THE ACTION OF NINHYDRIN ON THE ENZYMATIC AND ACTIN-COMBINING PROPERTIES OF MYOSIN

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[*Manuscript received April* 4, 1949]

Summary

Ninhydrin at 5 μM per mg. myosin produces 90 per cent. inhibition of myosin-ATP-ase at pH 9.0 in 3 hours at 5°C. At pH 7.0 the same inhibition is produced by 35 μM ninhydrin per mg. myosin.

This inhibition is reversed by cysteine, the release being complete after one hour's treatment of the myosin by ninhydrin, but incomplete, except with very high cysteine concentrations, after three hours' treatment of the myosin. Other amino acids did not release the inhibition produced by ninhydrin.

The addition of actin to myosin treated with ninhydrin at concentrations which produced over 90 per cent. inhibition of the ATP-ase resulted in a rise in viscosity similar to that encountered with untreated myosin. The addition of ATP to ninhydrin-treated actomyosin, however, usually caused a precipitation of the actomyosin rather than a dissociation into myosin and actin.

The removal of -SH groups of the myosin by ninhydrin was demonstrated by the disappearance of the nitroprusside reaction, but attempts to titrate -SH quantitatively in the presence of ninhydrin were unsuccessful.

I. Introduction

The association of the muscle proteins myosin and actin to form a complex characterized by high and anomalous viscosity has been studied by Szent-Györgyi and his colleagues (for reviews see Szent-Györgyi 1945, 1947). The addition of small amounts of adenosine triphosphate (ATP) to the actomyosin complex dissolved in 0.5M KCl has been shown to result in a drop in viscosity due to dissociation into the myosin and actin components. Recently Bailey and Perry (1947) have demonstrated that the presence of -SH groups on the myosin is necessary for the combination of myosin with actin, and that the same -SH groups are connected with the adenosine triphosphatase activity. Using ninhydrin as an -SH reagent, we have been able to demonstrate an inhibition of enzyme activity at concentrations which do not prevent the interaction of myosin and actin.

There is some evidence in the literature that ninhydrin has a strong affinity for -SH groups. Brückmann and Wertheimer (1947) have shown that both ninhydrin and the structurally related alloxan cause the disappearance of reduced glutathione from the blood of rodents. Alloxan is a recognized -SH reagent, and has been used to inhibit a number of enzymes (Lehmann 1939; Hopkins, Morgan, and Lutwak-Mann 1938; Naganna and Narayana Menon 1948; Walsh

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and Walsh 1948). Lieben and Edel (1932) suggested an interaction between alloxan and thiol groups, and Lazarow, Patterson, and Levey (1948), and Patterson, Lazarow, and Levey (1949) have shown that alloxan oxidizes cysteine and apparently forms an addition compound with the -SH of glutathione and protein. Recently Griffiths (1949) has shown that both alloxan and ninhydrin inhibit muscle hexokinase, and that this inhibition is released completely in the case of alloxan, partially in the case of ninhydrin, by cysteine. Zittle (1948) has demonstrated inhibition of ribonucleinase by ninhydrin (90 per cent. at 0.01M), but ascribes the effect to a reaction between ninhydrin and the amino groups of the enzyme.

II. Methods

(*a*) *Preparation of Myosin, Actin, and ATP*

Crystalline myosin, prepared from rabbit muscle by the method of Szent-Györgyi (1945), was used in most of the enzyme tests and all the viscosity measurements. For some enzyme tests actomyosin, thrice precipitated by the method of Bailey (1942), was used. Glass-distilled water only was used in all enzyme preparations. Actin was prepared by the method of Straub (1943). ATP was isolated as the dibarium salt by Kerr's (1941) method, either from fresh rabbit muscle or from a dehydrated powder prepared from sheep muscle by the method of Szent-Györgyi (1947). Before use it was purified and converted to the sodium salt by being passed through a column of Amberlite IR-100, by the method recommended by Polis and Meyerhof (1947).

