3. $Na_2{}^{35}SO_4$ was injected intraperitoneally on the fifth day after carrageenin and animals were killed at several intervals of time afterwards. The highest specific activity found was in a neutral salt-soluble extract at 10 hr. after isotope administration. The activity of this fraction then fell rapidly with time. Sulphate extracted by papain digestion had a maximal specific activity about 30 hr. after isotope administration. Loss of activity in this fraction was less rapid than in the neutral salt-soluble extract. A residual sulphate fraction had low specific activities throughout and an indefinite half-life.

4. It is suggested that neutral salt (0·2 M-NaCl) extract of the granuloma tissue may contain a precursor sulphated polysaccharide. The very high rate of metabolism of this neutral-salt extract parallels that of neutral salt-soluble collagen and points to a close metabolic interrelationship of these two connective-tissue components.

I wish to thank Professor Kellgren for many stimulating discussions and never-failing encouragement during the course of this work, and also Drs D. S. Jackson and G. Williams for their support and collaboration throughout.

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Studies on Glucosaminidase

N-ACETYL-β-GLUCOSAMINIDASE IN RAT KIDNEY

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The occurrence of *N*-acetyl-β-glucosaminidase in mammalian tissues was described by Watanabe (1936*a*) and noted by Hahn (1945) and Roseman & Dorfman (1951). Watanabe (1936*b*) and East, Madinaveitia & Todd (1941) studied the properties of the enzyme in partially purified preparations from ox liver and testis.

Hahn (1945) and East *et al.* (1941) showed that *N*-acetyl-β-glucosaminidase and hyaluronidase, which are present together in crude testicular extracts, are different enzymes. Linker, Meyer & Weissmann (1955) have shown that the oligosaccharides formed from hyaluronic acid by hyaluronidase ('poly-*N*-acetyl-β-glucosaminidase') are further degraded stepwise by β-glucuronidase and *N*-acetyl-β-glucosaminidase. In view of the probable importance of *N*-acetyl-β-glucosaminidase in the biochemistry of mucopolysaccharides and in studies on the structure of derivatives of *N*-acetylglucosamine, further work on the preparation and properties of this enzyme seemed desirable. A preliminary survey of rat tissues showed that kidney was the most active source of the enzyme.

The kinetics of hydrolysis of phenyl *N*-acetyl-β-D-glucosaminide by a partially purified preparation of *N*-acetyl-β-glucosaminidase from rat kidney have been investigated and a study has been made of some factors which influence the activity of the enzyme.

METHODS

Preparation of substrate. Phenyl *N*-acetyl-β-D-glucosaminide was prepared by a modification of the method of Helferich & Iloff (1933).

Preparation of the enzyme. Fresh rat kidney was ground for 1 min. with water in a stainless-steel homogenizer. The homogenate (2%, w/v) was brought to pH 4·8 with citrate buffer (final concn. 0·1 M) and centrifuged for 15 min. at 1500 *g*. The supernatant was fractionated at 0–4° by addition of saturated ammonium sulphate. The fraction precipitated between 20 and 50% saturation was dissolved in water and dialysed overnight at 0–4°. After centrifuging to remove inactive, insoluble material, the preparation was diluted to the original volume of homogenate to give an enzyme concentration suitable for assay by the method described below. Such preparations contained about 70–80% of the activity of the homogenate. The activity fell about 10% on storage for 1 week at 0–4°.

Estimation of enzyme activity

This was carried out by a modification of the method of East *et al.* (1941). Tubes containing substrate (0·01 M), buffer (0·05 M citrate, pH 4·3) and enzyme (0·1 ml.) in a final volume of 1·0 ml. were incubated at 37° for 1 hr. Each

estimation was carried out in duplicate. Control tubes, in which enzyme and substrate respectively were omitted, were also incubated. The selection of these standard conditions for the estimation was based on the results of the experiments described below.

Estimation of liberated phenol. At the end of the incubation period, the reaction was stopped by the addition of 2·0 ml. of Folin–Ciocalteu reagent (British Drug Houses Ltd., diluted 1 in 3). After centrifuging to remove precipitated protein, 2·0 ml. of the supernatant was added to 3·0 ml. of Na_2CO_3 (12%, w/v). Colour was developed by incubation at 37° for 20 min. and the absorption at 650 mμ. was measured in a Unicam SP. 500 spectrophotometer. After correcting for the enzyme and substrate blanks, which were very low, the amount of phenol liberated by the enzyme was read from a calibration curve constructed with a standard solution of phenol. Any additional component included in the reaction mixture was tested to see that it did not affect the colour reaction for phenol. The enzyme concentration was such that the amount of substrate hydrolysed did not exceed 5% of that initially present.

