Studies in the Biochemistry of Micro-organisms

75. DEHYDROCAROLIC ACID, A METABOLIC PRODUCT OF *PENICILLIUM CINERASCENS* BIOURGE

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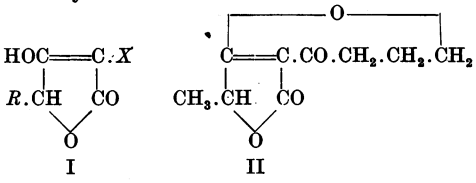
A series of acidic mould metabolic products, each of which is a derivative of tetronic acid (I, $R=H$; $X=H$) has been reported previously from this laboratory:

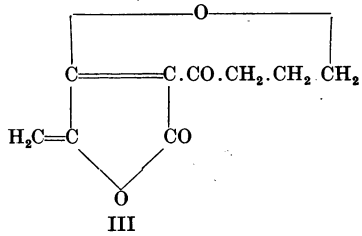


I II

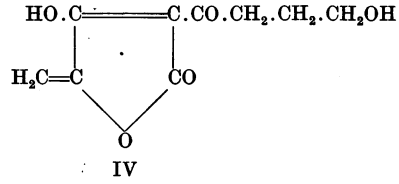
These products are: (*a*) *l*-γ-Methyltetronic acid. [I, $R=$—CH_3; $X=H$. Clutterbuck, Raistrick & Reuter, 1935*c*.] (*b*) Carolic acid. [II; hydrated form, I, $R=$—CH_3; $X=$—$CO.CH_2.CH_2.CH_2OH$. Clutterbuck, Haworth, Raistrick, Smith & Stacey, 1934; Clutterbuck *et al.* 1935*a*.] (*c*) Carolinic acid. [I, $R=$—CH_3; $X=$—$CO.CH_2.CH_2.COOH$. Clutterbuck *et al.* 1934, 1935*a*.] (*d*) Carlic acid. [I, hydrated form, $R=$—$CH_2.COOH$; $X=$—$CO.CH_2.CH_2.CH_2OH$. Clutterbuck *et al.* 1934, 1935*b*.] (*e*) Carlosic acid. [I, $R=$—$CH_2.COOH$; $X=$—$CO.CH_2.CH_2.CH_3$. Clutterbuck *et al.* 1934, 1935*b*.] (*f*) Terrestric acid. [I, hydrated form, $R=$—CH_3; $X=$—$CO.CH_2.CH_2.CHOH.CH_2.CH_3$. Birkinshaw & Raistrick, 1936.]

Products *a*, *b*, *c*, *d* and *e* were isolated from culture filtrates of *Penicillium Charlesii* G. Smith, and product *f*, terrestric acid, from culture filtrates of *P. terrestre* Jensen.

The present communication deals with a hitherto undescribed member of this series which was isolated from culture filtrates of a strain of *P. cinerascens* Biourge and for which the name *dehydrocarolic acid* is proposed. This substance has been shown to have the structural formula III or, in the hydrated form, IV.



III



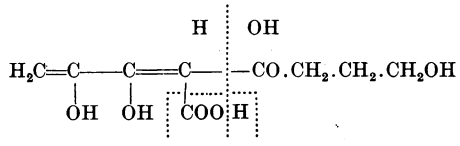
IV

Hence dehydrocarolic acid hydrate is α-(γ-*hydroxy*-n-*butyryl*)-γ-*methylenetetronic acid*.

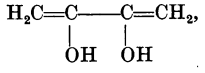
The experimental evidence on which this structural formula is based is as follows: (1) Dehydrocarolic acid, $C_9H_8O_4$, is a colourless, crystalline, monobasic acid. (2) Like carolic acid its aqueous solution turns Congo red paper a deep blue colour, and gives the same characteristic bright orange colour with ferric chloride. (3) It gives finally the same deep bluish-red colour on warming with concentrated sulphuric acid as does carolic acid. (4) Like carolic acid it is readily soluble in chloroform, but unlike this acid, which in aqueous solution is strongly dextrorotatory, dehydrocarolic acid is optically inactive. (5) On hydrolysis with boiling 2N-sulphuric acid some polymerization takes place and carbon dioxide, diacetyl and butyrolactone are formed though not in a theoretical yield. No polymerization is evident when carolic acid is hydrolyzed under the same conditions and the products of hydrolysis are one molecule each of carbon dioxide, acetoin and butyrolactone. (6) On catalytic reduction in hydrogen with a palladium-charcoal catalyst 2 atoms of hydrogen are absorbed and good yields of crystalline dl-*carolic acid*, $C_9H_{10}O_4$, are obtained. This substance, on acid hydrolysis, gives one molecule each of carbon dioxide, acetoin and butyrolactone (cf. dehydrocarolic acid). (7) On treatment with ozonized oxygen, formaldehyde is formed and was isolated as the dimedone derivative in a yield of 64 % of the theoretical for the formation of one molecule. Hence it is clear that dehydrocarolic acid contains the $CH_2{=}C$ grouping.

