Creatine Kinase Isoenzymes and the Role of Thiol Groups in the Enzymic Mechanism*

B. T. Hooton

ABSTRACT: Creatine kinase has been prepared from chicken hearts and is the form (enzyme III) electrophoretically characteristic of the brain. It was unnecessary to include mercaptoethanol in the preparation, and the pure enzyme was perfectly stable over several months. The enzyme appears as two bands, running close together on starch gel electrophoresis. The slower of the two bands was isolated and showed no tendency to interconvert with the faster band. In 8 M urea–0.05 M cysteine the double-banded enzyme dissociated into two subunits of mol wt 3.9×10^4. The purified single-band enzyme migrated as a single species on 8 M urea gels whereas the double-band form of the enzyme appeared to be composed of two protein species. Two active-site thiols per molecular weight of 80,000 and nine total thiols were measured by dithionitrobenzoic acid. For isoenzyme I (the enzyme characteristic of skeletal muscle) two active-site thiols and seven total thiols were detected. Unfolding of the enzymes I and III revealed marked differences in stability. Guanidine-HCl (0.5 M) exposed all thiols in enzyme III where 1 M guanidine-HCl was needed to unfold enzyme I completely. The inhibition of chicken enzyme I by iodoacetamide occurred at the same rate and to the same extent, approaching 100% inhibition, as is observed with rabbit creatine kinase I. Enzyme III lost only 70–75% of its activity after exposure to 100-fold M excess of iodoacetate and iodoacetamide. Dithionitrobenzoic acid gave similar inhibition. Iodomethane was also used and gave only partial inhibition. Complete inhibition by iodoacetamide was achieved only by reaction in 0.5 M guanidine-HCl when all thiols are blocked. Using [α-^{14}C]iodoacetamide, high-voltage electrophoresis of labeled tryptic peptides showed that to extend inhibition to 84% extensive blocking had occurred at buried sites leading to five major peptides being labeled with radioactivity. The mobility of enzyme III inhibited by iodoacetamide showed a slight decrease in mobility consistent with the fully blocked enzyme being partially active. It is concluded that the so-called essential thiols of creatine kinase are not directly involved in the catalytic mechanism but are probably involved in maintaining the geometry of the active-site region. Also, in enzyme III either the thiols are not so important in maintaining structure or precise conformation is not so critical as in the other isoenzymes.

The number of enzymes found to be composed of two or more noncovalently linked units increases steadily (Klotz, 1967), and creatine kinase has proved to be no exception. In 1963 Eppenberger *et al.* discovered three electrophoretically different forms of creatine kinase, and by hybridization of the two extreme forms the intermediate type appears (Dawson *et al.*, 1965), indicating that in the simplest case there are two subunits giving rise, by random combination, to three possible forms of enzyme. All three types have a molecular weight of 80,000 but differ in their kinetic behavior toward high and low concentrations of creatine phosphate and ADP[1] and have different Michaelis constants and markedly different peptide maps (Dawson *et al.*, 1965, 1967; Eppenberger *et al.*, 1967a). The intermediate form (enzyme II) was also intermediate in its kinetic properties and amino acid content. Despite the considerable data concerning these enzymes their metabolic or physiological significance is without an explanation, unlike the lactic dehydrogenase isoenzymes whose varying sensitivity to inhibition by pyruvate serves to fit them for different roles in anaerobic or aerobic muscle (Di Sabato and Kaplan, 1964).

Creatine kinase has two relatively reactive thiols which are associated with the enzymic activity in that their reaction with a number of thiol reagents leads to inhibition (Mahowald and Kuby, 1960; Mahowald *et al.*, 1962a). Dawson *et al.* (1967), while examining the chemical properties of creatine kinase isoenzymes I and III from rabbit and chicken tissues, found that all the enzymes behaved normally, giving extrapolated values of two thiols per mole of enzyme and complete inhibition except for the chicken enzyme III which gave only partial inhibition (70%). They suggested that this represented the reaction of only one thiol per enzyme molecule. The present work has confirmed the observation and examined in detail the nature of this anomalous behavior for the isoenzyme III from chicken hearts.

