canted, the latter dialyzed for five days at 5° against distilled water and lyophilized to give 14.1 g. (66%) of fraction R20-F1. The precipitate was reprecipitated five times by dissolving the precipitate in 500 ml. of water, adding 50 ml. of 0.012 *F* hydrochloric acid and centrifuging. The solutions in every case were 0.0011 *F* in hydrochloric acid with the *p*H varying between 3.0 to 3.1. The precipitate obtained after the fifth reprecipitation was dissolved in distilled water, the solution dialyzed for four days at 5° against the same solvent, filtered to remove a few suspended particles and lyophilized to give 3.0 g. (14%) of fraction R20-F2.

Acknowledgment.—The authors wish to express their indebtedness to Dr. W. T. J. Morgan for the data given in Table III and to Dr. D. H. Brown and Dr. E. L. Bennett for their assistance during the course of this investigation.

Summary

The isolation of two forms of A-substance, one soluble and the other insoluble in aqueous solutions at *p*H 3 and low ionic strength, from hog gastric mucin is described.

PASADENA, CALIFORNIA RECEIVED JULY 7, 1948

[CONTRIBUTION FROM THE BIOCHEMISTRY DEPARTMENT, UNIVERSITY OF WISCONSIN]

Precursors for Aliphatic Penicillins[1]

BY J. A. THORN AND M. J. JOHNSON

Penicillins so far listed in the literature include both aromatic and aliphatic types.[2] Exclusive of the biosynthetic penicillins described by Behrens, *et al.*,[3] the aromatic penicillins are benzylpenicillin (G) and *p*-hydroxybenzylpenicillin (X). Aliphatic penicillins include 2-pentenylpenicillin (F), *n*-amylpenicillin (dihydro F) and *n*-heptylpenicillin (K). Flavicidin, once reported to be 3-pentenylpenicillin, is now considered to be *n*-amylpenicillin.[4]

Introduction of the use of precursors stimulated considerable research on the problem of producing different penicillins, and many new ones were obtained by the Lilly group under Behrens.[3] Among the types of compounds effectively used as precursors were substituted mercaptoacetic, hydroxyacetic, polycyclic acetic, heterocyclic acetic and phenylacetic acid derivatives. Biosynthesis of penicillins having *n*-alkyl side-chains has not been reported, although several fatty acids were tested as possible precursors.[5,6]

As pointed out by Behrens, *et al.*,[5] yield stimulation data are not conclusive in testing for the precursor activity of compounds since increased formation of a penicillin (or formation of a new penicillin) can occur without a concurrent increase in total yield. This test was not used in the work to be described. Instead, the precursor effects, if any, of the different compounds examined were ascertained by determining quantitatively each of the penicillins formed in experimental and control broths. Comparison of the values so obtained gave the required information. The filter paper chromatographic method of Karnovsky and Johnson[7] was employed for the penicillin analyses.

Since precursors for the natural aliphatic penicillins had not been described, it was thought of interest to investigate the problem. It appeared reasonable to begin by studying the effects of various triglycerides and fatty acids on penicillin formation.

Experimental

Fermentation Techniques.—*Penicillium chrysogenum* Q176 was used exclusively in the work to be described. All fermentations were conducted at 25°, and the 500-ml. Erlenmeyer flasks containing the inoculated medium were agitated by means of a reciprocating shaker operating at 92 cycles per minute and having a horizontal displacement of four inches.

The inoculum medium employed was that of Jarvis and Johnson,[8] except that the ammonium sulfate was replaced by ammonium phosphate and copper sulfate was omitted. The fermentation medium[9] employed contained in g. per l.: lactose, 20; glucose, 10; ammonium acetate, 4; ammonium lactate, 5; KH_2PO_4, 3; $MgSO_4 \cdot 7H_2O$, 0.25; $ZnSO_4 \cdot 7H_2O$, 0.02; $FeSO_4 \cdot 7H_2O$, 0.02; $MnSO_4 \cdot H_2O$, 0.02; and Na_2SO_4, 0.5. In each case, the sugars were autoclaved separately and were added to the salt solution immediately before inoculation. In the case of the inoculum medium, the calcium carbonate was also sterilized separately in distilled water. The inoculum medium, 80 ml. per flask, was seeded with a spore suspension prepared by the method of Gailey, *et al.*,[10] and placed on a rotary shaker (270 cycles per minute, and imparting a motion such that all points on each flask described a horizontal circle 2.25 inches in diameter). When a thick vegetative growth was obtained (after about forty to forty-four hours); it was used to inoculate the fermentation medium. The fermentation flasks each contained 100 ml. of medium, to which was added 5 ml. of vegetative inoculum.

Compounds Tested for Precursor Activity.—All compounds, unless otherwise specified, were obtained from Eastman Kodak Co., Rochester, and were not further purified.

(1) Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from Lederle Laboratories, Pearl River, N. Y., and E. R. Squibb and Sons, New Brunswick, New Jersey.

(2) Wintersteiner, Boon, Carrington, MacCorquodale, Stodola, Wachtel, Coghill, Risser, Philip and Touster, in Clarke, Johnson and Robinson, "The Chemistry of Penicillin," Princeton University Press, Princeton, N. J., chapter 5, 1949.