(*b*) *Determination of Phosphorus and Nitrogen*

Inorganic phosphorus was measured by the method of Fiske and Subbarow (1925). Nitrogen was determined by nesslerization after sulphuric acid digestion, using the reagent of Vanselow (1940). Weight of protein was calculated from the nitrogen estimations by using Bailey's (1942) figure for the nitrogen content of myosin, and Straub's (1943) figure for the nitrogen content of actin.

(*c*) *Enzyme Tests*

For tests of ATP-ase activity myosin was dissolved in 0.02M veronal-HCl buffer containing 0.5M KCl, of pH either 9.0 or 7.0. Neither glycine nor borate buffers could be used in inhibition studies with ninhydrin, as they reacted with the inhibitor. In one series of tests in which Na_2CO_3-$NaHCO_3$ buffer was used, results obtained were similar to those with veronal-HCl buffer, but the ATP-ase activity was lower. Enzyme activity was measured at 37°C. The test mixture usually contained 3 ml. of a myosin solution containing 0.12-0.43 mg. protein per ml., 1.5 ml. of an ATP solution containing about 0.28 mg. 7 min. P per ml., and 0.15 ml. of 0.1M $CaCl_2$. Samples of 1 ml. volume for the estimation of inorganic P were withdrawn at intervals, usually at 5, 10, and 30 minutes after the commencement of incubation. At least three phosphate determinations were made for every enzyme test, although comparisons of activity were all made on the basis of the inorganic P produced in five minutes. In all tests the concentration of myosin was the rate-determining factor, and was such that usually about half

the ATP available was decomposed in five minutes. Myosin was fairly unstable at 37°C. in veronal buffer, particularly at the higher pH. In our experiments all the available ATP was never broken down; the curve of inorganic P production flattened out after 80-90 per cent. of the ATP had been used up, and at this time it was usually seen that the myosin had precipitated out. Unlike Mommaerts and Seraidarian (1947), however, we obtained higher activities at pH 9.0 than at pH 7.0, although the difference was not great.

The Q_P (cf. Bailey 1942) of crystalline myosin in our preparations varied from 800 to 2400, and that of actomyosin, prepared according to Bailey, varied from 400 to 1000. We confirmed the finding of Polis and Meyerhof (1947) that fresh myosin preparations tested against purified ATP showed no enhanced activity in glycine buffer, and no activation by cysteine. A stock solution of myosin (about 10 mg. protein per ml.) kept a few days at 5°C., however, lost some activity, which could be restored by cysteine. This loss of activity was hastened in dilute solutions. Myosin at 0.25 mg. per ml. stored at 5°C. lost up to 30 per cent. of its activity in 24 hours.

Ninhydrin, cysteine, and other agents to be added to the enzyme were dissolved immediately before use in 0.02M veronal buffer containing 0.5M KCl, the solutions being adjusted to the appropriate pH, 7.0 or 9.0, before being mixed with the enzyme solution. In testing the release of inhibition, ninhydrin was first allowed to act on the enzyme for varying lengths of time, after which the agent to be tested for release was added to the enzyme-ninhydrin mixture and allowed to act for approximately as long as the ninhydrin had acted alone.

(*d*) *Viscosity Determinations*

Viscosities were measured in Ostwald viscometers equilibrated in a water-bath at 7°C. Since the thixotropic property of myosin causes a gradual decrease in the time of outflow with successive readings, the first reading was used in the calculation of viscosities. The figures for specific viscosity quoted must thus be regarded as approximations only. However, since the changes we were concerned with were of considerable magnitude, any uncertainties due to the limitations of the Ostwald technique did not affect our conclusions.

