The explanation of this appears to be that Substance "A" is a dibasic acid containing two carboxyl groups and a lactone ring. When it is boiled with dilute alkali the lactone ring is opened, giving rise to a hydroxy tricarboxylic acid (subsequently referred to as hydrolysed "A") which in its turn on boiling with dilute acid loses water with the consequent closing of the lactone ring and the re-formation of the dicarboxylic acid, Substance "A." The experimental evidence on which these conclusions are based is given in tabular form in Table VII.

The results given in Table VII were obtained as follows :—

The weight of Substance "A" indicated (column 1) was first titrated with standard sodium hydroxide (column 2), and a measured excess (column 3) of sodium hydroxide was then added and the mixture boiled under a reflux for eight hours. The following day the cooled hydrolysis mixture was titrated back with standard H_2SO_4 (column 5). A measured excess of standard acid (column 7) was now added and the mixture boiled under reflux for about an hour. The cooled solution was titrated with standard sodium

Downloaded from rstb.royalsocietypublishing.org

TABLE VII.

Weight of Substance " A " Hydrolysed.	Volume of Sodium Hydroxide needed for Neutralisation.	Excess of Sodium Hydroxide added for Hydrolysis.	Total Volume of Sodium Hydroxide Added.	c.c. Sulphuric Acid needed to Neutralise.	Excess of Acid Produced during Hydrolysis.	Excess of Sulphuric Acid added for Acid Boil.	c.c. Sodium Hydroxide required to Neutralise after Acid Boil.	Loss in Acidity on Acid Boil.
Gm. 0·4789	29·26 c.c. N/10	50·02 c.c. N/10	79·28 c.c. N/10	37·97 c.c. N/10	50·02–37·97 = 12·05 c.c. N/10	61·61 c.c. N/10	49·73 c.c. N/10	61·61–49·73 = 11·88 c.c. N/10
0·4788	29·26 c.c. N/10	50·08 c.c. N/10	79·34 c.c. N/10	39·70 c.c. N/10	50·08–39·70 = 10·38 c.c. N/10	62·86 c.c. N/10	50·00 c.c. N/10	68·86–50·00 = 12·86 c.c. N/10
2·5085	30·60 c.c. N/2 (calculated)	69·44 c.c. N/2	100·04 c.c. N/2	27·54 c.c N/2	69·44–55·08 = 14·36 c.c. N/2	—	—	—
2·5046	15·48 c.c. N/1	34·54 c.c. N/1	50·02 c.c. N/1	27·60 c.c. N/1	6·94 c.c. N/1	—	—	—

Downloaded from rstb.royalsocietypublishing.org

hydroxide (column 8). These figures show fairly conclusively that the increase in acidity on boiling with dilute sodium hydroxide (column 6) is approximately half of the initial acidity (column 2), and that this increase in acidity is lost by subsequent boiling with dilute sulphuric acid (column 9). The alkaline hydrolysis is evidently not quite so complete when N/10 sodium hydroxide is used as when N/2 sodium hydroxide is used for the purpose (Experiment 3).

Isolation of hydrolysis product of Substance "A."

The neutralised solution from Experiment 3 in Table VII was cleared by shaking in the cold with a little Merck's blood charcoal, filtered, and 22·48 c.c. (column 4—column 5) of N/1 H_2SO_4 added to neutralise exactly the sodium hydroxide combined with the hydrolysis product. A stiff gelatinous precipitate was obtained and was extracted with ethyl ether in which it was readily soluble. The ether extract was evaporated to dryness *in vacuo* and the dried residue recrystallised by dissolving in the minimum amount of boiling ethyl ether and adding to this boiling light petroleum. The hydrolysis product readily separated in shining white plates.

Yield = 2·5 gm., from which it is evident that, since this product is homogeneous, there is only one product of hydrolysis and that no deep seated change has taken place during this process.

General properties of hydrolysed Substance "A."

(1) It crystallises from ethyl ether-light petroleum in flat, square plates, the corners of which are cut off.

(2) It melts sharply at 134°–135° C. On heating above its melting point bubbles of gas are given off which have been shown to be due to loss of water.

(3) The crystals obtained from ether and light petroleum contain solvent of crystallisation which is lost very slowly on exposure to air, but much more rapidly at 100° C.

Determination of combining weight of hydrolysed substance "A" by titration.

It was recognized that if hydrolysed Substance "A" is a hydroxy-tricarboxylic acid and if Substance "A" is the lactone corresponding to it, then considerable difficulty might be experienced in preparing a sample of perfectly pure hydrolysed "A," because of the ease with which hydroxy acids of certain types are known to revert to the lactone. This difficulty was further intensified by the fact that hydrolysed "A" was known to cling tenaciously to the solvent when a mixture of ether-light petroleum, which is the best crystallising agent, was used in its preparation. An attempt to overcome this difficulty by using chloroform as a solvent instead of ether gave the same result since hydrolysed "A" clings to chloroform as tenaciously as to ether. The problem therefore resolved itself into that of finding a method of removing the solvent from hydrolysed "A" without at the same time removing water and hence re-forming Substance "A."

Downloaded from rstb.royalsocietypublishing.org

A sample of hydrolysed Substance " A," free from solvent of crystallisation, and containing a minimum of re-formed Substance " A," was finally obtained as follows :—The air-dried sample of hydrolysed Substance " A," whether recrystallised from ether-light petroleum, or from chloroform, was dried at 60° C. at atmospheric pressure. Most of the solvent is driven off in a few hours and subsequent drying only results in a very slight loss in weight.

0·2124 gm. of hydrolysed Substance " A," crystallised from chloroform, and dried at 60° C. for 185 hours, was dissolved in absolute alcohol and titrated with N/10 sodium hydroxide to phenolphthalein. 17·73 c.c. of N/10 sodium hydroxide were required for neutralisation corresponding to a combining weight of 119·8 (combining weight of the tribasic acid $C_{17}H_{30}O_7 = 115{\cdot}3$). An excess of N/10 sodium hydroxide was now added, the mixture heated at 60° C. for 24 hours, cooled and titrated with N/10 acid. A further 0·67 c.c. of N/10 sodium hydroxide was neutralised during the hydrolysis corresponding to a total amount of 18·40 c.c. of N/10 sodium hydroxide and a *corrected* combining weight of 115·4.

0·1961 gm. of hydrolysed Substance " A " recrystallised from ether-light petroleum, and dried at 60° C. for 164 hours required 16·30 c.c. of N/10 sodium hydroxide for the primary titration, and a further 0·70 c.c. during the secondary hydrolysis, corresponding to an initial combining weight of 120·3 and a *corrected* combining weight of 115·3.

A portion of the same dried sample of hydrolysed Substance " A " gave the following results on micro-combustion (Schoeller, Berlin) as indicated in Table VIII.

Table VIII.

Weight of Hydrolysed " A " Analysed.	Weight of CO_2.	Weight of H_2O.	Percentage Carbon.	Percentage Hydrogen.
Mgm.	Mgm.	Mgm.		
4·821	10·430	3·70	59·02	8·59
4·454	9·630	3·50	58·97	8·79
Theoretical for $C_{17}H_{30}O_7$ ...	...	...	58·96	8·67

Determination of combining weight of hydrolysed Substance " A " by analysis of the silver salt.

Samples of the silver salt of hydrolysed Substance " A " were prepared by adding silver nitrate solution to the titrated alcoholic solution of hydrolysed Substance " A," filtering off the amorphous silver salt in a subdued light, washing carefully and estimating the silver content by ignition after drying.

Three samples of hydrolysed Substance " A," prepared under different conditions,

Downloaded from rstb.royalsocietypublishing.org

were used and the details are given in Table IX. It is evident from these results that the silver salt contained a small amount of the silver salt of Substance " A " and was therefore probably the salt of the equilibrium mixture of hydrolysed Substance " A " and Substance " A."