(3) O. K. Behrens, J. Corse, J. P. Edwards, L. Garrison, R. G. Jones, Q. F. Soper, F. R. Van Abeele and C. W. Whitehead, *J. Biol. Chem.*, **175**, 793 (1948).

(4) M. Adler and O. Wintersteiner, *ibid.*, **176**, 873 (1948).

(5) O. K. Behrens, J. Corse, D. E. Huff, R. G. Jones, Q. F. Soper and C. W. Whitehead, *ibid.*, **175**, 771 (1948).

(6) O. K. Behrens, chapter 19, in ref. 2.

(7) M. L. Karnovsky and M. J. Johnson, *Anal. Chem.*, **21**, 1125 (1949).

(8) F. G. Jarvis and M. J. Johnson, THIS JOURNAL, **69**, 3010 (1947).

(9) M.-S. Wong, B. S. Thesis, University of Wisconsin, 1947.

(10) F. B. Gailey, J. J. Stefaniak, B. H. Olson and M. J. Johnson *J. Bact.*, **52**, 129 (1946).

β,γ-Hexenoic acid (b. p. 122–123° (30 mm.)) and α,β-hexenoic acid (b. p. 128–130° (30 mm.)) were prepared by the methods of Boxer and Linstead.[11] β,γ-Pentenoic acid (b. p. 82–84° (11 mm.)) was obtained by the same method as β,γ-hexenoic acid, with propionaldehyde instead of butyraldehyde.

Liquid acids and triglycerides were sterilized by autoclaving at 15 p. s. i. for twenty minutes and were added to the fermentations by means of sterile pipets. In the case of solid compounds, appropriate amounts were sterilized in 500-ml. Erlenmeyer flasks and, after cooling, the fermentation medium was poured aseptically into these flasks.

Since fatty acids and triglycerides are toxic when added early in the course of the fermentation, the optimal time of addition was determined as follows. A fermentation was begun with five control flasks and five pairs of experimental flasks. To three pairs of experimental fermentations, lard oil (a mixture of triglycerides) was added at 0.05% levels every twelve hours after a given initial lard oil-free growth period (twenty-four, thirty-six and forty-eight hours, respectively). To the other two pairs, lard oil was added at 0.10% levels every twenty-four hours after initial lard oil-free growth periods of twenty-four and forty-eight hours, respectively. Addition of lard oil at twenty-four hours was found to lower the *p*H of the fermentations and also adversely to affect the penicillin yields (average units per ml. at ninety-six hours: control, 202; experimental, 80). Lard oil was not toxic, however, if added at thirty-six hours or later and, in fact, its addition resulted in increased yields (average units per ml. at ninety-six hours: control, 202; experimental, 345). From these data, it was apparent that the triglycerides to be tested for precursor activity should not be added before the fermentations were thirty-six hours old. In practice, it was found convenient to add them at twenty-four-hour intervals starting at forty-eight hours.

Determination of Penicillins.—The method of filter paper chromatography of Karnovsky and Johnson[7] was employed, in which the developed filter paper tapes were cut into 0.25 inch squares and plated out on agar medium seeded with *B. subtilis*. All figures to be given concerning penicillin yields and percentage composition of penicillin mixtures are therefore based on the *B. subtilis* assay, unless otherwise specified. Fermentation broths were kept until time of analysis by first adjusting them to *p*H 6.0–6.3 with phosphoric acid and then freezing. Broth samples were filtered through cotton before being applied to the tapes.

As has been reported by the authors, the chromatographic method gives variable, but usually low, recoveries for penicillins in synthetic broths. The average recovery obtained in the work to be described was 63% (average of 47 analyses), with values ranging from 32 to 111%. The effect of low recoveries on the estimation of the penicillins in broth samples is not known. However, it is thought that no great preferential inactivation of any one penicillin occurs during analysis, since analyses of two similar broths gave very closely the same results (on a percentage basis), although the recoveries were different in each case. This is illustrated by the data in Table I which lists the proportions of penicillins found upon chromatographic analysis of two pairs of broths from different fermentations. Penicillins i, iii and iv, to be discussed later, are penicillins which are less ether soluble than F, dihydro F and K. Values of the proportions of individual penicillins found in broths were therefore calculated on the assumption that inactivation during chromatography was not selective.

The data in Table I also illustrate the differences found in the relative amounts of the various penicillins formed in separate fermentations. Fairly close agreement, however, was obtained between individual broths of the same fermentation.

Several penicillins are produced when *P. chrysogenum* Q176 is grown in synthetic medium in shake-flasks,[7] the principal ones being F, dihydro F and K. The various

(11) S. E. Boxer and R. P. Linstead, *J. Chem. Soc.*, 740 (1931).

