DISCUSSION

The method described is specific for β -alanylhistidine peptides, since, of the substances containing the histidine group that are precipitable by the mercuric chloride reagent, only those which yield β -alanine or subsequent hydrolysis are determined. In biological products this group includes only carnosine and anserine.

SUMMARY

1. A reasonably rapid and accurate method is described for the determination of β -alanylhistidine peptides in biological materials.

2. The peptides are precipitated with a mercuric chloride reagent, the precipitate is hydrolysed with 6N-hydrochloric acid, and the β -alanine formed is oxidized to a product which forms a dinitrophenylhydrazone.

3. The recovery of the carnosine added to a meat extract was $99 \pm 1.90\%$.

4. Meat extract from various sources contains 5.6–6.6% (average 6.2%) of β -alanylhistidine peptides containing 90% of carnosine and 10% of anserine.

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Rutilantin: an Antibiotic Substance with Antiphage Activity

By I. N. ASHESHOV*

Lister Institute of Preventive Medicine, London, S.W. 1

AND J. J. GORDON†

Medical Research Council Antibiotics Research Station, Clevedon, Somerset

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Asheshov, Strelitz, Hall & Flon (1954) examined 1000 actinomycete cultures for antiphage activity to determine whether bacterial viruses could be used as simulants of animal or plant viruses, since antiphage tests can be handled more easily and give more rapid results than antiviral tests. Of these cultures, 201 showed antiphage activity. One of them, which was bright red in colour (Serial No. A220), had a wide range of activity against phages of various strains of staphylococci, enterococci, *Bacillus cereus*, *Bacillus megatherium*, *Escherichia coli*, *Salmonella paratyphi* and *Vibrio cholerae*. Extracts prepared from cultures of this organism were also active against the viruses of Eastern and Western Equine encephalomyelitis, PR8 influenza, mumps and Y-SK poliomyelitis.

It was of interest to investigate the chemistry of the active substance, to which the name

'rutilantin' was given. It was recently shown by Ollis, Sutherland & Gordon (1959) that mild acid hydrolysis of rutilantin picrate, or of the purified base, yielded a bright-red crystalline aglycone ('rutilantinone'), and a water-soluble basic carbohydrate fraction, neither of which showed antibacterial or antiphage activity. Chemical investigations led to structure (I) for rutilantinone.

(I)

* Member of the external staff, the Medical Research Council. Present address: 259 Swakeleys Road, Ickenham, Middlesex.

† Present address: Chemical Defence Experimental Establishment, Porton Down, Salisbury, Wilts.

This constitution was of particular interest because it was later established by Brockmann *et al.* (1960) that it is identical with the structure of

ϵ -pyrromycinone described by Brockmann & Lenk (1959*a*) derived from the cinerubins (Ettlinger *et al.* 1959) and also from pyrromycin (Brockmann & Lenk, 1959*b*), both of which are isolated from various streptomyces strains.

The present paper describes the isolation and partial purification of the active material.

EXPERIMENTAL

Culture conditions

Medium. This consisted of (g./l.) baker's yeast (20), glycerol (20), K_2HPO_4 (1.0), $MgSO_4 \cdot 7H_2O$ (0.5). Trace elements were included, as follows (mg./l.): boric acid (57), $CuSO_4 \cdot 5H_2O$ (157), $FeSO_4(NH_4)_2SO_4 \cdot 24H_2O$ (1400), $MnSO_4 \cdot 5H_2O$ (81), and $ZnSO_4 \cdot 7H_2O$ (79). Silicone A (23 p.p.m.) and triamyl citrate (340 p.p.m.) were also present, as antifoam. Fermentations were carried out in 100 gal. stainless-steel fermentors at 24° for 96 hr. with a stirring rate of 250 rev./min. and an aeration rate of 16 ft.³/min.

Inoculum. This was produced in 10 l. aspirators containing 8 l. of yeast medium, stirred at 300 rev./min., aerated at 18 l./min., and incubated at 24° for 96 hr. The amount of inoculum added was 1% (v/v).

