Table 1. *Rotation of the dextro-isocitrate-citrate-molybdate complex*

(For test conditions see text; α_D is the rotation in degrees observed in a 2 dm. tube, $[\alpha]_D$ the specific rotation calculated for isocitric acid.)

Concentration of isocitric acid (mg./ml.) ...	Temp.	0.48		0.96		1.92		3.84	
...	(°)	α_D	$[\alpha]_D$	α_D	$[\alpha]_D$	α_D	$[\alpha]_D$	α_D	$[\alpha]_D$
10		-0.80°	-834°	-1.56°	-812°	-2.98°	-776°	-5.39°	-701°
15		-0.77°	-802°	-1.50°	-781°	-2.82°	-735°	-5.18°	-675°
20		-0.75°	-781°	-1.43°	-745°	-2.66°	-693°	-4.96°	-645°
25		-0.73°	-760°	-1.38°	-719°	-2.51°	-654°	-4.75°	-618°
30		-0.70°	-729°	-1.32°	-688°	-2.35°	-612°	-4.54°	-591°
35		-0.68°	-709°	-1.26°	-657°	-2.19°	-570°	-4.33°	-564°

Table 2. *Effect of the concentration of dextro-isocitric acid on $[\alpha]_D$ in solutions containing the isocitrate-citrate-molybdate complex*

(Standard conditions. 2 dm. tubes. 20°. $[\alpha]_D$ calculated for isocitric acid.)

isoCitric acid concentration (mg./ml. solution tested)	α_D observed	$[\alpha]_D$
0.240	-0.40°	-834°
0.480	-0.75°	-781°
0.576	-0.89°	-772°
0.672	-1.02°	-758°
0.768	-1.15°	-750°
0.864	-1.29°	-747°
0.960	-1.43°	-745°
1.44	-2.09°	-726°
1.92	-2.66°	-693°
3.84	-4.96°	-645°

In the previous experiments (Krebs & Eggleston, 1943) the rotation measured was about -1.80° (2 dm. tubes) at

approximately 18° . According to Table 1 $[\alpha]_D^{18^\circ}$ for these conditions is -735° , whilst it was previously assumed to be -780° . A small correction of the previously calculated isocitric acid values is therefore required. The equilibrium mixture of citrate, isocitrate and *cis*-aconitate had been taken to contain 6.2% isocitrate (at 38°). The recalculation gives a value of 6.6% isocitrate.

SUMMARY

The optical rotatory powers of solutions containing the isocitrate-citrate-molybdate complex have been measured under varying conditions, in order to obtain reference values for the polarimetric determination of dextro-isocitric acid.

We are indebted to Dr H. B. Vickery, Connecticut Agricultural Experiment Station, New Haven, Conn., for a sample of dextro-isocitric acid prepared by the late Dr G. H. Pucher.

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The Use of Buffered Columns in the Chromatographic Separation of 2:4-Dinitrophenyl Amino-acids

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During an investigation of the action of papain on wool keratin, the elegant method of Sanger (Sanger, 1945; Porter & Sanger, 1948) was used to determine the end-groups of the peptides liberated by the enzyme. In this method, the terminal amino groups of proteins or polypeptides are condensed with 1:2:4-fluorodinitrobenzene and the amino-acids originally carrying free amino groups are isolated

chromatographically as their coloured *N*-2:4-dinitrophenyl (DNP) derivatives after acid hydrolysis.

It was observed at an early stage that silica gels which had been successfully employed for the separation of *N*-acetyl amino-acids were of little use for the separation of DNP amino-acids. Using chloroform as the solvent, for example, practically

all the DNP amino-acids ran together as one fast band. Consden, Gordon, Martin & Synge (1947), who had a similar experience, were of the opinion that the action of Sanger's gels must be attributed to the occurrence of strong adsorption effects in addition to the effect of distribution between the phases. Sanger (1945) originally found the *R* values (Martin & Synge, 1941) of DNP amino-acids to vary considerably with the batch of silica, and considered that they were adsorbed on the silica to a certain extent. Phillips & Stephen (1948) have also commented on the variation between different batches of gel.

The successful use of buffered columns of silica gel in the separation of the penicillins by partition chromatography (Boon, 1948) suggested that the expedient might be useful in the separation of DNP amino-acids.