Estimation of liberated N-*acetylglucosamine.* The addition of 0·1 ml. of 3N-Na_2CO_3 at the end of the incubation period brought the pH to 10·8 and stopped the enzyme reaction. The estimation of *N*-acetylglucosamine was completed as described by Aminoff, Morgan & Watkins (1952). The absorption was read at 560 mμ. and the amount of *N*-acetylglucosamine liberated by the enzyme was estimated from a standard curve made from a solution of *N*-acetylglucosamine in 0·05M citrate buffer, pH 4·3. The colour in the control tubes was very low and it was shown that the colour reaction for *N*-acetylglucosamine was not affected by the presence of either substrate or enzyme solution.

RESULTS

Nature of the enzyme reaction

The molar ratio of phenol to *N*-acetylglucosamine liberated on incubation of phenyl *N*-acetyl-β-glucosaminide with *N*-acetyl-β-glucosaminidase under the standard conditions described above was 0·99 ± 0·02 (mean ± S.E.) in three experiments, each carried out with a different preparation of the partially purified enzyme. Other experiments showed that under the same conditions neither phenol nor *N*-acetylglucosamine underwent any destruction on incubation with the enzyme. These results provide strong support for the assumption that the reaction involved is in fact a simple hydrolysis of the substrate.

Effect of pH

The effect of pH on the rate of hydrolysis of phenyl *N*-acetyl-β-glucosaminide by the enzyme was determined over the range pH 3–6·5 in 0·05M citrate buffer at a substrate concentration of 0·01M. Incubation and phenol estimation were carried out as described above. The results are shown in Fig. 1. A smooth curve with a single pH optimum at 4·3 was obtained. Watanabe (1936*b*) reported a pH optimum of about 4 for the enzyme in ox liver.

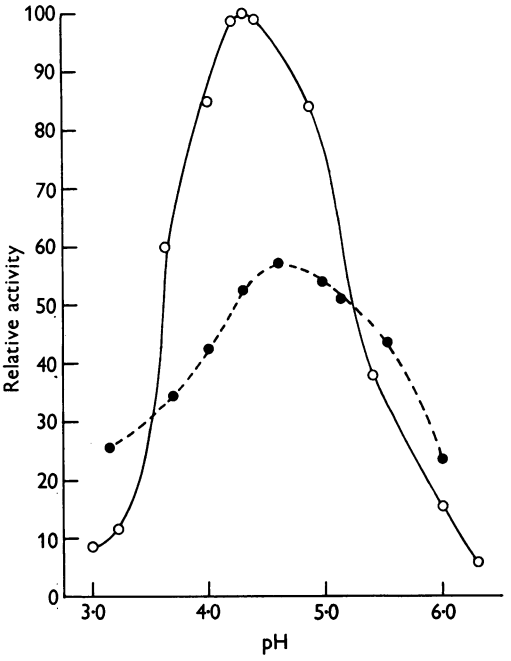

Fig. 1. Effect of pH on reaction velocity. Incubation in 0·05M citrate or acetate buffer for 1 hr. at 37°. Substrate: 0·01M phenyl *N*-acetyl-β-glucosaminide. ○, Citrate buffer; ●, acetate buffer.

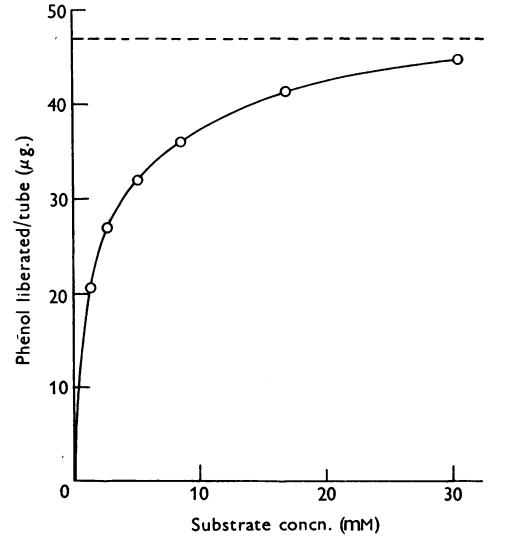

Fig. 2. Effect of varying substrate concentration on reaction velocity. The enzyme solution was incubated for 1 hr. at 37° in 0·05M citrate buffer, pH 4·3, with varying concentrations of phenyl *N*-acetyl-β-glucosaminide. Final vol. of reaction mixture was 1·0 ml. containing 0·1 ml. of enzyme solution. ○—○, Plot of reaction velocity against substrate concn.; – – – –, theoretical maximum velocity derived as described in the text.