TABLE IX.—Silver content of silver salt of hydrolysed " A " prepared under different conditions.

Weight of Silver Salt Ignited.	Weight of Silver.	Percentage of Silver in Silver Salt.	Equivalent calculated on Silver Content.	Remarks.
Gm. 0·3611	Gm. 0·1714	47·46	120·6	This silver salt was made from a sample of hydrolysed " A," which had been dried in air for five days and had an equivalent of 121·2.
0·3595	0·1707	47·50	120·5	This sample, crystallised from ether-light petroleum, had been dried for 164 hours at 60° C. and subsequently hydrolysed in aqueous solution at 60° C. for 24 hours with an excess of N/10 sodium hydroxide.
0·3895	0·1861	47·78	119·0	This sample, crystallised from chloroform, had been dried for 185 hours at 60° C. and subsequently hydrolysed as in previous experiment.

Acetylation of Hydrolysed "A."

(*a*) *With acetic anhydride and pyridine.*—A quantitative acetylation of hydrolysed " A " was attempted by the method of PETERSON and WEST (1927). 1·004 gm. of hydrolysed " A " (m.p. 132° C.) were dissolved in 20 c.c. of a mixture of 8 c.c. of acetic anhydride and 40 c.c. of pure pyridine and incubated at 37° C. for five days in a tube fitted with a glass stopper. The contents of the tube were then washed out with 200 c.c. of chilled water and titrated with NaOH.

TABLE X.

Weight of Hydrolysed " A."	c.c. N/1 NaOH required to Neutralise.	c.c. N/1 NaOH required to Neutralise Blank.	c.c. N/1 NaOH. Excess of Estimation over Blank.	c.c. N/1 NaOH due to Original Acidity.	c.c. N/1 NaOH equivalent to Acetic Anhydride used up.	Number of Acetyl Groups in 1 Mol. of Hydrolysed " A."
1·004	70·49	66·08	4·41	6·12	1·71	0·58

Downloaded from rstb.royalsocietypublishing.org

The figure for the number of acetyl groups (0·58) is almost identical with those obtained in similar experiments with Substance "A" (0·55, 0·57, see p. 308), but in both cases very considerable darkening in colour occurred and the products consistep for the most part of tar. It seems unlikely, therefore, that this reaction represents a true acetylation.

(*b*) *Acetylation of hydrolysed "A," with acetyl chloride.*—4·2 gm. of hydrolysed "A" (m.p. 134° C.) were boiled on a water bath under reflux for three-quarters of an hour with 40 c.c. of redistilled acetyl chloride. Hydrochloric acid was evolved and the substance gradually dissolved. The excess of acetyl chloride was removed and the residue transferred by washing out with ether to a large weighed crystallising dish. After removal of the ether the viscous material was spread out in a thin layer over the dish, and was then dried *in vacuo* over strong potassium hydroxide solution and sulphuric acid. The product weighed 4·21 gm. and was partially crystalline.

It was dissolved in boiling ether and a little boiling light petroleum added. The addition of light petroleum precipitates first a quantity of Substance "A," and if sufficient is added brings down also the acetyl compound. By repeated fractionation with ether-light petroleum 2·24 gm. of Substance "A" were recovered, the melting point of the different fractions varying from 140° to 142° C. From the ether mother-liquor, 1·53 gm. of a sticky non-crystalline material were obtained after drying. This material was boiled three times with light petroleum, the petroleum poured off and evaporated. A little oily matter was thus separated and after drying for two days 1·22 gm. of acetyl derivative remained. This was dissolved in absolute alcohol and made up to 50 c.c. Of this 25 c.c. were pipetted and boiled under reflux with 20 c.c. of N/1 NaOH for $2\frac{1}{2}$ hours. The hydrolysed material was acidified with H_2SO_4 and the acetic acid distilled off *in vacuo*. The combined distillates from 0·61 gm. of the acetyl derivative required 15·46 c.c. of N/10 NaOH. The theoretical value for a monoacetyl derivative is 15·72 c.c. A white precipitate which formed in the boiling flask was separated, dried and weighed (weight = 0·481 gm.). On recrystallising from ether-light petroleum, it melted at 145° C. and was evidently Substance "A." The theoretical amount of Substance "A" obtainable from 0·61 gm. of a monoacetyl derivative of hydrolysed "A" is 0·515 gm.

In a second experiment the distillate from 1·0798 gm. of the acetyl derivative required 27·45 c.c. of N/10 NaOH (theory for a monoacetyl derivative = 27·8 c.c.), and the weight of Substance "A" recovered by extraction with ether of the liquid remaining in the hydrolysis flask was 0·910 gm. (theory = 0·913 gm.). On recrystallising from ether-light petroleum and finally from water it melted at 145° C. and gave a mixed m.p. with a pure specimen of Substance "A" of 145° C.

The action of acetyl chloride on hydrolysed Substance "A" is therefore to form the mono-acetyl derivative, and also, to almost an equal extent, to dehydrate hydrolysed "A" giving Substance "A."

The acetyl derivative is an intensely sticky substance which does not crystallise on

Downloaded from rstb.royalsocietypublishing.org

keeping *in vacuo* for several weeks, but which on standing in the open air readily decomposes, the smell of acetic acid becoming evident and the material crystallising in the course of three days. This product, on recrystallising twice from ether and light petroleum, melted at 145° C. and gave a mixed m.p. of 145° C. with a pure sample of Substance " A." The acetyl derivative breaks down therefore on standing in air into Substance " A " and acetic acid.

Conversion of hydrolysed Substance " A " into Substance " A " by heating at 100° C.

About 0·5 gm. of hydrolysed Substance " A " was dried at 100° C. for 168 hours. At the end of this time the material had shrunk considerably and showed signs of oily droplets on the surface of the weighing bottle. The material was crystallised twice from ether-light petroleum and then dried for one week at 60° C. This recrystallised material then had the following characteristics: It melted at 145·5°–146° C. and its melting point was unchanged on mixing with Substance "A." It crystallised from water in the shining plates characteristic of Substance " A." 0·1981 gm. of it required 11·82 c.c. of N/10 sodium hydroxide corresponding to an equivalent of 167·6. Excess of alkali was now added and the alkaline mixture incubated overnight at 60° C. when a further 5·95 c.c. of N/10 acid had been produced. Thus the acidity of the material had been increased 50 per cent. by heating with dilute sodium hydroxide solution exactly as with Substance " A."

The ether-light petroleum mother-liquors left, on evaporation, a waxy material reminiscent of dehydrated " A " (p. 317), and it appears that on heating at 100° C. hydrolysed Substance "A" which has been crystallised from ether-light petroleum, three reactions take place :—

(*a*) A very quick reaction, *i.e.*, loss of solvent by hydrolysed " A."
(*b*) A quick reaction, *i.e.*, hydrolysed " A " ⟶ Substance " A."
(*c*) A slow reaction, *i.e.*, Substance " A " ⟶ dehydrated " A."

DEHYDRATED SUBSTANCE " A."

It has been noted above that when hydrolysed Substance " A " is heated at 100° C. for a week, Substance " A " is re-formed together with a small amount of a waxy material to which the name " dehydrated Substance ' A ' " is given. The quantitative relations between Substance " A," hydrolysed Substance " A," and dehydrated Substance " A " will now be dealt with, and later (p. 317) a description of the preparation and properties of dehydrated Substance " A " is given.

(*a*) *Effect of heating Substance " A " at* 180° *C.*—0·7470 gm. of air-dried Substance " A " was heated for one hour at 180° C. in a castor oil bath in a stream of dry CO_2-free nitrogen. The nitrogen, carrying products of decomposition, was passed through a calcium chloride tube to absorb any water given off and then through a baryta bubbler to absorb CO_2.