The structural formulae, III and IV, explain satisfactorily the following experimentally established facts. (1) From its general chemical properties dehydrocarolic acid is clearly a derivative of tetronic

acid. (2) The optical inactivity of dehydrocarolic acid, because of the absence of an asymmetric carbon atom, in contrast to the optical activity of all the other tetronic acid derivatives listed. (3) The products of acid hydrolysis are carbon dioxide, diacetyl and butyrolactone. Thus if the rings are opened, structures III and IV become:



and this structure on breakdown, would give the di-enol form,



of diacetyl, carbon dioxide, and, initially, γ-hydroxybutyric acid. (4) The ready conversion of dehydrocarolic acid to *dl*-carolic acid on catalytic reduction through the change of $CH_2{=}C$ to $CH_3.C.$ (5) The presence of the terminal $CH_2{=}C$ grouping and because of this grouping the tendency of dehydrocarolic acid to polymerize.

In addition to dehydrocarolic acid smaller amounts of carlosic acid, product *e* (p. 569) were isolated.

The culture filtrate of this strain of *Penicillium cinerascens* grown on Czapek-Dox solution completely inhibited the growth of *Staphylococcus aureus* at a dilution of 1:80. This was satisfactorily explained by the isolation of the antibiotics, gliotoxin (Weindling & Emerson, 1936; Johnson, Bruce & Dutcher, 1943; Johnson, McCrone & Bruce, 1944) and spinulosin (Birkinshaw & Raistrick, 1931; Oxford and Raistrick, 1942).

EXPERIMENTAL

Culture and cultural conditions

The culture used throughout this investigation was isolated in 1943 from Cumberland soil by Mrs J. M. Webb and was identified by Mr George Smith as a strain of *Penicillium cinerascens* Biourge. It bears the London School of Hygiene and Tropical Medicine (L.S.H.T.M.) catalogue number M. 538.

The culture medium used was 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.; $FeSO_4.7H_2O$, 0·01 g.; distilled water, to 1 l. The medium was distributed in 350 ml. amounts in a number of 1 l. conical flasks plugged with cotton wool, sterilized, inoculated with a spore and mycelium suspension of *P. cinerascens* and incubated at 24°.

ISOLATION OF GLIOTOXIN AND SPINULOSIN

Individual flasks were harvested from time to time and tested for antibacterial activity against the L.S.H.T.M. strain of *Staphylococcus aureus*, employing the serial dilution method described by Clutterbuck, Lovell & Raistrick (1932) using 2% glucose heart broth, and recording the results obtained after 24 hr. incubation at 37°.

The mould culture filtrate gave, after 12–15 days incubation, a maximum inhibition of the growth of *Staph. aureus* at a dilution of 1:80 and, after this period, 37 flasks were harvested, yielding a total volume of culture filtrate of 11·4 l., pH 3·8–3·9. This filtrate, which was pale yellow in colour with a slight purple tint, was thoroughly extracted once, without further treatment, with an approximately equal volume of chloroform. The chloroform-extracted solution now showed no antibacterial activity. The chloroform solution was washed with a little water and was evaporated *in vacuo* to dryness giving 2·39 g. of a dark oil which was fractionated by the method of Menzel, Wintersteiner & Hoogerheide (1944). It was dissolved in ether (900 ml.), filtered from a trace of brown amorphous matter and extracted four times with 50 ml. of saturated aqueous $NaHCO_3$ solution each time (extract A). The ethereal solution was now re-extracted with a total of 100 ml. of 6% aqueous Na_2CO_3 solution in four portions (extract B).