METHODS

Preparation of silica gel. The general procedure of Gordon, Martin & Synge (1943, 1944) and Tristram (1946) for the preparation of silica gel was followed. After precipitation of the gel, a considerable excess of HCl was added to the mixture, which was then allowed to stand for 3 hr. After filtration and washing, the gel was allowed to stand for 3 days in distilled water, and was again washed and dried. The gel prepared in this way was a 'non-adsorbent' gel.

Preparation of DNP amino-acids. The DNP derivatives of glycine, DL-alanine, DL-valine, DL-isoleucine, DL-leucine, L-proline, DL-phenylalanine, DL-methionine, DL-aspartic acid, DL-threonine, DL-serine and L-tryptophan were prepared by the method of Abderhalden & Blumberg (1910). Bis-DNP-L-lysine was prepared by the same method and ϵ -DNP-L-lysine according to Porter & Sanger (1948). All these derivatives were recrystallized from appropriate solvents before use and had melting points in good agreement with those in the literature. As reported by Sanger (1945), DNP-L-glutamic acid did not crystallize. DNP-DL-glutamic acid was prepared by the method of Abderhalden & Blumberg (1910). The cooled reaction mixture, after concentration *in vacuo* to remove the greater part of the ethanol, was diluted and extracted with ether. The heavy oil which precipitated on acidification solidified after standing overnight. It was recrystallized twice from ethyl acetate-cyclohexane and had m.p. 172°. (Found: C, 42.3; H, 3.6; N, 13.6; $C_{11}H_{11}O_8N_3$ requires C, 42.2; H, 3.5; N, 13.4%.) All the above compounds were found to give single bands on appropriate silica-gel columns. The reaction between L-tyrosine and 1:2:4-chlorodinitrobenzene gave a substance forming a single band on a buffered $CHCl_3$ column. This was presumably bis-DNP-L-tyrosine, since the compound formed by the action of 1:2:4-fluorodinitrobenzene on L-tyrosine had a similar rate (Sanger, 1945).

Preparation of columns. In general the customary technique of partition chromatography was employed, 66% by weight of a suitable buffer solution being added to the dry gel before pouring the column. The buffers employed are conveniently distinguished as A, B, F and G. Buffer A was a 2M-solution of NaH_2PO_4 . Buffers F, B and G were Sørensen's phosphate buffers (Britton, 1942, Table 76) of pH 6.47, 6.98 and 6.24 respectively. The concentrations of salts in these buffers were increased so that the molar con-

centrations of H_2PO_4 in them were respectively 0.25, 0.5 and 0.25. The pH of buffers A, B, F and G, as measured by the glass electrode, were 3.72, 6.61, 6.16 and 5.95 respectively.

It was observed during the course of this work that DNP amino-acids tended to decompose if exposed to sunlight on the column. Solutions of DNP amino-acids in solvents such as $CHCl_3$ also tended to decompose when exposed for long periods to light, particularly sunlight. The solutions then gave rise to additional bands on the chromatogram. The practice was therefore adopted of evaporating the solvent as soon as the band under consideration had run out of the column, and where necessary refractionation was carried out without undue delay. Ethanol solutions of DNP amino-acids left for some weeks in a cupboard were found to give rise to fast yellow bands on columns in addition to the bands due to the DNP amino-acid.

RESULTS

Using buffered columns of silica gel and water-saturated chloroform as solvent, mixtures of DNP amino-acids can readily be separated into distinct bands. By varying the pH of the buffer, and hence the pH of the aqueous phase, the band rate of any particular DNP amino-acid can be varied within wide limits. As the pH of the aqueous phase of the column is increased, the band rate of any particular DNP amino-acid decreases. The band rates can easily be made slow enough to separate DNP amino-acids by increasing the pH of the buffer.

Table 1 gives the band rates of a number of DNP amino-acids on columns buffered to different pH values in chloroform and *n*-butanol-chloroform mixtures. The behaviour of DNP-hydroxyproline was not studied, since it was known to be absent from the mixtures it was desired to investigate. Over the pH range studied, the variation of band rate with pH of the column is most marked in the case of the slower moving DNP amino-acids. Using a single solvent such as chloroform in conjunction with different buffered columns, it is possible to effect separation of DNP amino-acids which cannot be accomplished by the use of a single unbuffered column with the same solvent. The use of buffered columns largely eliminates the variations in behaviour between batches of silica gel which previous workers have commented on. A number of solvent systems have been found useful when employed with buffered columns. These systems and the type of separation they will accomplish are briefly described below. A description is also given of the method of separation of DNP amino-acid mixtures based on these solvents and buffers.