TABLE I

CHROMATOGRAPHIC ANALYSES OF SYNTHETIC BROTHS

No precursor added; the assay organism was *B. subtilis*

Ferment. no.	Flask	Total penicillin, u./ml.	Anal. recov., %	Penicillin, % i	iii	iv	F	FH_2	K
1	1	90	111	2.8	0.0	0.0	55.1	8.2	33.8
	2	105	80	2.9	0.5	0.0	48.7	8.3	39.7
2	1	94	42	4.3	3.0	0.6	39.6	10.0	44.3
	2	94	54	2.7	1.9	1.9	43.4	10.1	41.4

penicillins are listed below with their characteristic "position constants." The position constant of a penicillin may be defined as the relative distance traversed along the tape by the particular penicillin during development of the chromatogram. The distance is measured from the point of application to the point of maximum concentration, and the position constant is calculated by taking as 100 the distance moved by penicillin dihydro F. The constants listed below were calculated from data obtained by cutting the tapes into uniform squares and assaying each square for penicillin content. They differ slightly from those listed by Karnovsky and Johnson[7] since the latter were calculated from measurements of zone positions obtained upon plating out the entire (uncut) tapes.

Penicillin	Position constant	Penicillin	Position constant
i	3.5 ± 2	FH_2	100
ii	18.7 ± 2	K′	145 ± 5
iii	36.0 ± 3	K	167 ± 10
iv	50.1 ± 4	K″	185 ± 5
F	79.5 ± 3		

Unless otherwise specified, values given for the relative amounts of penicillin K will include those for penicillin K′ and K″. Penicillin in K′ generally accounted for less than 1% of the total penicillin in the broths. The separation of penicillins K and K″ was never sufficient to allow calculation of the individual proportions of the two substances.

Characterization of Biosynthetic Penicillins.—The general method used for the characterization of new penicillins was that of Higuchi and Peterson,[12] which involved isolation and identification of the R-group acids. About 3 l. of filtered broth, adjusted to *p*H 2.2 with sirupy phosphoric acid, was thoroughly shaken with cold amyl acetate and the resulting emulsion broken by means of a cream separator. The penicillin was then extracted from the solvent into 150 ml. of 0.1 *N* sodium hydroxide. The latter solution was heated on a steam-bath for seven minutes to break the β-lactam ring of the penicillins, making them less susceptible to extraction in the next step.[12]

The cooled solution of inactivated penicillins was adjusted to *p*H 1.5 with sulfuric acid, and, after addition of 15 g. of sodium sulfate, was subjected to continuous extraction with ether. The purpose of this extraction was to remove all of the free precursor acid originating from the triglyceride added during the fermentation. The R-group acids of the penicillins were then liberated by adding 30 g. of sodium hydroxide to the extracted solution and refluxing for three hours.[12] After cooling, the alkaline solution was adjusted to *p*H 1.5 with concentrated sulfuric acid and the R-group acids extracted with petroleum ether (boiling range, 30–75°) for twenty-four hours.

The petroleum ether solution of the R-group acids was concentrated by distillation to a volume of 2 to 4 ml. and the acids separated and presumptively identified by chromatography. The column used was analogous to that described by Ramsey and Patterson.[13] Seven grams of Celite 535 was employed as the supporting material, with

(12) K. Higuchi and W. H. Peterson, *Anal. Chem.*, **21**, 659 (1949).

(13) L. L. Ramsey and W. I. Patterson, *J. Assn. Official Agr. Chem.*, **31**, 139 (1948).

TABLE II

FATTY ACIDS AND TRIGLYCERIDES TESTED AS PRECURSORS

B. subtilis was used as the assay organism. The method of assay was sufficiently sensitive to allow determination of a penicillin present in an amount corresponding to about 0.2% of the total penicillin. Figures placed between two columns are the sums of the penicillins concerned.

