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Degradation of ^{14}C-Labelled Carolic and Carlosic Acids from *Penicillium Charlesii* G. Smith

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From cultures of *Penicillium Charlesii* G. Smith, grown on glucose and ^{14}C-carboxyl-labelled acetate as an additional carbon source, labelled carlosic and carolic acid have been isolated. Itaconic acid, not earlier reported for this species, has also been isolated. Degradation procedures for the two tetronic acids have been worked out and on the basis of the observed isotopic distribution, the synthesis of the tetronic acids is suggested to take place as a condensation between a high labelled 6-carbon unit of β-keto acid type derived directly from acetate by head-to-tail coupling and a low labelled 4-carbon member of the citric acid cycle, the latter being comparatively more diluted by unlabelled carbon from glucose during the incorporation of the acetate added.

It is known from the work of Raistrick and his collaborators [1,2] that *Penicillium Charlesii*, when grown on glucose, produces five closely related acids of the tetronic acid type: carolic, carlosic, carolinic, carlic and γ-methyltetronic acid. From other *Penicillium* species and molds in general a vast number of different organic compounds have been isolated and structurally determined, to a great extent by the work of the Raistrick school, but relatively little is known about the metabolic pathways which lead to the synthesis of these substances. The use of radioactive isotopes, added to the culture medium in the form of labelled glucose, acetate or other intermediary metabolites, is here, as has been the case in the studies on bacterial metabolism, one way of approaching the problem of mold biosynthesis. Among the increasing number of investigations of this kind, the papers by Arnstein and Bentley [3] on the formation of kojic acid, the work by Bentley and Thiessen [4] on itaconic acid biosynthesis and Birch's [5] studies on the synthesis of aromatic compounds can be mentioned.

The study of *Penicillium Charlesii* was suggested by Professor G. Ehrensvärd * as an interesting parallel to a more extensive investigation on the

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production of phenols and phenol-carbonic acids by *Penicillium* molds belonging to the species *brevi-compactum*, *griseo-fulvum*, *patulum*, *urticae* and *Daleae*. The biosynthesis of aromatic seven- and eight-carbon compounds from glucose by *P. urticae* is discussed in a paper by Ehrensvärd [6].

In order to get a more detailed picture of the metabolic production pattern of *Penicillium Charlesii*, a systematic search for substances in the culture medium was made, using a chromatographic method specially worked out at the laboratory for the study of mold metabolites [7]. With this method new substances could be detected and chromatographically defined, even though their structures were not known.

From the medium of younger cultures grown on glucose (14 days) itaconic acid, not earlier reported for this species, could be isolated in fairly large amounts (0.5 g from six cultures). In older cultures itaconic acid was present in much smaller amounts, for example, only 0.4 g could be isolated from forty-five cultures grown on glucose for a period of 47 days. This suggested that itaconic acid during the incubation period was taken up again from the medium and might be used as an intermediary metabolite in the synthesis of the tetronic acids. To test this possibility ^{14}C-carboxyl-labelled acetate was added to culture medium containing glucose with the expectation of obtaining ^{14}C-labelled itaconic acid. The labelled acid was then intended to be used as an additional carbon source in cultures of the same age as those from which it was to be isolated in order to study its eventual incorporation in the tetronic acids. After two weeks, however, itaconic acid could only be found in very small amounts, while on the other hand carolic and carlosic acid could be isolated, yielding about 0.25 g of each acid from five cultures. The labelled acetate was incorporated in itaconic acid as well as in the two tetronic acids. Addition of acetate apparently increased the formation rate of the tetronic acids, which normally could not be isolated from 2 weeks old cultures.

As itaconic acid could not be isolated in amounts sufficient either for addition to growing cultures, or to permit a complete degradation, no conclusions as to its role in tetronic acid synthesis could be drawn. The purpose of this paper will be to present degradation procedures for carolic and carlosic acid and to report the results of these degradations.