Chloroform and butanol-chloroform columns

Buffer A- $CHCl_3$ columns are useful for the separation of DNP-monoaminomonocarboxylic acids from DNP-glutamic acid, DNP-aspartic acid, DNP-threonine and DNP-serine. These columns will also separate DNP-glutamic acid from

Table 1. *Band rates (R values) of DNP amino-acids in chloroform and butanol-chloroform*

DNP amino-acid	R values			
	CHCl ₃ , buffer A	CHCl ₃ , buffer B	3% butanol- CHCl ₃ , buffer G	17% butanol- CHCl ₃ , buffer B
DNP-leucine	Fast	0.7	—	—
DNP-valine	Fast	0.5	—	—
DNP-phenylalanine	Fast	0.7	—	—
DNP-methionine	Fast	0.5	—	—
DNP-proline	Fast	0.3	—	—
DNP-alanine	Fast	0.2	—	0.5
DNP-tryptophan	0.6	0.4	Fast	—
DNP-glycine	0.9	0.03	0.5	0.2
Bis-DNP-lysine	0.7	0.06	Fast	Fast
Bis-DNP-tyrosine	Fast	0.07	0.9	Fast
DNP-threonine	0.3	Slow	0.18	0.15
DNP-serine	0.11	Slow	0.08	0.07
DNP-glutamic acid	0.17	Slow	0.2	Slow
DNP-aspartic acid	0.06	Slow	Slow	Slow

DNP-aspartic acid and DNP-threonine from DNP-serine. Buffer *B*-CHCl₃ columns will separate DNP-alanine from DNP-glycine and DNP-valine from DNP-alanine. The band rates of DNP amino-acids in CHCl₃ on columns buffered to a more alkaline pH (Sørensen's pH 8 phosphate buffer, 0.25M (Britton, 1942, Table 76) pH measured by glass electrode 7.84) were similar to those on buffer *B*-CHCl₃ columns. Use of these columns did not separate those DNP amino-acids not separated on buffer *B*-CHCl₃ columns. The rates of DNP amino-acids on buffer *B*-CHCl₃ columns are roughly similar to those found by Sanger (1945) on CHCl₃ columns. Buffer *B*, however, probably gives less acid columns than does Sanger's silica gel.

In general, maximum separation is achieved with the least acid columns, although the band rates may then be inconveniently slow. Convenient separations of the slower-moving DNP amino-acids can often be achieved by employing butanol-CHCl₃ instead of chloroform as the solvent. A mixture of DNP-glycine and bis-DNP-lysine or bis-DNP-tyrosine does not separate readily on a buffer *B*-CHCl₃ column since all three move slowly under these conditions. On a buffer-*B*-3% butanol-CHCl₃ column, however, the two latter DNP amino-acids move faster than DNP-glycine and are readily separated from it.

Similarly, a mixture of DNP-threonine, DNP-serine and DNP-aspartic acid can be separated into three bands, emerging from the column in the above order, on buffer *G*-3% butanol-CHCl₃ columns or on buffer *B*-17% butanol-CHCl₃ columns. With a change in the solvent and buffer in this way, the order in which the DNP amino-acids emerge from the column may be altered. Thus on buffer *A*-CHCl₃ columns DNP-glutamic acid emerges ahead of DNP-serine. On more alkaline columns, with butanol-CHCl₃ as the mobile phase (on buffer *B*-17% butanol-CHCl₃ columns), the *R* value of DNP-serine is greater than that of DNP-glutamic acid. This behaviour may depend on the fact that the ionization of DNP-glutamic acid can involve two carboxyl groups, as opposed to one in the case of DNP-monoamino-monocarboxylic acids. On the gels used by Sanger with 1% butanol-CHCl₃ as the mobile phase, DNP-threonine and DNP-serine have faster rates than DNP-glutamic acid and DNP-aspartic acid (Sanger, 1945; Porter & Sanger, 1948).