Downloaded from rstb.royalsocietypublishing.org

No decomposition was apparent at the melting point of Substance " A " (145° C.) but as the temperature rose bubbles of gas were given off. The temperature was maintained at 180° C. until this gas evolution ceased (one hour's heating). On cooling, the liquid in the heating tube set to a mass of fine needles. The following results were obtained :—

	Gm.
Weight of Substance " A " heated	0·7470
Total loss in weight of Substance " A "	0·0452
Gain in weight of calcium chloride tube	0·0423
Weight of CO_2 evolved	0·0011

These results indicate that only a negligible amount of CO_2 is evolved and that the loss in weight is due to loss of water which amounts to 5·66 per cent. (calculated from the gain in weight of the calcium chloride tube). This agrees very well with the theoretical for the following equation : $C_{17}H_{28}O_6 \rightarrow C_{17}H_{26}O_5 + H_2O$ (= 5·49 per cent.).

(*b*) *Effect of heating hydrolysed " A " at* 180° *C.*—A quantity of hydrolysed Substance " A " which had been crystallised from ether-light petroleum and air-dried for 13 days was heated for one hour at 180° C., in the same apparatus and under the same conditions as in the previous experiment.

Moisture was given off as soon as the melting point was reached (133° C.) and the substance appeared to melt and then partially to re-solidify. It melted a second time at 145° C. and continued to give off gas bubbles as the temperature was raised. This gas evolution was completed some time before heating was stopped.

The following results were obtained :—

	Gm.
Weight of hydrolysed " A " heated..	0·6773
Total loss in weight of hydrolysed " A "	0·0942
Gain in weight of calcium chloride tube	0·0710
Weight of CO_2 evolved	Nil

Since no CO_2 at all was evolved it is evident that some substance has been given off which is neither water nor CO_2, and amounting to 0·0942 gm. — 0·0710 gm. = 0·0232 gm. = 3·43 per cent.

This was shown to be solvent and hence the weight of solvent-free hydrolysed " A " which was used is 0·6773 gm. — 0·0232 gm. = 0·6541 gm. Hence the percentage loss of water from solvent free material is

$$\frac{0{\cdot}0710 \times 100}{0{\cdot}6541} = 10{\cdot}85 \text{ per cent.}$$

This agrees well with the theoretical value for the equation $C_{17}H_{30}O_7 \rightarrow 2H_2O + C_{17}H_{26}O_5$ (= 10·41 per cent.).

Downloaded from rstb.royalsocietypublishing.org

Perfectly definite proof was now found by the following experiment for the belief that some material, probably solvent, other than water is evolved on heating air-dried hydrolysed " A." A quantity of hydrolysed " A " which had been dried in air for eight days was heated in an apparatus similar to that used in the two above experiments, the conditions of heating, length of time, etc., being exactly the same. The gases evolved on heating were passed through two calcium chloride tubes to absorb water. The residual vapours were then passed through an ordinary combustion tube filled with copper oxide and heated in a furnace. The products of combustion were absorbed in calcium chloride and potassium hydroxide exactly as in an ordinary combustion. The following results were obtained :—

	Gm.
Weight of hydrolysed " A " heated..	0·8117
Total loss in weight of hydrolysed " A "	0·1127
Gain in weight of calcium chloride tube	0·0825
	(= 10·56 per cent. calculated on solvent free material).
	Gm.
Therefore, weight of solvent oxidized	0·0302
Weight of water produced on combustion	0·0341
Weight of CO_2 produced on combustion	0·0661

These figures correspond to 3·72 per cent. of solvent in the original air-dried hydrolysed " A," the solvent containing 12·6 per cent. hydrogen and 59·7 per cent. carbon, which figures agree reasonably well with the theoretical for diethyl ether, viz., hydrogen = 13·5 per cent. carbon = 64·9 per cent.

To sum up then, the effect of heating hydrolysed " A " or Substance " A " is that one or more of the following reactions takes place :—

(*a*) *Loss of solvent by hydrolysed " A."*—This change takes place, though only slowly, at room temperature, more quickly at 60° C. and very quickly at 100° C.

(*b*) *Hydrolysed " A " → Substance " A."*—This change takes place fairly readily at 100° C., and by careful regulation it is possible to obtain almost pure Substance " A " from hydrolysed " A " by this method. The change takes place according to the equation $C_{17}H_{30}O_7 \rightarrow C_{17}H_{28}O_6 + H_2O$

(*c*) *Hydrolysed " A " → Substance " A " → Dehydrated " A."*—This change takes place quickly at 180° C., though there is evidence that it also takes place very slowly at 100° C. (p. 314). The change occurs according to the following equation :—

$$C_{17}H_{30}O_7 \xrightarrow{-H_2O} C_{17}H_{28}O_6 \xrightarrow{-H_2O} C_{17}H_{26}O_5$$

The reverse reaction from Substance " A " to hydrolysed " A " is readily brought about by boiling with dilute alkali.

Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Preparation and properties of dehydrated Substance "A."

A quantity of Substance " A " was heated for an hour at 180° C. On cooling, the liquid set to a mass of fine needles which were crystallised by dissolving in a small amount of ether, adding about three volumes of light petroleum, filtering and allowing the filtrate to stand in a rapid current of air. When a sufficient degree of cooling had been obtained, the substance crystallised out, was filtered, recrystallised and air-dried. It was noted that the solubility-temperature gradient is very steep and that once the material has crystallised, rapid filtration must follow, otherwise the substance redissolves.

Dehydrated Substance " A " crystallises in fine white needles melting at 41° C.

1·9007 gm. of dehydrated Substance " A," dissolved in alcohol, required 12·27 c.c. of N/1 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 154·9. (Theoretical for $C_{17}H_{26}O_5$, assuming this to be a dibasic acid = 155·0.)

The results of micro-combustion (Schoeller, Berlin) are given in Table XI.

Table XI.

Weight of Dehydrated Substance " A " analysed.	Weight of CO_2.	Weight of H_2O.	Percentage Carbon.	Percentage Hydrogen.
Mgm.	Mgm.	Mgm.		
4·618	11·125	3·67	65·71	8·92
4·491	10·845	3·51	65·86	8·75
Theoretical for $C_{17}H_{26}O_5$ …	…	… …	65·75	8·45

Hydrolysis of Dehydrated Substance "A."

It was expected that, since dehydrated Substance " A " ($C_{17}H_{26}O_5$) arises from Substance " A " ($C_{17}H_{28}O_6$) by heating, it should be possible to regenerate Substance " A," or hydrolysed Substance " A " ($C_{17}H_{30}O_7$), by hydrolysing dehydrated Substance " A " with dilute alkali. This change does not appear to take place, however, as, on hydrolysis of dehydrated Substance " A," a new product arises which has been called Hydrolysed Dehydrated " A," and there was no evidence of the formation of either Substance " A," or Hydrolysed Substance " A."

That dehydrated " A " is hydrolysed by boiling with dilute alkali was shown quantitatively as follows :—

0·9530 gm. of dehydrated " A " was neutralised with N/2 NaOH and required 12·97 c.c. An excess of N/2 NaOH was now added, the mixture boiled and titrated back.

Downloaded from rstb.royalsocietypublishing.org

Acid equivalent to a further 5·68 c.c. of N/2 NaOH was formed, so that the equivalent of the hydrolysis product is 108·1.

In a repeat experiment on similar lines an equivalent of 111·0 was obtained.

The values agree with the theoretical value for a compound having the same empirical formula as Substance " A " ($C_{17}H_{28}O_6$) but possessing *three* carboxyl groups (theoretical = 109·3).

Isolation and properties of hydrolysed dehydrated "A."