EXPERIMENTAL

Cultivation: Penicillium Charlesii P146, obtained by the courtesy of Professor H. Raistrick at the London School of Hygiene and Medicine, was grown at a temperature of 24°C on a modified Czapek-Dox solution of the following composition: glucose 50.0 g, $NaNO_3$ 2.0 g, KH_2PO_4 1.0 g, KCl 0.5 g, $MgSO_4 . 7H_2O$ 0.5 g, $FeSO_4 . 7H_2O$ 0.005 g, distilled water 1 000 ml and $ZnSO_4 . 7H_2O$ added to give a final concentration of 10^{-6} M of the Zn^{2+}-ion. 1 liter, flat bottom culture flasks, containing 500 ml of the modified Czapek-Dox solution, were inoculated with a spore suspension of about 2×10^6 spores per flask.

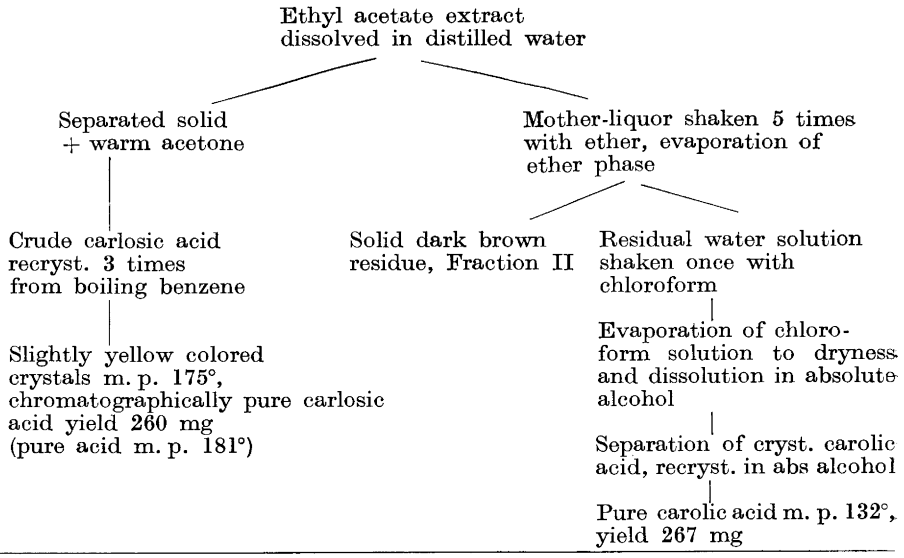
In the isotope experiment 50 mg of ^{14}C-carboxyl-labelled sodium acetate was added on the 3rd and 5th days after inoculation. Altogether 100 mg of labelled acetate (214 000 counts/min/mg carbon in the carboxyl group) was added to each of five culture flasks.

Isolation: After a growing period of two weeks, the culture medium was separated from the mycelium by filtration through cotton wool. The solution from five flasks, about 3 l including 500 ml distilled water used for washing of the mycelia, was evaporated

in vacuo to 200 ml. The solution was then, after acidification with 8 ml of concentrated hydrochloric acid, shaken 5 times with a total of 500 ml ethyl acetate. This extract was evaporated *in vacuo* to 50 ml and upon standing for a couple of days the solution solidified into a sticky mass of dark brown crystals, weighing 7.6 g. From this crude mixture of substances carolic and carlosic acid were isolated according to the procedure outlined in Table 1.

Table 1.

Table 1. Isolation of carolic and carlosic acid.



Both acids were chromatographically tested in six different systems [7], and were found to give R_F values identical with those of the corresponding acids obtained from Professor Raistrick's laboratory and also isolated here from larger cultures.

Chromatography of different fractions during the isolation procedure did reveal the presence of itaconic acid only in the crude mixture called fraction II in Table 1.