Compound added, amt. per 100 ml. medium	Total penicillin, units/ml.	Percentages i	ii	iii	iv	F	FH_2	K′	K
None	60	2.9	0.0	6.0	0.0	51.4	7.8	..	32.0
Sodium formate, 0.15 g.	33	2.1	.0	0.7	0.0	43.7	10.7	..	42.6
None	94	2.5	.0	3.5	0.8	79.4		..	14.0
Triacetin, 0.25 ml.	116	5.7	.0	0.0	0.0	57.5	10.7	..	26.1
None	86	3.4	.0	0.6	2.0	48.4	10.2	0.4	34.9
Propionic acid, 0.10 ml.	74	17.5	.0	0.4	5.8	35.7	8.8	3.3	28.4
None	94	4.3	.0	3.0	0.6	39.6	10.0	0.7	43.6
Tributyrin, 0.20 ml.	98	2.6	33.9	1.0	1.2	25.2	7.0	1.7	27.5
None	154	3.6	0.0	1.6	1.0	57.0	8.1	1.4	27.4
Tributyrin, 0.45 ml.	170	3.3	35.4	1.6	1.0	40.0	6.0	0.0	12.8
None	60	2.9	0.0	6.0	0.0	51.4	7.8	.0	32.0
β,γ-Pentenoic acid, 0.15 ml.	132	0.6	.0	83.1	0.0	8.5	1.4	.0	6.3
None	86	3.4	.0	0.6	2.0	48.4	10.2	.4	34.9
Tri-*n*-valerin, 0.05 ml.	104	2.5	.0	1.2	50.7	22.6	2.9	.5	19.6
None	94	2.5	.0	3.5	0.8	79.4		..	14.0
Tri-*n*-valerin, 0.18 ml.	148	3.1	.0	0.0	68.0	18.1	4.4	..	6.3
None	100	3.4	.9	3.9	1.5	58.1		..	32.2
α,β-Hexenoic acid, 0.15 ml.	102	4.4	10.1	2.4	2.5	55.5		..	25.2
None	89[a]	← 5.2 →				47.4	15.6	..	32.1
β,γ-Hexenoic acid, 0.10 ml.	110[a]	← 8.6 →				65.7	9.0	..	16.8
β,γ-Hexenoic acid, 0.05 ml.	104[a]	← 6.5 →				89.0	2.0	..	2.9
β,γ-Hexenoic acid, 0.15 ml.	99[a]	← 5.3 →				80.5	2.6	..	11.7
None	94	4.7	0.0	3.1	2.0	56.5	7.8	..	25.9
Tricaproin, 0.30 ml.	86	2.1	7.6	3.1	..	29.8	51.1	..	6.3
None	90	2.6	0.0	2.4	1.0	60.7	6.0	0.3	26.9
Heptanoic acid, 0.20 ml.	58	3.9	.0	6.9	16.0	33.2	5.7	7.2	27.0
None	78	3.7	.0	..	0.8	38.2	11.8	..	44.5
Tricaprylin, 0.20 ml.	110	1.3	.0	0.9	2.5	15.0	35.7	..	44.6
None	90	2.6	.0	2.4	1.0	60.7	6.0	0.3	26.9
Nonanoic acid, 0.20 ml.	39	4.1	.0	2.1	19.6	34.8	7.1	10.4	22.2
None	83	4.7	.0	3.1	2.0	56.5	7.8	..	25.9
Tricaprin, 0.20 ml.	89	5.6	.0	..	2.9	24.4	35.4	..	31.6
None	94	2.7	.0	1.9	1.9	43.4	10.1	..	41.4
Trilaurin, 0.15	104	4.6	.0	1.0	1.7	38.5	10.8	..	43.7
None	154	3.6	.0	1.6	1.0	57.0	8.1	1.4	27.4
Trimyristin, 0.45 g.	138	3.5	.0	1.0	2.5	43.7	9.7	0.8	39.0
None	86	3.4	.0	0.6	2.0	48.4	10.2	0.4	34.9
Ethyl linoleate, 0.10 ml.	98	2.1	1.2	1.1	2.0	20.9	27.5	1.3	43.8
None	83	4.7	0.0	3.1	2.0	56.5	7.8	..	25.9
Oleic acid, 0.20 ml.	170	7.6		1.4	3.0	30.4	25.8	..	31.8

[a] Chromatographic data obtained through the courtesy of Dr. M. L. Karnovksy.

7 ml. of 90% methanol (containing brom cresol green as indicator) as the stationary phase and Skellysolve C as the mobile phase. The isolated acid, derived from the R-group of the biosynthetic penicillin, was then identified by means of its *p*-phenylphenacyl ester.

Results

Table II shows the relative amounts of the different penicillins formed when various straight-chain fatty acids or their triglycerides were added to fermentations. In each case, values are given for the penicillins formed in a control fermentation.

Precursors for the Known Natural Penicillins

Penicillin Dihydro F.—As may be seen from Table II, addition of tricaproin to the fermentation medium resulted in a sixfold increase in formation of penicillin dihydro F. Formation

of the unknown penicillin ii was also stimulated. It appeared, therefore, that the mold was capable of utilizing the acid of the triglyceride as the R-group acid for the penicillin.

To determine whether or not the total precursor activity of tricaproin was due to the constituent caproic acid, a fermentation was conducted in which tricaproin was added in 0.15-ml. amounts at forty-eight, sixty-eight and ninety-six hours. At the same times were added to other fermentations caproic acid, glycerol and caproic acid plus glycerol, all in amounts approximately equivalent to the tricaproin employed in the first fermentation. The effect of these adjuvants upon penicillin formation is shown in Table III. It is seen that addition of tricaproin, caproic acid and caproic acid plus glycerol resulted in more than tripling the percentage of penicillin dihydro F found in the control. Addition of glycerol alone had no effect.

The values obtained for penicillin F in this experiment were unusually low. However, there appears little doubt that caproic acid is a precursor for penicillin dihydro F.

Since tricaproin was found to cause increased production of penicillin dihydro F, it was thought that the addition of tricaprylin to fermentations would perhaps result in increased formation of penicillin K. However, as may be seen from Table II, no such increase occurred. Instead, the formation of penicillin dihydro F was stimulated considerably. Similarly, addition of tricaprin to fermentations resulted in increased formation of penicillin dihydro F, the proportion of the latter in the experimental broth being about four times that in the control broth. These results would indicate that the mold is able to β-oxidize fatty acids and, in certain cases, use the resultant smaller fragments as penicillin precursors. Additional examples of this phenomenon will be discussed in following sections.

