forms taurocholic acid. A number of explanations are possible. Either there exists a different pathway which does not require the presence of L-cysteine sulphinic acid, or the decarboxylase occurs in an organ other than the liver, or these animals depend upon the taurine taken in with the diet. At present there is no means of distinguishing between these possibilities. The problem of taurine formation has become of greater interest since it has now been established that free taurine occurs in the central nervous system (Roberts, Frankel & Harman, 1950).

The activating effect of pyridoxal phosphate on liver extracts from pyridoxine-deficient animals establishes the chemical nature of the coenzyme. Earlier attempts at reactivation had been unsuccessful; this can now be understood as it has been shown that reactivation is possible only during the first few days of deficiency. This result can best be interpreted by assuming that in the pyridoxine-deficient rat the depletion in apoenzyme follows the loss of coenzyme with only a brief time lag. It would seem that reactivation is possible only while some enzymic activity remains; the enzymic activity is rarely more than trebled.

Successful reactivation of the enzyme L-dopa decarboxylase of the pyridoxine-deficient rat liver has previously been reported (Blaschko, 1950); this enzyme disappears at a slower rate than L-cysteic acid decarboxylase and reactivation can be more easily demonstrated. It thus appears that in the deficient animal there exists a time sequence with which the different pyridoxal phosphate enzymes disappear. The work here reported shows that there are three different sequences of this kind. The first is the disappearance of coenzyme bound to apoenzyme; the second, the disappearance of the enzyme protein; the third sequence is the disappearance of co-decarboxylase in the liver. It has already been shown that with both decarboxylases the loss of enzymic activity is either complete or almost complete when large amounts of coenzyme are still present (Blaschko *et al.* 1948).

SUMMARY

1. The decarboxylation of L-cysteic and L-cysteine sulphinic acids by extracts of mammalian liver has been studied; the evidence reported suggests that one enzyme is responsible for the decarboxylation of both these amino acids.

2. Enzymic activity is rapidly lost in pyridoxine-deficient rats; reactivation by the addition of pyridoxal 5-phosphate *in vitro* has been obtained in the early stages of the deficiency; this observation establishes pyridoxal phosphate as the coenzyme of the decarboxylase.

3. The biosynthesis of taurine is discussed in the light of these findings.

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Comirin: Isolation and Properties

By W. G. C. FORSYTH

Colonial Microbiological Research Institute, Trinidad, British West Indies

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Comirin, a new fungistatic antibiotic, is produced by a bacterium found accidentally on a medium used for the cultivation of a species of *Aspergillus*. The bacterium was identified as a new species, related to *Pseudomonas fluorescens*, and given the name of *P. antimycetica* n.sp. (Thaysen & Thaysen, 1953).

The characteristics of the bacterium and the culture conditions for comirin production will be reported separately (Thaysen & Thaysen, in preparation). Seven days' growth in peptone (0.5%)—sucrose (0.5%) medium was generally used.

In the following pages, the method of assay elaborated for following the efficiency of the ex-

traction procedure, the method of isolation, and some chemical and physical properties of comirin are described.

ISOLATION

Assay. Activity is measured by an 'agar-cup' plate diffusion method. The test organism is a strain of *Aspergillus flavus* (Hankey Culture Collection, F9) added as a standard suspension of spores to a medium containing glucose (0.5%), marmite (0.25%), peptone (0.5%), agar (2.0%).

Plates containing fifty 8 mm. holes are used to compare five solutions, each solution being added to ten holes at random. The solutions are heated before testing (to 90° from room temperature within 10 min.) in a water bath to pasteurize and also to disperse the sparingly soluble comirin. Four drops are added to each hole from a standard dropper. The plates are incubated at 30° for 24 hr. The ring sizes are measured to the nearest 0.5 mm. The main error is in the measurement. This error was more or less constant and could not be reduced by magnifying the zones since it was a function of the irregularity of the inhibition edge of the zones. The average standard deviation between replicates under these conditions is ± 0.7 mm. and between means is ± 0.3 mm. when measuring zones of 18–25 mm. diameter. This corresponds to an accuracy within the fiducial limits at the 5% level of probability of 75–125%. Between 18 and 25 mm. the diameter of the zone of inhibition is directly proportional to the logarithm of the concentration.