The residual ether solution was washed with a little water, dried and evaporated to dryness giving a pale yellow oil which crystallized to a semi-solid mass (1·24 g.), and was crystallized from ethanol (5 ml.) giving finally 0·18 g. of colourless square-ended rods, m.p. 200° (decomp.). This substance, which contained nitrogen and sulphur, had the same properties and optical rotation as gliotoxin and did not depress the melting-point of an authentic specimen of this compound m.p. 200° (decomp.) kindly supplied by Dr J. R. Johnson of Cornell University, U.S.A.

The deep red coloured $NaHCO_3$ solution (extract A) was acidified and re-extracted with chloroform. This yielded, on evaporation, a small amount of a purple solid, the total weight of which was 83 mg. from 180 flasks. It was fractionally sublimed in a high vacuum and gave a crystalline purple sublimate, m.p. 201°, alone or admixed with authentic spinulosin. It also gave the same colour reactions with alkalis and concentrated sulphuric acid as are given by spinulosin. (Found: C, 52·7; H, 4·6. $C_8H_8O_5$ requires C, 52·2; H, 4·4%.)

The Na_2CO_3 solution (extract B) was acidified and extracted five times with an equal volume of benzene. Removal of the benzene *in vacuo* yielded only a trace of oil indicating the absence of helvolic acid.

DEHYDROCAROLIC ACID

Preparation

The culture, culture medium and cultural conditions used were the same as are described in the previous section, but it was found that the best yields of dehydrocarolic acid were obtained if malt agar slope cultures of the mould, incubated at 24° and not more than 8 days old, were employed. It is advisable to use a heavy sowing of mycelium and spores.

The optimum time of harvesting the flasks was 20–24 days (see Table 1, batches 6*a*–6*e*), by which time the glucose, as determined by polarimeter had fallen to less than 1·0 %, the pH was 3·3–3·5 and the ferric chloride colour, obtained by adding 1 ml. of 10 % aqueous $FeCl_3$ to 4 ml. of culture filtrate was a distinct orange brown. Under these conditions the yield of crude dehydrocarolic acid obtained averaged about 1·0 g./l. of culture filtrate.

Experimental details of a number of preparations are given in Table 1.

Table 1. *Production of dehydrocarolic acid*

Batch no.	No. of flasks	Incubation period (days)	Vol. of metabolism solution (l.)	pH	Residual glucose by polarimeter (%)	Total wt. of crude acid (g.)	Crude acid (g./l. of culture filtrate)
1*a*	20	12	6·25	3·8	1·89	7·71	0·68
1*b*	17	15	5·15	3·9	1·14		
2*a*	25	20	7·7	3·3	0·94	5·58	0·73
2*b*	25	21	7·65	3·3	0·63	7·01	0·92
2*c*	22	24	6·8	3·3	0·46	11·98	1·76
3*a*	25	20	7·6	3·3	0·73	8·8	1·16
3*b*	25	23	7·7	3·4	0·25	10·1	1·31
4	30	22	8·8	3·5	0·45	7·21	0·82
5	30	26	8·9	4·1	0·08	4·22	0·48
6*a*	15	12	4·75	3·7	1·97	1·50	0·32
6*b*	15	20	4·4	3·5	0·90	3·19	0·72
6*c*	30	24	8·6	3·7	0·37	6·35	0·75
6*d*	30	26	8·4	3·9	0·25	4·55	0·54
6*e*	27	34	7·2	4·8	Nil	2·69	0·37

The culture filtrate was acidified with conc. HCl (10 ml./l.) and extracted once with one-quarter of its volume of chloroform and then three times with one-eighth of its volume of chloroform each time. The combined chloroform extracts were evaporated *in vacuo* to 200–300 ml. This concentrate was shaken for a few moments with saturated $NaHCO_3$ solution (5 ml./l. of original culture filtrate) to remove spinulosin and other colouring matters. The chloroform solution, now almost colourless, was stirred vigorously with a mechanical stirrer for 1–1½ hr. with double the above quantity of saturated $NaHCO_3$ solution. At the end of this time all the dehydrocarolic acid had passed into the $NaHCO_3$ layer. It is a notable fact that dehydrocarolic acid is only slowly extracted from chloroform solution by aqueous $NaHCO_3$, the most probable explanation being that this substance is present in chloroform solution mainly in the form given in structure III and in $NaHCO_3$ solution as structure IV.