Penicillin F.—Since the R-group acid of this penicillin is β,γ-hexenoic acid, the latter was tried as a precursor. Addition of the acid (0.10 ml. per 100 ml. of fermentation medium), with glycerol, raised the amount of penicillin F formed from 47% of the total penicillin in the control broth to 66% of that in the experimental broth. In another experiment, addition of β,γ-hexenoic acid resulted in the formation of the penicillin in amounts equal to 81 and 89% of the total penicillin.

TABLE III

EFFECT OF TRICAPROIN AND ITS CONSTITUENTS UPON PENICILLIN FORMATION

The assay organism was *B. subtilis*. Figures between two columns are the sums of the penicillins concerned.

Compound added, amt. per 100 ml. medium	Total penicillin, u./ml.	Penicillin, % i	ii	F	FH_2	K
(1) None	64	5.5	0.0	16.4	20.5	57.4
(2) Tricaproin, 0.45 ml.	80	14.2		2.8	77.2	5.8
(3) Caproic acid, 0.42 ml.	38	4.8	8.8	4.8	71.2	10.4
(4) Glycerol, 0.108 g.	70	10.3	0.0	9.7	20.7	59.3
(5) (2) + (3)	75	8.0		7.0	72.5	12.5

Penicillin K.—As noted above, caprylic acid (as tricaprylin) was found not to cause increased formation of penicillin K but, instead, to cause stimulation of dihydro F production. However, it should be noticed that the percentage of penicillin K was not decreased although that of penicillin dihydro F was increased (as contrasted to the decrease of penicillin K when precursors such as tributyrin, tri-*n*-valerin and β,γ-hexenoic acid were used). It would therefore appear that tricaprylin acted as a precursor for penicillin K. Similarly, when tricaprin was employed, the percentage of penicillin K was increased slightly even though that of dihydro F was increased by a factor of more than four. It would seem probable that the mold β-oxidized the capric acid and utilized the caprylic acid fragment as a precursor.

The same effect was found when *dl*-β-hydroxydecanoic acid was employed, the penicillin dihydro F being increased from 7.8% of the total penicillin in the control broth to 20.6% in the experimental broth whereas the values for penicillin K were 32.0 and 39.2%, respectively.

Precursors for the Unidentified Natural Penicillins

In addition to penicillins F, dihydro F and K, at least six unidentified penicillins are formed when *P. chrysogenum* Q176 is grown in a synthetic medium containing lactose, glucose, acetate, lactate and salts (see list in Experimental section). Four of the six penicillins together account for about 10% of the total penicillin in broths. Penicillin K″, as mentioned before, has not been separated sufficiently from penicillin K to allow its quantitative estimation. The sixth penicillin, ii, is only occasionally found upon chromatographic analysis of broths, probably because it is formed in very small amounts.

Since these penicillins are formed in such small quantities, their identification by isolation procedures would be difficult. It was therefore thought of interest to determine whether precursors could be found which would stimulate their formation. As will be discussed below, precursors were indeed found for penicillins having the same position constants as some of the natural unidentified penicillins. It is realized, of course, that two penicillins having the same position constant are not necessarily identical and it is therefore necessary to distinguish between the natural penicillin and the possibly identical biosynthetic penicillin.

Biosynthetic Penicillin i.—As may be seen from Table II, the use of propionic acid as a precursor resulted in the formation of a penicillin having about the same position constant

as penicillin I (penicillin i, 3.5 ± 2; biosynthetic penicillin i, 5.1).

It cannot be stated with certainty that penicillin i is a single penicillin since it moves such a short distance along the tape that separation of two or more possible constituent penicillins would not be attained.

Biosynthetic Penicillin ii.—Use of tributyrin as a precursor (added in 0.10-ml. amounts per 100 ml. of medium at fifty-three and seventy-five hours) resulted in the formation of a penicillin having the same position constant as natural penicillin ii. The biosynthetic penicillin accounted for 34% of the total penicillin produced by the mold. Figure 1 shows the chromatograms obtained from (1) 12 μl. of control broth, and (2) 12 μl. of experimental broth.

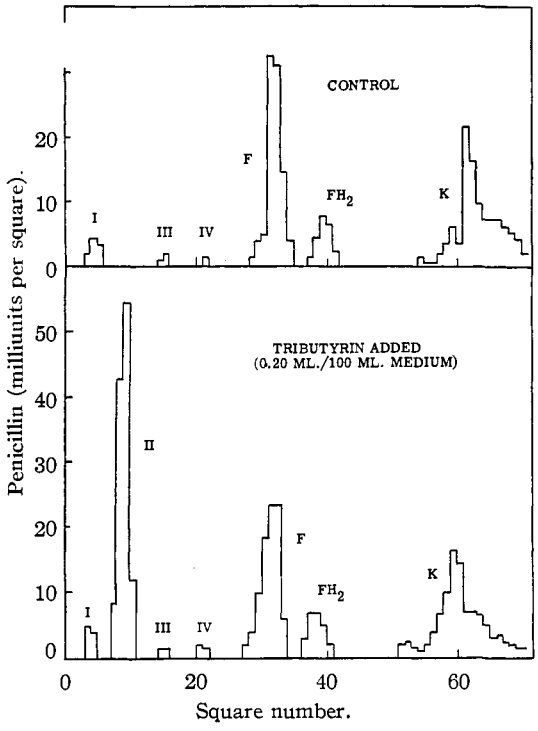

Fig. 1.—Chromatograms showing the effect of tributyrin on penicillin formation in synthetic medium. The amount of penicillin found in each square is plotted against the position of the square in the uncut tape. Samples were applied at points between squares 4 and 5.