Preliminary experiments. It is found that when active culture solutions are centrifuged, only a fraction of the activity remains in the cleared solutions. Over a pH range of 3–9 there is no significant variation in the amount of activity retained by the bacteria ($41 \pm 4\%$). It is immaterial whether the solutions are pasteurized before or after centrifuging, i.e. either comirin is sparingly soluble in the media in this pH range, or it is retained adsorbed on inactive cell debris. The adsorbed or water-insoluble, active fraction can be brought into solution with aqueous alcohols, acetone, dioxane or pyridine.

Comirin cannot be extracted from active media with light petroleum, benzene, ether, chloroform, amyl acetate or ethyl acetate at any pH. In fact, shaking with these solvents partly precipitates the activity at the interface. Comirin can be extracted by *n*-butanol.

The activity is precipitated by heavy metal acetates, picric acid, tannic acid, phosphotungstic acid, and other general protein precipitants. It is also precipitated on part saturation with $(\text{NH}_4)_2\text{SO}_4$, Na_2SO_4 or MgSO_4 or with NaCl. A method of isolation via initial $(\text{NH}_4)_2\text{SO}_4$ precipitation was worked out but discarded, since it was much more laborious and considerably less effective than the simple method finally adopted. It will not therefore be reported.

The activity is completely adsorbed by activated charcoal or alumina. It cannot, however, be eluted from them by buffers, alcohols, or acid alcohols, and is only recovered in part on elution with aqueous pyridine. A striking property of comirin is its adsorption by cellulose. On simply filtering 10 ml. of a purified aqueous solution containing 0.4 mg. of comirin through a 10 cm. Whatman no. 1 filter paper in a conical filter, over 90% is adsorbed. This can be eluted with aqueous alcohols or pyridine.

Extraction with n-butanol. Although it is possible to concentrate comirin either by initial precipitation or by adsorption, the fact that the activity is partly adsorbed by the

bacterial debris limits the usefulness of these methods. Extraction with an immiscible solvent would seem the most likely approach. Early efforts were expended in extracting the activity from salt-saturated media with ethanol or *n*-propanol, but this was abandoned in favour of direct butanol extraction.

The active culture medium has a pH *ca.* 7, and no advantage is gained by altering the pH. Extremes of pH increase the losses during extraction. Using a 1/4 volume of butanol does not give complete extraction. Although practically all the comirin is removed from the aqueous layer, it is apparently not very soluble in water-saturated butanol and much remains at the interface. Using continuous, or batch extractions on the countercurrent principle, better extraction could of course be obtained. It was found, however, that by raising the temperature of the butanol–water gel, a much larger proportion of comirin passed into the butanol. In this way very high extraction is obtained in a single step, readily adaptable to working with large volumes. There is an optimum temperature at 70°, because at higher temperatures a gel-forming material passes into the butanol and decreases the recoveries at the later stages.

Recovery from butanol. (1) Concentration at low pressure. It is found that, when the butanol extract is concentrated at low pressure at room temperature to small volume, the active material precipitates from the concentrate as a white powder. Generally about 70% of the activity is recovered. (2) Displacement with immiscible solvents. An equal volume of solvent is added to the water-saturated butanol, giving an organic and aqueous layer. The activity can be recovered from the interfacial precipitate in the following yields: light petroleum (72%); ether (50%); amyl acetate (86%). (3) Dehydration with salt (cf. Fevold, Dimick & Klose, 1948). The butanol is shaken with 4% (w/v) of NaCl and left overnight. An inactive saline layer separates off. The butanol layer contains 2% of the activity and 91% can be recovered from the interfacial precipitate. This method is routinely used.

