Notatin: an Anti-bacterial Glucose-aerodehydrogenase from *Penicillium notatum* Westling and *Penicillium resticulosum* sp. nov.

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In studying the anti-bacterial properties developing in cultures of *Penicillium notatum* on the lines already pursued by Fleming (1929), Clutterbuck, Lovell & Raistrick (1932), Abraham, Chain, Fletcher, Florey, Gardner, Heatley & Jennings (1941) and others, progress was at first difficult owing to the erratic results of the bacteriological assays. It was soon found that when culture filtrates from various strains were assayed against *Staphylococcus aureus*, the addition of glucose to the test medium profoundly affected the results. Some cultures produced a substance active in the absence of glucose (penicillin) and showed the characteristics already described in the papers cited above. Other cultures produced a substance active only in the presence of glucose; these cultures showed development of acidity up to about pH 3.5, which was maintained for some time, the culture finally becoming alkaline. The substance responsible for anti-bacterial activity in presence of glucose has been isolated and was originally named 'Penicillin A' (Boots Pure Drug Co. Ltd., Coulthard, Michaelis & Short, 1941), but the name was later changed to 'notatin' to avoid confusion with penicillin. Notatin has formed the subject of brief notes in the literature (Coulthard, Michaelis, Short, Sykes, Skrimshire, Standfast, Birkinshaw & Raistrick, 1942; Birkinshaw & Raistrick, 1943), but no complete account of its production, isolation, properties, nature and mode of action has hitherto been published by us, the details having been reserved for the present communication.

Notatin is isolated from the culture filtrate by evaporation of the culture solution and precipitation with acetone, or by producing a complex with tannic acid from which notatin is regenerated by extracting the tannic acid with acetone (The British Drug Houses Ltd. & Skrimshire, 1942). The crude substance can be purified by regeneration from the complexes which it forms with tannic acid or Reinecke salt.

Notatin is an enzyme of a flavoprotein nature, catalyzing the oxidation of glucose to gluconic acid by means of atmospheric oxygen, with the production of hydrogen peroxide according to the equation:

Notatin is in fact similar in its enzymic properties to the 'glucose oxidase' obtained by Müller (1928, 1929, 1936) from *Aspergillus niger* mycelium and later obtained in a purer form by Franke & Lorenz (1937) and Franke & Deffner (1939); it is, however, much more potent than the preparation of these authors and is believed to represent the same product in a much higher state of purity. Other investigators (Kocholaty, 1942, 1943; Roberts, Cain, Muir, Reithel, Gaby, Van Bruggen, Homan, Katzman, Jones & Doisy, 1943; Van Bruggen, Reithel, Cain, Katzman, Doisy, Muir, Roberts, Gaby, Homan & Jones, 1943; Waksman & Horning, 1943) have also obtained from the culture fluid of *Penicillium notatum* an anti-bacterial substance with the properties of a glucose aerodehydrogenase which is almost certainly identical with notatin.

Van Bruggen *et al.* (1943) call attention to what they consider a point of discrepancy between the properties of their 'Penicillin B' and notatin. Penicillin B was found to promote production of acid and H_2O_2 in presence of *d*-xylose, *d*-mannose and *d*-galactose as well as *d*-glucose, whereas notatin was stated (Coulthard *et al.* 1942) to show anti-bacterial action only in presence of glucose. The latter brief note summarized our observations on the replacement, in the broth substrate employed in the anti-bacterial test, of glucose by various other carbohydrates and related compounds. None showed any capacity to replace glucose with the exception of maltose, which was about one-tenth as effective as glucose. Since, however, maltose is a readily hydrolyzed disaccharide, any hydrolysis during sterilization would in this case produce glucose, which would then show inhibition in the normal manner.

We have examined the enzymic action of notatin on other sugars by measuring the oxygen uptake and acidity produced on shaking in presence of oxygen. Xylose and mannose showed a definite though slow uptake of oxygen, but with maltose, arabinose and galactose no oxygen was absorbed. These results are sufficiently close to the observations of Franke & Deffner (1939) and Van Bruggen *et al.* (1943) to encourage the belief that the oxidase

of Müller, notatin, and penicillin B are identical; a slight difference in purity could account for the observed difference in respect of galactose. With all preparations the rate of attack on glucose is of a much higher order.

Hirsch (1943) observed the secretion of a glucose-oxidizing enzyme by *P. notatum* Fleming (*sic*), and claimed that whilst it bore a general resemblance to notatin it differed in two respects: formation of H_2O_2 in the oxidation of glucose was not detected, and the enzyme was bacteriostatic, not bactericidal. Hirsch worked with dialyzed culture filtrates, his only recorded attempt at isolation of the enzyme giving a water-insoluble product. It is possible that any differences in behaviour between Hirsch's enzyme and notatin are due to the difference in purity of the two products.

That the anti-bacterial action of notatin is due to its aerodehydrogenase activity and in particular to the production of hydrogen peroxide is evident from the following facts: (1) similar conditions are required for anti-bacterial activity and for aerodehydrogenase action; (2) in presence of catalase, which destroys hydrogen peroxide, notatin has no anti-bacterial activity.

Since the actual anti-bacterial agent in notatin activity is hydrogen peroxide it follows that any flavoprotein enzyme system which gives rise to hydrogen peroxide should show the same anti-bacterial action. This has been demonstrated by Green & Pauli (1943) for the flavoprotein of milk, which catalyzes the oxidation of hypoxanthine to uric acid. They conclude that there is little to choose between the flavoproteins from cow's milk and *P. notatum* respectively as far as anti-bacterial action is concerned, the sole difference being that notatin is a somewhat more stable enzyme.

Birkinshaw, Raistrick & Smith (1942) noted the anti-bacterial activity of the culture fluid from *P. resticulosum*. In examining the metabolic products of this mould they isolated a new mould metabolic product, fumaryl-*DL*-alanine, which had no anti-bacterial activity, and a substance with high anti-bacterial titre which was precipitated by acetone from the culture solution in a crude form. The nature of this substance was not determined. As the properties of notatin showed certain similarities to those of this earlier product the *P. resticulosum* active substance was further examined and it was indeed found to act as a glucose-aerodehydrogenase. It could be isolated in more active form than hitherto by the tannic acid precipitation as developed for notatin, although the yield was so small that no attempt was made to purify to the same degree as notatin. The evidence is sufficient, however, to indicate that it is in all probability identical with notatin.

EXPERIMENTAL

PRODUCTION OF NOTATIN

Organism. The most satisfactory culture of *Penicillium notatum* Westling for the production of notatin was derived from a strain supplied by Sir Alexander Fleming. It has been maintained on a liquid medium and subcultured at 10–20-day intervals, single-spore isolations being carried out at intervals.

Medium and method of culture. In one laboratory the Czapek-Dox medium (A) was employed: $NaNO_3$, 2.0 g.; $MgSO_4 \cdot 7H_2O$, 0.5 g.; $FeSO_4 \cdot 7H_2O$, 0.01 g.; KH_2PO_4 , 1.0 g.; KCl, 0.5 g.; glucose monohydrate, 40.0 g.; tap water, 1 l. This was filled (in 500 ml. amounts) into 40 oz. white flint-glass panelled bottles, plugged with cotton-wool and sterilized by steam at 115°. The final pH value is 6.0–6.5. The culture filtrate from medium A has a pH value of about 3.5 and is inhibitory for *Staph. aureus* up to a titre of 70,000.