Considerable difficulty was experienced in isolating the substance obtained by boiling neutralised dehydrated " A " with excess of sodium hydroxide. The substance very readily decomposes in presence of acid. Thus, acidification, with *normal* hydrochloric or sulphuric acid, of the alkaline solution after hydrolysis and shaking out with ether, gave a product consisting almost exclusively of dehydrated " A." When, however, the diluted hydrolysed solution was acidified with N/10 HCl in presence of ether, the acid being added in small quantities with continuous shaking, it was found that on evaporating the ethereal solution in a current of air to small bulk and adding an equal volume of light petroleum, a good yield of an acid was precipitated, having a melting point of 87° C.

The action of heat on hydrolysed dehydrated "A."

0·4789 gm. of hydrolysed dehydrated " A " (m.p. 87° C.) was heated for one hour at a temperature rising from 100° to 160° C. in a castor oil bath in a stream of dry CO_2-free nitrogen. The substance decomposed almost immediately after melting, and the water formed was carried in the stream of nitrogen through weighed calcium chloride tubes. On cooling, the liquid in the heating tube set to a mass of fine needles. The residual material on crystallising from ether-light petroleum melted at 41° C. and gave an equivalent of 148·9, and was therefore dehydrated Substance " A." The following results were obtained :—

	Gm.
Weight of hydrolysed dehydrated " A "	0·4789
Total loss in weight	0·0379
Gain in weight of the calcium chloride tubes	0·0264

The gain in weight of the calcium chloride tubes corresponds to a loss of water amounting to 5·51 per cent., which agrees very closely with the theoretical for the following equation (5·49 per cent.): $C_{17}H_{28}O_6 \rightarrow C_{17}H_{26}O_5 + H_2O$. It would appear, therefore, that hydrolysed dehydrated " A " is a substance having the same empirical formula as Substance " A," but containing three carboxyl groups and on heating above 100° C. it reverts by loss of 1 mol. of water to dehydrated " A."

The presence of solvent of crystallisation, as indicated by the difference between the *total* loss in weight on heating (0·0379 gm.) and the gain in weight of the $CaCl_2$ tubes (0·0264 gm.), together with the extreme instability of hydrolysed dehydrated " A "

Downloaded from rstb.royalsocietypublishing.org

even at room temperature, have rendered it impossible to obtain satisfactory combustion results. The instability of the material is indicated by the fact that, while a freshly prepared sample has a melting point of 87° C., this had fallen to 82° C. after standing for three days at room temperature and to 42°–43° C. after standing for three weeks at the same temperature.

Alkylation Products.

It was hoped by studying the alkylation products of Substance " A " and hydrolysed Substance " A " to obtain confirmation of the observation already made (see p. 313) that hydrolysed " A " contains one hydroxyl group. The alkylation products were, however, disappointing, and it was found impossible to alkylate the hydroxyl group by any method tried, although the three carboxyl groups were relatively easily esterified. Further, all the products obtained were oily liquids which refused to crystallise and were difficult to purify.

1. *Methylation of Substance " A " with diazomethane.*

One gram of pure Substance " A " was dissolved in dry ether and an excess of an ethereal solution of diazomethane was added. An immediate reaction took place. After standing for one hour the excess of diazomethane was removed by shaking with a little water. The ethereal solution was separated, evaporated and dried to constant weight over H_2SO_4 in a vacuum desiccator. 1·02 gm. of a colourless oil were obtained, which did not crystallise on standing for several weeks.

Zeisel estimations on this material gave the following results :—0·1353 gm. and 0·1204 gm. respectively gave 0·1770 gm. and 0·1574 gm. of silver iodide, corresponding to 17·26 per cent. and 17·25 per cent. OCH_3 (theoretical for $C_{17}H_{26}O_4\ (OCH_3)_2$ = 17·42 per cent.).

2. *Methylation of Hydrolysed Substance " A " with dimethyl sulphate.*

A quantity of hydrolysed " A " was treated with a large excess of methyl sulphate, sodium hydroxide added and the mixture boiled for about an hour, the reaction being kept alkaline by the addition of more sodium hydroxide. The mixture was cooled, filtered and acidified, giving rise to a white gelatinous precipitate which was extracted with ether and fractionally crystallised from ether and light petroleum. It was shown to consist of unchanged hydrolysed Substance " A," and there was no indication of the formation of any methoxyl compound.

3. *Ethylation of hydrolysed Substance " A " with diazoethane.*

2 gm. of pure hydrolysed Substance " A " were dissolved in dry ether and a considerable excess of an ethereal solution of diazoethane added. An immediate reaction

Downloaded from rstb.royalsocietypublishing.org

took place and the mixture was allowed to stand overnight. The excess of diazoethane was then removed in the ether vapour by evaporation and the resultant pale yellow oil was dried over sulphuric acid. ZEISEL estimations on this material gave the following results :—0·1356 gm. and 0·1284 gm. respectively gave 0·2197 gm. and 0·2073 gm. of silver iodide, corresponding to 31·08 per cent. and 30·96 per cent. OC_2H_5 (theoretical for $C_{17}H_{28}O_5\ (OC_2H_5)_2 = 22{\cdot}39$ per cent. and $C_{17}H_{27}O_4\ (OC_2H_5)_3 = 31{\cdot}39$ per cent.). The ethylation product is thus either a triethyl ester or a monoethyl ether of a diethyl ester.

It was shown that the product does not contain an ethoxyl group, by hydrolysing a quantity of it with boiling alcoholic sodium hydroxide. The regenerated acid melted at 132° C., gave an equivalent of 121·2, and was unchanged hydrolysed Substance "A." Hence, this ethylation product is the triethyl ester of hydrolysed Substance "A." This conclusion is confirmed by the fact that this product is insoluble in sodium hydroxide.

4. *Attempted methylation of the triethyl ester of hydrolysed "A" with methyl iodide and silver oxide.*

An attempt was made to methylate the triethyl ester of hydrolysed Substance "A" by the method of PURDIE and IRVINE (1903). 1 gm. of the triethyl ester was treated with 2·18 gm. of methyl iodide and 1·79 gm. of silver oxide in 7 gm. of methyl alcohol. After refluxing for two hours the ester was recovered. It still gave a ZEISEL value of 31·18 per cent. OC_2H_5, and on hydrolysis by heating with alcoholic sodium hydroxide, unchanged hydrolysed "A" was obtained. Methylation, therefore, did not appear to have taken place. It is interesting to note that a similar difficulty was experienced by ANSCHÜTZ (1903), in an attempt to methylate the hydroxyl group of citric acid.

Amides of Substance "A" and Hydrolysed Substance "A."

(*a*) *Amides from distilled methylated Substance "A."*—1 gm. of the *twice distilled* dimethyl compound of Substance "A" was treated with about ten volumes of aqueous ammonia solution saturated at 0° C., shaken and kept in a stoppered tube overnight at 0° C. In the morning the mixture appeared as a thin, gelatinous, soapy mass which was dried completely over sulphuric acid and then crystallised from boiling absolute alcohol. On cooling, a small amount of material (fraction 1) separated, consisting of very fine microscopic needles. The mother-liquors from fraction 1 were evaporated to dryness and crystallised from a mixture of ether and light petroleum, giving rise to a crystalline material (fraction 2). The mother-liquors from fraction 2 were freed from solvent, re-treated with 10 volumes of ammonia, and the fractionation carried through again, as described above. By this means further quantities of fractions 1 and 2 were obtained.

Downloaded from rstb.royalsocietypublishing.org

Fraction 1.—0·2 gm. of crude fraction 1 was obtained. This was recrystallised from absolute alcohol and was finally obtained as a white powder crystallising in fine white needles and having a melting point of 178·5° C. It gave the following results on analysis (Schoeller, Berlin) :—

5·188 mg. gave 11·450 mg. CO_2 and 4·40 mg. H_2O. C = 60·20 per cent., H = 9·50 per cent. (Duplicate C = 60·51 per cent., H = 9·66 per cent.)