Further purification. Aqueous pyridine (50%, v/v) is found to be by far the best extractant of the activity from the saline precipitate. The activity can then be recovered in high yield from the aqueous pyridine by addition of amyl acetate or ethyl acetate and once again recovering the interfacial precipitate.

Final procedure. The following is a typical extraction flow sheet. The figures in brackets are the amounts of comirin in each fraction as determined by assay:

The active culture in 90 l. lots (≈ 2 g. comirin) is stirred vigorously for 3 hr. with 22.5 l. *n*-butanol. After standing overnight, the aqueous layer (130 mg. comirin) is siphoned off and discarded. The gel remaining is heated to 70° for 1 hr. and separated on a Sharples centrifugal separator at 50–70°. The water from the gel (120 mg. comirin) may be added to the next batch if desired. The interfacial precipitate (150 mg. comirin) is discarded.

The butanol extract (1.6 g. comirin, 80%) with 4% (w/v) NaCl added, is stirred for 2 hr., and allowed to settle. The butanol layer is siphoned off and the interfacial precipitate (1.43 g. comirin) separated from the aqueous solution by centrifuging. The precipitate is extracted, by shaking and centrifuging, with three successive lots (500, 300, 200 ml.) of 50% (v/v) pyridine (freshly redistilled)–water. The combined pyridine extracts are then shaken with 4 vol. of amyl acetate and the organic layer discarded. The white

gelatinous interfacial precipitate is collected, washed with a small volume of absolute ethanol, then acetone, and dried *in vacuo*. The comirin is thus obtained as a white amorphous powder. Yield; 1.28 g. (64%).

1 mg. of this preparation in 37 ml. water gave a clearing zone of 23 mm. in the standard assay. By the above method it is readily obtained in 60–70% yield.

Evidence of homogeneity

Paper chromatography. Comirin was chromatographed by a simple ascending-solvent one-dimensional strip technique and detected by laying the developed air-dried strips on agar media seeded with the *Aspergillus flavus* used as test organism (Forsyth, 1953).

Comirin moves as a single discrete spot in water-saturated amyl alcohol (R_F , 0.05); water-saturated *n*-butanol (R_F , 0.16); *n*-butanol (2 parts)–pyridine (1 part)–water (4 parts), (R_F , 0.42); aqueous ammonia sp.gr. 0.880 (R_F , 0.75); water-saturated phenol (R_F , 0.81); 60% (v/v) aqueous dioxane (R_F , 0.88); 25% (v/v) monoethylamine (R_F , 0.92); 60% (v/v) pyridine–water (R_F , 0.96). It remains on the starting line with water. In no case is more than one spot obtained, even with two-dimensional descending solvent systems using pairs of the above.

Countercurrent distribution. The distribution of the activity during a ten-tube diagonal countercurrent distribution analysis (Bush & Densen, 1948), using 30 mg. of comirin and a mixture of *n*-butanol (1 part) and water containing 10% pyridine (5 parts) is shown in Fig. 1.

Other evidence. It would appear that the biological effect is due to one substance. The following experiments suggest that no inactive impurities are present: (a) Dissolving in aqueous pyridine and reprecipitating at the interface does not increase the activity. (b) Fractional precipitation from pyridine or acetic acid solution with ether, ethyl acetate or amyl acetate, does not give any fractions of increased activity. (c) Comirin is much more soluble in boiling water than in cold. Fractionation by dissolving in boiling water and cooling does not lead to any increase in activity. (d) Pre-

cipitates obtained by cooling hot saturated aqueous pyridine solutions do not show an increase in activity. (e) Comirin was recovered from fractions 8, 9 and 10 of the above countercurrent analysis. The material isolated has the same activity as the original. (f) Comirin, chromatographed in a column of packed cellulose pulp with the top layer of a mixture of *n*-butanol–pyridine–water (2:1:4, v/v), moves as a single band. On recovery from the active fraction of the eluate, the recovered material is of the usual activity.