Throughout this paper the anti-bacterial activity or titre of solutions or solid products expresses the number of ml. to which 1 ml. of the solution must be diluted or in which 1 g. of solid must be dissolved to attain the limiting active dilution, i.e. the maximum dilution completely inhibiting growth of *Staph. aureus* in the standard glucose-broth medium when incubated for 18 hr. at 37° (see 'Assay of notatin', p. 30).

Kept in a cool place, free from mycelium, and saturated with chloroform, culture filtrates retain activity for periods of at least 3 months and even at times appear to increase in potency.

In another laboratory the Czapek-Dox medium was modified by the addition of sodium acetate (+3H₂O), 1.0 g.; trisodium citrate (+3H₂O), 1.0 g.; and $CuSO_4 \cdot 5H_2O$, 0.0175 g./l. (medium B). For the composition of this medium we are indebted to Dr C. G. Pope of the Wellcome Physiological Research Laboratories (private communication). In medium B adjusted to pH 7.8 before sterilization by autoclaving at 10 lb. for 30 min. or by steaming at 100° for 45 min., notatin was produced regularly to an anti-bacterial titre of 10,000–25,000.

After the addition of a small volume of an aqueous suspension of the culture, the bottles were inclined on racks to give a shallow liquid layer with maximum surface, and incubated at 20–22°, although good yields have been obtained at 12–15° and sometimes at higher temperature. The liquor was poured from the mycelium and filtered lightly through cloth. A little chloroform was added as preservative and the solution treated as described in the section on isolation.

Table 1. Effect of age of culture on anti-bacterial titre and pH

(Cultures grown on medium A; initial content of glucose monohydrate = 4 %.)

Age of culture (days)	Glucose content (%)	Anti-bacterial titre	pH
9	1.85	15,000	—
11	1.11	40,000	—
14	0.63	70,000	—
11	—	>20,000	3.5
16	—	>20,000	3.5
22	—	>20,000	>8
30	—	< 1,000	>8

Growth becomes visible in the first 2-3 days, when the pH falls to about 3.5 where it remains throughout the normal incubation period of about 14 days. During this period the anti-bacterial titre of the culture solution rises steadily as the glucose content falls; beyond this time the yield of notatin decreases and the pH value tends to become alkaline, penicillin rather than notatin being produced (Table 1).

Observations on medium and strains employed

Medium. As the inoculated medium B became acid after a few days' incubation it seemed that there was no point in starting at a pH as high as 7.8. Trials with the unadjusted medium at an initial pH of about 6.0 and inoculated with the same spore suspension as controls on the standard medium B showed, however, that there was a definite alkaline swing after a short acid phase and penicillin was produced, with only traces of notatin. In this way it was found possible to produce at will penicillin or notatin from the same inoculum by merely changing the pH before sterilization (Table 2).

Thus the condition of the glucose has no effect on notatin production; further, the amount of heating is not the determining factor, since batches of medium at pH 7.8, (a) steamed at 100° for 30 min., (b) steamed three times on successive days, (c) autoclaved once at 10 lb. for 30 min., (d) autoclaved twice, (e) filtered sterile through a Seitz filter, all gave similar results in notatin production.

No satisfactory substitute was found for glucose in the medium; mannose, malt extract, lactose, levulose and brown sugar did not yield a trace of notatin. Notatin up to a titre of 100 or less was obtained from sodium glutamate (0.2%), sodium maleate (0.2%), but not from sodium tartrate, fumarate, gluconate, or malate. A normal growth of mould was obtained and penicillin was also produced from all these substrates. The variations described above were not observed in medium A.

Necessity for acid swing of medium, for production of notatin. Notatin is normally produced during the acid phase of the culture when the pH is between 3.5 and 4.0, usually between the seventh and fourteenth day of incubation. On nine occasions in

Table 2. *Production of notatin or penicillin as influenced by initial pH*

Culture	Initial pH	Cultures in medium B (autoclaved)					
		Notatin production			Penicillin production		
		Time (days)	Titre	Final pH	Time (days)	Titre	Final pH
G 592 22/4	5.9	7	0	—	20	1000	7.67
	7.3	7	20,000	3.88	—	—	—
G 592 6/5	6.0	11	0	—	14	1000	6.86
	7.8	11	20,000	4.12	—	—	—

Further investigation showed that, in order to get satisfactory and consistent yields of notatin in medium B, the medium should be made up adding the glucose first and the trace metals last, after the phosphate, the order of addition being: glucose, NaNO_3 , KCl, Na citrate, Na acetate, KH_2PO_4 , MgSO_4 , CuSO_4 , FeSO_4 . Other orders of make-up produced satisfactory quantities of penicillin but did not produce notatin at pH 7.8 or 6.5.

It was considered that the marked decomposition which the glucose undergoes when autoclaved at the higher pH was probably sufficient to account for the great difference between the acid and alkaline media. Thus in one experiment the glucose before sterilization was 4.03%—after sterilization at pH 7.8 the determined value was 3.96 by Cu reduction and 2.68 by rotation, whereas with an initial pH of 5.83 the values after sterilization were 4.17 and 4.05% respectively. However, when glucose, sterilized by filtration, was added to the salt solutions, made up to pH 7.8 and 6.5 respectively, the previous results were duplicated.

192 batches on medium B, notatin was being produced at quite alkaline pH values and sometimes at the same time as penicillin. Notatin was never obtained, however, unless there had been a marked acid phase, although production might not start until this phase was over. Substances that tended to suppress the acid swing prevented the formation of more than traces of notatin; this effect was seen if the content of sodium acetate or sodium citrate was increased above 1 g./l.

Influence of copper, iron and zinc on the production of notatin. In order to examine the separate and combined effects of these trace metals on the production of notatin an experiment was carried out using the basal medium B to which known amounts of salts of the trace metals were added after sterilization. The control medium contained 1.0 p.p.m. of Fe, 0.1-0.2 p.p.m. of Cu and no demonstrable zinc. This medium was divided into six portions after sterilization, and the following additions made: (a) nil; control, (b) 2 p.p.m. of Fe, (c) 4 p.p.m. of Cu, (d) 1 p.p.m. of Zn, (e) 4 p.p.m. of Cu and 2 p.p.m.

of Fe, (f) 4 p.p.m. of Cu, 2 p.p.m. of Fe and 1 p.p.m. of Zn. Growth of *P. notatum* was normal in all bottles but notatin was only produced in quantity in the control series and in those bottles to which Fe (b) and Cu and Fe (e) had been added; the bottles with Cu only (c) produced a low notatin titre and no bottle with Zn added produced any notatin. The bottles with Zn also failed to show the usual acid swing, the lowest pH attained being 5.57 on the fourth day, whereas the others were normal in this respect.

Comparison of strains; production of penicillin after notatin

Forty-six subcultures derived from various sources have been investigated for notatin production on medium B. Some of these no doubt represent different strains of *P. notatum* but many were in fact the same strains which have been cultured in different laboratories for varying lengths of time. Of these 46 cultures, only five failed to produce notatin to a titre of at least 100 when the initial pH was 7.8; there were 13 failures at an initial pH of 6.0.

With four exceptions, which may not have been incubated long enough, all the notatin-producing strains investigated eventually produced penicillin in bottles in which notatin had been produced, though this might take a considerable time. Certain bottles started at an alkaline pH produced demonstrable penicillin during the first 3 or 4 days, provided the pH had not fallen too far; the production of notatin was not in any way affected by this and was no greater or no less than in bottles in which this had not occurred. There appears to be no direct correlation between notatin production and penicillin production and it is not necessary for the culture to produce notatin before penicillin or vice versa.