The viscosities of a myosin solution and of the same myosin solution treated with actin were measured simultaneously in two viscometers. The actomyosin solutions were prepared by mixing 0.5-1.1 ml. of a solution containing 2.7 mg. of actin per ml. with 10 ml. of various myosin solutions containing 0.35-0.70 mg. per ml., the final ratio of myosin to actin being about 5:2 by weight in each instance. The effect of ATP on the viscosity of actomyosin was then measured after the addition, by means of a capillary pipette, of a small amount of ATP solution (0.3 ml., 0.2 mg. 7 min. P per 10 ml. actomyosin solution) to the viscometer containing actomyosin.

III. Inhibition of the ATP-ase

Inhibition of ninhydrin was studied first at pH 9.0. It was found that the inhibition was established rather slowly at 5°C. (Fig. 1). After this time relation had been established, inhibition studies were made after pretreatment with

ninhydrin for three hours at 5°C. The relation between concentration of inhibitor

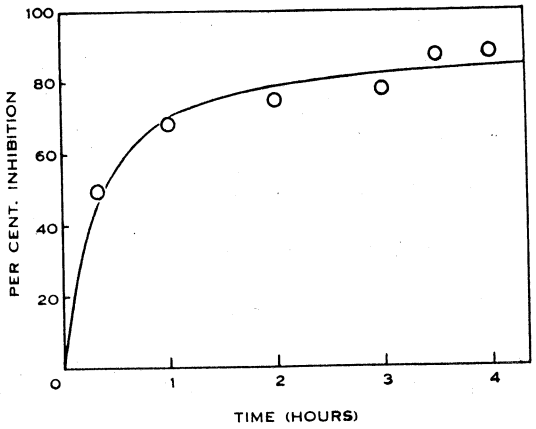

Fig. 1.—Time curve of ninhydrin inhibition of myosin-ATP-ase at pH 9.0. Ninhydrin concentration 0.001M (4.5 μM per mg. myosin).

and degree of inhibition is illustrated in Figure 2. At pH 9.0 ninhydrin inhibited myosin-ATP-ase at quite high dilutions (90 per cent. at 5 μM ninhydrin per mg. myosin, or 0.001M ninhydrin, at usual myosin concentrations). Since at a lower pH myosin was more stable and viscosity tests were more conveniently carried out, inhibition of ATP-ase was also studied at pH 7.0. At this pH about seven times the concentration of ninhydrin was needed to produce the same inhibition, as shown in Figure 2.

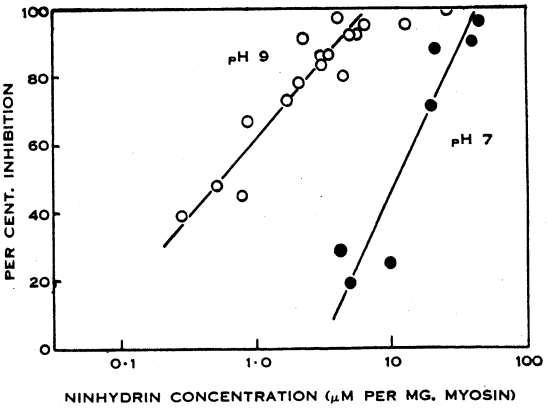

Fig. 2.—Inhibition of myosin-ATP-ase by ninhydrin at pH 7.0 and pH 9.0. All points are derived from experiments in which the ninhydrin had been allowed to act on the enzyme for three or more hours at 5°C.

Cysteine released the inhibition produced by ninhydrin, but complete release was not usually obtained when the ninhydrin had been allowed to act on the myosin for three hours. Figure 3 shows the release of inhibition at pH 9.0 plotted

against relative cysteine concentration, the higher curve being derived from experiments in which cysteine was added after the inhibitor had acted for one hour at 5°C., the lower from experiments in which the inhibitor had acted for three hours at 5°C. Complete release of inhibition after three hours' treatment with ninhydrin was obtained in one experiment when the concentration of cysteine was ten times that of the ninhydrin. The experiments from which Figure 3 is derived were all done with concentrations of ninhydrin of 3-5 μM per mg. myosin. In two experiments in which ninhydrin at 26 μM per mg. was allowed to act on myosin for three hours at pH 9.0, cysteine at four times the concentration of the ninhydrin produced only a 25 per cent. release of inhibition.