The $NaHCO_3$ solution was now acidified with conc. HCl and dehydrocarolic acid separated as almost colourless crystals which were washed with water and dried. The acid thus obtained is sufficiently pure for most purposes. Chloroform extraction of the filtrate and washings gave a second crop of somewhat less pure material. The sum of the weights of these two crops is referred to in Table 1 as 'crude' acid.

The further purification of dehydrocarolic acid is rendered more difficult by the ease with which the substance polymerizes particularly on heating in polar solvents. It can be recrystallized in small amounts from a large volume of light petroleum (b.p. 60–80°). For larger amounts the following method proved to be satisfactory. The finely powdered dry acid (12 g.) was added to a boiling mixture (1·5 l.) of chloroform (17–20 % by vol.) and light petroleum (b.p. 60–80°), Δ of mixed solvents, 0·822–0·851 at 20°. After boiling for a few moments under reflux with vigorous agitation the solution was quickly filtered and cooled. 2·5 g. of the crude acid remained undissolved and there separated from the filtrate 7·5 g. of pure dehydrocarolic acid as colourless fine platelets. Evaporation *in vacuo* of the mother liquors to one-third vol. yielded an only slightly impure second crop.

Isolation of carlosic acid

The culture filtrate from batches 1*a* and 1*b*, Table 1 (11·4 l.) after separation of dehydrocarolic acid with chloroform, was re-extracted five times with ether, using 2 l. of solvent each time. On removal of the solvent there remained 0·76 g. of a reddish semi-crystalline residue. This was dissolved

in dry ether and fractionally crystallized by the graded addition of light petroleum (b.p. 60–80°). An almost colourless crystalline fraction (0·08 g.; m.p. 178°) was obtained and was recrystallized from benzene from which it separated in colourless needles identical in form with those of authentic carlosic acid crystallized in the same way. This substance was identified as carlosic acid as follows: m.p. 178°, not depressed on admixture with authentic carlosic acid. Rotation of free acid ($c = 0{\cdot}99\,\%$) in water: $[\alpha]^{19°}_{5461} = -160°$, unchanged overnight; carlosic acid has the same rotation, −160° (Clutterbuck *et al.* 1934). (Found: C, 52·51; H, 5·26; equivalent by titration, 113·6. $C_{10}H_{12}O_6$ requires C, 52·61; H, 5·31 %; equivalent as a dibasic acid, 114.)

Properties of dehydrocarolic acid

Dehydrocarolic acid crystallizes in colourless fine platelets. It has no melting-point, since because of its great tendency to polymerize, it changes to a glassy or rubbery material on heating at temperatures above 80° whether alone or in many solvents, particularly polar solvents. Its empirical formula is $C_9H_8O_4$. (Found: C, 59·95, 59·95; H, 4·58, 4·41; neutralization equivalent to phenolphthalein (sharp end-point), 181·1. $C_9H_8O_4$ requires C, 59·98; H, 4·47 %; equivalent, titrating as a monobasic acid, 180·2.) An aqueous solution of the sodium salt ($c = 0{\cdot}50\,\%$) is optically inactive. The substance does not inhibit the growth of *Staph. aureus* (L.S.H.T.M. strain) at a concentration of 1:500.

It is almost insoluble in light petroleum, slightly soluble in ether, ethanol and ethyl acetate but dissolves readily in cold chloroform though it is not quite so soluble in this solvent as is carolic acid.

It dissolves to the extent of about 2 % in cold water and this aqueous solution gives the following reactions. (*a*) It is strongly acidic turning Congo red paper blue. (*b*) With $FeCl_3$ a characteristic bright orange colour. (*c*) No colour with $NaNO_2$ solution. (*d*) It readily reduces Fehling's solution, immediately decolorizes $KMnO_4$ solution and fairly quickly gives a precipitate with Brady's reagent, 0·3 % 2:4-dinitrophenylhydrazine in 2N-HCl. A neutral 0·1N-aqueous solution of the sodium salt gives the following reactions. (*a*) With $CaCl_2$ or $BaCl_2$, no precipitate even after the addition of 4 vol. of ethanol. (*b*) $AgNO_3$ or $HgCl_2$, no precipitate but some darkening overnight. (*c*) Lead acetate, slight precipitate on standing. (*d*) Basic lead acetate, heavy white precipitate. (*e*) Copper sulphate, nickel sulphate or uranyl acetate, no precipitate but on standing overnight well formed nodular crystals develop, blue, blue-green and yellow respectively.