3·047 mg. gave 2·12 mg. Ag I. OCH_3 = 9·19 per cent. (Duplicate 8·90 per cent.)

3·065 mg. gave 0·211 c.c. nitrogen at 24·5° C. and 762 m.m. Nitrogen = 7·92 per cent. (Duplicate 7·78 per cent.)

Calculated for $C_{18}H_{34}O_5N_2 = C_{17}H_{31}O_4N_2\,(OCH_3)$. C = 60·34 per cent., H = 9·56 per cent., N = 7·82 per cent., OCH_3 = 8·66 per cent.

Fraction 2.—0·60 gm. of fraction 2 was obtained, which on recrystallisation from ether-light petroleum separated in beautiful white prisms melting at 78·5° C. which gave the following results on analysis (Schoeller, Berlin) :—

4·382 mg. gave 9·850 mg. CO_2 and 3·65 mg. H_2O. C = 61·31 per cent., H = 9·32 per cent. (Duplicate C = 61·31 per cent., H = 9·45 per cent.)

3·041 mg. gave 4·24 mg. AgI. OCH_3 = 18·42 per cent. (Duplicate 18·08 per cent.)

2·925 mg. gave 0·098 c.c. nitrogen at 23·5° C. and 762 m.m. Nitrogen = 3·87 per cent (Duplicate 3·95 per cent.)

Calculated for $C_{19}H_{35}O_6N = C_{17}H_{29}O_4N\,(OCH_3)_2$. C = 61·08 per cent., H = 9·45 per cent., N = 3·75 per cent., OCH_3 = 16·63 per cent.

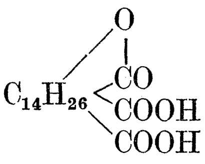
(*b*) *Amides from undistilled methylated Substance "A."*—5 gm. of crude, *undistilled* methylated Substance "A" were treated exactly as described in paragraph (*a*) for the distilled material. The yields of amides obtained were in marked contrast to those obtained with the distilled methyl compound since, while 3·1 gm. of fraction 1 were obtained, it was found impossible to isolate any fraction 2. The 3·1 gm. of fraction 1, on recrystallisation from absolute alcohol melted at 178·5° C. and admixture with a little of fraction 1 from the distilled methyl compound produced no lowering of the melting point. The two fractions may therefore be taken to be identical.

(*c*) *Amides from triethyl ester of hydrolysed Substance "A."*—On treating the triethyl ester of hydrolysed Substance "A" with cold saturated aqueous ammonia the greater part of the triethyl ester was recovered unchanged even after lengthy treatment. A small amount of the diamide was, however, obtained having a melting point of 178·5° C. and a mixed melting point of 178° C. with fraction 1.

From the analytical results it appears that fraction 1 (m.p. 178·5° C.) is the diamide of the monomethyl ester of hydrolysed Substance "A," while fraction 2 (m.p. 78·5° C.)

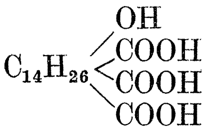
Downloaded from rstb.royalsocietypublishing.org

is the monoamide of the dimethyl ester of hydrolysed Substance " A " Their relations to Substance " A " and to hydrolysed Substance " A " are indicated as follows :—



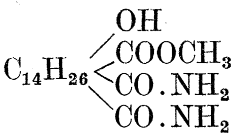
$C_{17}H_{28}O_6$

Substance A.



$C_{17}H_{30}O_7$

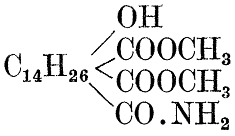
Hydrolysed Substance A.



$C_{18}H_{34}O_5N_2$

Fraction 1.

M.Pt. 178·5° C.



$C_{19}H_{35}O_6N$

Fraction 2.

M.Pt. 78·5° C.

Fusion of Substance " A " with Potassium hydroxide. Isolation of Lauric Acid ($C_{12}H_{24}O_2$).

1 gm. of Substance " A," 5 gm. of potassium hydroxide and 3 c.c. of water were warmed until the material was completely dissolved, and then heated in a metal bath at 310° C. for one hour. After cooling, the melt was dissolved in about 15 c.c. of water and acidified with 110 c.c. of N/1 H_2SO_4. Carbon dioxide was liberated and a substance precipitated, which on drying weighed 0·5 gm. It was dissolved in ether, filtered, the ethereal solution treated with a little animal charcoal to remove a trace of yellow oil, and the clear filtrate evaporated. The residue melted at 41° C. and gave an equivalent by titration of 196·5 (0·1031 gm. required 5·42 c.c. of N/10 NaOH). It was crystallised to constant melting point from light petroleum and air-dried. This product then melted at 43·6° C. and gave an equivalent of 199·2 (0·1998 gm. required 10·03 c.c. of N/10 NaOH). Lauric acid, $C_{12}H_{24}O_2$, melts at 43·6° C., and has an equivalent of 200. The combustion figures are as follows (SCHOELLER, Berlin) :—

TABLE XII.

Weight of Fusion Product	Weight of CO_2.	Weight of H_2O.	Percentage Carbon.	Percentage Hydrogen.
Mgm.	Mgm.	Mgm.		
5·070	13·430	5·50	72·26	12·14
3·708	9·770	4·02	71·88	12·13
Theoretical for $C_{12}H_{24}O_2$ ...	... ...	... ...	71·96	12·09

The mixed melting point of the product with a pure sample of lauric acid kindly supplied by Professor R. ROBINSON, F.R.S., was also 43·6° C., and lauric acid is therefore one product of fusion.

The solution remaining after filtering off the lauric acid was extracted with ether in a continuous extractor for four days and gave 0·18, 0·16, 0·06 and 0·03 = 0·43 gm. of dried material. This material was shown to consist of a mixture of oxalic and succinic

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Downloaded from rstb.royalsocietypublishing.org

acids. It was dissolved in water and the oxalic acid removed as calcium oxalate. The oxalic acid was regenerated from the calcium oxalate and sublimed in a high vacuum. The sublimate melted at 187° C. and had an equivalent of 45·5 (0·1021 gm. required 22·44 c.c. of N/10 NaOH). The oxalate-free solution was now treated with barium chloride, heated, the precipitated barium succinate filtered off, acidified, and the succinic acid extracted with ether and sublimed. It had a melting point of 184° C. and an equivalent of 60·1 (0·0119 gm. required 1·98 c.c. of N/10 NaOH).

The products obtained by fusion of 1 gm. of Substance " A " with potassium hydroxide are therefore 0·5 gm. of a material consisting almost entirely of lauric acid, a considerable amount of carbon dioxide, and 0·43 gm. of a mixture of succinic and oxalic acids. The oxalic acid probably arises from succinic acid under the conditions of fusion while the carbon dioxide is probably produced from formic acid, since sodium formate is known to break down readily under these conditions. It seems probable, therefore, that the initial products obtained by fusion from 1 molecule of Substance " A " are 1 molecule each of lauric, formic and succinic acids.

Oxidation of Substance " A " with acid permanganate.

2 gm. of Substance " A " were dissolved in 75 c.c. of N/1 H_2SO_4 and heated under reflux in a boiling water bath. 50 c.c. of a solution containing 0·77 gm. of $KMnO_4$ were now added, a few drops at a time. The permanganate solution was decolorised almost immediately, providing the addition was not too rapid. A considerable amount of oily material was gradually precipitated as the reaction proceeded. After all the permanganate had been added, the reaction was stopped and the flask cooled, when the oil readily solidified and was filtered off, dried and weighed. Weight = 1·54 gm. This material softened at 74° C. and completely melted at 103° C. By dissolving in ether and adding light petroleum 1·1 gm. of the material were precipitated as a white solid having a m.p. of 140° C., which on recrystallisation from water had a m.p. of 145° C. and was unchanged Substance " A." The material remaining in solution was again fractionated from ether-light petroleum and a further 0·1 gm. of impure Substance " A " separated. The ether-light petroleum filtrate on evaporating in a current of air now gave a residue having a weight of 0·29 gm., a m.p. of 87° C. and an equivalent of 229·8 (0·0878 gm. required 3·82 c.c. of N/10 NaOH).