Assay

Diffusion assay. The agar diffusion technique, with *V. cholerae* and phage (Asheshov, Hall & Flon, 1955), was used. The medium used for the plates consisted of papain digest broth (Asheshov, 1941) containing agar (0.75%). The inoculum was a 4 hr. culture of *V. cholerae* in papain broth to which the phage (filtered free of bacteria) was added immediately before use. The plates were flooded with a mixture of host cells and phage in papain digest broth buffered to pH 7, the excess of fluid was pipetted off, and the plates were returned to the incubator for 30 min. Filter-paper disks (1.3 cm. diameter) were soaked in test solution, dried if an organic solvent was being used, and deposited on the plate. Plates were incubated for 18 hr. at 37°. Antiphage activity appeared as a zone of normal bacterial growth in the clear agar. Attempts to develop a reliable unit assay were unsuccessful because the zones obtained were irregular in shape, and had an uneven and not very well-defined edge; the slope of the plot of zone diameter against log concentration for this reason was not very reproducible. Results for diffusion assays are accordingly expressed as mean zone diameter (cm.) multiplied by 5.7. The figures thus obtained could only give a rough indication of the activity of a solution, and where a more quantitative assessment of antiphage activity was required, broth dilution tests were employed.

Broth dilution assay. The procedure was that of Asheshov, Hall & Flon (1955) with the demonstrable lysis of host cells in liquid medium as an indicator of normal phage activity, antiphage activity being indicated by growth of host cells. Tests were made in 5 ml. of broth of the following composition (/l.): papain digest (400 ml.), glucose (10 g.), NaCl (8.5 g.), bromocresol purple (10 mg.), trace elements (as already described); pH 7.3. *V. cholerae* cells (0.1 ml.) were suspended in broth, phage was added to each tube, and the tubes were incubated at 37° for 18 hr. The end point was

the greatest dilution of active substance at which growth was normal, accompanied by liberation of acid indicated by change in colour.

Isolation

Preliminary experiments. At the end of the fermentation period, cultures showed antiphage zone diameters of 11.6–12.4 cm. No apparent loss of activity occurred on standing at room temperature at pH values between 2 and 10 for 12 hr. On centrifuging at neutral pH, a rather cloudy supernatant was obtained, but at pH 2 the supernatant was clear. The antiphage activity, as well as the red pigment, was present both in the mycelium and the supernatant. Tests showed that colour and activity could be transferred readily from the supernatant into various water-immiscible organic solvents, after making them slightly alkaline with dilute aqueous NH_3 (NaOH or KOH were not so convenient for this purpose, since they tended to form precipitates at pH values above 9, with irreversible loss of activity). Effective solvents included ethyl and *n*-butyl acetate, $CHCl_3$, trichloroethylene, and butan-1-ol. Light petroleum removed some of the colour, but none of the activity.

Active material could be extracted from mycelium with acetone or methanol. From this, it could be transferred to a water-immiscible solvent, such as $CHCl_3$ or trichloroethylene.

From trichloroethylene or $CHCl_3$, activity could be transferred back into aqueous solution by adding 1 vol. of light petroleum, extracting the mixture with 0.1*N*-HCl, and neutralizing with dilute aqueous NH_3 . This left only a trace of activity in the solvent mixture but a large amount of the colour remained, indicating that part of the red material is inactive.

Precipitation as picrate or reineckate. When an aqueous extract was treated with excess of 1% picric acid, or with ammonium reineckate, a bright-red precipitate was formed. The supernatant in either case was completely free of antiphage activity and showed only slight colour. This reaction accorded a convenient means of isolating the active material as solid.

Procedure for isolation as picrate

Culture (320 l.) was adjusted to pH 2 and centrifuged through a Sharples centrifuge.

Supernatant. The supernatant showed antiphage activity at 1:100 dilution. After adjusting to pH 9.5 with 2.5*N*- NH_3 solution it was stirred vigorously for 5–10 min. with trichloroethylene (0.25 vol.). The emulsified mixture was passed at a rate of 150 l./hr. through an Alfa-Laval centrifuge, the bowl of which was assembled for emulsion separation. The aqueous phase, which was practically colourless and inactive, was rejected and the trichloroethylene phase evaporated down to 2–3 l. at 10 mm. in a van Heyningen (1949) distillation apparatus. The concentrate was active at a dilution of 1:5000, and the activity of the solid content 1:480 000. It was treated with 1 vol. of light petroleum (b.p. 80–100°), the mixture filtered if necessary, and extracted twice with 0.1*N*-HCl (0.5 vol.). The combined HCl extracts were treated with excess of aqueous 1% picric acid, and the precipitate was filtered off and dried in air at 30°. Yield 40 g.