Propanol-cyclohexane columns

Propanol-cyclohexane (5%) used with buffered columns is a very useful solvent for the separation of DNP-monoamino-monocarboxylic acids. The rates of the DNP amino-acids on these columns are also dependent on the pH of the aqueous phase. Table 2

Table 2. *Band rates (R values) of DNP amino-acids in 5% propanol-cyclohexane*

DNP amino-acid	R values (5% propanol-cyclohexane)		
	Buffer A	Buffer B	Buffer F
DNP-leucine	—	0.4	0.5
DNP-valine	Fast	0.15	0.4
DNP-phenylalanine	—	0.2	0.3
DNP-methionine	—	0.06	0.08
DNP-proline	—	—	0.06
DNP-alanine	Fast	0.04	0.10
DNP-tryptophan	—	—	0.11
DNP-glycine	—	0.02	0.02

gives the rates of DNP amino-acids in this solvent. From the band rates quoted by Sanger (1945) for propanol-cyclohexane columns it is evident that buffer *F* gives less acid columns than did Sanger's gels with this solvent. It has been found that buffer *F* columns are suitable for the separation of the majority of the DNP amino-acids of this group. Buffer *B* columns may be used for the separation of the longer-chain DNP amino-acids. The shorter-chain DNP monoamino-acids move rather slowly on buffer *F*-5% propanol-cyclohexane columns, but buffer *G*-10% propanol-cyclohexane columns gives rates suitable for convenient separation.

On buffer *F*-5% propanol-cyclohexane columns DNP-valine can be separated from DNP-alanine, and DNP-alanine from DNP-glycine. DNP-isoleucine and DNP-valine can be readily separated on a buffer *B*-5% propanol-cyclohexane columns. A mixture of DNP-isoleucine and DNP-phenylalanine can also be separated on the latter

column, as can a mixture of DNP-valine and DNP-methionine. A mixture of DNP-isoleucine, DNP-valine, DNP-alanine and DNP-glycine separates into four bands on this column, but the rates of DNP-alanine and DNP-glycine are rather slow.

These columns thus generally affect the separations that can be achieved on ethanol-ligroin columns made according to Sanger (1945). It is noteworthy that both these solvent systems contain alcohols. The type of separation which can be brought about by a given solvent system may therefore be correlated with the chemical character of the solvent. Martin (1948) has previously pointed out the relationship existing between the chemical constitution of the solvent and the extent of separation of amino-acids and their derivatives in partition chromatography.

Ether columns

Buffer *A*-ether columns can be used to free ether extracts of hydrolysates of DNP-proteins and polypeptides from HCl (cf. Sanger, 1945) as all the DNP amino-acids travel fast on these columns. On one occasion when an ether extract of DNP amino-acids from the hydrolysate of a protein treated with 1:2:4-fluorodinitrobenzene was passed through a buffer *G*-ether column to remove HCl, yellow bands, presumably DNP amino-acids, were observed passing down the column. Investigation showed that the DNP amino-acids moved on buffer *B*-ether columns with characteristic rates, which are given in Table 3. These columns are useful for separating the slower moving DNP amino-acids from each other.

Table 3. *Band rates (R values) of DNP amino-acids in ether and 33 % ether-ligroin*

DNP amino-acid	Ether, buffer <i>B</i>	33 % ether-ligroin, buffer <i>B</i>
DNP-leucine	Fast	0.5
DNP-valine	Fast	0.2
DNP-phenylalanine	Fast	0.1
DNP-methionine	1.0	0.1
DNP-proline	0.5	0.04
DNP-alanine	0.5	0.08
DNP-tryptophan	Fast	0.09
DNP-glycine	0.2	0.04
Bis-DNP-lysine	0.6	(Sparingly soluble)
Bis-DNP-tyrosine	0.9	(Sparingly soluble)
DNP-threonine	0.14	—
DNP-serine	0.10	—
DNP-glutamic acid	0.04	—
DNP-aspartic acid	0.01	—

A mixture of DNP-threonine, DNP-serine and DNP-aspartic acid separates into three bands, whilst DNP-threonine or DNP-serine can be separated from DNP-glutamic acid. DNP-glutamic acid separates from DNP-aspartic acid, but the band rates are rather slow for convenient separation. DNP amino-acids which do not separate readily on buffer *B*-CHCl₃ columns will often do so on buffer *B*-ether columns. Thus bis-DNP-tyrosine or bis-DNP-lysine will separate from DNP-glycine. The faster

moving DNP amino-acids also separate on buffer *B*-ether columns. The band rates are somewhat high, but if more alkaline buffers are employed the bands 'tail' into each other. A mixture of DNP-valine, DNP-alanine and DNP-glycine separates into three bands, and DNP-alanine can be separated from DNP-methionine or DNP-tryptophan.