Variation of culture in notatin-producing power

Successive generations of a culture might vary in the type of anti-bacterial substance produced. Thus a culture derived from one supplied by Sir Alexander Fleming, grown in medium A at 26°, gave after 6-7 days a culture filtrate of notatin activity >20,000 for the first, 15,000 for the second, and <1000 for the third generation, whereas the penicillin activity increased in the same sequence. Attempts to discover morphological or cultural characteristics which would permit the selection of the most suitable culture for our purpose were unsuccessful, although it was observed that cultures producing penicillin tended to liquefy gelatin more rapidly. The mould in the notatin-producing cultures often failed to spore freely in liquid or on solid medium and might appear almost white to light grey-green. In liquid medium it often grew in a

half-submerged waterlogged state. Cultures grew well at temperatures between 10° and 30° but not at 37°. Variant colonies sometimes appeared, often characterized by non-production of notatin or penicillin.

By careful maintenance of a notatin-producing strain in the same medium from generation to generation, with subculture at appropriate short intervals, the anti-bacterial titre of the culture filtrate was increased considerably, in some cases up to 70,000. The culture fluids varied from light brownish yellow to almost colourless but this did not appear to correlate with the activity.

ISOLATION

150 l. of culture filtrate, having an anti-bacterial titre of 30,000-70,000, are concentrated to one-fifth of their original volume *in vacuo*, care being taken that the internal temperature does not rise above 25°. After concentration the activity sometimes rises and may increase by as much as 400% (cf. glucose aerodehydrogenase, Franke & Deffner, 1939). The reddish-brown concentrate (pH 3.1-3.5) is then mixed with twice its volume of acetone at 0° and allowed to stand at 0° overnight. The supernatant solution is decanted from the precipitate, which is usually sticky. The precipitate is dissolved in 5 l. of water, and the notatin is precipitated by adding an equal volume of acetone with cooling by ice. The product is dissolved in 2.5 l. of water and precipitated with 2.5 l. of acetone in the same way. At this stage a solid can be obtained by triturating the sticky, or sometimes oily, precipitate with acetone and drying in a vacuum desiccator, yielding a product of activity 40-150 × 10⁶. The recovery of anti-bacterial activity was 30-50%, calculated on the original culture filtrate. However, the solid is not usually isolated at this stage, the crude precipitate being dissolved in 200 ml. of cold water and spun clear or almost clear in a Sharples centrifuge, giving an orange-red concentrate (pH c. 5) which is worked up by one of the following methods.

(a) *Precipitation with tannic acid.* The concentrate is diluted with cold water to 700 ml. and the notatin precipitated at 0° with a saturated aqueous solution of 8 g. of tannic acid (B.P.). After standing for ½ hr. at 0°, the precipitate, which is usually purple in colour, is spun on a Sharples centrifuge. The notatin-tannate precipitate is decomposed by triturating with two or three 50 ml. portions of acetone and centrifuged. The crude notatin (still purple in colour) is then extracted successively at the centrifuge with 40, 35 and, if necessary, 20 ml. portions of water. The combined aqueous extracts are precipitated at 0° with an equal volume of acetone. The yellow precipitate is collected, triturated three times with acetone, and dried for a short time in air and then *in vacuo* at 5 mm. over absorbent charcoal and calcium chloride. The solid is powdered and stored at 0° in sealed nitrogen-filled ampoules. Yield, 3-4 g. of activity, 3-4 × 10⁶; recovery 20-40%, calculated on the assay of the original culture filtrate.

(b) *Precipitation of notatin with Reinecke salt.* 100 ml. of the above concentrate are mixed with a solution of 2 g. of Reinecke salt in 90 ml. of water. The solution is cooled in ice and acidified to pH 3.5-4 with N-acetic acid. The pinkish precipitate is collected at the centrifuge and the

red supernatant liquid decanted and rejected. The residual solid is suspended in 20–30 ml. of water and the turbid solution spun clear. Notatin is precipitated from the supernatant solution by adding 3 vol. of acetone at 0° and the resulting precipitate worked up as described above. The yield and the potency are the same as those obtained by the tannic acid process.

Purification of notatin

(a) *By reprecipitation with acetone.* Although notatin as prepared according to the above process was of high potency in some batches, further purification was necessary in others. Several batches of solid were combined and extracted with water (10–20 ml./g.), and the brownish solution was precipitated with an equal volume of acetone. Recovered activity, c. 90%.

(b) *By ammonium sulphate fractionation.* A 2.5% notatin solution in water or phosphate buffer (pH 7), prepared from a solid of titre 290×10^6 , was brought to half saturation with saturated ammonium sulphate solution, which removes a little inactive protein. Notatin was then precipitated by bringing the concentration of ammonium sulphate to within 80–83% of saturation. The canary yellow precipitate was dissolved in water, dialyzed for 56 hr. against distilled water in the refrigerator, and the notatin precipitated with acetone at 0°. The extinction ratio $E_{280}/E_{460} m\mu$, which is a measure of the amount of protein impurity in a preparation of flavoprotein (Corran, Dewan, Gordon & Green, 1939), was 8.6 and the activity of the solid 3.5×10^6 .

Remarks on the above processes. Purification of notatin by adsorption on C_γ aluminium hydroxide (Willstätter & Kraut, 1923) or on tricalcium phosphate gel, or adsorption of impurities (leaving notatin in solution), does not result in any considerable increase in the potency of notatin of good initial potency, nor does it remove catalase completely. On the other hand, adsorption with either gel can be used with advantage for the isolation of notatin, in place of precipitation with tannic acid or Reinecke salt. Notatin is easily adsorbed on these gels at pH 3–3.5 and is eluted with a pH 6–7 phosphate buffer or dilute ammonium sulphate solution.

Notatin forms a chromatogram on aluminium oxide (Savory & Moore Ltd.) which has previously been boiled twice for 3.5 hr. with hydrochloric acid (1 vol. conc. acid + 1 vol. water). A solution of notatin in sodium phosphate—citric acid at pH 3 (McIlvaine's buffer) when passed through the column gives a broad buff-coloured band, from which notatin can be eluted with phosphate buffer solution at pH 5. Starting with material having an activity of 250×10^6 , the recovered notatin had an activity of 350×10^6 ; recovery 20–35%.

Dialysis of the concentrate (against distilled water) before precipitation with tannic acid or Reinecke salt was not usually carried out because facilities for conducting the operation at 0° were not available and at room temperature the prosthetic group tends to dialyze with consequent loss

of potency. On the other hand, dialysis at 0° for 17 hr. and subsequent precipitation of the dialysate with tannic acid results in a purification of notatin. Thus, the activity of one preparation was raised from 290×10^6 to 400×10^6 ; recovery 55%.

The mother liquors from the first acetone precipitation yield a slightly coloured amorphous precipitate on standing, which, on reprecipitation from water by acetone, yields white crystals, having no anti-bacterial activity and probably consisting of a polysaccharide. There is no absorption of oxygen when an aqueous solution containing this sugar and notatin is shaken in an atmosphere of oxygen.

PROPERTIES OF NOTATIN

Physical and chemical

Notatin having an activity of $300\text{--}400 \times 10^6$ is buff to yellow in colour. The solid is stable for at least 8 months and usually for 2 years when stored at 0° in ampoules filled with nitrogen or air. It is freely soluble in water, concentrated solutions which froth on shaking being brownish green, and dilute solutions yellowish green. Notatin will not pass through a nitro-cellulose membrane as used by Seibert (1934) for the purification of tuberculin, and concentration and purification of notatin solutions may be effected by ultra-filtration.