In a repeat experiment 2·14 gm. of Substance " A " gave rise to 0·2 gm. of the oxidation product having a m.p. of 87° C. This was recrystallised to constant melting point from boiling light petroleum from which it separates very readily on cooling in glistening plates having a m.p. of 92·6° C. and an equivalent of 250·7 (0·1070 gm. required 4·27 c.c. of N/10 NaOH). On combustion it gave results as shown in Table XIII (Schoeller, Berlin).

Both the equivalent and the results of combustion correspond very closely with those required for ketopentadecoic acid $C_{15}H_{28}O_3$ (equivalent 256).

Downloaded from rstb.royalsocietypublishing.org

TABLE XIII.

Weight of Substance Analysed.	Weight of CO_2.	Weight of H_2O.	Percentage Carbon.	Percentage Hydrogen.
Mgm.	Mgm.	Mgm.		
4·758	12·235	4·70	70·14	11·05
4·243	10·920	4·21	70·19	11·10
Theoretical for $C_{15}H_{28}O_3$ ...	...	... ...	70·26	11·01

In view of the small yields of oxidation product obtained a further experiment was carried out in which considerably larger amounts of permanganate were used. In this case, however, none of the product $C_{15}H_{28}O_3$ was obtained, the main oxidation product proving to be succinic acid.

Oxidation of hydrolysed "A" with alkaline permanganate.

The amount of ketopentadecoic acid obtained by acid oxidation was never large (0·1–0·2 gm. from 2 gm. of Substance "A") and other methods of oxidation were tried in order to obtain a better yield so that complete identification would be more readily possible.

Oxidation by alkaline permanganate, however, did not yield more than a trace of the keto-acid. In one experiment 2·5 gm. of Substance "A" were boiled with 50 c.c. of N/1 NaOH for one hour in order to open the lactone ring, diluted to 1,500 c.c. and 1,500 c.c. of permanganate (containing 15 gm. of $KMnO_4$) and 3 gm. of sodium hydroxide were added. The solution was heated in a boiling water bath for one hour, cooled, sufficient sulphur dioxide passed just to clear the liquid and 20 c.c. of N/1 H_2SO_4 added. A white precipitate was obtained which on filtering off and drying weighed 1·2 gm. and consisted almost entirely of hydrolysed "A" together with a trace of the keto-acid which was separated by its solubility in boiling light petroleum. From the aqueous solution, after neutralising, evaporating *in vacuo* to a small bulk, acidifying and extracting in a continuous extractor with ether, there was obtained 1·18 gm. of material consisting of a yellow oil, together with a small amount of volatile acid reminiscent of butyric and caproic acids, and a larger amount of succinic acid which, after subliming twice, melted at 184° C. and gave an equivalent of 59·9 (0·01706 gm. required 2·85 c.c. of N/10 NaOH).

It appears, therefore, that oxidation with alkaline permanganate probably gives rise to the keto-acid, but that this is further oxidized yielding successively the series of fatty acids containing an even number of carbon atoms from lauric acid downwards, together with succinic acid.

Downloaded from rstb.royalsocietypublishing.org

Oxidation of hydrolysed " A " with permanganate in acetone solution. Preparation of γ-ketopentadecoic acid.

4 gm. of hydrolysed " A," m.p. 134° C., were dissolved in 60 c.c. of acetone and 16 c.c. of water added. The solution was then warmed in a water bath until the acetone began to boil and 0·732 gm. of powdered potassium permanganate added, a little at a time. The permanganate immediately disappeared and carbon dioxide was evolved. When all the permanganate had been added, the acetone was evaporated, the residue cooled, 50 c.c. of N/10 H_2SO_4 added and sufficient sulphur dioxide passed, just to clear the solution. The product was then extracted with ether, the ether removed and the residue dried and weighed (Wt. = 2·8 gm.). This was then digested with a large volume of boiling light petroleum in which it almost completely dissolved. On filtering the hot solution the keto-acid immediately crystallised in plates melting at 88° C. It was then dissolved in ether, treated with a little animal charcoal to remove a small amount of a yellow oil and after filtering and removing the ether, was crystallised twice from boiling petroleum. The product then melted at 92·6° C. Its melting point was unchanged on admixture with the keto-acid prepared by oxidation with permanganate in sulphuric acid solution. It also had the same equivalent, 0·1287 gm. requiring 5·10 c.c. of N/10 NaOH corresponding to an equivalent of 252·3 (theoretical for $C_{15}H_{28}O_3$ = 256). It will be noticed that the yield of crude keto-acid obtained by this method from hydrolysed " A " is over 90 per cent.

Reduction of γ-ketopentadecoic to n*-pentadecoic acid.*

0·5 gm. of γ-ketopentadecoic acid, prepared from hydrolysed " A " as described above, was reduced by CLEMMENSEN's method (1914). 50 gm. of zinc were immersed for one hour in 300 c.c. of 5 per cent. mercuric chloride solution. The aqueous layer was poured off and the Zn-Hg couple used without further washing. The Zn-Hg couple and the ketopentadecoic acid were now boiled for one hour with dilute hydrochloric acid, further quantities of acid being added from time to time. After cooling, the product was extracted with ether, the ether removed and the residue dried (weight = 0·48 gm.). The dry residue dissolved almost completely in cold light petroleum and on blowing a current of air over the solution, *n*-pentadecoic acid crystallised out. It was recrystallised from light petroleum and had the following properties: it melted at 53° C. and its melting point was not altered by admixture with a pure sample of *n*-pentadecoic acid prepared from material received through the courtesy of Prof. A. C. CHIBNALL. The reduction product had an equivalent of 243·4, 0·1478 gm. requiring 6·06 c.c. of N/10 NaOH—while the synthetic product had an equivalent of 242·6, 0·2382 gm. requiring 9·82 c.c. of N/10 NaOH—and the theoretical for $C_{15}H_{30}O_2$ is 242·2. The reduction product gave the following figures on combustion (Table XIV, SCHOELLER, Berlin) :—

Downloaded from rstb.royalsocietypublishing.org

TABLE XIV.

Weight of Reduction Product Analysed.	Weight of CO_2.	Weight of H_2O.	Percentage Carbon.	Percentage Hydrogen.
Mgm.	Mgm.	Mgm.		
2·948	8·030	3·37	74·29	12·78
4·554	12·410	5·09	74·35	12·51
Theoretical for $C_{15}H_{30}O_2$	...	...	74·35	12·49

Preparation of γ-ketopentadecoic acid from synthetic n-*pentadecoic acid.*

The keto-acid was prepared by the method of CLUTTERBUCK and RAPER (1925) by oxidation of the ammonium salt of *n*-pentadecoic acid with hydrogen peroxide. The *n*-pentadecoic acid was purchased from Messrs. Eastman Kodak, and purified by repeated crystallisation from light petroleum.