Mycelium. This was stirred for 20 min. with acetone (20 l.) and filtered by suction. The solid residue was stirred 20 min. with a further 10 l. of acetone, 2.5*N*- NH_3 solution

being added to the mixture to bring the solution to a dark red. The mixture was filtered again and the solid residue rejected. The pooled acetone extracts were stirred with 1 vol. of trichloroethylene and 0.5 vol. water, sufficient aqueous NH_3 solution being added to bring the pH to 9.5, and the mixture was allowed to settle. The aqueous layer was siphoned off and re-extracted with 0.5 vol. of trichloroethylene. The spent aqueous phase was inactive, whereas the pooled organic extract was active at a dilution of 1:1500; activity of solids was 1:200 000. The volume of the solvent mixture was then reduced at 10 mm. to 6–8 l. The concentrate was treated with 1 vol. of light petroleum, and the mixture extracted with 0.5 vol. of 0.1N-HCl. A red precipitate formed; this was filtered off, stirred with 0.1N-HCl (1–2 l.) and treated with excess of aqueous 1% picric acid. The organic layer was then re-extracted with 0.1N-HCl (0.5 vol.), and the pooled HCl extracts were treated with aqueous 1% picric acid. The picrate precipitates were filtered off and dried as previously. Yield 50 g.

Preparation of soluble salts from picrate

The antibiotic was readily converted into the water-soluble hydrochloride or citrate, as follows: Picrate (0.75 g.) was suspended in 15 ml. of CHCl_3 , and a slow stream of NH_3 gas passed through the solution for 1–2 min. The mixture was centrifuged, and the supernatant washed three times with water (1 vol.), the first wash water being adjusted to pH 8. The CHCl_3 solution was treated drop by drop with a solution of HCl gas in ethyl acetate (2N) until precipitation was complete, and the precipitate centrifuged off and washed with ether. The hydrochloride preparation thus obtained was light red in colour and showed antiphage activity at a dilution of $1:5 \times 10^6$ (i.e. 1 part in 5 million).

To prepare the citrate the hydrochloride was redissolved in water, adjusted to pH 6 with aqueous NH_3 solution and extracted twice with ethyl acetate (1 vol.). The extract was concentrated to about one-third vol. and treated drop by drop with saturated citric acid in dry ethyl acetate, until precipitation was complete. The bright-red precipitate was centrifuged down and washed with ether. Different batches of citrate preparations were active at dilutions varying between $1:5 \times 10^6$ and $1:7 \times 10^6$.

Yields of hydrochloride or citrate were approximately 20–30% of the picrate used.

PROPERTIES

Rutilantin is evidently a basic substance having little solubility in water at neutral pH. The citrate is soluble in water to the extent of approximately 1 mg./ml., and the hydrochloride much more soluble.

Stability. Aqueous solutions retain full activity for 12 hr. at room temperature at pH values between 1 and 10, but in N-HCl a slight fall in activity occurs. At 40° activity remains the same for 1 hr. at neutral pH, but is completely destroyed at pH 2 or 10. Above 40°, activity is rapidly lost, even at neutral pH.

Toxicity to mice. Citrate preparations were tested for toxicity by injecting them intraperitoneally into mice, 12 mice being used for each dose. From

these experiments it was found that a citrate preparation of activity $1:5 \times 10^6$ had a LD_{50} of 45 mg./kg. Another preparation with antiphage activity $1:7 \times 10^6$ showed LD_{50} of 55 mg./kg.

Antibacterial spectrum. Tests of antibacterial activity were also carried out on solutions of the citrate by using the agar diffusion technique. At a concentration of 1:10 000 there was appreciable inhibitory action against the following organisms: *Aerobacter cloacae*, eight strains of *Micrococcus*, three strains of *Staphylococcus albus*, four strains of *Staphylococcus aureus*, *Sarcina lutea*, two strains of *Streptococcus pyogenes*, *Str. cremoris*, *Str. lactis*, *Str. faecalis*, two strains of *Str. agalactiae*, *Bacillus anthracis*, *B. megatherium*, *B. mycoides*, *B. cereus*, six strains of *B. subtilis* and *Salmonella typhi*. At a concentration of 1:100 000 the antibacterial activity was negative or doubtful. It would appear therefore that antiphage activity is at least 50 times as great as antibacterial activity.