This fraction was further purified by repeated crystallisation from chloroform, giving 100 mg of a crystalline substance, by chromatography test found to be a mixture of carlosic and itaconic acids. 50 mg of this mixture was run on a silicic acid column and eluted with chloroform and 5—15 % solutions of butanol in chloroform, according to the scheme worked out by Marvel and Rands [8]. The effluent peak volumes for carlosic and itaconic acid were 200 ml and 290 ml, respectively, identical with the values obtained for a test mixture of the same substances. The eluent was collected in 10 ml fractions, which were titrated with 0.2 N NaOH using phenol red as an indicator. The fractions containing itaconic acid were evaporated to dryness but could not be separated from the indicator substance without losing most of the acid at the same time. The remaining 50 mg of the ^{14}C-labelled carlosic-itaconic acid mixture was run on a silicic acid column in exactly the same way as the first portion with the exception that the collected fractions were not titrated. The fractions known to contain itaconic acid were evaporated to dryness, giving 11.8 mg of practically pure itaconic acid.

Degradation: Hydrolysis of the tetronic acids in 2 N sulphuric acid is known [2] to break up the ring to give carbon dioxide and acetoin, one molecule of each, and a third molecule of a 4-carbon compound formed by the side chain attached to the α-position of the ring.

The side chains of carolic and carlosic acid give one molecule of γ-butyrolactone and butyric acid, respectively.

The common procedure for the different steps worked out for the degradation of carolic and carlosic acid is outlined in the following.

1 mmole of the acid is hydrolyzed for 24 h in 10 ml of 2 N sulphuric acid at a temperature of 140°C. A stream of CO_2-free nitrogen is passed through the hydrolysis solution into a flask with saturated $Ba(OH)_2$ solution to take up the CO_2 formed, on its way passing another flask with 2,4-dinitrophenylhydrazine to trap any acetoin leaving the solution. The CO_2 coming from the ring is easily split off during the first three hours of hydrolysis, while on the other hand the second molecule of CO_2 from the carboxy-methyl group in the γ-position of carlosic acid is more slowly released. By prolonged hydrolysis for 23 h about 60 % of this carboxyl group is recovered as $BaCO_3$ as seen from the values in Table 2. Another 6 h of hydrolysis does not increase the yield. The hydrolysis flask is supplied with an efficient reflux-condenser to decrease the amount of acetoin carried along with the nitrogen stream.

The hydrolysate was then neutralized (to phenolphthalein) with a 33 % solution of NaOH and steam distilled in order to blow over the acetoin. 100 ml of distillate was collected, a large enough volume for all the acetoin to be carried over. The acetoin solution was further treated, according to the method of Clutterbuck and Reuter [9], with 10 ml of 2 N H_2SO_4 and 300 mg (1.3 mmole) of potassium periodate for the oxidation of acetoin to acetaldehyde and acetic acid. Merely keeping the mixture at room temperature for 23 h, as suggested by Clutterbuck and Reuter, was in this case not very effective. A more complete splitting of the molecule was obtained by keeping the solution at 60°C for about 70 h. During this time nitrogen was continuously bubbled through the solution and the acetaldehyde formed was passed *via* an alkaline trap (2 ml 0.01 N NaOH) to take up any acetic acid carried along, into a chromic acid solution (6 N H_2SO_4 + 0.8 N CrO_3) kept at 70°C. The chromic acid solution was later steam distilled and the acetic acid (from acetaldehyde) was titrated with 0.2 N NaOH and put aside for further degradation.

In order to recover the acetic acid split off from acetoin, the reaction mixture was neutralized, evaporated to about 10 ml and after acidification with H_2SO_4 solid sodium sulphite was added in amounts just enough to reduce all the iodine to iodide. The solution could then be steam-distilled and the acetic acid titrated and stored for later Schmidt degradation.

The neutralized hydrolysate contained after steam-distillation sodium butyrate or sodium γ-hydroxybutyrate, depending upon whether carlosic or carolic acid was degraded. Alkaline titration of butyrolactone at a temperature of 80 – 90° did within about 2 h lead to the complete opening of the lactone ring and the formation of sodium γ-hydroxybutyrate. Butyrolactone and butyric acid were separated from the neutralized hydrolysis mixture by steam-distillation after acidification with conc. H_2SO_4.

Butyric acid was after titration with 0.2 N NaOH and evaporation to dryness ready for Schmidt degradation. Butyrolactone on the other hand, or rather Na-γ-hydroxybutyrate obtained after alkaline titration, had to be reduced to butyric acid. This could be done in one operation by heating the sodium γ-hydroxybutyrate (about 0.5 mmole) in a bomb tube containing 1 ml of hydriodic acid, d 1.96, for 5 h at a temperature of 230°C. At this temperature the γ-iodo-butyric acid, formed already at lower temperatures [10], was further converted to butyric acid. The acid was separated from the reaction mixture after dilution to about 20 ml with distilled water and addition of solid sodium sulphite until all the iodine was reduced to iodide. 3 g of silver sulphate dissolved in 3 ml of 85 % H_2SO_4 was then added in order to precipitate the iodide-ions as silver iodide. The precipitate was filtered off, the filtrate steam-distilled and the distillate, containing butyric acid, titrated and evaporated to dryness.

In this manner all the degradation products from carolic and carlosic acid, with the exception of the CO_2 formed during hydrolysis, were obtained in the form of fatty acids suitable for further degradation by the Schmidt procedure [11].

The total ^{14}C-content of the labelled substances was determined by combustion to CO_2 in a stream of oxygen passed through a quartz tube over a platinum gauze heated to 800°C.

RESULTS

The results of the degradations are presented in Tables 2 and 3 and Fig. 1. 1 mmole of each acid was taken for hydrolysis. Carlosic acid was mixed with non-active acid in the proportion 1:1, while carolic acid was degraded without being diluted. The CO_2 formed, either by hydrolysis, by Schmidt degradation or by total combustion, was trapped in a saturated barium hydroxide solution as $BaCO_3$. The ^{14}C-content was determined on 15 mg samples of uniform layers or $BaCO_3$ on 1 cm^2 discs under standard conditions, using a Tracergraph Geiger counter with an automatic sample changer. The ^{14}C-content is given in counts per min per mg carbon counted as $BaCO_3$. The ^{14}C-values of the different carbon atoms of carlosic acid are multiplied by two in order to get values comparable to those obtained for undiluted carolic acid.

Table 2. Degradation of carlosic acid.

Substance formed	time after start of hydrolysis	mmole of CO_2 collected during the diff. time intervals	^{14}C-content counts/min/ mg carbon	amounts in mmole of the degradation products
CO_2:	35 min	0.25	1 600	0.25
	1 h 10 min	0.35	1 564	0.60
	1 h 45 min	0.18	1 598	0.78
	2 h 20 min	0.09	1 278	0.87
	3 h 10 min	0.11	1 080	0.98
	11 h 40 min	0.43	560	1.41
	22 h 55 min	0.17	488	1.58
Acetoin:				
acetaldehyde → acetic acid (tot. combust.)			70	0.61
COOH-group			137	0.28
CH_3-group			22	0.23
acetic acid (total combustion)			72	0.69
COOH-group			130	0.43
CH_3-group			8	0.05 *
Butyric acid: (total combustion)			830 **	0.95
COOH-group			1 674	0.83
propionic acid			—	0.79
COOH-group			72	0.48
acetic acid			—	0.38
COOH-group			1 688	0.31
CH_3-group			16	0.23
Total combustion of carlosic acid			680	

* As this amount of $BaCO_3$ was not sufficient for the 1 cm^2 disc, the activity was measured on a uniform layer of 4 mg of $BaCO_3$ in a 0.4 cm^2 disc, giving values 1/3 of those obtained with discs of standard size.

** This value refers to the isotope content of CO_2 from total combustion of butyric acid obtained by hydrolysis of 0.1 mmole of undiluted carlosic acid.

Table 3. Degradation of carolic acid.