It would appear that, pending results from other physical methods (ultracentrifuge, electrophoresis, etc.), the material may be considered reasonably homogeneous.

Purified preparations of comirin are haemolytic. That this is a property of the antibiotic itself, and not due to a haemolytic contaminant can be readily shown. It is possible to detect haemolytic substances on paper chromatograms by placing the developed chromatograms on saline agar plates containing washed blood cells. Zones of lysis of the blood cells are formed under the influence of these separated haemolytic substances. The identity or otherwise of the antibiotic and haemolytic substances can then be found by covering the blood cell plates with a thin layer of nutrient agar containing a test organism and incubating. If the two activities are due to a single substance, then the centres of the two zones will coincide and the shape, but not necessarily the area, of the two zones will be identical. When comirin was tested in this manner using eight different solvent systems, in every case the zones of inhibition and lysis coincided. Moreover, the diameters of the two zones were practically equal.

PROPERTIES

Solubility. The solubility of comirin at 25° in various solvents is shown in Table 1.

Stability of comirin. Comirin (13 mg.) was dissolved in water (500 ml.) and divided into 9 × 50 ml. lots. Each lot was made up to 100 ml. with water and dilute HCl or NaOH to give the desired pH in the range 3–9 (Table 2). The solutions were then assayed after pasteurization (by heating to 90° from room temperature within 10 min. in a water bath) and after further heating (100° for 30 min.) under reflux.

While there is a loss at alkaline pH values on continued heating, there is also a highly significant apparent increase at pH ≤ 4 even with the heating necessary for assay only.

Table 1. Solubility of comirin at 25°

	mg./100 ml.
Water	4
50% Aqueous ethanol	12
50% Aqueous acetone	18
25% Monoethylamine	140
Pyridine	170
50% Aqueous pyridine	300
Glacial acetic acid	320

Insoluble in: anhydrous alcohols, acetone, dioxan, ether, ethyl acetate, amyl acetate, chloroform, benzene, light petroleum.

Fig. 1. Countercurrent distribution of comirin (30 mg.) in *n*-butanol–10% (v/v) aqueous pyridine (1:5, v/v). Ten-tube diagonal system. 90 ml. solvent/tube. Theoretical. $p=0.52$, $\odot$ — $\odot$ experimental (for definition of p see Bush & Densen, 1948).

Table 2. *Stability of comirin*

A comirin solution of known concentration (by weighing) adjusted to the pH values shown and assayed before and after heating (30 min. at 100°). Results as percentage original. For conditions of assay see text. A significant difference at the 5% level = +25%. *Italic type*, significant; *clarendon type*, highly significant difference from original.

	pH	...	...	9.0	8.0	7.5	7.0	6.5	6.0	5.0	4.0	3.0
Assay (includes pasteurization)				97	97	101	97	108	108	93	<i>144</i>	176
Assay after further heating (30 min. 100°)				35	<i>64</i>	101	108	104	104	97	176	206

Table 3. *Dispersion and aggregation*

A comirin solution of known concentration by weighing: *A*, pasteurized at pH 7; *B*, heated 15 min. at 100° at pH 3; *C*, *B* readjusted to pH 7. The solutions *A*, *B* and *C* then allowed to stand 24 hr. at 25°. Results as percentage original. (H.S., highly significant; N.S., not significant).

	pH	...	<i>A</i>	<i>B</i>	<i>C</i>
			7	3	3-7
Assay after treatment			100	178	180
After 24 hr. standing			85	115	80
After re-pasteurizing			88	185	85
Loss on standing			N.S.	H.S.	H.S.
Gain on re-pasteurizing			N.S.	H.S.	N.S.

A preparation of comirin shown to be homogeneous with regard to chromatography (4 mg.), was pasteurized with 100 ml. water. The pH was then adjusted to 3.0 and the solution boiled for 15 min. The solution was then adjusted back to pH 7. After 24 hr. the solutions were again tested before and after pasteurizing. The results are shown in Table 3.

Dialysis. When an active, aqueous solution of the antibiotic is suspended in a cellophan sac, no dialysis through the cellophan can be detected. However, when the organism is streaked on sterile cellophan, placed on glucose-marmite-peptone-agar containing fungal spores and incubated, a very slight zone of inhibition is observed directly below the streak. This zone was only the width of the streak (*ca.* 2 mm.), whereas spraying fungal spores over the top of the cellophan showed that an inhibition zone of *ca.* 15 mm. had been produced. The antibiotic must be very sparingly dialysable in aqueous solution through cellophan. Using a very active solution, only 0.2% dialysed in 24 hr. When, however, solutions which had been pasteurized at acid pH were dialysed, the following results were obtained after 24 hr. as percentage dialysed: (1) pasteurized at pH 7, 0.3%; (2) at pH 3, 3.1%; (3) pasteurized at pH 3 and adjusted back to pH 7, 3.0%.

Chemical analysis and tests. (a) Comirin contains no sulphur, halogens, phosphorus or ash. Air-dried samples contain 5-7% moisture. The following analyses were obtained on a moisture-free basis: C, 49.08, 48.76, 48.86; H, 6.70, 6.86, 7.00; N (Dumas), 11.88, 12.18, 11.73; N (Kjeldahl), 12.09,

11.91, 11.88%. The mean N value, 11.9%, is too low for a simple peptide. The antibiotic melts with decomposition over the range 230-235° depending on the rate of heating. All attempts at crystallization have been unsuccessful. (b) Comirin gives positive Millon-Cole, Pauly, Sakaguchi and xanthoproteic reactions. A positive biuret reaction is obtained but a negative ninhydrin reaction. The activity is unaffected on boiling with ninhydrin. Glyoxylic acid and Molisch tests were also negative. The activity is not destroyed by pepsin, papain or trypsin. (c) Comirin (6 mg.) was hydrolysed with 0.6 ml. 6N-HCl for 18 hr. in a sealed tube at 110°. An average of 90% of the total nitrogen is liberated as α -amino acid nitrogen (ninhydrin-carbon dioxide volumetric method). The original material contains no free α -amino acid N as measured by this method. The antibiotic is soluble in 6N-HCl to give a clear, colourless solution, but after 1 hr. heating, a copious precipitate separates out on cooling. At this stage ether-soluble material (*ca.* 20%) has been liberated. On further heating the hydrolysate darkens and 6% of dark 'humin' forms. An equal volume of water was added and this precipitate was centrifuged off. The supernatant was evaporated several times to dryness *in vacuo* with addition of water to remove HCl. The residue was taken up in 0.3 ml. of water and subjected to paper chromatography (Consden, Gordon & Martin, 1944).

The apparatus used for two-dimensional chromatography was essentially that of Dent (1948). However, the chromatogram was run in one direction with phenol in the presence of NH₃ and NaCN, and in the other with the butanol-acetic acid mixture of Partridge (1948). Whatman no. 1 paper was used throughout. The chromatograms were sprayed with ninhydrin (0.1%, w/v) in butanol and the strength of the spots detected was estimated visually from their area and colour intensity.

Identification of ninhydrin-reacting substances. Most of the amino acids were readily identifiable from the pattern of the spots, the chromatograms being compared with those of synthetic mixtures of pure amino acids run under identical conditions. In many cases identification was checked by adding pure amino acids to the hydrolysates. It was shown by the CuCO₃ technique of Crumpler & Dent (1949), which distinguishes α -amino acids from other

ninhydrin-reacting substances, that one spot present—no. 13—was not due to an α -amino acid. The nature of this substance is not known.

Leucines (no. 1). It was readily shown by one-dimensional chromatography with *tert*-amyl alcohol (Work, 1949) that both leucine and isoleucine were present. Methionine could not be detected by ninhydrin, or the H_2O_2 technique (Dent, 1947), even when the chromatograms were overloaded with the other amino acids.

Tyrosine (no. 3). Although the original hydrolysate gave quite a strong Millon-Cole reaction, most of the colour producing substances were in the black insoluble precipitate formed (cf. Gordon, Martin & Synge, 1943) and only a trace of tyrosine was detected in the solution after centrifuging. This was barely visible on the two-dimensional chromatograms, but its presence could be shown by a one-dimensional chromatogram run in butanol-acetic acid (Partridge, 1948) and sprayed with diazotized sulphanilic acid followed by Na_2CO_3 solution (Pauly, 1904). The absence of histidine was also shown with this reagent.

The basic amino acids (nos. 10, 11, and 12). These three substances were shown to be basic, since they were retarded when phenol was used with an acetic acid instead of an ammonia atmosphere. Spots nos. 10 and 11 were readily identified by conventional methods as arginine and lysine. Spot no. 12 was separable from added ornithine. Other possi-

bilities are α - γ -diaminobutyric acid which has been found in the polymyxin antibiotics (Jones, 1949) and α - ϵ -diaminopimelic acid which has been found in *Corynebacterium diphtheriae* (Work, 1950) and soils (Bremner, 1950). α - ϵ -Diaminopimelic acid, however, moves in a different position, appearing under glutamic acid (Bremner, 1950). α - γ -Diaminobutyric acid synthesized from glutamic acid could not be distinguished from the unknown on the above chromatogram. The two spots coincided when the chromatograms were run both in ammonia and in acetic acid atmospheres.

'Unknown' (no. 13). This spot gave an atypical ninhydrin reaction. A brownish yellow spot first appeared which slowly changed to the normal purple. Although qualitative tests on the original antibiotic for sulphur were consistently negative, and methionine was absent, tests were made for cystine or sulphur-containing breakdown products. The H_2O_2 technique failed to reveal cystine, although cystine was readily detected when added to the hydrolysate. Also, spraying with potassium iodoplatinate (Winegard, Toennies & Block, 1948) failed to detect any sulphur-containing amino acids. The sensitive spot test for cystine described by Dent & Rose (1949) was also negative. The substance did not form a salt on treatment of the paper with $CuCO_3$, and is not therefore an α -amino acid.

Dinitrophenyl (DNP) derivative of the antibiotic. Comirin readily reacts with 2:4-dinitrofluorobenzene (DNFB) to form a DNP derivative (Sanger, 1945). The antibiotic (0.1 g.) was suspended with 0.1 g. $NaHCO_3$ in 1 ml. of water, 0.1 ml. DNFB in 2 ml. ethanol was added and the mixture shaken for 2 hr. It was then extracted successively with water, ethanol and ether leaving a bright yellow solid practically insoluble in water and ethanol, but readily soluble in pyridine. That the reaction was complete could be shown by paper chromatography with 60% (v/v) acetone. Under these conditions comirin has an R_F of about 0.8, whereas both the colour and activity (the DNP derivative is active) remain on the starting line.

The DNP comirin was hydrolysed for 18 hr. with 5N-HCl and the hydrolysate then extracted with ether. The ether extract and the water layer were prepared as usual (Sanger, 1945) and subjected to paper chromatography. All the colour was confined to the aqueous layer. The aqueous layer gave two water soluble spots, major component (R_F , butanol-acetic acid, 0.64) minor component (R_F , 0.73). They are found close to the leucines in the two-way chromatogram.

Substrate. To determine whether there was any correlation between the amino acid composition of the comirin produced and the protein substrate, 20 amino acids were tested individually as the sole source of nitrogen. The medium used was as follows:

Fig. 2. Diagram of two-way chromatogram of the amino acids in a comirin hydrolysate. Solvents: water-saturated phenol; *n*-butanol-acetic acid-water (4:1:5, v/v). First run in phenol from left to right, then in butanol-acetic acid downwards. Intensity of spots approximately represented by area of circles. (Provisional identification: 1, leucines; 2, valine; 3, tyrosine; 4, alanine; 5, threonine; 6, glycine; 7, serine; 8, glutamic acid; 9, aspartic acid; 10, arginine; 11, lysine; 12, α - γ -diaminobutyric acid; 13, unknown).

Table 4. *Production of comirin on amino acids as sole source of nitrogen*

		For conditions see text.										
Inoculum	{ Slope... Colony	...	<i>A</i>		<i>B</i>		<i>C</i>		<i>D</i>			
		...	1	2	1	2	1	2	1	2		
Nitrogen substrate			Inhibition zones (mm.)								Active cultures (%)	Mean clearing zones
Peptone			22	23	22	20	19	19	17	18	100	20.0
Ammonium citrate			21	20	17	17	18	19	17	16	100	18.1
Ammonium phosphate			18	19	19	18	19	19	12	14	100	17.2
L-Histidine			22	20	17	16	19	18	20	19	100	18.8
L-Arginine			18	21	20	17	19	17	21	—	88	17.6
L-Asparagine			22	19	19	20	15	—	16	—	75	15.9
DL-Alanine			22	9	9	12	15	—	12	—	75	11.9
DL-Glutamic acid			20	—	—	18	—	—	—	13	38	—
DL-Aspartic acid			20	—	—	—	—	—	—	—	12	—
L-Lysine			—	—	—	—	9	17	15	17	50	—
DL-Tryptophan			—	—	—	—	—	19	—	16	25	—

Glucose, 0.5 %; K_2HPO_4 , 0.1 %; KH_2PO_4 , 0.1 %; $MgSO_4$, 0.04 %; $FeSO_4$, 0.002 %. The nitrogen compounds were added in the proportion of 75 mg. N per 100 ml. and the pH adjusted to 6.5.

Loopfuls from four different slopes of *Ps. anti-mycetica* were plated out at high dilutions and two colonies picked at random from a suitable plate from each slope. These were suspended in eight 10 ml. lots of saline and one saline tube was used to inoculate each set of media in 10 ml. lots in test tubes. One drop was used for each inoculum. In this way the effect of strain variation (Thaysen & Thaysen, in preparation) could be overcome. After incubation for 7 days at 30° growth was noted and the activity determined. In Table 4 are listed the nitrogen compounds which supported good growth and gave some activity.

It would appear that the organism is able to utilize under these conditions, only those amino acids which either play a major part in protein synthesis (alanine, glutamic acid and aspartic acid), or contain a nitrogen grouping in addition to the α -amino N (histidine, asparagine, arginine, lysine and tryptophan).

DISCUSSION

A simple method for the isolation of comirin in an apparently homogeneous form has been developed, based on its rather peculiar solubility properties. Comirin is readily isolated by repeated precipitation at the interface between organic and aqueous solutions. Throughout this investigation the technique of paper chromatography has proved extremely useful as a guide to isolation procedures.

Comirin on hydrolysis gives 14 ninhydrin positive substances of which 12 are readily identified as common amino acids. An uncommon basic amino acid is present which is probably α - γ -diaminobutyric acid. The fourteenth ninhydrin-reacting substance

is not an α -amino acid. Glycine, serine, and to a lesser extent, aspartic acid, make up the bulk of the peptide moiety. There is no simple relationship between the amino acids useful as a substrate and the constituents of the peptide. The different strains vary greatly (Table 4) in their ability to produce comirin from various amino acids.

Comirin solutions when heated at pH ≤ 4 show a large increase in apparent activity. On standing the activity returns to normal. There is no evidence of a chemical dissociation. On paper chromatography the same R_f values are obtained whether the material has been 'activated' at pH 4 or not. Comirin probably exists in aqueous solution as a colloidal suspension, which is dispersed on heating at acid pH values and reaggregates on standing. It is very sparingly dialysable through cellophan.