Various samples of notatin gave the following figures on analysis, for which we are indebted to Dr Sidney Smith of the Wellcome Foundation: C, 50.0–52.5; H, 6.7–7.2; N, 11.8–14.2; P, 0.17–0.58%; the results being corrected for moisture and ash. Specimens containing less than 1.4% of ash have been obtained. Notatin usually contains catalase but catalase-free preparations are occasionally obtained (qualitative test) and it therefore appears unlikely that catalase forms a compound with notatin (cf. Gordon, Green & Subrahmanyam, 1940). Some attempts to remove catalase by simple fractionation with ammonium sulphate or by adsorption were not successful but the operations were not systematically repeated. Aqueous solutions of notatin have pH 6–7 and a single specimen examined had a small *laevo* rotation: $[\alpha]_D^{22}, -4.8^\circ$ ($c = 0.0123$).

The anti-bacterial activity is rapidly destroyed at pH less than 2 and above 8 or on heating to 60°. Addition of dilute acetic acid to notatin solution causes a greenish fluorescence. When sodium hydro-sulphite is added to an aqueous solution of notatin which has been made alkaline with sodium carbonate, the colour is discharged but is completely restored by shaking in air.

The absorption spectrum in aqueous solution exhibits maxima at 270/280, 375/380 and 450/460 $m\mu$, which characterize it as a 'yellow enzyme'. In contrast to the typical flavoproteins, which in the visible region have a main peak at 450/460 $m\mu$,

notatin has its main peak in this region at 380m μ . In this respect it shows some resemblance to the liver aldehyde oxidase (Gordon *et al.* 1940), the xanthine-aldehyde-dihydrocoenzyme I oxidase of

On the assumption that notatin has a molecular weight of 73,000, which is that of the majority of the flavoprotein enzymes, and contains one flavin unit per molecule, it can be calculated from the extinction

Fig. 1.

Fig. 2.

Fig. 1. Adsorption spectrum of notatin purified to the stage where $E_{1\text{ cm}}^{1\%}$, 450/460m μ = 1.3 (see Table 3).

Fig. 2. Adsorption spectrum, in the near ultra-violet and visible region, of notatin purified to the stage where $E_{1\text{ cm}}^{1\%}$, 450/460m μ = 1.3 (see Table 3).

The absorption spectra were measured by Dr Elvidge and Mr Voss.

milk (Corran & Green, 1938), and a flavoprotein obtained from yeast (Green, Knox & Stumpf, 1941). The extinction coefficient of the absorption band at 450/460m μ runs parallel with the anti-bacterial activity (Table 3).

Table 3. Relation between extinction coefficient and anti-bacterial titre of notatin

Sample	Anti-bacterial titre	$E_{1\text{ cm}}^{1\%}$			Ratio $E_{280} : E_{460}$
		450/460 m μ	375/380 m μ	270/280 m μ	
A 38	400×10^6	1.61	1.98	14.4	8.9
405-409	400×10^6	1.45	1.84	15.6	10.7
A 170	350×10^6	1.30	1.68	11.24	8.6
162-167	350×10^6	1.30	1.60	19.0	14.6
233-237	330×10^6	1.18	1.42	12.4	10.5
A 14	320×10^6	1.15	1.30	50.0	43.0
168-72	310×10^6	1.08	1.38	15.6	15.0
A 24	250×10^6	1.05	1.35	22.0	21.0
136-138	100×10^6	1.00	1.20	16.0	16.0
A 61	50×10^6	0.93	1.35	17.0	18.2

coefficient of the absorption band at 450/460m μ ($E_{1\text{ cm}}^{1\%} = 1.54$; this figure is based on the measurements of Warburg & Christian (1938), for riboflavin and alloxazine-adenine dinucleotide) that the purity of our best preparations is about 90% ($E = 1.45 \times 100/1.54$). It is also assumed that no other coloured group, in addition to flavin, participates in the peak at 450/460m μ .

Stability and anti-bacterial activity

Solid notatin possesses very high anti-bacterial activity when tested by *in vitro* methods. The purest samples usually have an activity of 400,000,000, several have given a titre of 1,000,000,000. Samples of the solid material have retained very high potency when kept in sealed containers for periods up to 8 months, although after keeping for a few weeks it has not been found possible to demonstrate activity greater than 400,000,000.

Table 4. *Effect of pH and dilution fluid on anti-bacterial activity of notatin*

pH	Length of exposure at given pH																							
	0 hr.								4 hr.								24 hr.							
	Dilution of notatin (in millions)								Dilution of notatin (in millions)								Dilution of notatin (in millions)							
	10	20	50	100	200	500	1000	10	20	50	100	200	500	1000	10	20	50	100	200	500	1000			
In buffer:	Effect on growth of <i>Staph. aureus</i>																							
2.0	-	-	-	-	-	x	x	-	-	-	-	-	?	x	-	-	-	-	-	x	x	x		
2.5	.	.	.	.	.	.	.	-	-	-	-	-	?	x	-	-	-	-	-	x	x	x		
3.3	.	.	.	.	.	.	.	-	-	-	-	-	?	x	-	-	-	-	-	x	x	x		
5.0	-	-	-	-	-	x	x	-	-	-	-	-	x	x	-	-	-	-	-	x	x	x		
7.0	.	.	.	.	.	.	.	-	-	-	-	-	x	x	-	-	-	-	-	x	x	x		
7.5	.	.	.	.	.	.	.	-	-	-	?	x	x	x	-	-	?	x	x	x	x	x		
8.0	-	-	-	-	x	x	x	-	-	?	x	x	x	x	x	x	x	x	x	x	x	x		
In broth:																								
2.1	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		
2.4	.	.	.	.	.	.	.	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		
2.6	.	.	.	.	.	.	.	-	-	x	x	x	x	x	x	x	x	x	x	x	x	x		
3.0	-	-	-	-	-	x	x	-	-	-	-	-	?	x	-	-	-	-	-	-	?	x		
5.0	.	.	.	.	.	.	.	-	-	-	-	-	x	x	-	-	-	-	-	-	?	x		
6.9	.	.	.	.	.	.	.	-	-	-	-	-	x	x	-	-	-	-	-	-	?	x		
7.5	.	.	.	.	.	.	.	-	-	-	-	-	x	x	-	-	-	-	-	-	x	x		
8.0	-	-	-	-	?	x	x	-	-	-	-	x	x	x	-	-	-	x	x	x	x	x		
	- = no growth.								? = slight growth.								x = growth.							

- = no growth.

? = slight growth.

x = growth.

Sample 162-7 was reported, on 12 June 1942, as having a titre of 10^6 ; tests in July and at the beginning of August confirmed this potency, but later in August a series of figures of 400×10^6 or 300×10^6 was obtained. Sample A38 gave in August an activity of 1000×10^6 and a similar value in September. Fourteen further tests carried out at intervals from 16 October to 18 March following gave in one test a value of 700×10^6 , in the other 13, about 400×10^6 .

Solutions of notatin (1 mg./ml. in chloroform-water) often do not exert maximum activity until they have been prepared for a few hours; they have been stored in the refrigerator for up to 40 days without change.