DISCUSSION

Isolation of rutilantin in the solid form from large volumes of culture proved to be a relatively simple matter, advantage being taken of the partition between certain organic solvents and water at different pH values, and the insolubility of the picrate salt in water. The quantities of picrate obtained separately from the supernatant and the mycelium indicate that the active substance is distributed fairly evenly between the two phases of the culture.

Biologically, this substance is of fundamental interest on account of its high antiphage activity. In view of the earlier report of Asheshov *et al.* (1954) on antiviral activity, it would be of great interest to test the purified preparations against viruses. The mouse tests indicate it to be a toxic substance, but this may perhaps not necessarily exclude its ultimate therapeutic use, since the LD_{50} is still very high in relation to the antiphage activity.

SUMMARY

1. A simple method has been developed for the isolation and partial purification of rutilantin from cultures of an actinomycete (A 220).
2. The antiphage activity of the isolated material is at least 50 times its antibacterial activity.
3. The partly purified substance is toxic to mice.

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The Oxidation of D- α -Hydroxy Acids in Animal Tissues

BY P. K. TUBBS AND G. D. GREVILLE*

Department of Biochemistry, University of Cambridge

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It was realized by 1913 that the lactate formed by mammalian tissues from various hexoses is entirely of L-configuration (Levene & Meyer, 1913). Dakin & Dudley (1913a) and Neuberger (1913), however, showed that animal tissues form lactate from methylglyoxal, which, until the work of Lohmann and others in 1931-33 (e.g. Meyerhof, 1933), was thereupon believed to be an intermediate in glycolysis. That methylglyoxal gives rise to the 'unnatural' D-lactate caused surprisingly few misgivings. This reaction is now thought to be part of an amino ketone cycle (see Discussion).

It has been almost universal practice to use DL-lactate in experiments with animal tissues, the D-isomer being ignored on the assumption that it is metabolically inert, despite various reports to the contrary (e.g. Dakin & Dudley, 1913b; Abramson & Eggleton, 1927; Cori & Cori, 1929; Brin, Olson & Stare, 1952).

Huennekens, Mahler & Nordmann (1951a, b) reported that fresh, crude mitochondrial suspensions from rabbit liver and kidney could oxidize D-lactate, but that the L-enantiomorph was not oxidized unless the particles were supplemented with a 'factor', supposed to be a lactate racemase, from the soluble cytoplasm. Mahler, Tomisek & Huennekens (1953) continued this work, using both manometric and dye reduction (Thunberg tube) techniques; no evidence was obtained for the involvement of coenzymes in the formation of pyruvate from D-lactate, and attempts to obtain D-lactate oxidation in non-particulate systems

were unsuccessful. Baker (1952) and Winitz *et al.* (1956) reported oxidation of D- α -hydroxy acids by rat-tissue preparations; again no coenzyme requirement was found. With kidney only the mitochondrial fraction was effective, and the slight activity in the soluble fraction of liver homogenates was perhaps due to glycollate oxidase (Kun, Dechary & Pitot, 1954), an enzyme virtually absent from kidney.

The present work has extended these various findings. Mitochondrial D- α -hydroxy acid dehydrogenase has been obtained in soluble form and partially purified. This communication is mainly devoted to the properties of this enzyme, which, it appears, should be added to the short list of mitochondrial 'cytochrome-reducing dehydrogenases' (Singer & Kearney, 1960).

Preliminary reports have appeared (Tubbs & Greville, 1959; Tubbs, 1960a), a detailed account has been given elsewhere (Tubbs, 1960b), and effects of metal-complex-forming agents on the dehydrogenase have been described (Tubbs, 1960c).

MATERIALS AND METHODS

Abbreviation. DEAE, Diethylaminoethylcellulose.

Water redistilled in Pyrex apparatus was used, and, when possible, AnalAR reagents were used. Solutions of substrates and labile compounds were stored at -10° unless prepared immediately before use. KCN solutions were neutralized with HCl.

Buffers. Tris buffers were prepared from the 'Sigma 121' product (Sigma Chemical Co., St Louis, Mo., U.S.A.) and HCl. Glycylglycine (Roche Products Ltd.) and KH_2PO_4 were brought to the required pH with KOH, as was $\text{Na}_4\text{P}_2\text{O}_7$ with HCl. Measurements of pH were made at room temperature with a Pye glass electrode and meter.

* Present address: Biochemistry Department, Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge.