the oxygen uptake and acetoacetate oxidation when added alone or with fumarate, but not in the presence of α -oxoglutarate. The reasons for these differences are discussed. Anaerobically, relatively slight inhibitions of the reduction of acetoacetate were observed.

6. The oxidation of L(+)- β -hydroxybutyrate is inhibited by dinitrophenol, whereas that of the D(-)-form is not. This is related to the fact that only the L(+)-form requires conversion into the coenzyme A derivative. The differences in the behaviour of the L- and D-forms towards dinitrophenol were used to examine the configuration of the β -hydroxybutyrate formed in sheep-heart muscle aerobically and anaerobically. In both cases the D-form only was found.

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REFERENCES

- Aisenberg, A. C. & Potter, V. R. (1955). *J. biol. Chem.* **215**, 737.
 Aldridge, W. N. (1957). *Biochem. J.* **67**, 423.
 Birt, L. M. & Bartley, W. (1960). *Biochem. J.* **76**, 328.
 Burton, K. (1957). *Ergebn. Physiol.* **49**, 275.
 Chance, B. & Hollunger, G. (1960). *Nature, Lond.*, **185**, 666.
 Chance, B. & Williams, G. R. (1955*a*). *Fed. Proc.* **14**, 190.
 Chance, B. & Williams, G. R. (1955*b*). *J. biol. Chem.* **217**, 383, 409.
 Cross, R. J., Taggart, J. V., Covo, G. A. & Green, D. E. (1949). *J. biol. Chem.* **177**, 655.
 Davies, R. E. & Krebs, H. A. (1952). *Symp. biochem. Soc.* **8**, 77.
 Edson, N. L. (1935). *Biochem. J.* **29**, 2082.
 Green, D. E., Goldman, D. S., Mii, S. & Beinert, H. (1953). *J. biol. Chem.* **202**, 137.
 Green, D. E., Needham, D. M. & Dewan, J. G. (1937). *Biochem. J.* **31**, 2327.
 Greenberg, L. A. & Lester, D. (1944). *J. biol. Chem.* **154**, 177.
 Hunter, F. E. & Hixon, W. S. (1949). *J. biol. Chem.* **181**, 73.
 Klingenberg, M., Slenczka, W. & Ritt, E. (1959). *Biochem. Z.* **332**, 47.
 Krebs, H. A. (1959). In *Ciba Foundation Symposium on the Regulation of Cell Metabolism*, p. 1. Ed. by Wolstenholme, G. E. W. & O'Connor, C. M. London: J. and A. Churchill Ltd.
 Krebs, H. A. (1960). *Proc. Roy. Soc. Med.* **53**, 71.
 Krebs, H. A. & Eggleston, L. V. (1945). *Biochem. J.* **39**, 408.
 Krebs, H. A. & Eggleston, L. V. (1948). *Biochem. J.* **42**, 294.
 Krebs, H. A. & Kornberg, H. L. (1957). *Ergebn. Physiol.* **49**, 212.
 Lardy, H. A. & Wellman, H. (1952). *J. biol. Chem.* **195**, 215.
 Lehninger, A. L. & Greville, G. D. (1953). *Biochim. biophys. Acta*, **12**, 188.
 Lester, D. & Greenberg, L. A. (1948). *J. biol. Chem.* **174**, 903.
 McCann, W. P. (1957). *J. biol. Chem.* **226**, 15.
 Martius, C. & Nitz-Litzow, D. (1953). *Biochim. biophys. Acta*, **12**, 134.
 Simon, E. W. (1953). *Biol. Rev.* **28**, 453.
 Stern, J. R., Coon, M. J., del Campillo, A. & Schneider, M. C. (1956). *J. biol. Chem.* **221**, 15.
 Stern, J. R. & Ochoa, S. (1951). *J. biol. Chem.* **191**, 161.
 Tyler, D. B. (1950). *J. biol. Chem.* **184**, 711.
 Utter, M. F. & Swim, H. E. (1957). In *Methods in Enzymology*, vol. 4, p. 584. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
 Walker, P. G. (1954). *Biochem. J.* **58**, 699.
 Witter, R. F., Newcomb, E. H. & Stotz, E. (1953). *J. biol. Chem.* **202**, 291.

Biochem. J. (1961) **78**, 106

Studies on Glucosaminidase

2. SUBSTRATES FOR N-ACETYL- β -GLUCOSAMINIDASE*

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Methods available for the estimation of the activity of N-acetyl- β -glucosaminidase have been limited by a lack of suitable substrates. Those with an alkyl N-acetyl- β -glucosaminide as substrate (Neuberger & Pitt Rivers, 1939; Kuhn & Tiedemann, 1954) depend on the relatively non-

specific estimation of reducing sugar liberated. Phenyl (Helferich & Iloff, 1933) and p-nitrophenyl (Westphal & Schmidt, 1952) N-acetyl- β -glucosaminide were used as substrates by Watanabe (1936), Neuberger & Pitt Rivers (1939) and Pugh, Leaback & Walker (1957*b*), and by Conchie, Findlay & Levvy (1959) respectively, in methods depending on the colorimetric or spectrophoto-

* Part I: Pugh, Leaback & Walker (1957*b*).

metric estimation of the liberated phenol. The development of a general method for the synthesis of alkyl and aryl *N*-acetyl- β -glucosaminides (Leaback & Walker, 1957) has extended the range of substrates available. This paper describes the kinetics of hydrolysis of an alkyl and four aryl *N*-acetyl- β -glucosaminides by enzymes from two sources. The suitability of these compounds as substrates for the estimation of *N*-acetyl- β -glucosaminidase is compared.

METHODS AND MATERIALS

Preparation of substrates. Methyl, ethyl, phenyl, *o*- and *p*-nitrophenyl and *p*-acetylphenyl *N*-acetyl- β -glucosaminides were prepared as described by Leaback & Walker (1957). Approximate solubilities of the aryl glycosides (mm) in water at 20–22° are: phenyl, 40; *o*-nitrophenyl and *p*-acetylphenyl, 23; *p*-nitrophenyl, 15.

Buffers. Citrate buffers (0.5M): these were prepared from stock solutions of sodium citrate and citric acid. In order to obtain the desired final pH in reaction mixtures 0.05M with respect to citrate, allowance was made for the rise of approx. 0.2 pH unit which takes place on dilution (Kolthoff & Bosch, 1927). Pyrophosphate buffer, pH 9.2: sodium pyrophosphate (10 g.), semicarbazide (2.5 g., recrystallized from methanol) and glycine (0.5 g.) were dissolved in water and made up to 300 ml. after adjusting to pH 9.2 with 2N-NaOH.

Diphosphopyridine nucleotide. This was a 2% (w/v) solution of diphosphopyridine nucleotide-75 (Boehringer und Söhne, Mannheim, Germany).

Alcohol dehydrogenase. Alcohol dehydrogenase suspension (Boehringer und Söhne) was used.

Enzyme preparations

Ram testis. A solution (1%, w/v) of freeze-dried extract of ram testis (obtained from Bengel Ltd., Holmes Chapel, Cheshire) was fractionated by addition of saturated ammonium sulphate solution, pH 4.9. The fraction precipitated between 30 and 50% saturation, which contained about 60% of the initial enzyme activity, was taken up in a small volume of water and dialysed for 24 hr. against

distilled water at 4°. The activity of this preparation fell by 20% on storage for 4 weeks at 0–4°.

Sweet-almond meal. The meal (obtained through the courtesy of Professor H. N. Rydon) was extracted with 10 ml. of 0.05M-citrate buffer (pH 4.4)/g. The extract was fractionated as described above except that the fraction precipitated between 30 and 60% saturation with ammonium sulphate was taken. This preparation was extremely stable on storage at 0–4°.

Rat tissues. The freshly dissected tissue was blotted, weighed and then ground with water for 1 min. in a glass homogenizer before diluting to a final concentration of 1% (w/v).

Enzyme estimations

Unit of *N*-acetyl- β -glucosaminidase. One unit of *N*-acetyl- β -glucosaminidase is defined as the amount of enzyme liberating 1 μ mole of phenol from 10 mM-phenyl *N*-acetyl- β -glucosaminide in 0.05M-citrate buffer, pH 4.4, in 1 hr. at 37°.

Kinetic experiments. Substrate and a suitable dilution of enzyme were incubated at 37° in 0.05M-citrate buffer. The final volume was 0.4 ml. with ethyl *N*-acetyl- β -glucosaminide as substrate and 1.0 ml. with the aryl glucosaminides. Each estimation was carried out in duplicate. Control tubes, in which enzyme or substrate was omitted, were also incubated. In these experiments substrate concentration, enzyme concentration, pH and duration of incubation were independently varied.

Activity in tissues. The conditions described by Pugh, Leaback & Walker (1957a, b) were used. The substrate concentrations and periods of incubation were: phenyl *N*-acetyl- β -glucosaminide, 10 mM, 60 min.; *p*-nitrophenyl and *p*-acetylphenyl *N*-acetyl- β -glucosaminide, 3.6 mM, 20 min.

Estimation of liberated aglycone

Phenols. Phenol was estimated as described by Pugh *et al.* (1957b); *o*- and *p*-nitrophenol and *p*-acetylphenol were estimated directly in a Unicam SP. 500 spectrophotometer after stopping the enzyme reaction by the addition of 2.0 ml. of 0.25M-sodium carbonate. The wavelength used was that at which the anion of the phenol showed maximum absorption. The molecular extinction coefficients of the phenols under these conditions are shown in Table 1.

Table 1. Absorption characteristics of substituted phenols and the corresponding aryl *N*-acetyl- β -glucosaminides

Compound	Solvent	$\lambda_{\max.}$ (m μ)	$10^{-3} \times \epsilon_{\max.}$
<i>p</i> -Nitrophenol	0.1N-HCl	318	9.95
	Na ₂ CO ₃ *	400	18.10
	0.1N-NaOH	300	10.80
<i>p</i> -Nitrophenyl <i>N</i> -acetyl- β -glucosaminide	0.1N-HCl	280	6.56
	Na ₂ CO ₃ *	412	4.60
	0.1N-NaOH	260	3.34
<i>o</i> -Nitrophenyl <i>N</i> -acetyl- β -glucosaminide	0.1N-HCl	275	14.10
	Na ₂ CO ₃ *	324	22.9
	0.1N-NaOH	265	16.84

* 2.0 ml. of 0.25M-Na₂CO₃ was added to a solution of the phenol in 1.0 ml. of 0.05M-citrate buffer, pH 4.4.

The substrates in alkaline solution had absorption bands corresponding to those of the un-ionized phenol, and their extinction coefficients at λ_{max} of the phenol anion were so low that unhydrolysed substrate did not interfere in the spectrophotometric estimation of the phenol liberated in an enzyme assay (cf. Robinson, Smith, Spencer & Williams, 1952). After correcting for enzyme and substrate blanks, the amount of phenol liberated by the enzyme was read from a calibration curve constructed with a standard solution of phenol.

Ethanol. A modification of the method of Bücher & Redetski (1951) was used (cf. Kuhn & Tiedemann, 1954). The enzyme reaction was stopped by the addition of 3.0 ml. of pyrophosphate buffer, pH 9.2. Diphosphopyridine nucleotide (0.1 ml.) and 0.02 ml. of alcohol dehydrogenase were added and, after 1 hr. at room temperature, the tubes were centrifuged at 1500 *g* for 15 min. The above-mentioned steps were all carried out in the same stoppered tube. The absorption of the supernatant was measured at 340 *mμ* in a Unicam SP. 500 spectrophotometer. After correction for the enzyme and substrate blanks, the amount of ethanol liberated by the enzyme was read from a calibration curve constructed from a standard solution of ethanol. Calculation, by using the value 6.3×10^3 for ϵ of reduced diphosphopyridine nucleotide, showed that oxidation of ethanol was 90 % complete under these conditions. The calibration curve was reproducible, and ethanol incubated with the enzyme preparations in the absence of substrate was completely recovered.

RESULTS

Effect of pH

The effect of pH on the rate of hydrolysis of each substrate by *N*-acetyl- β -glucosaminidase from testis was determined over the range pH 3.0–5.6. A smooth curve with a single pH optimum was obtained in each case (Table 2). The optimum pH for the hydrolysis of *o*-nitrophenyl *N*-acetyl- β -glucosaminide was higher (4.8) than for the other substrates (4.4–4.5). Similar results were obtained with *o*- and *p*-nitrophenyl *N*-acetyl- β -glucosaminide with the enzyme from sweet-almond meal.

Table 2. *Optimum pH for N-acetyl- β -glucosaminidase*

Activity towards each substrate was determined for seven to ten values within the range pH 3.0–5.5 in 0.05M-citrate buffer. Period of incubation 30 min. For methods of assay see text.

Enzyme preparation	Substrate (<i>N</i> -acetyl- β -glucosaminide, mm)	Optimum pH
Testis	Phenyl, 10.0	4.40
	<i>o</i> -Nitrophenyl, 8.0	4.80
	<i>p</i> -Nitrophenyl, 3.6	4.45
	<i>p</i> -Acetylphenyl, 3.6	4.50
	Ethyl, 10.0	4.45
Sweet-almond meal	<i>o</i> -Nitrophenyl, 8.0	4.75
	<i>p</i> -Nitrophenyl, 3.6	4.40

The peaks of the curves obtained with this enzyme preparation were broader than those of the corresponding curves for the testis enzyme.

Effect of substrate concentration

The effect of various substrate concentrations on the reaction velocity in 0.05M-citrate buffer, pH 4.4, was studied. The results are shown in Table 3. Typical Michaelis–Menten curves for each substrate were obtained by plotting velocity of hydrolysis against substrate concentration. Values for the Michaelis constant (K_m) and theoretical maximum velocity of hydrolysis (V_{max}) were derived graphically by the method of Lineweaver & Burk (1934). Several preparations of each enzyme were used in the course of these experiments. To allow comparison of the results the activity of these preparations was estimated under standard conditions (for definition of units see above) and rates of hydrolysis in Table 3 are expressed as μ moles of substrate hydrolysed/hr./unit of enzyme. At the highest substrate concentrations used the reaction mixtures were approximately half-saturated with respect to glycoside, and the observed rates of hydrolysis were close to the theoretical maximum. There was no indication of inhibition by excess of substrate.

The value for the rate of hydrolysis of each substrate relative to phenyl *N*-acetyl- β -glucosaminide is shown. Ethyl *N*-acetyl- β -glucosaminide was hydrolysed much more slowly than the aryl substrate, whereas the effect of the ring substituent in the phenolic substrates was to increase the rate of hydrolysis.

The sensitivity of a method for the estimation of *N*-acetyl- β -glucosaminidase with one of these substrates depends not only on the rate of hydrolysis of the substrate but also on the extinction obtained per μ mole of liberated aglycone in the final spectrophotometric estimation (Table 1) and on the fraction of the incubation mixture available for this estimation (66 % for phenyl *N*-acetyl- β -glucosaminide but 100 % for the other substrates where no deproteinization is necessary before colour development). The estimation of *N*-acetyl- β -glucosaminidase with *p*-nitrophenyl *N*-acetyl- β -glucosaminide as substrate is some 12 times as sensitive as with the phenyl glycoside.

Competition between substrates

Although Neuberger & Pitt Rivers (1939) showed that *N*-acetyl- β -glucosaminidase hydrolysed both an alkyl and an aryl *N*-acetyl- β -glucosaminide, there is no evidence to show whether the same enzyme is responsible for both these activities. The effect of varied substrate concentrations on the rate of hydrolysis of three aryl *N*-acetyl- β -glucosaminides was studied in the presence and absence

Table 3. Activity of N-acetyl-β-glucosaminidase towards various substrates

Activity was measured at five concentrations of each substrate within the range indicated. For methods of assay, evaluation of K_m and V_{max} and definition of enzyme unit, see text. Relative rates of hydrolysis are referred to the value for phenyl N-acetyl-β-glucosaminide.

Enzyme preparation	...	Testis					Emulsin				
		Phenyl	o-Nitro-phenyl	p-Nitro-phenyl	p-Acetyl-phenyl	Ethyl	Phenyl	o-Nitro-phenyl	p-Nitro-phenyl	p-Acetyl-phenyl	
<i>N</i> -Acetyl-β-glucosaminide	...	6	3	4	4	7	4	3	5	3	
No. of experiments		1-10	0-3-10	0-1-4	0-3-7	1-50	1-10	0-2-10	0-1-4	0-1-7	
Range of substrate concentration (mM)		2.9±0.1	2.2±0.1	1.5±0.2	1.9±0.1	4.6±0.2	1.1±0.1	0.40±0.02	0.41±0.04	0.51±0.03	
K_m (mM ± s.e.)		1.31±0.17	2.60±0.12	3.45±0.10	3.23±0.12	0.13±0.02	1.15±0.11	2.25±0.21	2.55±0.32	2.34±0.10	
V_{max} (μmoles of substrate hydrolysed/unit of enzyme/hr. ± s.e.)											
Relative rate of hydrolysis	1		2.0	2.7	2.5	0.1	1	2.0	2.2	2.1	

of a fixed concentration of methyl or ethyl N-acetyl-β-glucosaminide. The results, plotted according to Lineweaver & Burk (1934), showed that hydrolysis of the aryl substrates was inhibited competitively by the alkyl compounds. The inhibitor constants (K_i) are shown in Table 4. K_i for ethyl N-acetyl-β-glucosaminide agrees well with its K_m as a substrate (Table 3). These results indicate that one enzyme is responsible for the hydrolysis of both aryl and alkyl N-acetyl-β-glucosaminides.

N-Acetyl-β-glucosaminidase in rat tissues

Dodgson & Spencer (1953) found that several aryl sulphates were unsuitable for the estimation of arylsulphatase in crude tissue preparations, because the phenol liberated by the enzyme underwent further metabolic changes which depended on the nature of the tissue, the substituent group of the phenol and the pH of the reaction mixture. N-Acetyl-β-glucosaminidase activities in various rat tissues were compared with phenyl, p-nitrophenyl and p-acetylphenyl N-acetyl-β-glucosaminide as substrates. Recoveries of the phenols added to tissue homogenates under the conditions of assay were 98-102 % complete. Table 5 shows that the relative amounts of substrate hydrolysed by each tissue were almost constant. Comparison of the columns in Table 5 shows that the relative activities of tissues measured with any one substrate were closely similar.

Table 4. Inhibition of hydrolysis of aryl N-acetyl-β-glucosaminides by alkyl N-acetyl-β-glucosaminides

Various concentrations of each substrate (for ranges see Table 3) were incubated with testis enzyme in the absence and in the presence of the indicated concentration of inhibitor. Each result is the mean of two experiments.

Substrate (N-acetyl-β-glucosaminide)	Inhibitor (N-acetyl-β-glucosaminide, mM)	K_i (mM)
Phenyl	Ethyl, 6.0	4.8
p-Nitrophenyl	Ethyl, 8.0	5.3
p-Acetylphenyl	Methyl, 14.2	6.7

Table 5. Activity of N-acetyl-β-glucosaminidase in rat-tissue homogenates estimated with various substrates

Substrates used were phenyl, p-nitrophenyl and p-acetylphenyl N-acetyl-β-glucosaminide. For conditions of incubation and methods of assay, see text. Results are expressed as μg. of phenol liberated/mg. of tissue (wet wt.)/hr.

	Phenol	p-Nitro-phenol	p-Acetyl-phenol
Kidney	26.9	108.0	98.5
Liver	6.9	23.8	19.4
Spleen	6.1	22.1	17.2
Testis	3.3	10.8	10.0

DISCUSSION

With a general method for the preparation of *N*-acetyl- β -glucosaminides available, the selection of substrates for examination was based on the work of Williams and co-workers (Spencer & Williams, 1951; Robinson *et al.* 1952) and Dodgson & Spencer (1953) on substrates for β -glucuronidase and arylsulphatase. The advantages in convenience and simplicity of basing the enzyme assay on the direct spectrophotometric estimation of the anionic form of the liberated phenol were demonstrated. The absorption spectra of the substituted phenols and of their glycosides showed that in alkaline solution the absorption of the conjugated phenol was negligible at $\lambda_{\max}$ for the phenol anion. Unhydrolysed substrate did not interfere in the estimation of liberated phenol, and substrate blanks in the assays were low. Dodgson & Spencer (1953) emphasized the additional advantage of using a phenol with anionic $\lambda_{\max} > 310 \text{ m}\mu$ so that absorption due to protein is small; a low enzyme blank is achieved without deproteinizing the reaction mixture.