Effect of pH. Solutions of notatin possess maximum stability at pH values between about 3.5 and 6.0. The stability is dependent on the nature of the solvent; in glucose broth, solutions are quite unstable at pH 2-2.5, whereas in a phosphate-acetate-borate universal buffer they show little signs of inactivation. On the other hand, glucose-broth solutions are quite stable up to pH 7.5, whereas in some buffer solutions there may be a decrease in activity at pH 7.0. In several experiments, culture fluids allowed to become faintly alkaline have retained their activity for at least several days. The effect of pH on stability at normal room temperature is illustrated in Table 4. It has not been found possible to regenerate activity by simple readjustment of pH value.

Action of enzymes. Pepsin (at pH 2.5) and trypsin (at pH 7.5), when in contact with notatin for periods up to 8 hr., do not appear to affect its activity.

Assay of notatin. The standard method employed for the anti-bacterial assay of notatin depends on

the determination of the inhibitive effect against *Staph. aureus* in glucose broth. Inhibition tests were carried out in tryptic digest broth or Hartley broth containing 0.5% of glucose. Serial dilutions of notatin were made in 10 ml. volumes contained in 5 or 6 in. $\times \frac{3}{4}$ in. tubes, sown with a small number of test organisms from a 24 hr. culture, the result being read after 18 hr. incubation at 37°. The potency of a sample is assessed according to the dilution which will just prevent the growth of bacteria, growth being detected by production of turbidity in the medium.

In some cases lethal tests were also carried out in various media in a similar way. Small volumes were plated at intervals and the number of viable organisms counted. The two methods of assay gave comparable results (Table 5).

Table 5. *Comparison of visual growth test with bacterial counts. Viable S. aureus after treatment with notatin in glucose broth*

Time of exposure to notatin (hr.)	Notatin dilutions (in millions)				
	100	200	300	500	700
	Relative numbers of organisms surviving				
0	x	x	x	.	x
2	x	x	x	x	x
6	75	100	x x	x x x x	x x x x
18	0	0	∞	∞	∞
Visual broth reading at 18 hr.	No growth	No growth	Slight growth	Growth	Growth

x = 200-300 organisms.

x x, x x x x and ∞ = progressively larger numbers.

These tests of lethal effect are of interest from another aspect; they prove definitely that the anti-bacterial action of notatin is bactericidal rather than bacteriostatic.

Glucose concentration in assay. In the absence of glucose pure notatin shows no activity against the test organism or only a very slight one, attributable to the small amounts of 'glucose' found in the normal digest broth, which may be as much as 0.05 %. The apparent activity was found to increase with increasing amounts of added glucose up to a maximum of 0.5 %, beyond which no further change in activity was shown. The figure of 0.5 % glucose was for this reason adopted in the standard method of assay. Several other carbohydrates and polyhydric alcohols have been tried in place of glucose in the test medium, namely sucrose, lactose, maltose, galactose, mannose, arabinose, mannitol and glycerol. None showed any capacity to replace glucose with the exception of maltose, which was about one-tenth as effective as glucose.

Assay of notatin and penicillin occurring together. In some of the work described penicillin and notatin were separately assayed in solutions in which both were present. The solution was in this case extracted with ether, the ethereal extract being shaken with barium carbonate suspension. The ether-extracted aqueous solution and the barium carbonate extract were then tested separately, the anti-bacterial titre of the two solutions giving the notatin and penicillin content respectively.

Activity of notatin against other organisms. Notatin inhibits growth of a number of bacteria including both Gram-positive and Gram-negative species. In Table 6 the figures give the highest dilution of notatin producing total inhibition of growth from a series of tests with each organism. At least two inoculating doses were used. With staphylococci the second dose was 100 times the first, with other organisms the second dose was 10 times the first; there was no difference in end-point between the two doses. All tests were carried out in Hartley glucose-broth incubated at 37° for 17–18 hr.

Enzymic nature of notatin—identification as a glucose aerodehydrogenase

Preliminary experiments on the effect of incubating notatin with glucose indicated that under aerobic conditions acid was produced (shown by titration and pH determination) and that the optical rotation of the solution sank to a low value, whereas anaerobically (in a closed polarimeter tube) the same solution showed no change in rotation after 24 hr., and no production of acid. This suggested that notatin was an enzyme capable of catalyzing the oxidation of glucose by oxygen.

In order to test this hypothesis quantitative experiments were performed at room temperature in

Table 6. *Anti-bacterial action of notatin against various micro-organisms*

Organism	Size of inoculum. No. of organisms/ ml. of broth in tube under test		Highest dilution of notatin giving complete inhibition of growth
	(i)	(ii)	
<i>Staph. aureus</i> , 1	10	1000	10 ⁸
<i>Staph. aureus</i> , 2	10	1000	10 ⁸
<i>Strep. haemolyticus</i> , 1	—	5000	10 ⁹
<i>Strep. haemolyticus</i> , 2	—	5000	10 ⁷
<i>Strep. pneumoniae</i>	—	5000	10 ⁸
<i>Esch. coli</i>	100	1000	10 ⁶
<i>Shigella paradysenteriae</i> Flexner	40	400	10 ⁸
<i>Shigella sonnei</i>	80	800	10 ⁶
<i>Ebert. typhosa</i> , 1	40	400	10 ⁷
<i>Ebert. typhosa</i> , 2	40	400	10 ⁷
<i>Ebert. typhosa</i> , 3	40	400	10 ⁹
<i>Ebert. typhosa</i> , Vi, 4	40	400	10 ⁷
<i>Ebert. typhosa</i> , Vi, 5	40	400	10 ⁷
<i>Ebert. typhosa</i> , Vi, 6	40	400	10 ⁷
<i>Bact. paratyphosum</i> A	100	1000	10 ⁸
<i>Bact. paratyphosum</i> B	100	1000	10 ⁷
<i>Bact. typhi murium</i>	30	300	10 ⁷
<i>Bact. enteritidis</i> Gaertner	100	1000	10 ⁷
<i>Proteus vulgaris</i> , 1	50	500	10 ⁸
<i>Proteus vulgaris</i> , 2	50	500	10 ⁹
<i>Proteus vulgaris</i> , 3	50	500	10 ⁸
<i>V. comma</i> (<i>V. cholerae</i>)	50	500	10 ⁶ –10 ⁸
<i>B. anthracis</i>	20	200	10 ⁷
<i>Brucella abortus</i>	—	—	10 ⁷
<i>Klebsiella pneumoniae</i>	—	—	10 ⁶ –10 ⁸
<i>Salmonella cholerae suis</i>	—	—	10 ⁴ –10 ⁵

a catalytic hydrogenation apparatus with facilities for constant shaking, the usual hydrogen atmosphere being replaced by one of air or oxygen. With dilute solutions of glucose containing notatin it was found that 1 mol. of glucose rapidly takes up one atom of oxygen and produces one equivalent of acid. With more concentrated glucose solutions but with no increase in the amount of notatin the oxygen uptake was greater than one atom but this extra oxygen was found to be present in the oxidized solution as hydrogen peroxide.

Uptake of oxygen by notatin in presence of dilute glucose. The solution, containing 0.2 g. glucose and 0.02 g. notatin in 100 ml. of water, was shaken in presence of air in the catalytic hydrogenation apparatus at room temperature (19°) and the uptake of oxygen was measured. This was apparently complete in 166 min., the total uptake being 12.7 ml. (corr. N.T.P.). The pH of the solution was now 3.5, and the power to reduce Benedict qualitative reagent was lost. Titration to permanent end-point (phenol phthalein) required 11.10 ml. of 0.1N-NaOH. The rotation of the solution in a 4 dm. tube (Hg green) was +0.09°. Oxidation of 1 mol. of glucose to 1 mol. of monobasic acid with uptake of 1 atom of oxygen requires: titratable acidity = 11.10 ml.; uptake of oxygen = 12.4 ml. The end-point is reached more quickly in presence of a buffer. When the above was repeated in presence of 1 % of sodium acetate the oxidation was complete in 45 min. Titratable acid 11.2 ml. (theoretical

11.1 ml.)—final pH 5.8. Oxygen uptake 12.4 ml. (i.e. theoretical). Final rotation in 4 dm. tube $\alpha_{5461}=0.13^\circ$. (No reduction of Benedict.) A repetition in presence of pure oxygen gave similar results.

Uptake of oxygen with stronger glucose solutions. Production of H_2O_2 . A solution of 5 g. glucose, 0.02 g. notatin, 5 g. sodium acetate, in water to 100 ml., was shaken in oxygen at room temperature.

The oxygen uptake in 125 min. was 449 ml. (at N.T.P.). Theoretical for glucose present = 311 ml. Excess of actual over theoretical uptake = 138 ml. The acid produced was 27.4 ml. N by titration. Theoretical for 1 equivalent = 27.8 ml. The final pH was 4.8.

A qualitative test with acid titanium sulphate indicated the presence of hydrogen peroxide. 5 ml. of the oxidized solution were treated with 5 ml. 2N- H_2SO_4 and excess KI and the iodine was titrated after half an hour. Used 12.15 ml. 0.1N- Na_2SO_3 , or 24.3 ml. N for 100 ml., which is equivalent to 135 ml. of oxygen (cf. excess O_2 uptake of 138 ml.).

Identification of gluconic acid. The acid formed from glucose by oxidation in presence of notatin was isolated as the Ca salt, which was shown to be Ca gluconate by calcium determination (Ca on sulphated ash, 9.36%; (calcium gluconate requires 9.30%), and by preparation of the phenylhydrazide of the acid, m.p. 201–202° (decomp.); no depression of m.p. when mixed with an authentic sample of gluconic acid phenylhydrazide also of m.p. 201–202° (decomp.).

Glucose is therefore oxidized by gaseous oxygen in presence of notatin in accordance with the equation

The fact that no hydrogen peroxide was detected in oxidation of glucose at low concentration was no doubt due to the presence of catalase in the sample of notatin used: owing to the relatively high ratio of notatin to glucose in this case the amount of catalase present was sufficient to decompose the hydrogen peroxide completely.

Estimation of oxygen coefficient of notatin. As it had now become evident that notatin was very similar to, although much more potent than, the glucose aerodehydrogenase examined by Franke & Deffner (1939), an attempt was made to determine the oxygen coefficient Q_{O_2} ($=\mu l. O_2/mg. enzyme \times hours$) on the 'optimal substrate' of Franke & Deffner.

A solution consisting of 0.1 mg. of notatin in 1 ml. of water, 2 ml. of 0.2M-phosphate buffer (pH 6.0) and 2 ml. of 2M-glucose was shaken for 1 hr. at 15° in presence of oxygen. The corrected oxygen uptake was 5.84 ml., the H_2O_2 corresponded with 1.75 ml. of 0.1N-thiosulphate ($\equiv 0.98$ ml. extra oxygen). Deducting this extra oxygen, $Q_{O_2}=48,600$ at 15°, or applying a temperature coefficient calculated from the graph on p. 8 of the paper of Franke & Lorenz (1937), Q_{O_2} at 30° would be of the order of 120,000. The activity obtained by Franke & Lorenz for their most active preparation was 8000 units (at 30°); on the basis of the flavin content of their enzyme they calculate that the pure enzyme would have an activity of 240,000 units.

Sugars other than glucose as substrates

In order to determine whether other sugars could act as hydrogen donors in presence of notatin,

1 g. of the sugar (used as purchased from The British Drug Houses Ltd.) and 20 mg. of notatin (activity 4×10^8) were dissolved in 40 ml. of phosphate buffer of pH 7.1. The solution was shaken in an atmosphere of oxygen at atmospheric pressure and temperature (c. 23°). In every case the activity of the notatin was confirmed after the test by addition of glucose, when the oxygen uptake was always approximately theoretical. The results are shown in Table 7.

Table 7. *Replacement of glucose by other sugars in the enzymic reaction*

Sugar	Time shaken in oxygen with sugar (hr.)	pH	Vol. of O_2 absorbed at N.T.P. (ml.)	Vol. of O_2 at N.T.P. equiv. to 1 atom/mol. of sugar (ml.)
d-Glucose	1	6.8	58	62
d-Xylose	12	6.6	72	75
d-Mannose	3	6.8	32*	62
Maltose	2	7.1	0	31
l-Arabinose	3	7.1	0	75
d-Galactose	4.5	7.1	0	62

* Absorption ceased after 3 hr.

Evidently d-xylose and d-mannose can act as hydrogen donors in the reaction catalyzed by notatin, whereas maltose, l-arabinose and d-galactose cannot act in this manner. Even with d-xylose and d-mannose, however, the rate of reaction is much slower and is in fact of quite a different order from that observed with d-glucose.

Nature of prosthetic group in notatin. Resolution into flavin and protein

Lumino-flavin test (cf. Franke & Deffner, 1939). Notatin (50 mg.) dissolved in N-NaOH (50 ml.) was exposed to light from 100-watt lamp for 90 min. in a test-tube cooled with running water. The solution was acidified with 2N-HCl and extracted with 10 ml. of $CHCl_3$. The solution had strong greenish-yellow fluorescence, visible in daylight, stronger in ultra-violet, indicating the presence of flavin in notatin.

Fission. Solutions of the non-protein part of the notatin molecule have been prepared by three methods. (1) By acidification to pH 1.8. (2) By treatment with trichloroacetic acid as described by Haas (1938). (3) By heating an aqueous solution of notatin at 85–90° for 10 min.

The solutions obtained by methods (1) and (2) were subjected to chromatographic analysis on a column of aluminium oxide prepared by digesting aluminium oxide (Savory & Moore Ltd.) twice with cold concentrated hydrochloric acid for 5 min. and washing with water until the suspension remained just acid to Congo red. A canary-yellow ring was produced at the head of the column and on washing with N/30-HCl a second zone (purple) was developed when the solution was prepared by method (2), but not in the case of solution prepared by method (1). The zones were eluted by phosphate buffer (pH 7), but the purple zone afforded a solution showing weak general ab-

sorption, too weak for accurate measurement. The solution obtained from the yellow zone, however, showed strong absorption. In the case of the solution obtained by method (3) the absorption spectrum was measured without further purification. The molecular extinction coefficients shown in Table 8 are calculated on the assumption that the molecular extinction coefficient at $450\text{ m}\mu$ is $1.13 \times 10^7 \text{ cm}^2 \text{ mol}^{-1}$.

Table 8. *Molecular extinction coefficients of solutions of the flavin moiety prepared by three methods*

$\text{m}\mu$	Solu- tion 1	Solu- tion 2	Solu- tion 3	Ribo- flavin	Flavin- adenine dinucleo- tide*
260	4.57	4.46	7.5	2.56	3.7
375	1.21	0.96	1.35	0.98	0.89
	1.13	1.06	1.42	—	—
450	(1.13)	(1.13)	(1.13)	(1.13)	1.13

* Warburg & Christian (1938).

The flavin moiety of notatin maintains the higher peak at $375\text{ m}\mu$ in the visible range of the spectrum when separated from the protein by heating or by acidification to pH 1.8, indicating that this peak is not associated with another coloured group attached to the protein, as suggested by Green *et al.* (1941), in the three flavoproteins mentioned above. The height of the $375\text{ m}\mu$ peak is probably a feature of notatin-flavin itself and on this assumption it may well be that the notatin prosthetic group is different from those hitherto described. On the other hand, the $375\text{ m}\mu$ peak becomes 'normal' when isolated by means of trichloroacetic acid and subsequent chromatographic adsorption in which a second zone (purple) is eliminated.

In all three samples the extinction coefficient at $260\text{ m}\mu$ is higher than the coefficients of the corresponding bands both in riboflavin and in the flavin adenine-dinucleotide of Warburg. The coenzyme of notatin is probably a polynucleotide and, in agreement with this, the silver salt is insoluble and the coenzyme can be regenerated from it. The explanation of the high extinction coefficient of the band at $260\text{ m}\mu$ may be that a small quantity of protein is still present or, since absorption at $260\text{ m}\mu$ is associated with adenine, that the coenzyme contains more than 1 molecule of adenine. The first explanation agrees with the higher extinction coefficient at $260\text{ m}\mu$ observed in the case of the solution prepared by method 3, which does not include purification by chromatography. It could readily be determined whether notatin is of alloxazine-adenine-dinucleotide type by testing it for coenzyme activity in the *d*-amino-acid oxidase system.

The protein obtained by decomposition of notatin by the three methods described above is denatured and is insoluble in water but soluble in dilute alkali. Prolonged dialysis of notatin solution against running tap water also removes the prosthetic group (compare Ball, 1939) and a buff protein can be isolated by means of tannic acid from the slightly coloured solution remaining in the dialyzing sack after 5 days. This protein possessed only one-fifth of the activity of notatin and prolonging the dialysis caused incipient denaturation. A white protein can,

however, be obtained from notatin by a modification of the technique of Warburg & Christian (1938; cf. Corran, Green & Straub, 1939). When an equal volume of saturated ammonium sulphate is added to notatin solution at pH 1.7, a white precipitate is obtained which is soluble in water and is almost devoid of anti-bacterial activity. This protein can be purified via the tannate but is very easily denatured by precipitation with acetone and subsequent drying. In aqueous solution this protein shows a definite peak at $263\text{ m}\mu$ but only general absorption in the visible region of the spectrum. Many unsuccessful attempts have been made to reconstitute notatin from the protein (produced by dialysis or by means of acidification), using either the flavins obtained by methods 1, 2 or 3 or riboflavin itself.

Mechanism of anti-bacterial action of notatin

Notatin may possess anti-bacterial activity *per se* or it may exert such activity through the products formed by virtue of its enzymic action on glucose in presence of oxygen. There is a good deal of evidence for the latter view. As already stated, the anti-bacterial action is exerted only in the presence of glucose. It can also be shown that oxygen must also be present. This is not altogether easy since the reaction is very sensitive to oxygen, and is indeed far more delicate than any chemical test we were able to use. When, however, great care was taken to exclude all oxygen, then no appreciable activity could be demonstrated either by fall in pH, indicating production of gluconic acid, or by bactericidal action. McIntosh & Selbie (1943) have found that against the anaerobes *Cl. welchii*, *Cl. oedematiens* and *Cl. septicum* notatin is bacteriostatic *in vitro* at a dilution of 1/1,000,000,000. Since the test was of necessity carried out under anaerobic conditions it would seem that even the trace of oxygen obtaining access during the preparation of the serial dilution before evacuation was sufficient in this case to allow the notatin to exert its effect at the high dilution noted, suggesting that these anaerobes are even more sensitive to the anti-bacterial agent produced (hydrogen peroxide) than our test organism.

It is quite clear from the results shown in Table 9 that the anti-bacterial action of notatin is dependent on the presence of oxygen.

Further confirmation of the necessity of oxygen for anti-bacterial activity of notatin was obtained in the experiment recorded in Table 10. All the aerobic tubes showed no growth under the test conditions, whereas there was good growth in all tubes of the anaerobic series.

If the bactericidal action of notatin is bound up with its enzymic activity, this might be due to production of (a) an unfavourable pH due to gluconic

Table 9. *Effect of anaerobiosis on anti-bacterial action of notatin*

(A series of experiments was put up in Thunberg tubes (Keilin's modification) in which the notatin solution (1 ml. 0.02%) was placed in the hollow stopper and the glucose (1 ml. 0.2%) and the inoculum (1 ml. *Staph. aureus* dilution in broth) in the tube, the reagents being mixed after evacuation. The solutions were made up in oxygen-free water under pure nitrogen and the *Staphylococcus* suspension was made up in freshly boiled and rapidly cooled broth. The original tubes had pH 7.0 and approximately 400 staphylococci/ml. After 4 hr. incubation at room temperature the pH was determined and bacterial counts were made.)

	After 4 hr.	
	pH	No. of viable organisms/ml.
Control—Tubes not evacuated:		
Notatin + glucose + <i>Staph. aureus</i>	4.1	1
Notatin + glucose + <i>Staph. aureus</i>	4.6	0
Tubes evacuated rapidly and thoroughly:		
Notatin + glucose + <i>Staph. aureus</i>	7.0	435
Notatin + glucose + <i>Staph. aureus</i>	7.0	421
— Glucose + <i>Staph. aureus</i>	7.0	400
— Glucose + <i>Staph. aureus</i>	6.9	428
Notatin — + <i>Staph. aureus</i>	7.0	437
Notatin — + <i>Staph. aureus</i>	7.0	418
Tubes evacuated slowly:		
Notatin + glucose + <i>Staph. aureus</i>	6.6	403
Notatin + glucose + <i>Staph. aureus</i>	7.0	398
Tubes evacuated slowly after a few min. standing on bench:		
Notatin + glucose + <i>Staph. aureus</i>	5.1	124
Notatin + glucose + <i>Staph. aureus</i>	5.6	240

Table 10. *Effect of anaerobiosis on anti-bacterial action of notatin*

(Tubes containing glucose broth and graded dilutions of notatin were set up as for anti-bacterial assay. Anaerobic conditions were secured by boiling the broth for 1 hr. before use and by sealing with sterile paraffin after inoculation with *Staph. aureus*. A similar aerobic series served as controls.)

Dilution of notatin	10 ¹	10 ²	10 ³	10 ⁴	10 ⁵	10 ⁶	10 ⁷
	Visual growth						
Aerobic control	—	—	—	—	—	—	—
Anaerobic sealed after inoculation	±	+	+	+	+	+	+

acid, (b) gluconic acid, which may be toxic *per se*, or (c) hydrogen peroxide, or a combination of these factors. In an attempt to decide this question the toxicities of gluconic acid and hydrogen peroxide were separately investigated.

Toxicity of gluconic acid. It is evident from Table 11 that gluconic acid concentrations up to 0.25% and pH down to 5.8 do not inhibit the

Table 11. *Effects of gluconic acid*

(To sterile tubes of glucose broth (4 ml.) were added sterile solutions of gluconic acid (1 ml.) to give the following percentages of gluconic acid in the medium: 2, 1, 0.5, 0.25, 0.125. The tubes were then inoculated with *Staph. aureus* and incubated at 37°. A similar series, uninoculated, served as controls. The tubes containing 2 and 1% of gluconic acid gave an immediate heavy ppt. (protein), the unsown tube containing 0.5% gave a ppt. after short incubation. The pH of the uninoculated tubes was determined and the ppt. or turbidity in all tubes observed after 24 hr. incubation.)