Substance formed	time after start of hydrolysis	mmole of CO_2 collected during the diff. time intervals	^{14}C-content counts/min/mg carbon	amounts in mmole of the degradation products
CO_2:	1 h	0.60	1 617	0.60
	3 h	0.34	1 650	0.94
	3 h 30 min	0.03	—	0.97
Acetoin:				
acetaldehyde → acetic acid				0.67
0.15 mmole for total combustion			55	(0.52)
COOH-group			179	0.38
CH_3-group			13	0.32
acetic acid (total combustion)			75	0.65
COOH-group			158	0.34
CH_3-group			11	0.43
Butyrolactone:				
(0.42 mmole * → 0.32 mmole butyric acid)				
butyric acid			—	0.32
COOH-group			1 299	0.24
propionic acid			—	0.20
COOH-group			101	0.14
acetic acid			—	0.16
COOH-group			909	0.08
CH_3-group			23	0.13
Total combustion of carolic acid			550	

* About 0.5 mmole of the butyrolactone was used in order to try the Schmidt degradation on sodium γ-hydroxybutyrate without previous reduction to butyric acid. The yield of the degradation was, however, only about 15 %.

The isotope content of the CO_2 split off from the tetronic acid ring is in the case of carolic acid a mean value of the two determinations made, in the case of carlosic acid a mean value of the first three determinations, containing undiluted CO_2 from the ring. The following fractions, obtained from carlosic acid, have a continuously decreasing isotope content depending upon a mixing with low labeled CO_2 from the carboxy-methyl group at the γ-position. The ^{14}C-content given for this carboxyl carbon might be regarded as a maximum value due to contamination by heavily labelled CO_2 from the ring.

In the above-mentioned paper by Clutterbuck and Reuter [9] it is indicated that by periodate oxidation of acetoin the carbonyl carbon and its adjacent methyl group yield acetic acid and the carbinol group and its adjacent methyl group are split off as acetaldehyde. Whether the symmetrical labelling found for acetoin corresponds to the original isotopic distribution between the β- and γ-carbon atoms in the tetronic acid ring (see Fig. 1) is doubtful. The asymmetrical carbon atom in the carbinol group in the γ-position is responsible for the optical activity of the tetronic acids. As hydrolysis of an ester linkage

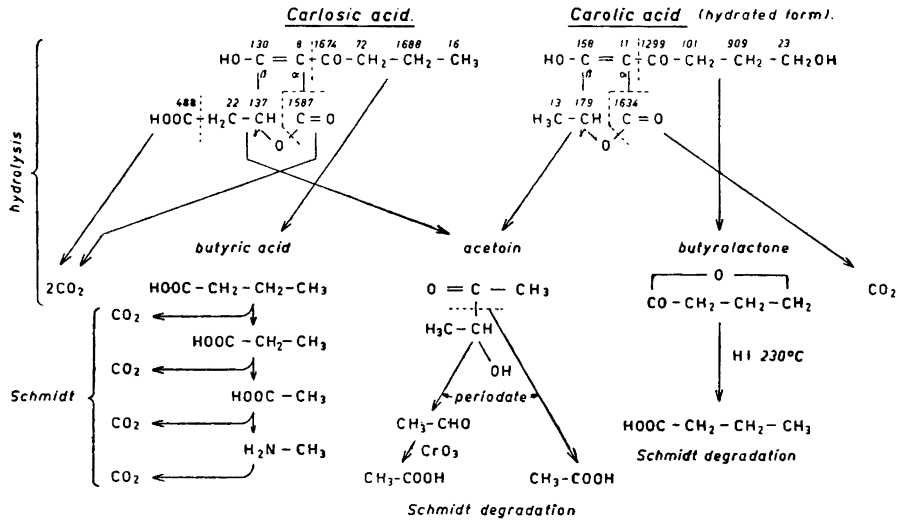


Fig. 1. Degradation scheme for carlosic and carolic acid. The values refer to the isotope content in counts/min/mg C, showing the incorporation of $CH_3{}^{14}COOH$ (214 000 counts/min/mg C in the carboxyl group).