Ether-ligroin columns

The longer chain DNP monoamino-acids have characteristic rates on buffer *B*-33 % ether-ligroin columns (Table 3). These acids move fast on buffer *B*-ether columns. A mixture of DNP-valine, DNP-alanine and DNP-glycine separates into three bands on a buffer *B*-33 % ether-ligroin column. An extensive investigation has not yet been made, but these columns seem to effect the same separations as buffer *B*-CHCl₃ columns.

Columns employing organic solvents as the stationary phase

When 'non-adsorbent' gels were employed it was found that certain of Sanger's columns, which use liquids such as aqueous ethanol or aqueous acetone as the stationary phase, did not separate the appropriate mixtures of DNP amino-acids clearly. Thus with the gels prepared by the present author a mixture of DNP-valine and DNP-phenylalanine was not clearly separated on acetone-cyclohexane columns prepared according to Sanger (1945). When one volume of acetone was mixed with one volume of buffer *G* instead of water and the column then prepared as before, the expected separation was readily obtained.

The separation of DNP-alanine from DNP-proline was also difficult on glycol-benzene columns using 'non-adsorbent' gels. Using the same solvent system both DNP-proline and DNP-alanine moved extremely slowly on a gel which had been prepared by precipitating sodium silicate with acid and washing with buffer (buffer *G*) before drying.

Band rates of acid soluble DNP amino-acids

The band rates of the acid-soluble DNP amino-acids were also found to vary with the pH of the aqueous phase of the column. No systematic investigation of this variation was made, but it was found that columns buffered with buffer *A* gave the desired separations with 66 % methyl ethyl ketone-ether and 30 % butanol-chloroform as the moving phase. These are the solvent systems used by Sanger for these separations.

Separation of mixtures of DNP amino-acids

From the present experiments, and those of Sanger, it is evident that a mixture of DNP amino-acids can be separated into its constituents in a number of different ways, as can a mixture of *N*-acetyl amino-acids (Tristram, 1946). The number of

fractionations necessary to effect a complete separation obviously depends on the number of DNP amino-acids present in the mixture being studied. Buffered columns have been successfully employed by the present author in separating DNP amino-acid mixtures, and the scheme employed differs from that elaborated by Porter & Sanger (1948). The initial fractionation is generally carried out on buffer *B*-ether columns, although buffer-*G*-3% butanol-chloroform columns were employed in the earlier stages of this work. On buffer *B*-ether columns the longer chain DNP monoamino-acids move rapidly down the column followed by DNP-glycine, DNP-threonine and DNP-serine, which can be separated and collected. DNP-glutamic and DNP-aspartic acids form a slow-moving band at the top of the column which splits into its constituents on continued development. Elution of these two bands separately with ether usually required too long a time and it is convenient to elute them together with ether containing a few drops of glacial acetic acid to make the column acid. These DNP amino-acids then run rapidly through the column and, after taking the solution to dryness, can be fractionated on a buffer *A*-chloroform column. The fast-moving first band obtained on buffer *B*-ether columns contains the DNP monoaminomonocarboxylic acids. After taking to dryness, it is refractionated on a buffer *B*-chloroform column. The bands from this fractionation are then further refractionated on buffer *G*-10% propanol-*cyclohexane* columns for the slower-moving DNP amino-acids and on buffer *B*-5% propanol-*cyclohexane* columns for the faster-moving DNP amino-acids.

Sanger (1945) observed that partition chromatography on paper was not successful in separating mixtures of DNP amino-acids, owing to 'tailing' of the spots. Phillips & Stephen (1948) claimed some success, particularly with the slower-moving DNP amino-acids, using a two-dimensional technique. Experiments by the present author with a wide variety of solvents led to similar conclusions to those of Sanger (1945), only a partial separation of the different DNP amino-acids being achieved. It is probable that adsorption of the DNP amino-acids on the paper occurs and this effect is superimposed on the partition effects. There is some evidence that adsorption does occur, since when DNP amino-acids were run in distilled water under conditions similar to those of capillary analysis (Rheinboldt, 1925) fairly well-defined spots were formed at some distance from the solvent front. The difference in rate between the different DNP amino-acids was not sufficient to effect separations.

In a few experiments employing partition chromatography, paper previously soaked in buffer solution and then dried was used, but no marked increase in the effectiveness of the separation was achieved.

Alumina-treated paper (Datta & Overell, 1949) similarly showed no marked improvement in separation over ordinary paper in a limited number of experiments. Columns of powdered cellulose saturated with buffer solutions effected some degree of separation of DNP amino-acids, but were not as successful as silica-gel columns.