5 gm. of recrystallised *n*-pentadecoic acid, 3 c.c. of 0·88 ammonium hydroxide and 250 c.c. of water were heated under reflux in a large flask in the water bath. When the temperature had been raised to 60° C. and the acid completely converted to soap, 10 c.c. of " perhydrol " were added and the temperature raised gradually to 90° C. After the reaction had ceased, two further amounts of 10 c.c. of peroxide were added. The whole reaction lasted about four hours. The mixture was cooled, acidified with phosphoric acid, the products filtered off, the acids converted into the soaps with a little alcoholic potassium hydroxide and the ketone extracted with light petroleum. The residue was acidified and the acids were filtered off and crystallised twice from methyl alcohol, the liquid being cooled by blowing a current of air over the alcoholic solution. In this way 75 per cent. of the pentadecoic acid was recovered unchanged. The acid obtained in the mother liquors of three such experiments was then crystallised from light petroleum, and gave an acid which, after several recrystallisations from boiling petroleum, melted at 92·6° C. and gave a molecular weight by titration of 254 (0·1250 gm. required 4·92 c.c. of N/10 NaOH). The m.p. was not depressed by mixing with the γ-ketopentadecoic acid obtained from Substance " A " by oxidation with permanganate in acetone solution.

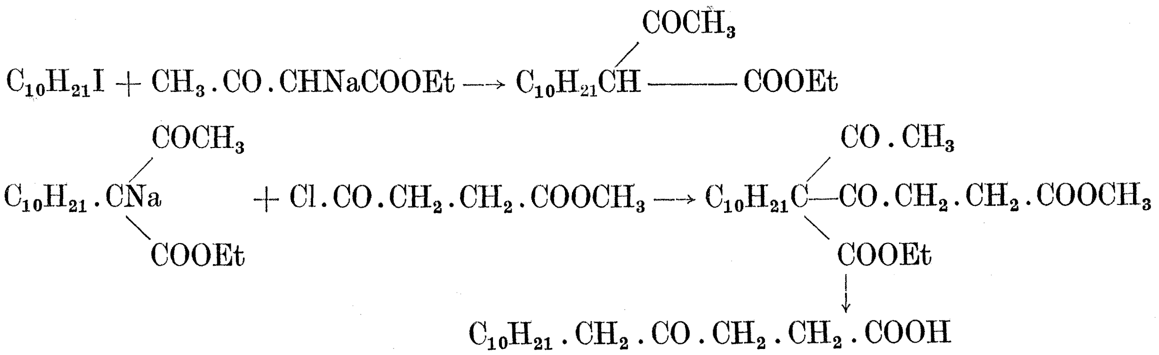
Synthesis of γ-Ketopentadecoic Acid.

The starting point for the synthesis was *n*-octyl iodide, which was first condensed with ethyl malonate, the ethyl octylmalonate hydrolysed and the free acid distilled to remove CO_2 thus forming *n*-decoic acid. This acid was then esterified and the ester reduced to decyl alcohol by the method of BOUVAULT and BLANC (1903) and this on treatment with hydrogen iodide (KRAFFT, 1886) gave decyl iodide. The synthesis

Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

was then carried out by a similar method to that used by Robinson and Robinson (1925) for the synthesis of other keto-acids. Decyl iodide was condensed with sodioacetoacetate to give ethyl α-acetyl-*n*-dodecoate which was treated with sodium and condensed with γ-carbomethoxypropionyl chloride. The product after alkaline and then acid hydrolysis gave finally γ-ketopentadecoic acid.



$$C_{10}H_{21}I + CH_3.CO.CHNaCOOEt \longrightarrow C_{10}H_{21}CH\begin{matrix}\diagup COCH_3 \\ \text{———} COOEt\end{matrix}$$

$$C_{10}H_{21}.C\begin{matrix}\diagup COCH_3 \\ Na \\ \diagdown COOEt\end{matrix} + Cl.CO.CH_2.CH_2.COOCH_3 \longrightarrow C_{10}H_{21}C\begin{matrix}\diagup CO.CH_3 \\ \text{—}CO.CH_2.CH_2.COOCH_3 \\ \diagdown COOEt\end{matrix}$$

$$\downarrow$$

$$C_{10}H_{21}.CH_2.CO.CH_2.CH_2.COOH$$

Ethyl n-*octylmalonate.*—2·3 gm. of sodium were dissolved in 25 gm. of alcohol and 16 gm. of ethyl malonate added and allowed to stand; 31 gm. of octyl iodide were then added and the mixture heated on the water bath for two hours. The alcohol was removed, the flask cooled and water added to dissolve the separated iodide. The ester was extracted with ether, the ethereal solution dried over $CaCl_2$ and fractionated *in vacuo*. Yield 18·5 gm. of an oil, b.p. 169° C./17 mm.

n-*Octylmalonic acid.*—The ester was hydrolysed by boiling for two hours with alcoholic potassium hydroxide and after removing the alcohol and cooling, the acid was precipitated with hydrochloric acid, filtered off and dried on a porous plate. The crude acid weighed 15 gm. (m.p. 108° C. decomp. at 140° C.).

n-*Decoic acid.*—The octylmalonic acid was then distilled *in vacuo* and gave 9 gm. of solid decoic acid m.p. 31° C.

Ethyl n-*decoate.*—13 gm. of *n*-decoic acid were esterified by the method of Fischer and Speier (1895) using 90 c.c. of absolute alcohol and 3 gm. of dry gaseous hydrochloric acid. The alcohol was distilled off and the residue poured into water. The ester was extracted with ether, the ethereal solution washed and dried over $CaCl_2$ and distilled. The ester boiled at 131° C./17 mm. Yield = 11 g.

Decyl alcohol.—22 gm. of ethyl decoate were dissolved in 80 gm. of absolute alcohol (redistilled over a little sodium) and the solution dropped slowly by way of a condenser on to 16 gm. of sodium. The sodium melted and was shaken whilst the alcoholic solution was being added. After reduction was completed, a little more alcohol was added and the flask was heated in a boiling $CaCl_2$ bath to remove any small pieces of sodium, cooled, water added and the ethyl alcohol distilled off. The residue was then acidified and extracted with ether. The ethereal solution was then dried and distilled, the decyl alcohol being collected at 127° C./16 mm., and the decoic acid, resulting from hydrolysis of the ester, at 170° C./16 mm. The yield of distilled alcohol was 10 gm.

Downloaded from rstb.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Decyl iodide.—The alcohol was saturated with gaseous hydrogen iodide, heated on the water bath, cooled and again saturated, allowed to stand overnight, again saturated and allowed to stand. The iodide was then decolorised with sodium carbonate and extracted with ether, the ethereal solution dried over $CaCl_2$ and distilled. The decyl iodide boiled at 132° C./15 mm.

Ethyl α*-acetyl-*n*-dodecoate.*—0·93 gm. of sodium was dissolved in 12 c.c. of absolute alcohol and 7·9 gm. of ethyl acetoacetate and subsequently 10·7 gm. of decyl iodide were added. The mixture was heated on the water bath under reflux for three hours. Sodium iodide separated. The alcohol was removed in a stream of air, the residue cooled, water added to dissolve the iodide and the ester extracted with ether. The ethereal solution was dehydrated over $CaCl_2$ and distilled. The dodecoate boiled at 170° C./16 mm.

γ*-Carbomethoxypropionyl chloride.*—This was prepared by the method of CLUTTERBUCK and RAPER (1925) by converting succinic anhydride into the monomethyl ester, treating with thionyl chloride and fractionating; the methyl ester chloride boiled at 93° C./18 mm.

γ*-Ketopentadecoic acid.*—A solution of γ-carbomethoxypropionyl chloride (4 gm.) in ether (20 c.c.) was added to the sodio derivative from ethyl α-acetyl-*n*-dodecoate (7 gm.) and sodium (0·6 gm.) in ether (220 c.c.). The mixture was cooled in ice water, kept overnight and boiled under reflux for one and a half hours. The washed and isolated product was hydrolysed by boiling for six hours with 5 per cent. KOH (200 c.c.), then for 30 hours with 5 per cent. H_2SO_4 (160 c.c.) and finally for three hours with 5 per cent. KOH. The solution was then acidified and extracted with ether. The ethereal solution was reduced to a small volume and the keto-acid precipitated with light petroleum. It was finally recrystallised four times from boiling light petroleum and then melted at 92·6° C. and did not depress the m.p. of the keto-acid obtained by oxidation of hydrolysed "A" with permanganate. 0·1029 gm. required 4·05 c.c. of N/10 NaOH for neutralisation, corresponding to an equivalent of 254 (theoretical for $C_{15}H_{28}O_3 = 256$).