Effect of substrate concentration

Fig. 2 shows the effect of varying substrate concentration on the reaction velocity in 0·05 M citrate buffer, pH 4·3. The rate of hydrolysis increased with increase in the substrate concentration, giving a typical Michaelis–Menten curve. A true maximum in the rate of hydrolysis was not obtained even at the highest substrate concentration used, but it is not possible to extend the curve further since the reaction mixture is almost saturated with substrate at a concentration of 0·03 M. The theoretical maximum velocity of hydrolysis at infinite substrate concentration, shown in Fig. 2, was found by plotting the results according to the equation derived by Lineweaver & Burk (1934):

$$1/v = 1/V_{max.} + K_m/V_{max.}S,$$

where S is the substrate concentration, v and $V_{max.}$ the observed and maximum velocity respectively and K_m the Michaelis constant (cf. Fig. 5). The mean value for K_m found in ten experiments was 1·78 mM phenyl N-acetyl-β-glucosaminide (Table 3), which is of the same order as the value of 3 mM found by Watanabe (1936*b*) for the enzyme in ox liver.

A substrate concentration close to the optimum cannot be conveniently employed in routine estimations owing to the limited solubility of the substrate. The value 0·01 M which was selected is arbitrary but, as can be seen from Fig. 2, it is high enough on the curve to ensure that substrate concentration is not a critical variable in the results obtained.

Effect of enzyme concentration

Fig. 3 shows that under standard conditions for the estimation of N-acetyl-β-glucosaminidase activity the amount of phenol liberated was proportional to the amount of enzyme present over an eightfold range of enzyme concentration. The amount of substrate hydrolysed by the highest concentration of enzyme was only 15% of that initially present and there was no indication of a decline in the rate of hydrolysis due to inhibition by reaction products.

Effect of time of incubation

The effect of varying the period of incubation on the hydrolysis of phenyl N-acetyl-β-glucosaminide, under conditions which were otherwise standard, is shown in Fig. 4. The rate of liberation of phenol was proportional to the time of incubation up to 1 hr. but after this it declined progressively. It is probable that this fall is due to instability of the enzyme on prolonged incubation, even in the presence of substrate, and not to either substrate exhaustion or inhibition by reaction products (cf. Fig. 3). In contrast to these results Watanabe (1936*b*) found that a partially purified enzyme preparation from ox liver showed no loss of activity on incubation for 10 hr. even though 87% of the substrate initially present was split.

Stability of the enzyme

The partially purified preparation of N-acetylglucosaminidase from rat kidney was comparatively stable to dialysis and subsequent storage at 0–5° (see above). At 37° in 0·05 M citrate buffer, pH 4·3, however, the enzyme was unstable in the absence of

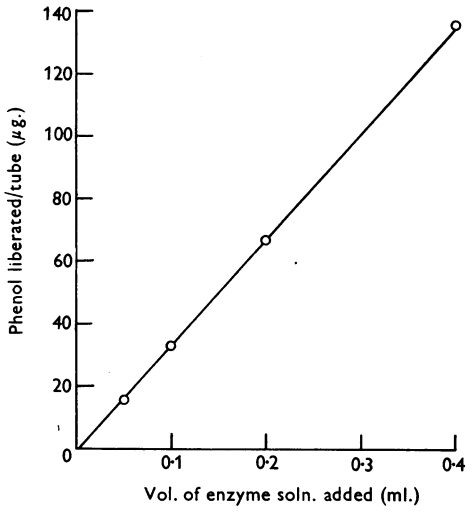

Fig. 3. Effect of enzyme concentration on reaction velocity. Varying amounts of enzyme solution were incubated in 0·05 M citrate buffer, pH 4·3, for 1 hr. at 37°. Substrate: 0·01 M phenyl N-acetyl-β-glucosaminide; final vol. of reaction mixture was 1·0 ml.

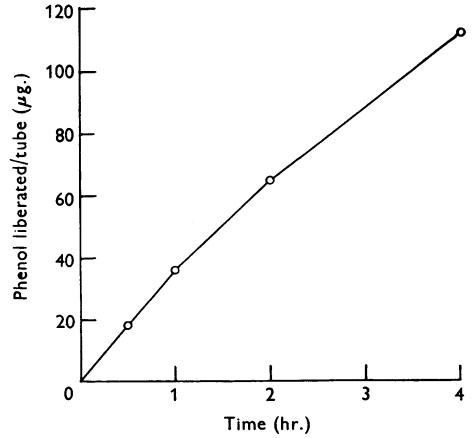

Fig. 4. Effect of time of incubation on degree of hydrolysis. The reaction was allowed to proceed for varying periods at 37° in 0·05 M citrate buffer, pH 4·3. The substrate was 0·01 M phenyl N-acetyl-β-glucosaminide.

substrate, the activity falling to 50% of its initial value in about 90 min. (Table 1). The stability of the enzyme in acetate buffer of the same molarity and pH was much greater. This difference in the effect of the two buffers on the stability of the enzyme was also observed with crude tissue homogenates and is almost certainly due to combination between the enzyme and acetate, which results in stabilization of the enzyme in the absence of substrate and inhibition of its activity in the presence of substrate (see below).

Effect of various substances on the activity of N-acetyl-β-glucosaminidase

The following substances, present in the assay mixture at a final concentration of 0·01 M, had no effect on the enzyme activity: NaCl, KCl, $MgCl_2$, NaF, KCN, ethylenediaminetetraacetic acid, glucosamine hydrochloride.

Activity in acetate and other buffers

Acetate, which was the buffer employed by East *et al.* (1941) and Hahn (1945) in their work on β-glucosaminidase, was also used in the early stages of this work. In acetate buffer the pH optimum of the enzyme from rat kidney was 4·6 (Fig. 1), in agreement with the value found by East *et al.* (1941) for a partially purified preparation from ox testis. The substrate concentration curve in acetate buffer was abnormal. Enzyme activity increased irregularly with increasing substrate concentration and showed no sign of flattening off even at the highest attainable substrate concentration. It was then found that the activity of the enzyme was considerably higher in citrate buffer than in equimolar acetate of the same pH, and that in citrate the activity was independent of the molarity of the buffer, whereas in acetate activity fell progressively with increasing acetate concentration (Table 2). The effect of other buffers, at two different molarities, on the activity of the enzyme was also tested. The results (Table 2) show that at a final concentration of 0·1 M the activity in formate, propionate, lactate and succinate was 80–100% of the activity in citrate. A fourfold reduction in the molarity of the buffer resulted in a 10–15% fall in the activity of the enzyme in each case.

Table 1. *Stability of* N-*acetyl-β-glucosaminidase at* pH 4·3 *and* 37°

Activity was measured after pre-incubation in 0·05 M buffer solution for varying periods. Results are expressed as percentage residual activity.

Period of pre-incubation (hr.)	Residual activity (%)	
	Citrate buffer	Acetate buffer
1	59	91
2	39	88
4	33	87

Table 2. *Effect of nature of buffer and concentration of buffer on* N-*acetyl-β-glucosaminidase activity*

Incubation lasted 1 hr. at 37°, pH 4·3. The substrate was 0·01 M phenyl *N*-acetyl-β-glucosaminide. Results are expressed as percentage of the activity in 0·1 M citrate buffer.

Buffer	Activity (%)			
	0·1 M	0·05 M	0·025 M	0·01 M
Citrate	100	100	99	95
Acetate	44	51	59	65
Formate	97	—	85	—
Propionate	82	—	75	—
Succinate	99	—	83	—
Lactate	91	—	78	—

Inhibition by acetate, acetamide and N-*acetylglucosamine*

It seemed possible that the inhibition of the enzyme produced by acetate might be competitive. Fig. 5 shows the results of an experiment in which the effect of varying substrate concentration on the activity in citrate buffer was tested in the presence and absence of a fixed concentration of acetate, the final pH being the same in both cases. The results, plotted according to Lineweaver & Burk (1934), show that acetate behaves as a typical competitive inhibitor.