Concentration of gluconic acid present (%)	Precipitate or turbidity		pH (un-inoculated medium)
	Inoculated medium (ppt. due to acid and/or growth)	Uninoculated medium (ppt. due to acid)	
2.0	+++	+++	4.3
1.0	+++	+++	4.6
0.5	+++	+++	5.0
0.25	++	—	5.8
0.125	+++	—	6.6

organism. It seems that the production of gluconic acid or the lowering of pH produced by it cannot be responsible for the anti-bacterial action of notatin.

Toxicity of hydrogen peroxide. Hydrogen peroxide is well known to have anti-bacterial properties but it became of interest to determine the minimum concentration which would inhibit the growth of the test organism (Table 12).

Table 12. *Inhibition of growth of Staph. aureus by hydrogen peroxide*

(Sterile broth tubes, containing known amounts of added H₂O₂ in a descending series, were inoculated with *Staph. aureus* and incubated for 24 hr. at 37°.)

H ₂ O ₂ concentration (%)	Growth
0.004	—
0.002	—
0.001	—
0.0005	++
0.00025	++

Thus hydrogen peroxide at a concentration of 0.001%, or 1 in 10⁵, inhibits completely the growth of *Staph. aureus* in glucose broth. It seems probable that such a concentration could readily be produced in the medium by the limiting active dilution of notatin, namely 1 in 10⁵, particularly having regard to the possibility of adsorption of the enzyme on the surface of the bacteria with resultant local concentration of hydrogen peroxide far in excess of the general concentration throughout the medium. More direct evidence as to the responsibility of hydrogen peroxide for the bactericidal effect of notatin is

provided by a study of the addition of catalase to the medium.

Effect of catalase on the bactericidal activity of notatin. A series of tubes was set up containing 4.0 ml. of glucose- (2%) broth, 0.5 ml. of a solution of notatin at the required dilution (except where 'none added') and 0.5 ml. of a phosphate buffered catalase solution (kindly supplied to us by Prof. Keilin) or of phosphate buffer pH 7. The additions of catalase or phosphate buffer were made before those of notatin. As there was no means of sterilizing the catalase solution, although we believed it to be sterile, a series of unsovn tubes with glucose broth and catalase was included to exclude the possibility of adventitious organisms vitiating the test. The tubes were incubated for 24 hr. at 37° and then examined; the effects on growth are indicated in

Table 13. *Effect of catalase on anti-bacterial action of notatin*

Concentration of notatin present	Inoculated		Uninoculated + catalase
	+ catalase	+ buffer	
None added	+++	+++	-
1/50,000	+++	-	-
1/100,000	+++	-	-
1/200,000	+++	-	-
1/400,000	+++	-	-
1/800,000	+++	-	-
1/1.6 million	+++	-	-
1/3.2 million	+++	-	-

(The control: catalase + uninoculated buffer gave no growth.)

Table 13. It is clear that in presence of catalase the anti-bacterial activity of notatin is destroyed, which is a strong indication that the activity is due to hydrogen peroxide formation. This was further supported by the experiment recorded in Table 14.

Table 14. *Hydrogen peroxide production in presence of notatin*

(Tubes from the above experiment containing glucose-broth + buffer + notatin (inoculated) and glucose-broth + catalase + notatin (uninoculated), all of which showed no growth, were examined for H_2O_2 . 2.0 ml. from each tube were treated with 2 ml. of 2N- H_2SO_4 and 0.25 g. KI. After 30 min. the solution was titrated with 0.01N-thiosulphate.)

Notatin concentration	H_2O_2 found		
	Catalase added—uninoculated (ml. 0.01N- $Na_2S_2O_3$)	Buffer added— inoculated	
		ml. 0.01N- $Na_2S_2O_3$	% H_2O_2
1/50,000	0.04	0.68	0.0116
1/100,000	0.04	0.45	0.0080
1/200,000	0.04	0.56	0.0095
1/400,000	0.03	0.46	0.0078
1/800,000	0.03	0.34	0.0058
1/1.6 million	0.05	0.30	0.0052
1/3.2 million	0.07	0.34	0.0058

In Table 14 the small titration figures for H_2O_2 in the medium with catalase probably represent experimental error. It is evident that the hydrogen peroxide formed by notatin in absence of catalase in this test at all dilutions of notatin employed are well above the limiting dilution (0.001%) for growth of *Staph. aureus*.

Anti-bacterial product from Penicillium resticulosum. Identity with notatin

Enzymic action. The crude anti-bacterial product isolated from *P. resticulosum* had characteristics resembling those of notatin, although, as found by Birkinshaw, Raistrick & Smith (1942), it had a much lower activity against *Staph. aureus* in glucose broth. In order to decide whether it had enzymic properties similar to notatin, a preparation of activity 40,000 was shaken aerobically with glucose in the manner described for notatin.

The 'optimum substrate' of Franke & Deffner (1939) was used; it contained 5.7 mg. of the active preparation in 1 ml. of water, 2 ml. of 0.2M-phosphate buffer (pH 6.0) and 2 ml. of 2% glucose. The mixture was shaken for 1 hr. in presence of oxygen at 18°. The total oxygen uptake was 6.9 ml. (at N.T.P.). A hydrogen peroxide determination showed that 3.4 ml. of oxygen were used for production of hydrogen peroxide according to the equation

The amount of oxygen used for production of gluconic acid according to the equation

was therefore 3.5 ml. Thus the amount of hydrogen peroxide formed was 97% of theoretical, a quantitative value within the limits of experimental error. The activity coefficient (Q_{O_2}), calculated according to Franke & Deffner (1939) and corrected to 30°, is therefore about 1000. This would represent a notatin content of less than 1%. The product from *P. resticulosum* has thus similar enzymic properties to notatin. Since it produces H_2O_2 almost quantitatively it is probably free from catalase.

Purification by tannic acid. Since the activity of the active *P. resticulosum* product was of a much lower order than that of notatin an attempt was made to purify it by the tannic acid method.

The culture filtrate from 50 flasks of *P. resticulosum* (14 l.) was treated with 5.0 g. of tannic acid in 50 ml. of water. After keeping in cold room overnight the precipitate was spun off. The dark solid was extracted and washed well with acetone and the residue was extracted twice with 25 ml. of water. The aqueous solution (50 ml.) was precipitated by 100 ml. of acetone, the precipitate was spun off and dried *in vacuo*. The weight of the recovered solid (25.5 mg.) was not sufficient to allow

of further treatment. The anti-bacterial activity, however, even at this stage was between 1 and 10 million, considerably better than the best previously recorded activity of 160,000. It seems likely that further purification would yield a product having an activity of the same order as that of notatin.

SUMMARY

1. The production, isolation and properties of notatin, an anti-bacterial substance obtained from culture solutions of *Penicillium notatum* Westling, are described in detail.

2. Notatin is an enzyme of flavoprotein nature, catalyzing the oxidation of glucose to gluconic acid by means of atmospheric oxygen with the production of hydrogen peroxide, according to the equation:

3. Notatin has high anti-bacterial activity *in vitro*, the best preparations completely inhibiting the growth of *Staphylococcus aureus* at dilutions as great as 1 in 1,000,000,000. The anti-bacterial action of notatin is shown to be due to its power of producing hydrogen peroxide in presence of glucose and oxygen, the actual agent being hydrogen peroxide.

4. Sugars other than glucose (e.g. *d*-xylose and *d*-mannose) can function in the enzymic reaction, but glucose shows a much higher activity.

5. The anti-bacterial substance previously isolated in a crude form from *Penicillium resticulosum* nov.sp. is in all probability identical with notatin.

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