DISCUSSION

Sanger (1945) and Consden *et al.* (1947) considered that the DNP amino-acids are adsorbed to a certain extent on the silica gel. The theory of the partition chromatogram, as initially worked out by Martin & Synge (1941), assumes that the partition coefficient of the solute remains constant with varying concentration, but, as they pointed out, this is seldom the case. If appreciable ionization of the solute, e.g. a DNP amino-acid or *N*-acetyl amino-acid, occurs in the aqueous phase the partition coefficient will vary appreciably with the concentration. As a result the forward boundary of the chromatogram band will become sharper and the back more diffuse, a result commonly observed in ordinary 'adsorption' chromatography where the adsorption follows a Langmuir type isotherm (Tiselius, 1947; Cannan, 1946). Tiselius emphasized that there was hardly any difference in principle between ordinary and partition chromatography. By making the pH of the aqueous phase of the column sufficiently high, the ionization of the DNP amino-acid in the aqueous phase becomes appreciable and the band formed on the chromatogram will be of the adsorption type. The adsorption effects noted by Sanger (1945) and Consden *et al.* (1947) are thus probably due to small amounts of alkali retained by the gel during its preparation. Porter & Sanger (1948) found that adsorbent gels giving low *R* values produced maximum resolution for any given solvent, but gave 'tailing' bands. The present author believes that variations in behaviour between different batches of gel using unbuffered columns are due in large part to variations in the pH of the aqueous phase in contact with the gel. Using a given buffered column, no significant variation has been observed in the rate of a particular DNP amino-acid when different batches of gel are employed. This indicates that variations between different batches of gel, e.g. in the amount of acid or alkali retained, do not cause significant variations in the pH of the aqueous phase of a buffered column. Nevertheless, the band rate of any particular DNP amino-acid varies with the amount of material placed on the column and with the distance the band has travelled down the column. It is possible that some of the variations between batches of gel observed in the separation of *N*-acetyl amino-acids (Tristram, 1946; Martin, 1948) might be eliminated by the use of buffered columns.

The present buffers and solvent systems may not necessarily be the best or the most convenient for separating mixtures of DNP amino-acids, and other schemes of separation using buffered columns could almost certainly be devised. The primary object of the present work was not centred in devising the best analytical method, but in eliminating some of the difficulties encountered using unbuffered columns. The application of these methods will be described in a forthcoming paper.

SUMMARY

The use of buffered silica-gel columns in the partition chromatographic separation of 2:4-dinitrophenyl amino-acids is described.

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The Mechanism of the Action of Notatin

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Notatin is an enzyme discovered by Müller (1928) in *Aspergillus niger* and in *Penicillium glaucum*, and named by him glucose oxidase. Müller showed that this enzyme catalyses the oxidation of glucose by molecular oxygen to an acidic product which he identified as gluconic acid. Subsequently it was found by Franke & Lorenz (1937), using a more highly purified preparation, that hydrogen peroxide was formed in this reaction; these authors also suggested that the enzyme was a flavoprotein. A more complete purification was achieved by Coulthard, Michaelis, Short, Sykes, Skrimshire, Standfast, Birkinshaw & Raistrick (1942, 1945), who named the enzyme notatin. Independently, Van Bruggen, Reithel, Cain, Katzman, Doisy, Muir, Roberts, Gaby, Homan & Jones (1943) also purified the same enzyme, designated by these authors penicillin B. The experiments of the American workers suggested that the prosthetic group was the alloxazine-adenine dinucleotide. This was definitely proved by Keilin & Hartree (1946, 1948a, b), who examined the physical

and chemical properties of the enzyme in great detail and also determined its specificity range very thoroughly.

The overall reaction catalysed by notatin is generally represented as follows:

This equation does not make clear which is the essential, enzyme-catalysed step in the reaction and, indeed, there has been some discussion as to whether notatin is a dehydrogenase (Franke & Lorenz, 1937; Franke & Deffner, 1939), or an oxidase (Müller, 1936). The latter hypothesis assumes that the enzyme 'activates' molecular oxygen, one of the oxygen atoms combining with the aldehyde form of glucose, whilst the other reacts with water to form hydrogen peroxide. However, the fact that notatin contains alloxazine-adenine dinucleotide suggests that the enzyme is a dehydrogenase. According to this second concept notatin catalyses the transfer of two hydrogen atoms from glucose or from the