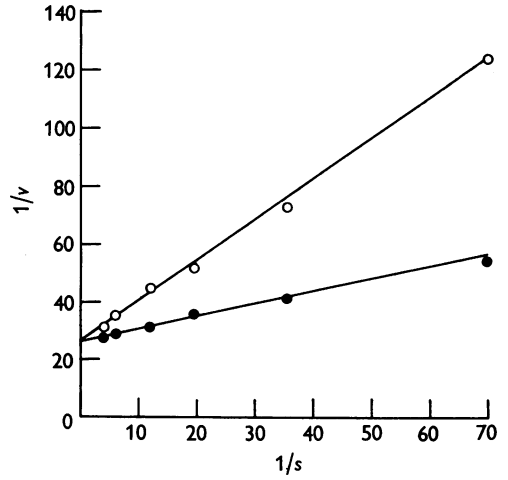

Fig. 5. Effect of varying substrate concentration on the rate of hydrolysis of phenyl *N*-acetyl-β-glucosaminide in the presence (○), and the absence (●), of 0·045 M acetate buffer, pH 4·3. Incubated for 1 hr. at 37° in 0·05 M citrate buffer, pH 4·3. Results are plotted according to Lineweaver & Burk (1934); *s*, 10(substrate concn.) (M); *v*, mg. of phenol liberated/tube.

Watanabe (1936*b*) showed that *N*-acetyl-β-glucosaminidase was inhibited by its reaction product, *N*-acetylglucosamine. This has been confirmed in the present work and it was found that the inhibition was competitive, as might be expected. Since acetate and *N*-acetylglucosamine had been found to inhibit competitively, it seemed likely, on structural grounds, that acetamide would behave similarly, and this was confirmed by experiment. Table 3 summarizes the results obtained. The values of K_m and K_i, which are assumed to measure the dissociation constants of the enzyme–substrate and enzyme–inhibitor complexes, were evaluated graphically according to Lineweaver & Burk (1934). The figures for the apparent relative affinities of the enzyme for the substrate and for the three inhibitors are derived from the reciprocals of the dissociation constants.

In addition to the theoretical assumptions implicit in the derivation of these figures, there are other considerations which have been left out of account. The mutarotation of *N*-acetylglucosamine, which is present in solution as a mixture of α- and β-forms, has been studied only in water at 20° (Kuhn & Haber, 1953). At pH 4·3 and 37° no difference between the inhibitory powers of a freshly prepared and an aged solution of *N*-acetylglucosamine could be demonstrated, even in experiments with an incubation period as short as 10 min. Nevertheless, it is probable that each of the anomers has a different affinity for the enzyme. With acetate the direction and amount of the change in the pH optimum from pH 4·3 in citrate to 4·6 in acetate is of the order predicted from the assumption that inhibition is due to un-ionized acetic acid and not to the acetate ion.

DISCUSSION

The results described above have extended the early work of Watanabe (1936*a*, *b*) on the properties of *N*-acetyl-β-glucosaminidase in mammalian tissues. The partially purified enzyme preparation from rat kidney showed no evidence of the presence of more than one active component. It was stable to dialysis, showed no activation or inhibition by common ions and in citrate buffer the kinetics of hydrolysis of phenyl *N*-acetylglucosaminide by the enzyme were simple.

In testicular extracts, β-glucosaminidase has been distinguished from β-glucuronidase (Hahn, 1945), hyaluronidase (Hahn, 1945; East *et al.* 1941) and α-glucosaminidase (Roseman & Dorfman, 1951), and from β-glucosidase in emulsin (Helferich & Iloff, 1933) and in extracts of snail digestive tract (Neuberger & Pitt Rivers, 1939). The specificity of the mammalian enzyme has not been studied. With the snail enzyme, Neuberger & Pitt Rivers (1939) showed that the requirements for hydrolysis were that the substrate should be a β-glucosaminide with an *N*-acetyl or *N*-formyl group on $C_{(2)}$ and with the hydroxyl groups on $C_{(3)}$, $C_{(4)}$ and $C_{(6)}$ unsubstituted. The inhibition of *N*-acetyl-β-glucosaminidase by acetate has not hitherto been described. We have confirmed that this inhibition can also be shown in a testicular enzyme preparation similar to that used by East *et al.* (1941). Although the results of Neuberger & Pitt Rivers (1939) do not suggest any inhibition by acetate of the snail enzyme, it is interesting to note that Roseman & Dorfman (1951) found that the activity of *N*-acetyl-α-glucosaminidase was greater in phosphate–citrate buffer than in acetate. Veibel & Lillelund (1940) observed that the rate of hydrolysis of *o*-cresol glucoside by β-glucosidase was about 10% less in acetate than in citrate buffer. However, a normal type of substrate-concentration curve was obtained in acetate and the lower activity was explained by the relatively greater affinity of enzyme for its reaction products in this buffer. Although inhibition due to such a mechanism would probably appear to be competitive in the type of analysis carried out by us, it seems much more likely that the marked inhibition of *N*-acetyl-β-glucosaminidase by acetate is due to a direct interaction of the enzyme with acetate to form an inactive complex. The increasing apparent relative affinity of the enzyme for acetate, acetamide and *N*-acetylglucosamine supports this interpretation, as does the greater stability of the enzyme incubated in the absence of substrate in acetate as compared with citrate buffer.