A second fermentation was carried out to determine whether or not an even greater proportion of biosynthetic penicillin ii might be formed if larger amounts of precursor were used. Addition of 0.45 ml. of tributyrin per 100 ml. of medium resulted, however, in the formation of the penicillin in the same proportion as before (see Table II).

To obtain sufficient biosynthetic penicillin ii for characterization purposes, a large scale fermentation was set up, employing 4 l. of medium (in 40 flasks). To each flask was added 0.05 ml. of tributyrin at forty-nine, seventy-five and ninety-six hours. After one hundred twenty hours, the combined broths were adjusted to *p*H 6.0 with phosphoric acid and filtered through Celite 545. The filtered beer was then treated as described in the experimental section. The free butyric acid, if any, in the solution of the inactivated penicillins, was removed by continuous extraction with ether for eighty minutes. The conditions of extraction were such that, in a control experiment using 4.80 g. of butyric acid in 150 ml. of solution, no residual acid could be demonstrated by chromatography after extraction for eighty minutes, although 1 mg. of butyric acid is sufficient to give a very marked band.

The petroleum ether solution of the liberated R-group acids was concentrated to a volume of about 4 ml. and placed on a chromatographic column. Three bands were observed. The first was stationary and did not leave the top of the column. The second and third bands separated well, the second one resembling in its rate of movement butyric acid and the third one a higher acid (probably caproic or a hexenoic acid). The acid from band 2 (estimated visually by band width to be 10 mg.) was mixed with 20 mg. of propionic acid and 19 mg. of valeric acid and the mixture applied to a new column. In this case, three well separated and moving bands were observed, corresponding in their rates of movement to propionic acid (highest band), butyric acid and valeric acid (lowest band). Titration of the eluted acids of the first and third bands gave recoveries of 95 and 98%, respectively, for the added propionic and valeric acids.

The solution of the acid (presumably butyric) from the second band was concentrated to a volume of 1 to 2 ml. and the *p*-phenylphenacyl ester prepared. After recrystallization from petroleum ether, the ester melted at 81–82° (required for butyric, 82°). A depression of 0.5° was obtained in a mixed melting point with an authentic sample of *p*-phenylphenacyl butyrate. The isolated R-group acid was therefore butyric acid, showing that biosynthetic penicillin ii must be *n*-propylpenicillin.

Since caprylic acid was not found in the final solution of isolated R-group acids, it is probable that the inactivated penicillin K was extracted by the ether under the exhaustive conditions used to free the penicillin concentrate of free butyric acid. It is probable that much of the inactivated penicillins F and dihydro F was also extracted since only an estimated 5 to 10 mg. of acid(s) was present.

Biosynthetic Penicillin iii.—Addition of 0.15 ml. of β,γ-pentenoic acid (with glycerol) to 100 ml. of fermentation medium resulted in the formation by the mold of biosynthetic penicillin iii in an amount corresponding to about 80% of the total penicillin. Since penicillin iii in the control broth amounted to only 6%, the pentenoic acid is obviously an effective precursor. The acid was effective also in raising the over-all yield of penicillin, the yield in the experimental broth being 132 units/ml. as contrasted to 60 units/ml. in the control.

Since biosynthetic penicillin iii has solubility characteristics such that, upon chromatography, it takes up a position between biosynthetic propyl- and butylpenicillins (butylpenicillin will be described in the next section), it would seem reasonable to believe that it is 2-butenylpenicillin (see Fig. 2).

Biosynthetic Penicillin iv.—Use of tri-*n*-valerin as a precursor resulted in the very marked production of a penicillin having the same position constant as natural penicillin iv (see Fig. 3). The biosynthetic penicillin accounted for nearly 50% of the total penicillin formed by the mold.

For characterization purposes, a fermentation was conducted employing 40 flasks of medium to each of which was added 0.20 ml. of tri-*n*-valerin. At the end of the fermentation, the combined and filtered broth was processed as previously described. Free valeric acid was disposed of by continuous extraction with ether for sixty minutes (a control experiment showed this to be sufficient to completely free a solution of 7.0 g. of valeric acid).