On warming with conc. H_2SO_4, dehydrocarolic acid dissolves giving a colourless solution which, on heating to 120°, becomes a deep bluish-red colour which is almost identical with that given by carolic acid at 140°. Both solutions show an intense brownish red fluorescence in ultra-violet light.

Dehydrocarolic acid mono-2:4-dinitrophenylhydrazone

Brady's reagent (0·3 % 2:4-dinitrophenylhydrazine in 2N-HCl; 160 ml.) was added to a solution of dehydrocarolic acid (0·5 g.) in water (50 ml.) acidified with conc. HCl (5 ml.). A yellow crystalline precipitate separated and was collected after 24 hr. (0·50 g.; m.p. 152°). Recrystallized from ethanol (25 ml.) it was obtained as clusters of yellow platelets (0·32 g.; m.p. 157° unchanged on further crystallization). The substance reacts acid to litmus and is at once soluble in very dilute Na_2CO_3 solution. An ethanolic solution gives with a trace of alkali a red colour characteristic of a mono-2:4-dinitrophenylhydrazone (Neuberg's reaction). Carolic acid, when treated with Brady's reagent under exactly the same conditions, gives no precipitate for some hours but finally yields a small amount of a dirty brown deposit. (Found: C, 47·4; H, 4·0; N, 14·6; equivalent by titration (Clift & Cook, 1932), 380. $C_{15}H_{14}O_8N_4$ requires C, 47·6; H, 3·7; N, 14·8 %; equivalent as a monobasic acid 378. $C_{15}H_{12}O_7N_4$ requires C, 50·0; H, 3·3; N, 15·6 %; equivalent, 360.) Hence this derivative is the 2:4-dinitrophenylhydrazone of the hydrated form of dehydrocarolic acid, structure IV on p. 569.

Catalytic reduction of dehydrocarolic acid. Formation of dl-*carolic acid*

Dehydrocarolic acid (1 g.) dissolved in water (80 ml.) was reduced with hydrogen by means of a catalyst prepared from $PdCl_2$ (0·2 g.) and norite charcoal (0·5 g.), the method used being that described by Clutterbuck *et al.* (1935*a*). 125 ml. of hydrogen was absorbed in 4 min. (theory for uptake of $1H_2 = 124{\cdot}4$ ml.) when absorption of hydrogen had become very slow. The catalyst was now removed by filtration and the filtrate was extracted three times with one-third its volume of chloroform. On removal of the solvent *in vacuo* there remained 0·85 g. of white solid, m.p. 95–105°. On continuous ether extraction of the chloroform-extracted solution, 0·10 g. of an oil was obtained which gave a red $FeCl_3$ reaction in water similar to that recorded for tetrahydrocarolic acid by Clutterbuck *et al.* (1935*a*). Larger quantities of the reduction product were more conveniently prepared as follows: dehydrocarolic acid (5 g.) was suspended in water and brought into solution by the addition of 0·2N-NaOH (112 ml.); total vol. 150 ml. This was reduced in 10 min. by a catalyst prepared from $PdCl_2$ (0·5 g.) and norite (1 g.). Uptake of hydrogen, 78 % of theory; 3·70 g. (74 % of theory) of reduction product

was isolated after acidification and extraction with chloroform. Catalytic reduction of the fully neutralized sodium salt of dehydrocarolic acid is slow but gives about the same yield of reduction product.

The reduction product (10 g.) was purified by dissolving in warm benzene (40 ml.) and adding warm dry ether (100 ml.). On repeated recrystallization colourless, globular, hard clusters of needles or plates were obtained. (Found: C, 59·57; H, 5·60. $C_9H_{10}O_4$ requires C, 59·31; H, 5·54 %.) The reduction product showed a similar anomalous behaviour in respect to melting-point as was recorded for *d*-carolic acid from *Penicillium Charlesii* by Clutterbuck *et al.* (1935*a*) with which it was compared. Reduction product, m.p. 117°; *d*-carolic acid, m.p. 130°; mixture of the two, m.p. 121°. All three samples reset on cooling and all remelted at 113°.

The reduction product is optically inactive. It gives chemical reactions, e.g. $FeCl_3$ colour, conc. H_2SO_4 colour, reactions with metallic salts, which are identical with those given by *d*-carolic acid when tested side by side. It is however more soluble in all solvents.

Its identity as dl-*carolic acid* was established by a study of its behaviour on acid hydrolysis since it gives the same hydrolytic products (CO_2, acetoin, and γ-butyrolactone) and in the same amounts (approximately one molecule of each) as does *d*-carolic acid. The reduction product (1·008 g.) was hydrolyzed by boiling its solution in 2N-H_2SO_4 (50·0 ml.) under nitrogen for 3 hr. Unlike dehydrocarolic acid (next section) there was little formation of colour and no evidence of polymerization. The method of estimating the hydrolytic products was that described in detail by Clutterbuck *et al.* (1935*a*, p. 313) and the following results were obtained.

(*a*) *Carbon dioxide*: equivalent to 10·28 ml. N-HCl, or 92·6 % of 1 mol.; *d*-carolic acid gave 95·4 %.

(*b*) *Acetoin*: confirmed qualitatively by strong reduction of Fehling's solution in the cold, a strong Voges-Proskauer reaction and the slow formation of the bis-dinitrophenylhydrazone of diacetyl on treatment with 2:4-dinitrophenylhydrazine in 2N-HCl; m.p. and mixed m.p. 318°. Estimation by the Wood-Ost method gave 98 % of 1 mol.; *d*-carolic acid gave 82·6 %.

(*c*) *γ-Butyrolactone*: confirmed qualitatively by micro-distillation of the lactone (0·50 g.) (see next section) and conversion into the phenylhydrazide, m.p. 94°, alone or admixed with authentic γ-hydroxy-*n*-butyric acid phenylhydrazide. It was estimated quantitatively by hot titration of the hydrolysis solution which had been previously neutralized in the cold: found, 105·4; *d*-carolic acid gave 100·7 %.

Thus acid hydrolysis of the reduction product proceeded exactly as for *d*-carolic acid according to the equation:

$$C_9H_{10}O_4 + 2H_2O \rightarrow \underset{\text{acetoin}}{CH_3.CHOH.CO.CH_3} + \underset{\text{butyrolactone}}{CH_2.CH_2.CH_2.CO + CO_2}$$

Acid hydrolysis of dehydrocarolic acid

Dehydrocarolic acid (2·036 g.) was hydrolyzed by boiling for 3 hr. with 2N-H_2SO_4 (100 ml.) in a current of CO_2-free nitrogen. The issuing gas was passed directly into a flask cooled in a freezing mixture, then through a bubbler containing Brady's reagent and finally through a bubbler containing standard baryta.

The dehydrocarolic acid dissolved when the bath temperature reached 70° giving initially a clear solution which, on raising the temperature, rapidly became brown and from which there quickly separated a dark brown, rubbery, polymerized product (total, 0·4 g.). Yellow vapours of diacetyl appeared in the upper portion of the hydrolysis flask and a yellow liquid, later setting to a yellow crystalline mass collected in the flask in the freezing mixture. Further, a heavy yellow precipitate quickly appeared in the bubbler containing Brady's reagent. Under similar conditions of hydrolysis neither *d*- nor *dl*-carolic acid gives any polymerized product, nor any appreciable colour, nor any precipitate in the Brady bubbler.

Identification and estimation of the products of hydrolysis gave the following results:

(*a*) *Carbon dioxide*: equivalent to 15·80 ml. N-HCl, or 92 % of 1 mol. if allowance is made for the weight of insoluble polymerized product formed.