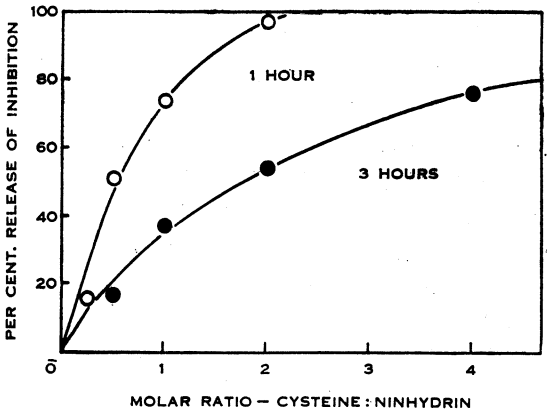

Fig. 3.—Release of ninhydrin inhibition of myosin-ATP-ase at pH 9.0. The upper curve is derived from experiments in which the ninhydrin had been allowed to act on the myosin for one hour at 5°C., the lower from those in which the ninhydrin had been allowed to act for three hours at 5°C. Ninhydrin concentrations in these experiments varied between 3 and 5 μM/mg. myosin, producing an inhibition of 77-88 per cent. after one hour and 83-92 per cent. after three hours. Each point is derived from at least three individual experiments.

Release of inhibition at pH 7.0, although not studied in as much detail, was apparently quite similar to that illustrated for pH 9.0.

Neither glycine at four times the concentration of the ninhydrin nor alanine at the same or ten times the concentration of the ninhydrin released the inhibition of ATP-ase produced by three hours' treatment by ninhydrin. Thioglycollic acid did release the inhibition, but was less effective than cysteine (50 per cent. release by a concentration four times that of the ninhydrin, after treatment with ninhydrin for three hours at pH 9.0). The addition of alanine at ten times the ninhydrin concentration to either cysteine or thioglycollic acid at four times the ninhydrin concentration did not improve the release of inhibition obtained by either cysteine or thioglycollic acid alone.

IV. Effect of Ninhydrin on Actomyosin Formation

In studying the viscosity of myosin solutions it is usual to use concentrations of about 2 mg. of protein per ml. At such high concentrations, however, the treatment of the myosin with ninhydrin at equivalently high concentrations caused profound changes in the physical properties of the protein. The solution became opaque, then set to a stiff gel, which gradually changed to an intense blue colour. With lower absolute concentrations of myosin and ninhydrin, the same relative concentration being maintained, these changes did not occur, and viscosities could be measured readily in the Ostwald apparatus, the viscosity of ninhydrin-treated myosin being very nearly the same as that of untreated myosin.

Table 1

EFFECT OF NINHYDRIN ON THE FORMATION AND DISSOCIATION OF ACTOMYOSIN AT pH 7.0*

Myosin Concentration (mg./ml.)	Treatment	Specific Viscosity			
		Myosin Alone	Myosin + Actin	Myosin + Actin + ATP	
0.37	Untreated	1.07	1.28	2 min.	1.09
	Ninhydrin at 45 μM/mg. myosin†	1.05	1.32	2 min.	1.21
				15 min.	1.12
0.50	Untreated	1.10	1.51	2 min.	1.14
	Ninhydrin at 45 μM/mg. myosin	1.06	1.64	2 min.	1.43
				15 min.	1.35
0.74	Untreated	1.17	1.83	2 min.	1.26
	Ninhydrin at 45 μM/mg. myosin	1.23	1.49	‡	

* In all experiments ninhydrin had been allowed to act on the myosin for three or more hours before measurement of the viscosity.

† An enzyme test of this sample revealed 96 per cent. inhibition of the ATP-ase.

‡ Actomyosin precipitated on addition of ATP.

In Tables 1 and 2 are shown the results of viscosity measurements at pH 7.0 and pH 9.0 respectively. It can be seen that the treatment of myosin with ninhydrin in concentrations which produced over 90 per cent. inhibition of the enzyme activity had relatively little effect on the combination of myosin with actin, as demonstrated by the rise in viscosity of myosin solutions on the addition of actin. The relative instability of the ninhydrin-treated actomyosin solutions, however, was demonstrated by their behaviour in the presence of ATP. The addition of ATP to untreated actomyosin resulted in a rapid fall in viscosity to a figure approximating that of the original myosin solution. The addition of ATP to ninhydrin-treated actomyosin at pH 9.0, however, caused the precipitation of the actomyosin. In fact, at the highest concentration recorded, 0.70 mg. per ml., the actomyosin precipitated soon after the addition of actin to the

myosin, before the ATP could be added.* At pH 7.0 and the lower myosin concentrations, the addition of ATP to ninhydrin-treated actomyosin resulted in a drop in viscosity, but the fall was a slow one. Even after 15 minutes the viscosity was still falling slowly, and had not yet reached the level attained by the controls at the first reading after the addition of ATP, i.e. at about two minutes.† This is illustrated in Table 1, where the viscosities of ninhydrin-treated actomyosins at 2 and 15 minutes after the addition of ATP are compared with those of control actomyosins at two minutes after the addition of ATP. Finally,

TABLE 2

EFFECT OF NINHYDRIN ON THE FORMATION AND DISSOCIATION OF ACTOMYOSIN AT pH 9.0*

Myosin Concentration (mg./ml.)	Treatment	Specific Viscosity		
		Myosin Alone	Myosin + Actin	Myosin + Actin + ATP
0.35	Untreated	1.07	1.27	1.07
	Ninhydrin at 5.7 μM/mg. myosin†	1.05	1.20	‡
0.46	Untreated	1.10	1.39	1.12
	Ninhydrin at 5.4 μM/mg. myosin	1.09	1.28	‡
0.70	Untreated	1.16	1.75	1.21
	Ninhydrin at 5.7 μM/mg. myosin	1.10	1.57**	–

* In all experiments ninhydrin had been allowed to act on the myosin for three or more hours before measurement of the viscosity.

† An enzyme test of this sample revealed 92 per cent. inhibition of the ATP-ase.

‡ Actomyosin precipitated on addition of ATP.

** Actomyosin precipitated soon after addition of actin.

at the highest myosin concentration studied at pH 7.0, the addition of ATP caused precipitation, as it had done at the higher pH. It is possible that the drop in viscosity recorded on the addition of ATP to ninhydrin-treated actomyosin at the lower concentrations at pH 7.0 is not a measure of the dissociation of myosin and actin, but represents merely an early stage of the process of aggregation which at higher concentrations is manifested as visible precipitation. When precipitation did not occur immediately it was preceded in our experiments by a drop in viscosity.

* Nitrogen estimations on the precipitated material and the supernatant solution indicated that both myosin and actin must have contributed to the formation of the precipitate, a fact which supports the conclusion that the combination to form actomyosin had preceded precipitation.

† This fall in viscosity with successive readings was much greater in magnitude than the slight successive falls in viscosity due to the thixotropic effect.

V. Direct Estimation of -SH Groups

Myosin treated with urea at a concentration of 1 mg. per ml. gave a positive reaction with nitroprusside. The nitroprusside colour could be eliminated by previous treatment of the myosin by ninhydrin, the concentration of ninhydrin required being lower at pH 9.0 than at pH 7.0 (e.g. the colour developed with nitroprusside was about half as strong as the control in myosin treated with ninhydrin at 0.07 μM per mg. at pH 9.0 or 1.4 μM per mg. at pH 7.0). Attempts to estimate -SH quantitatively in the presence of ninhydrin (by *o*-iodosobenzoate or ferricyanide titrations) failed. It is possible that either ninhydrin formed an addition compound which could still be oxidized by the reagents employed, or that reduced ninhydrin was itself oxidized.