N-Acetyl- β -glucosaminidase acted on alkyl and aryl glycosides, but differences were observed in the Michaelis constants and rates of hydrolysis of the substrates. If K_m is assumed to be an equilibrium constant and its reciprocal a measure of the affinity of enzyme for the substrate, then, in comparison with phenyl *N*-acetyl- β -glucosaminide, the *o*- and *p*-nitro and *p*-acetyl substituents in the aglycone produced similar increases in the rate of hydrolysis of the substrate and in its affinity for the enzyme. Analogous though larger effects on the activity of arylsulphatase towards the corresponding sulphates were observed by Dodgson, Spencer & Williams (1956). Nath & Rydon (1954) obtained similar results in comparing phenyl and *p*-nitrophenyl β -glucosides as substrates for β -glucosidase, but with *o*-nitrophenyl β -glucoside the increase in rate of hydrolysis was much greater. The lower affinity and rate of hydrolysis of ethyl as compared with phenyl *N*-acetyl- β -glucosaminide agrees with the results obtained with other glycosidases (Helferich, Lampert & Sparmberg, 1934). Further information about enzyme-substrate interaction and the nature of the active site is required in order to interpret these results in terms of a reaction mechanism for *N*-acetyl- β -glucosaminidase.

The differences between substrates are, however, relevant to the selection of a method for the estimation of *N*-acetyl- β -glucosaminidase; it was shown that the higher sensitivity and convenience

attainable in the spectrophotometric estimation of *p*-nitrophenol or *p*-acetylphenol make their glycosides the substrates of choice. Both phenols were recovered quantitatively after incubation at pH 4.4 with crude tissue preparations (cf. Dodgson & Spencer, 1953).

SUMMARY

1. The hydrolysis of ethyl, phenyl, *o*- and *p*-nitrophenyl and *p*-acetylphenyl *N*-acetyl- β -glucosaminide by preparations of *N*-acetyl- β -glucosaminidase from ram testis and sweet-almond meal has been investigated.
2. The same enzyme is responsible for the hydrolysis of alkyl and aryl *N*-acetyl- β -glucosaminides.
3. The effect of substituents in the benzene ring on K_m and $V_{\max}$ values was studied.
4. *p*-Nitrophenyl *N*-acetyl- β -glucosaminide has been selected as the most convenient and sensitive substrate for the estimation of *N*-acetyl- β -glucosaminidase in crude or purified preparations.

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REFERENCES

- Bücher, T. & Redetzki, H. (1951). *Klin. Wschr.* **29**, 156.
 Conchie, J., Findlay, J. & Levy, G. A. (1959). *Biochem. J.* **71**, 318.
 Dodgson, K. & Spencer, B. (1953). *Biochem. J.* **53**, 444.
 Dodgson, K., Spencer, B. & Williams, K. (1956). *Biochem. J.* **64**, 216.
 Helferich, B. & Iloff, A. (1933). *Hoppe-Seyl. Z.* **221**, 252.
 Helferich, B., Lampert, U. & Sparmberg, G. (1934). *Ber. dtsh. chem. Ges.* **67**, 1808.
 Kolthoff, I. M. & Bosch, W. (1927). *Biochem. Z.* **183**, 438.
 Kuhn, R. & Tiedemann, H. (1954). *Ber. dtsh. chem. Ges.* **87**, 1141.
 Leaback, D. H. & Walker, P. G. (1957). *J. chem. Soc.* p. 4754.
 Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
 Nath, R. L. & Rydon, H. N. (1954). *Biochem. J.* **57**, 1.
 Neuberger, A. & Pitt Rivers, R. U. (1939). *Biochem. J.* **33**, 1580.
 Pugh, D., Leaback, D. H. & Walker, P. G. (1957a). *Biochem. J.* **65**, 16p.
 Pugh, D., Leaback, D. H. & Walker, P. G. (1957b). *Biochem. J.* **65**, 464.
 Robinson, D., Smith, J. N., Spencer, B. & Williams, R. T. (1952). *Biochem. J.* **51**, 202.
 Spencer, B. & Williams, R. T. (1951). *Biochem. J.* **48**, 537.
 Watanabe, K. (1936). *J. Biochem., Tokyo*, **24**, 297.
 Westphal, O. & Schmidt, H. (1952). *Liebigs Ann.* **575**, 84.