(*b*) *Diacetyl*. The contents of the flask in the freezing mixture were diluted with a little water and there was added hydroxylamine hydrochloride (1·6 g.) and sodium acetate crystals (3·2 g.). A crystalline solid (0·28 g.) quickly separated which, after recrystallization from ethanol (7 ml.) gave colourless, thick, broad rods, wt. 0·17 g., m.p. 246° alone or on admixture with authentic dimethylglyoxime. (Found: C, 41·22; H, 6·97; N, 23·8. $C_4H_8O_2N_2$ requires C, 41·37; H, 6·94; N, 24·1 %.) A very dilute aqueous solution gave, on addition of nickel sulphate, the characteristic brilliant scarlet precipitate of nickel dimethylglyoxime. The heavy yellow precipitate in the Brady bubbler was collected after 3 days; wt. 0·44 g.; m.p. 318° decomp., alone or on admixture with authentic diacetyl bis-2:4-

dinitrophenylhydrazone. It gave a deep blue colour with Neuberg's reagent which is typical of a bis-2:4-dinitrophenylhydrazone.

The weights obtained of dimethylglyoxime and of diacetyl bis-2:4-dinitrophenylhydrazone correspond to 0·32 g. of diacetyl or 41 % of 1 mol. if allowance is made for the weight of insoluble polymerized product formed.

(*c*) *γ-Butyrolactone.* The cooled, brown, acid hydrolysis solution was filtered from the brown polymerized product and the filtrate was exactly neutralized in the cold with 2N-NaOH to phenolphthalein. It was then heated and neutralization continued to a permanent end-point, requiring 5·30 ml. N-NaOH or 60 % of 1 mol. of γ-butyrolactone if allowance is made for the weight of insoluble polymerized product formed.

The neutralized solution was now evaporated *in vacuo* to dryness several times. Only a faint trace of volatile product was detectable in the distillate either by the Voges-Proskauer test or with Brady's reagent indicating the absence of acetoin; cf. *d-* or *dl*-carolic acid.

The solid remaining after evaporation was dissolved in water (50 ml.), made strongly acid with 5N-H_2SO_4 and boiled for a few minutes. The small amount of polymerized product which was formed was separated by filtration and the clear yellow filtrate was continuously extracted with ether for 2 days. On removal of the ether there remained 0·79 g. of a clear yellow oil which was distilled at atmospheric pressure in a micro-distillation apparatus. The distillate, a clear yellow mobile oil, wt. 0·53 g., b.p. 195–200° (γ-butyrolactone boils at 203°) was treated with phenylhydrazine (0·66 g.) in ethanol (10 ml.) and the mixture was boiled under reflux for 2 hr. The ethanol was removed *in vacuo* and the semi-solid residue was crystallized from ethyl acetate giving colourless, lustrous plates, m.p. (after sublimation in a high vacuum) 96° alone or admixed with the phenylhydrazide prepared from authentic γ-butyrolactone. (Found: C, 61·84; H, 7·61; N, 14·1. $C_{10}H_{14}O_2N_2$ requires C, 61·83; H, 7·27; N, 14·4 %.)

Thus acid hydrolysis of dehydrocarolic acid proceeds, but not quantitatively, according to the equation:

$$C_9H_8O_4 + 2H_2O \rightarrow \underset{\text{diacetyl}}{CH_3.CO.CO.CH_3} + \underset{\text{butyrolactone}}{CH_2.CH_2.CH_2.CO} + CO_2$$

and shows that the extra centre of unsaturation in dehydrocarolic acid, as compared with carolic acid, cannot be in the butyrolactone side chain.

Action of ozone on dehydrocarolic acid. Formation of formaldehyde

Dehydrocarolic acid (0·205 g.) was dissolved in water (25 ml.) cooled in ice and a stream of ozonized oxygen was passed through for 3 hr. No formaldehyde could be detected in the issuing gases. The ozonized solution was left at room temperature overnight in a closed flask. The formaldehyde was precipitated direct from the solution by the method of Reeves (1941). After the addition of M-sodium acetate solution (22 ml.) and alcoholic dimedone solution (11 ml. of 8 % solution) the dimedone compound of formaldehyde separated at once. It was collected, washed with water and dried *in vacuo*; wt., 0·210 g. or 64 % of theory; m.p. 187–188°. It was recrystallized from ethanol (10 ml.) giving colourless, shining broad needles, m.p. 189°, alone or in admixture with the authentic formaldehyde dimedone compound, methylene-bis-dimethyldihydroresorcinol. (Found: C, 69·80; H, 8·30. $C_{17}H_{24}O_4$ requires C, 69·85; H, 8·25 %.)