VI. Discussion

Although quantitative data on the elimination of -SH by ninhydrin have not been obtained, the indirect evidence that a reaction with thiol groups occurs is quite strong. It comprises in the first place the reversal of ninhydrin inhibition of ATP-ase by cysteine and not by other amino acids, and secondly, the elimination of the nitroprusside colour. This conclusion is further supported by the analogy with alloxan and other evidence from the literature cited in the introduction. At the same time, ninhydrin is known to deaminate and decarboxylate amino acids (Ruhemann 1911), and the occurrence of a reaction of this type with myosin may be reflected in the lower effectiveness of cysteine in releasing the inhibition produced by prolonged treatment with ninhydrin or by higher ninhydrin concentrations. If this view is correct, we may conclude that ninhydrin reacts most rapidly and at the lowest concentrations with -SH, this reaction being much more complete at the higher pH, and that the combination with other groups is slower. As might be expected, the secondary inhibition, due presumably to the reaction with amino and carboxyl groups, is irreversible.

Finally, at quite high ninhydrin concentrations (> 0.02M at pH 9.0), the physical properties of myosin are affected. The formation of a gel* and development of blue colour are macroscopic evidences of changes in the protein molecule. They parallel closely the clotting and colouration of fibrinogen by ninhydrin, described by Chargaff and Bendich (1943) and ascribed by them to the oxidation of aminoacyl groups of the protein.

Although ninhydrin strongly inhibits the ATP-ase activity of myosin, and this inhibition can most reasonably be ascribed to the elimination of the -SH groups, it has little or no effect on the ability of myosin to combine with actin to produce a complex having a high viscosity. The rise in viscosity on the addition of actin to ninhydrin-treated myosin duplicates almost exactly actomyosin formation with untreated myosin, and it would be difficult to believe that the same phenomenon was not involved in both cases. Our results, however, can not easily be reconciled with those of Bailey and Perry. It is to be noted that Bailey and Perry measured only the viscosity of actomyosin solutions before and

* The consistency of this gel was not affected by the addition of ATP.

after the addition of ATP, and based their calculations of inhibition of actomyosin formation on these figures, whereas we measured also the rise in viscosity on the addition of actin to myosin solutions. It is conceivable that myosin and actin may combine through some groups other than the -SH of myosin, but since intact -SH groups are needed for the attachment of ATP, the displacement of actin by ATP would not be effected in the presence of -SH inhibitors. Evidence against this, however, is to be found in the statement by Bailey and Perry that in their experiments the viscosities of untreated and inhibited actomyosins in the presence of ATP did not differ significantly. Mommaerts (1948) has studied the reaction between ATP and actomyosin in detail, and has concluded that the effect of ATP on the physical behaviour of myosin is entirely independent of the ATP-ase activity, although the experiments on which this conclusion is based are rather uncertain. In view of this conflict in the data it seems that the conclusion of Bailey and Perry that "the viscosity decrease of actomyosin is related to -SH groups and to no other factor" is perhaps an over-simplification.

We have already indicated that the fall in viscosity on the addition of ATP to more dilute solutions of ninhydrin-treated actomyosin at pH 7.0 is probably not an indication of dissociation, but rather of the early stages of precipitation. ATP normally causes "super-precipitation" of actomyosin at KCl concentrations between 0.02M and 0.16M at pH 7.0 (Szent-Györgyi 1947), so that a ninhydrin-treated actomyosin in 0.5M KCl is behaving similarly to an untreated actomyosin at lower KCl concentrations, which may indicate that treatment with ninhydrin has reduced the K^+ combining power of the myosin. Since actin also reduces the K^+ combining power of myosin, precipitation following the addition of actin (Table 2) could also be explained in this way.

VII. Acknowledgments

The work described in this paper was carried out as part of the research programme of the Division of Economic Entomology, C.S.I.R. The authors are indebted to Mr. D. McIntosh for skilful technical assistance.

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