The petroleum ether solution of the liberated R-group acids was concentrated to a volume to 3 to 4 ml. and placed on a chromatographic column. Two moving bands were

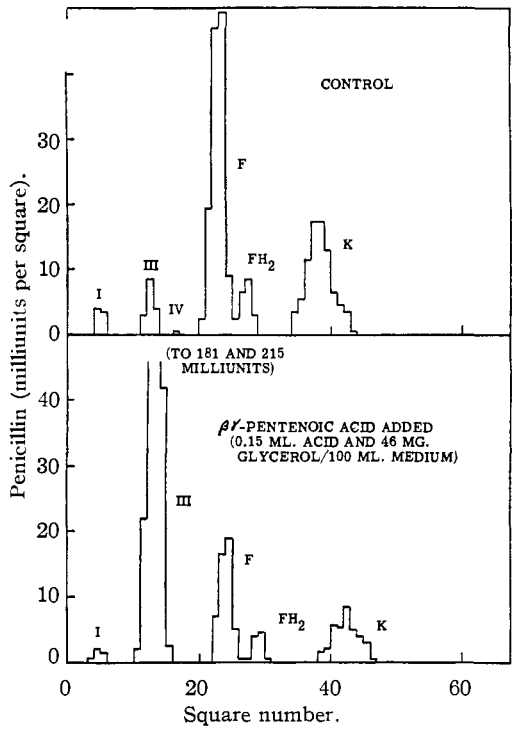

Fig. 2.—Chromatograms showing the effect of β,γ-pentenoic acid on penicillin formation in synthetic medium. Samples were applied at points between squares 4 and 5.

observed, the slower one resembling in its rate of movement valeric acid and the faster one caproic or a hexenoic acid. The acid from the slower band (estimated visually from the width of the band to be 10 to 15 mg.) was mixed with 19 mg. of butyric acid and 18.5 mg. of caproic acid. This mixture, upon chromatography, was resolved into three well separated bands, the fastest one corresponding to caproic acid and the slowest to butyric acid. Titration of the acids of these two bands gave recoveries of 96 and 99%, respectively, of the added butyric and caproic acids. The center band, corresponding to the isolated R-group acid, was presumably due to valeric acid. Its *p*-phenylphenacyl ester was prepared. The derivative melted at 69° (required for valeric acid, 69°), and a mixed m. p. with authentic *p*-phenylphenacyl *n*-valerate showed no depression.

The isolated R-group acid was therefore *n*-valeric acid, proving the biosynthetic penicillin iv to be *n*-butylpenicillin.

Biosynthetic Penicillin K′.—Addition of heptanoic and nonanoic acids to fermentations each caused considerable amounts of two penicillins to be formed. These penicillins had the same position constants as penicillins K′ and iv. The amounts produced are given in Table II. Heptanoic acid also caused formation of biosynthetic penicillin iii. Since biosynthetic penicillin iv is butylpenicillin, it is possible that the mold β-oxidized the two acids and used the resulting valeric acid as a precursor. It is thought probable that biosynthetic penicillin K′ is hexylpenicillin. If biosynthetic penicillin iii is 2-butenylpenicillin, its formation from heptanoic acid could easily be explained by assuming that the mold uses as its precursor the acid derived from heptanoic acid by an initial β-oxidation followed by dehydrogenation in the β,γ-position.

Heptanoic and nonanoic acids, added as the free acids with appropriate amounts of glycerol, are both considerably toxic to the mold, as may be seen in Table II.

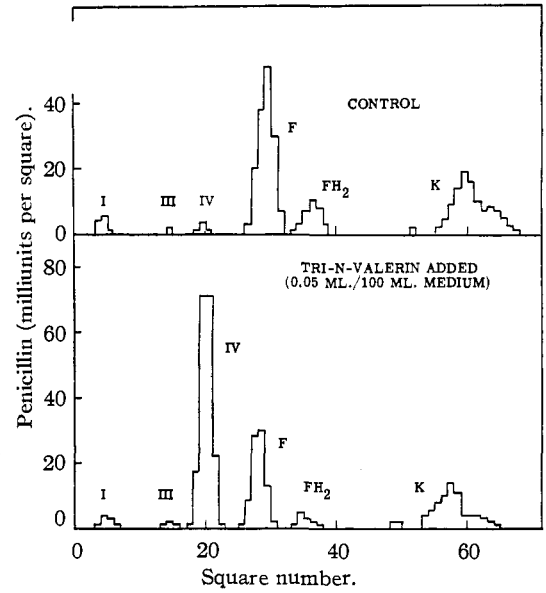

Fig. 3.—Chromatograms showing the effect of tri-*n*-valerin on penicillin formation in synthetic medium. Samples were applied at points between squares 4 and 5.

Branched-chain Acids as Precursors

dl-α-**Methylbutyric Acid.**—When this acid was used as a possible precursor, it was found that a penicillin having the same position constant as penicillin iv was formed to the extent of 63% of the total penicillin in the broth. This penicillin has been observed in repeated experiments. Since the position taken up by the penicillin upon chromatography eliminates the possibility that its R-group acid is formed by β-oxidation of the α-methylbutyric acid, it is quite probable that the R-group acid of the penicillin is indeed α-methylbutyric acid. It would seem, therefore, that the mold is capable of utilizing α-methyl substituted acids as penicillin precursors.

Isovaleric Acid.—Addition of isovaleric acid to the fermentation medium gave rise, once more, to the formation of a penicillin having the same position constant as penicillin iv. In this case, the penicillin formed accounted for about 23% of the total. It would appear, from the last two examples, that the chromatographic method employed is unsuitable for distinguishing among the isomeric butylpenicillins.