The only substances with which comirin might be confused are the various antifungal antibiotics from *Bacillus subtilis*, and *B. polymyxa*. However, the solubility, lack of activity against bacteria, and the amino acid composition of comirin, clearly differentiate it from these other substances. Comirin is readily distinguished from other antifungal agents produced by fungi and actinomycetes by paper chromatography (Forsyth, 1953).

No non-pigmented antifungal substance has previously been isolated from a non-spore-forming bacterium, although the closely related species *Ps. fluorescens* has been reported to have antifungal activity, which however can also be readily distinguished from comirin by paper chromatography.

SUMMARY

1. A method of isolating comirin from active culture media, in what is believed to be a homogeneous form, in 60-70 % yield is described.

2. The homogeneity was checked by chromatography and countercurrent analysis.

3. Comirin is a peptide containing an ether-soluble moiety.

4. The antibiotic shows a striking apparent increase in activity on heating in acid solution.

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A New Method for the Determination of the Amidase Activity of Trypsin: Kinetics of the Hydrolysis of Benzoyl-L-Arginineamide

By S. A. BERNHARD*

Department of Colloid Science, University of Cambridge

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The determination of the amidase activity of trypsin is complicated by the appreciable rate of self-digestion of the enzyme under optimum conditions for the hydrolysis. In order to obtain significant results it was necessary to find a kinetic procedure by which the hydrolysis could be followed rapidly relative to the rate of inactivation of the enzyme. In the course of tests of the formol titration method of Iselin & Niemann (1950) it was observed that the esterase activity of trypsin was unaffected by formaldehyde. Esterase activity was followed by the potentiometric titration of Schwert, Neurath, Kaufman & Snoke (1948) and of Schwert & Eisenberg (1949) with benzoyl L-arginine ethyl ester (BAEE) as substrate. The fact that the activity of trypsin is unaffected by formaldehyde (as determined by esterase activity) was made the basis of a potentiometric method for following the amidase activity. At the pH-activity optimum (pH 7.5–8.0), in the presence of formaldehyde the trypsin-catalysed hydrolysis of amides may be represented by the following equations:

Thus, in the case of trypsin, the hydrolysis of amides can be followed directly by measuring the

amount of oxonium ions liberated in the presence of formaldehyde. This method has been successfully carried out employing benzoyl-L-arginineamide (BAA) as substrate.

MATERIALS AND METHODS

Substrates. Benzoyl-L-arginineamide and ethyl ester were prepared from L-arginine hydrochloride (British Drug Houses Ltd., London) as described previously (Bergmann, Fruton & Pollok, 1939). Acetyl-L-phenylalanine (I) was prepared, according to the method of Huang & Niemann (1951), from L-phenylalanine (supplied by Roche Products Ltd., Welwyn, Herts). The ethyl ester was prepared by esterification of I in HCl-saturated ethanol at room temperature. After 2 days the reaction mixture was partially evaporated under vacuum and precipitated with water. The crude ester was recrystallized twice from hot water (pH 7).

Enzymes. Trypsin: the trichloroacetic acid precipitate of Northrop, Kunitz & Herriott (1948) was suspended in water and exhaustively dialysed against 0.01M-HCl until the protein all dissolved (about 3 days at 5°). Crystallization of this material resulted in no further increase in activity and hence the dialysed samples at pH 2 were stored and used in the rate experiments.

Chymotrypsin: chymotrypsin was prepared by Dr H. Gutfreund from the trypsin digest of a sample of chymotrypsinogen (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) by the method of Northrop *et al.* (1948). Before use a 0.1% solution was dialysed against 0.005M ethylenediamine (pH 7.5) solution.

* Present address: Naval Medical Research Institute, Bethesda, Md., U.S.A.