The conditions selected for the assay of the enzyme in partially purified preparations have been found suitable for use with unfractionated tissue homogenates, and further work on the specificity and distribution of the enzyme is being carried out.

Table 3. *Apparent affinity of inhibitors for* N-*acetyl-β-glucosaminidase compared with that of phenyl* N-*acetyl-β-glucosaminide*

Compound	Apparent dissociation constant* (mM)	Apparent relative affinity
Phenyl *N*-acetyl-β-glucosaminide	1·78±0·10 (10)	100
N-Acetylglucosamine	4·40±0·66 (3)	41
Acetamide	10·2±0·35 (3)	18
Acetate (pH 4·3)	17·0±2·2 (3)	11

* Mean±S.E. and, in parentheses, number of experiments.

SUMMARY

1. The hydrolysis of phenyl *N*-acetyl-β-D-glucosaminide by *N*-acetyl-β-glucosaminidase in a partially purified extract of rat kidney has been

investigated, and conditions for the estimation of the enzyme have been established.

2. In 0·05M citrate buffer, the enzyme shows optimum activity at pH 4·3. The optimum substrate concentration cannot be attained owing to the limited solubility of the substrate.

3. *N*-Acetyl-β-glucosaminidase is inhibited competitively by *N*-acetylglucosamine, acetamide and acetate.

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The Action of 2:4-Dinitrophenol on Myosin and Mitochondrial Adenosine Triphosphatase Systems

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Mitochondria freshly isolated from liver show low or negligible adenosine triphosphatase (ATPase) activity towards added substrate, but on addition of low concentrations of 2:4-dinitrophenol (DNP), adenosine triphosphate (ATP) is hydrolysed at a rapid rate (Hunter, 1951; cf. Lardy, 1945). Similar, though perhaps less striking, effects have been obtained with mitochondria isolated from skeletal muscle (Chappell & Perry, 1954). The precise nature of this effect of DNP is not understood, although, significantly, it is accompanied by the loss of oxidative phosphorylating activity (Loomis & Lipmann, 1948; Cross, Taggart, Covo & Green, 1949) and by the inability of ATP to reverse the swelling induced in mitochondria of pigeon breast muscle by hypotonic media (Chappell & Perry, 1954). In isolated mitochondria the action of DNP is complex and may involve, amongst other things, effects on the permeability of the membrane; but it appears from the work of Lardy & Wellman (1953) on the ATPase activity of aqueous extracts of mitochondrial acetone-dried powders that DNP has a specific stimulatory effect on the ATPase enzyme itself. Furthermore, the observation that DNP stimulates the ATPase activity of myosin (Webster, 1953) suggests that DNP can have an effect on the mechanism of ATP hydrolysis when it is catalysed by other enzymic systems.

In previous studies on the metabolism of ATP by intracellular components of muscle (Chappell, 1954; Chappell & Perry, 1953, 1954) we found that under the ionic conditions in which DNP stimulated the enzymic activity of L-myosin (Weber & Portzehl, 1952), 0·1–5 mM DNP failed to stimulate the myofibrillar ATPase activity of rabbit myofibrils. The present communication is concerned with a general investigation of the effects of DNP on the ATPase activity both of myosin and isolated myofibrils and shows that the presence of actin modifies the action of DNP on the enzymic activity of myosin. The response of the latter system to DNP has been compared with that of the mitochondrial ATPase.

Certain aspects of this work have already been reported (Chappell & Perry, 1955). In an associated communication, Greville & Needham (1955) reported similar findings on the DNP stimulation of L-myosin ATPase activity.

METHODS

Myofibrils. Myofibrils were prepared in a medium containing 0·039M borate buffer, pH 7·1, and 0·025M-KCl (borate–KCl) as previously described (Perry & Grey, 1956). Stock preparations were stored at 0°, and for enzyme experiments they were used within 10–12 days, diluted with borate–KCl to give suspensions containing 5–7 mg. of protein/ml.

Myofibrillar proteins. L-Myosin was prepared by a method described elsewhere (Perry, 1955) which combines the experience of a number of workers. The method of Guba & Szent-Györgyi (1945) was used for the preparation of G-actin solutions. G-Actin was purified by isoelectric