Relative Precursor Efficiencies of Tributyrin, Tri-*n*-valerin and Phenylacetic Acid

Phenylacetic acid is a commonly used precursor for benzylpenicillin. It was thought of interest to compare its efficiency with those of tributyrin and tri-*n*-valerin. Accordingly, tributyrin, tri-*n*-valerin and phenylacetic acid (with glycerol) were each added to different fermentations in 0.5 milliequivalent amounts (per 100 ml. of medium) at forty-eight, seventy-two and ninety-six hours, and the resultant broths chromatographed. To-

tal penicillin yields (in *B. subtilis* units) for the three broths were: (1) tributyrin addition, 92 units/ml.; (2) tri-*n*-valerin addition, 148 units/ml.; (3) phenylacetic acid addition, 280 units/ml. Chromatographic analyses showed that in the tributyrin broth, 23.9% of the total penicillin was propylpenicillin, in the trivalerin broth, 68.0% of the total penicillin was butylpenicillin, and in the phenylacetic acid broth, benzylpenicillin constituted 88.5% of the total. Then, if it is assumed that any inactivation occurring during chromatography is non-selective, it is seen that the addition of tributyrin caused the production of 22 units/ml. of propylpenicillin, whereas addition of tri-*n*-valerin caused the formation of 101 units/ml. of butylpenicillin and addition of phenylacetic acid (with glycerol) resulted in the formation of 248 units/ml. of benzylpenicillin. It is apparent, therefore, that on a unit basis phenylacetic acid is more than ten times as efficient a penicillin precursor as is tributyrin and more than twice as efficient as tri-*n*-valerin.

Discussion

It has been shown that *P. chrysogenum* Q176, growing in a synthetic medium, is capable of employing butyric, valeric, caproic and β,γ-hexenoic acids as precursors for the appropriate penicillins. For example, the addition of β,γ-hexenoic acid causes increased formation by the mold of 2-pentenylpenicillin (F), and addition of caproic acid results in increased formation of amylpenicillin (dihydro F). Two new biosynthetic penicillins obtained in this way, *n*-propylpenicillin and *n*-butylpenicillin, have the same apparent distribution coefficients (as evidenced by their position constants) as two penicillins which are normally formed by the mold in synthetic medium, and it is thought possible that they are identical with the latter. It is obvious, however, since other penicillins, for example penicillins with branched side-chains, could have the same chromatographic positions as *n*-propylpenicillin and *n*-butylpenicillin, that only suggestive evidence can be given by chromatographic position.

Two other penicillins, iii and K′, also occur normally in synthetic broths. Production of biosynthetic penicillins having the same position constants as iii and K′ may be brought about by use of β,γ-pentenoic acid and heptanoic (or nonanoic) acid, respectively. Since penicillin iii has solubility characteristics such that it takes up a position between propylpenicillin and butylpenicillin, it seems quite possible that penicillin iii is 2-butenylpenicillin. Similarly, since penicillin K′ lies between amyl- and heptylpenicillins, it is possible that it is hexylpenicillin. If it were an unsaturated penicillin, *e. g.*, β,γ-hexenylpenicillin, it would probably take up a position closer to amylpenicillin. There is, of course, a possibility that penicillin K′ is a heptenylpenicillin, but it is not clear how such a penicillin would be formed from precursors such as heptanoic and nonanoic acids.

That caproic acid acts a precursor for propylpenicillin, and that caprylic and capric acids act as precursors for amylpenicillin, is most easily explained by assuming that the mold is able to β-oxidize these acids. Further evidence for this assumption are the facts that nonanoic acid gives rise to the same penicillins as does heptanoic acid, and α,β-hexenoic acid is a precursor for propylpenicillin.

It is of interest that the best precursors found (in the sense of producing the appropriate penicillin with the practical exclusion of the other penicillins normally formed by the mold) were unsaturated in the β,γ-position, *i. e.*, β,γ-pentenoic and β,γ-hexenoic acids. As mentioned earlier, the most effective precursors found by Behrens, *et al.*, were substituted acetic acids, *e. g.*, *n*-propylmercaptoacetic acid.[3] It would thus appear that the presence of some atom or molecular grouping which could presumably block the β-oxidation of the acids is of importance if the acids are to be effective precursors.

Summary

Caproic and β,γ-hexenoic acids have been found to act as precursors for the natural penicillins dihydro F and F as formed by *P. chrysogenum* Q176 grown in a synthetic medium.

Two new biosynthetic penicillins, propylpenicillin and butylpenicillin, have been shown to be formed in good yields when tributyrin and tri-*n*-valerin are employed as the respective precursors. The R-group acids of these two penicillins were isolated and identified.

Propionic, β,γ-pentenoic, heptanoic and nonanoic acids have been shown to act as precursors for various penicillins.

The possibility is discussed that the biosynthetic penicillins obtained by use of various aliphatic acids as precursors may be identical with several of the uncharacterized naturally occurring penicillins.

MADISON 6, WIS. RECEIVED SEPTEMBER 26, 1949