normally does not lead to the rupture of the bond between the carbon atom and the attached hydroxyl group of a secondary alcohol [12], the acetoin formed by hydrolysis would be expected to show optical rotation. The fact that the optical activity of the solutions of carolic and carlosic acid dropped to zero during the hydrolysis, shows, however, that there must have been a change in the configuration of acetoin. As this change is probably not just a stereochemical alteration within the carbinol group [12], an exchange of substituents between the carbinol and carbonyl groups must be assumed to have taken place, with a mixing of the isotope contents as a consequence.

The isotope content found for the β- and γ-carbon atoms might then originally have been built into either one of these two carbon atoms.

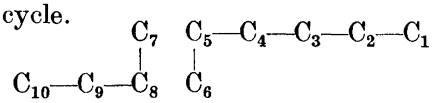
It has also been shown by Juni [13] that in unevenly labelled acetoin, produced by yeast, there occurs some mixing between the carbon atoms, for example by alkaline steam-distillation. In the present investigation it was not necessary to avoid alkaline steam-distillation for the separation of acetoin from the hydrolysis mixture, as at this stage acetoin was already optically inactive.

The only certain information about the ^{14}C-incorporation in itaconic acid was obtained by total combustion. Two determinations gave a mean value of 225 counts/min/mg carbon.

DISCUSSION

From the results of the incorporation of ^{14}C-labelled acetic acid in carolic and carlosic acid it is evident that, in the synthesis of the two acids, a common plan is followed. Considering carlosic acid, the distribution of the ^{14}C-isotope

indicates that the molecule can be regarded to be composed of two differently labelled parts, one 6-carbon and one 4-carbon unit, the former about 10 times as heavily labelled as the latter. The 6-carbon chain seems to be more directly built up from acetyl groups, whereas the lower isotope content of the other part points to a greater dilution with carbon from unlabelled glucose, probably over the citric acid cycle.



If this 4-carbon part, represented by the carbon atoms C_7 to C_{10} in the schematic formula given above (arbitrarily numbered), should at all be considered as a ^{14}C-labelled member of the citric acid cycle, it should have its ^{14}C-carbon mainly in C_7 and C_{10} corresponding to the carboxyl groups of one of the four dicarboxylic acids. The diverging results obtained, *i. e.* an equal distribution of the isotope between C_7 and C_8, can be accounted for, as was discussed earlier, by a change in the configuration of the acetoin molecule formed in the course of the hydrolytic breakdown of the tetronic acids. The isotope content of these two carbon atoms can thus be thought of as originating from C_7. The isotope content of this carbon is thereby increased to 267 counts/min (337 counts/min in the case of carolic acid), a value of the same order of magnitude as that of C_{10}, 488 counts/min.

If the total ^{14}C-content, $5 \times 225 = 1\ 125$ counts/min, obtained for itaconic acid, is assumed to be equally distributed between the two carboxyl groups, a distribution found in itaconic acid from *Aspergillus terreus*, fed carboxyl-labelled acetate [4], the ^{14}C-content of each group will be about 500 counts/min. This value is comparable in magnitude to the isotope contents of C_7 and C_{10}, supposed to originate from the carboxyl groups of a dicarboxylic acid, thus suggesting that itaconic acid in *Penicillium Charlesii* might also be formed over the citric acid cycle.

Even if the results do not suggest a definite scheme for the synthesis of the tetronic acids, they seem, however, to exclude a few pathways along which the acids probably not are formed.

A similar formation mechanism for ascorbic acid and the tetronic acid ring might be anticipated from the structural relationship between the two compounds. The tetronic acid ring would then in accordance with ascorbic acid be synthesized from glucose [14], so that the carbon sequence 6—1 in the glucose molecule would correspond to the carbon sequence 6, 5, 7, 8, 9, 10 in the schematic formula shown above. In glucose labelled by the incorporation of ^{14}C-carboxyl acetate the isotope will appear [15] in carbon atoms 3 and 4. Tetronic acid formed in this way from glucose would thus be labelled in C_7 and C_8. This is in accordance with the results, but the labelling of C_{10} and the high isotope content of C_6 cannot be accounted for by this scheme.