The substance gave the following results on micro-combustion (SCHOELLER, Berlin):—

TABLE XV.

Weight of Substance Analysed.	Weight of CO_2.	Weight of H_2O.	Percentage Carbon.	Percentage Hydrogen.
Mgm.	Mgm.	Mgm.		
4·737	12·140	4·63	69·91	10·94
4·991	12·820	4·90	70·03	10·98
Theoretical for $C_{15}H_{28}O_3$	…	…	70·26	11·01

Downloaded from rstb.royalsocietypublishing.org

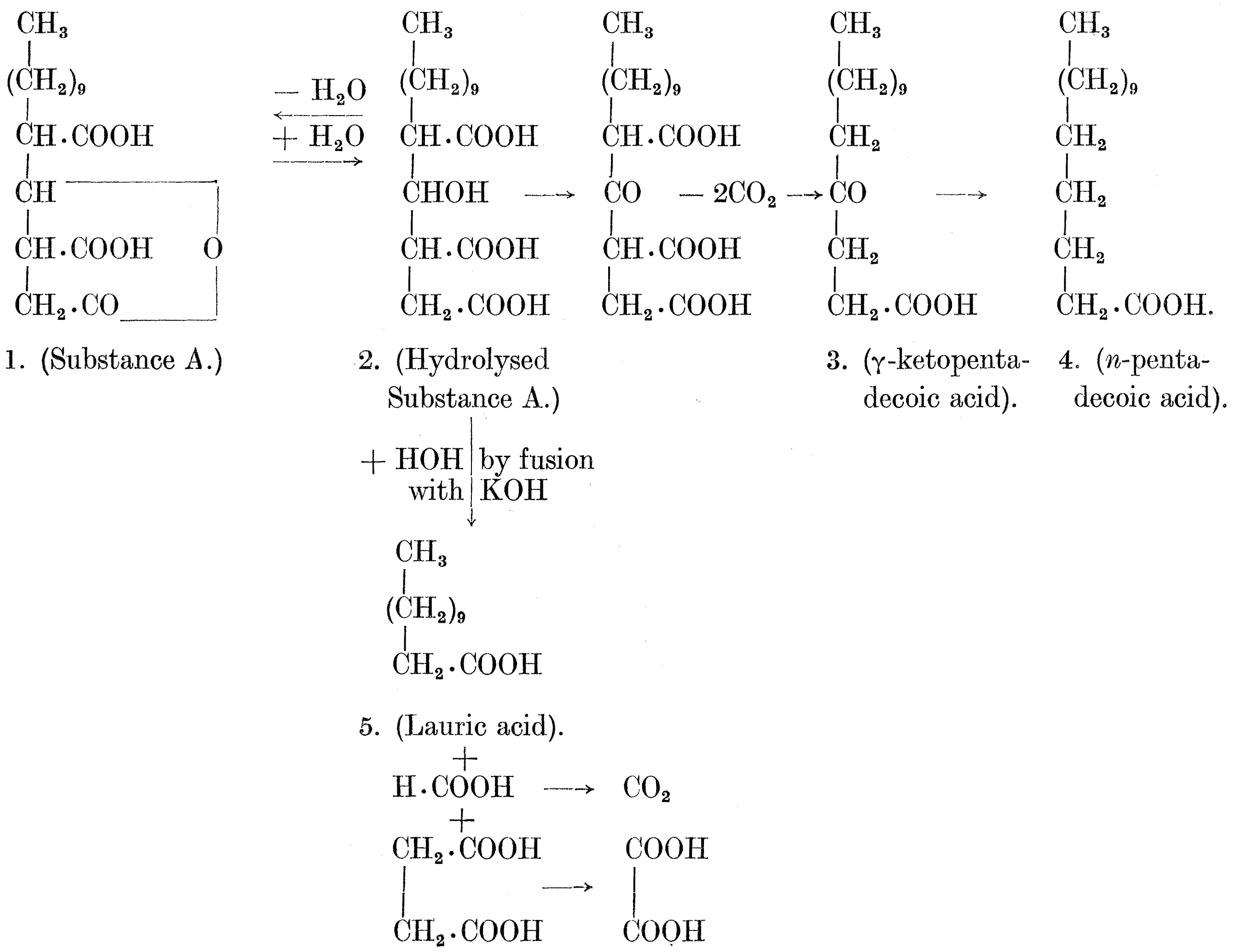
Discussion of Results.

The following results are of importance in arriving at a constitutional formula for Substance " A " :—

1. Substance " A," $C_{17}H_{28}O_6$, titrates as a dibasic acid, contains two COOH groups (p. 305), and an asymmetric carbon atom (p. 306).
2. On hydrolysis with dilute sodium hydroxide, Substance " A," $C_{17}H_{28}O_6$, gives rise to hydrolysed Substance " A," $C_{17}H_{30}O_7$, which titrates as a tribasic acid containing three carboxyl groups (p. 310). The seventh oxygen atom is present as a hydroxyl group since hydrolysed Substance " A " gives a mono-acetyl derivative (p. 313). The ease with which hydrolysed Substance " A " reverts to Substance " A " by the loss of a molecule of water on heating (p. 314), suggests that hydrolysed Substance " A " is a γ-hydroxy-tricarboxylic acid, and that Substance " A " is the lactone of this acid.
3. Substance " A " on methylation with diazomethane gives a lactone dimethyl ester (p. 319) while hydrolysed Substance " A " on ethylation with diazoethane gives a hydroxy-triethyl ester (p. 319).
4. On fusion with potassium hydroxide, hydrolysed Substance " A " gives lauric acid, $C_{12}H_{24}O_2$, succinic acid, $C_4H_6O_4$, and carbon dioxide (p. 322).
5. Hydrolysed Substance " A " is oxidized by permanganate, in acetone solution, almost quantitatively to a ketopentadecoic acid (p. 325), which on reduction gives an almost quantitative yield of *n*-pentadecoic acid (p. 325).
6. The ketopentadecoic acid arising as an oxidation product of hydrolysed Substance " A," was shown by synthesis to be the γ-keto-acid (p. 326), thus indicating that the hydroxyl group in hydrolysed Substance " A " is in the γ-position to the terminal carboxyl group.

These facts prove conclusively that Substance " A " is the lactone of γ-hydroxy-βδ-dicarboxypentadecoic acid of the formula (1) and hydrolysed Substance "A" is thus γ-hydroxy-βδ-dicarboxypentadecoic acid, formula (2). The breakdown of Substance " A " on oxidation to γ-ketopentadecoic acid, formula (3) and to *n*-pentadecoic acid formula (4), is shown in the following scheme in which is also given the relation of Substance " A " to its fusion products, lauric acid, formula (5), succinic acid and CO_2.

Downloaded from rstb.royalsocietypublishing.org



Summary.

P. spiculisporum LEHMAN, when grown on CZAPEK-DOX glucose solution, gives rise to a new metabolic product. This substance, which is a new polybasic fatty acid, $C_{17}H_{28}O_6$, is the lactone of γ-hydroxy-βδ-dicarboxypentadecoic acid. A description is given of its preparation, properties, derivatives and breakdown products. Other metabolic products of *P. spiculisporum* LEHMAN are succinic acid and γ-ketopentadecoic acid.