It is thus clear that dehydrocarolic acid contains the $CH_2{=}C{<}$ grouping.

SUMMARY

1. *Dehydrocarolic acid*, $C_9H_8O_4$, a hitherto undescribed mould metabolic product, is formed on Czapek-Dox glucose medium by a strain of *Penicillium cinerascens* Biourge. Its isolation, properties and breakdown products are described. It has been shown to be a dehydro derivative of carolic acid, $C_9H_{10}O_4$, a metabolic product of *P. Charlesii* G. Smith. Dehydrocarolic acid hydrate has been shown to be α-(γ-hydroxy-*n*-butyryl)-γ-methylenetetronic acid.

2. Culture filtrates of *P. cinerascens* show antibacterial activity due to the presence of the antibiotics gliotoxin and spinulosin. Dehydrocarolic acid has not any appreciable antibacterial activity.

3. Carlosic acid, previously reported as a metabolic product of *P. Charlesii* G. Smith, was also isolated in small amounts.

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The Action of Vitamin K in Hypervitaminosis A

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Moore & Wang (1943, 1945) have given a comprehensive review of the toxic effects produced in experimental animals by greatly excessive amounts of vitamin A. From their experiments with pure vitamin A acetate they concluded that the most characteristic lesions were skeletal fractures and haemorrhage, the first of these lesions being predominant in growing animals and the second in adult animals. The fractures caused by pure vitamin A have been confirmed by Herbst, Pavcek & Elvehjem (1944) and Pavcek, Herbst & Elvehjem (1945) who also found that telangiectatic bovine livers, rich in vitamin A, are toxic to rats. Light, Alscher & Frey (1944) approached the problem from a new angle, by showing that hypervitaminosis A in rats was associated with a pronounced hypoprothrombinaemia, which could be corrected by giving vitamin K as 2-methyl-3-phytyl-1:4-napthaquinone. The object of the present investigation has been to confirm and extend this important observation.

EXPERIMENTAL

Method

In a series of experiments, piebald rats were fed on massive doses of vitamin A for periods varying from 10 to 18 days, with or without the addition of vitamin K, or, in one experiment, of vitamin C. The source of the vitamin A was halibut-liver oil, containing 40,000 i.u./g., which was kindly supplied by Allen and Hanbury, Ltd. At the termination of each experiment the rats were killed under ether anaesthesia. The chest wall was opened and as much blood as possible withdrawn from the heart into an oxalated syringe. The livers were removed, weighed, and placed in 5% KOH solution, and the bodies were X-rayed for the presence of fractures. Vitamin A was estimated in the livers by the antimony trichloride method applied after the digestion of the tissues by alkali (Davies, 1933) and in the blood by the same method applied according to the technique of Yudkin (1941). Plasma clotting times were measured by the method of Campbell, Smith, Roberts & Link (1941) and an approximate measurement was made of the plasma/cell ratio by centrifuging the blood for a standard time in graduated centrifuge tubes. Unfortunately no haematocrit was available.

The action of vitamin K in hypervitaminosis A

Exp. 1–4. Piebald rats of both sexes, usually young but in one experiment nearing maturity, were given a basal diet of casein 20%, sugar 60%, fat 15% and salt mixture 5%, with dried yeast 10% (additional). Vitamin A was supplied as one drop of halibut-liver oil/rat/week, vitamin D as one drop of 'Radiostol', and vitamin E as 1 mg. of *dl*-α-tocopheryl acetate. Each experiment, except no. 4, included three groups which are described in Tables 1 and 2 as 'control', 'excess of vitamin A' and 'excess of vitamin A + vitamin K'. In the control groups the fatty component of the basal diet was supplied as arachis oil, whereas groups given excess of vitamin A received their fat as halibut-liver oil, which was freshly mixed each day with the solid components of the diet. In Exp. 1 the group given vitamin K in addition to vitamin A received it in the form of 'Synkavit' (tetrasodium 2-methyl-1:4-naphthahydroquinone diphosphoric acid ester). The Synkavit was added to the dry constituents of the diet so as to supply about 160 μg./rat daily. To avoid any possible criticism that the admixture