Another route for the formation of the tetronic acids would be a synthetic pathway over acetoin as an intermediary step. Such a mechanism is, however, not consistent with the results obtained, as it does not explain either the origin of the heavily labelled carbon in the carbonyl group in C_6 or the presence of the carboxyl group in C_{10}. Acetoin was furthermore never chromatographically detected in any of the isolated fractions.

Although tracer studies of this kind have their limitations, especially when a number of alternative pathways can be considered for the formation of a certain metabolite, the following speculations on tetronic acid synthesis are cautiously presented as consistent with the observed isotopic distribution.

The formation of the tetronic acids is thought to take place as a condensation between a 6-carbon chain, β-keto-caproyl-coenzyme A, formed *via* acetyl groups in fatty acid synthesis, and a 4-carbon compound of the citric acid cycle. If placed in the schematic formula shown above the β-keto acid will have its carboxyl group in C_6 and the carboxyl groups of the dicarboxylic acid will be in C_7 and C_{10}. The most suitable configuration of the dicarboxylic acid would be that of malic acid with the carbinol group in C_8 and a CoA-linked carboxyl group in C_7. The latter might then take part in a condensation reaction with the methylene group in C_5, activated and having a tendency to give off protons due to the influence of the adjacent carbonyl group and the CoA-linked carboxyl group. The hydroxyl group at C_7 would be formed by enolization, which also would give the double bond between C_5 and C_7. To complete the ring an ester linkage is then assumed to form between C_6 and C_8. Some support for the idea of malic acid as the 4-carbon unit is given by the findings of Larsen and Eimhjellen [16] that malic acid is accumulated with exclusion of itaconic acid in cultures of *Aspergillus terreus*, when the pH of the medium is increased by addition of $CaCO_3$. Whether an increase of the pH-value during the growth period is responsible for the observed accumulation of tetronic acids in preference to itaconic acid in younger cultures of *Penicillium Charlesii* cannot be settled. The pH-value of the culture medium was found to be almost the same before and after an incubation period of 14 days, 4.4 and 4.3 pH-units, respectively. Whatever the cause of the changed production pattern, the accumulation of tetronic acids with the exclusion of itaconic acid might be favored by an increased formation of malic acid. The assumption that malic acid would be present in a CoA-linked form is purely speculative.

The condensation might also take place between β-keto-caproyl-CoA and succinyl-CoA as the activated form of the dicarboxylic acid. It is known that succinyl-CoA by the action of the enzyme thiophorase preferentially gives off its coenzyme A to β-keto acids [17]. For the case that the β-keto acid already is activated by coenzyme A it seems not impossible that a condensation can take place instead. Once the 4-carbon part is attached to the methylene group in C_5, it might then be oxidized and hydrated in order to get a hydroxyl group in C_8, required for an ester linkage to be formed between C_6 and C_8.

Carlosic acid synthesized in this way by condensation of 6-carbon and 4-carbon units might then by decarboxylation of the carboxyl group in C_{10} and oxidation of the methyl group in C_1 to a hydroxy-methyl group be transformed to carolic acid. Oxidation of C_1 in carlosic acid to a hydroxy-methyl group would give carlic acid; decarboxylation of C_{10} together with an oxidation of C_1 to a carboxyl group would lead to the formation of carolinic acid. γ-Methyl-tetronic acid, finally, might be formed by hydrolysis of the bond between C_4 and C_5 from any of the other four tetronic acids, in the case of carlosic and carlic acid a decarboxylation of the carboxyl group in C_{10} would also be required.

γ-Methyltetronic acid might also be considered as the parent substance synthesized by a condensation of acetyl-CoA and activated malic acid or succinyl-CoA.

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