A multitude of enzymes are known to contain cysteine residues which are associated in some way with the function of the active site although the nature of their involvement is most often unknown. Before implicating

* From the Medical Research Council Research Group in the Structure and Biosynthesis of Macromolecules, The Department of Biochemistry, The University, Newcastle upon Tyne, England. *Received January 3, 1968.*

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); $IAcNH_2$, iodoacetamide.

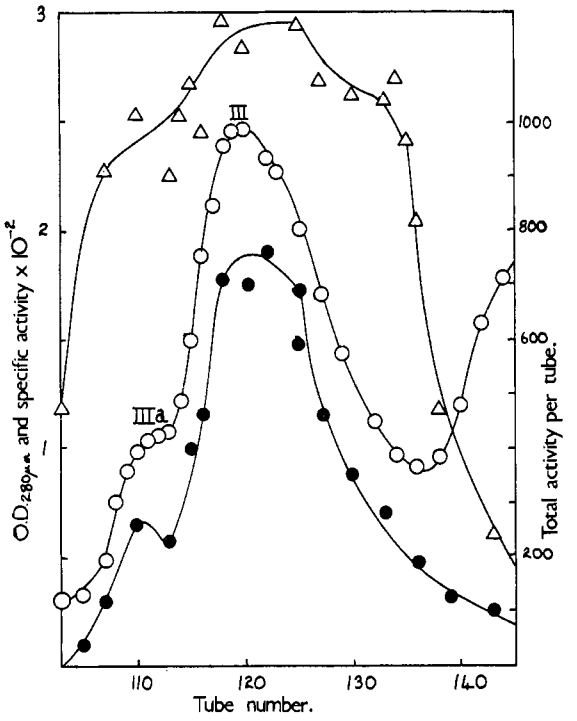

FIGURE 1: Elution profiles of the two bands of creatine kinase activity III. Column conditions are given in the text. Absorption at 280 mμ (○). Total activity per tube, μequiv of H^+/min per 10 ml (●). Specific activity, μequiv of H^+/min per $OD_{280m\mu}$ unit (△).

amino acid side chains in catalytic mechanisms, it is as well to consider first that their function may well be only auxiliary (Koshland, 1960). The two thiols of creatine kinase have been incorporated as an integral part of the mechanism of action (Watts and Rabin, 1962) but the evidence presented here tends to argue against this, namely, the thiols appear rather to be involved in some indirect way with the enzyme function.

Experimental Section

Materials. Urea was recrystallized from methanol according to Martin and Frazier (1963) with the addition of 1 mM EDTA at pH 7.0. Guanidine hydrochloride (obtained from B.D.H. Ltd., Poole, Dorset) was recrystallized from 1 mM EDTA three times. Both urea and guanidine hydrochloride were taken to dryness at 35° *in vacuo*. Iodoacetic acid was recrystallized from ether–hexane and used immediately (Noltmann *et al.*, 1962). Iodoacetamide was recrystallized twice as described (Watts *et al.*, 1961).

Preparation of Creatine Kinase I. In this paper creatine kinase isoenzymes are given the suffixes I, II, and III in order of increasing electrophoretic mobility on starch gel at pH 7.0 or 8.5, enzyme I being the slowest and enzyme III the fastest. Enzymes I, II, and III correspond to MM, MB, and BB, respectively (Dawson *et al.*, 1967). The preparative procedures adopted here are modifications of the procedure described earlier (Hooton and Watts, 1966) involving ethanol precipitation steps (Kuby *et al.*, 1954).

Enzyme I. Chicken breast muscle (800 g) was quickly removed from four chickens and homogenized in 3 l. of Tris-HCl buffer (pH 8.1, 20°), ionic strength 10^{-2}, containing 1 mM EDTA. The extract was centrifuged at 2000*g* for 60 min and the supernatant was concentrated to 500 ml by pressure dialysis using 5 ft of $^{3}/_{32}$ in. dialysis tubing. Any precipitate was removed by centrifugation at 5000*g* and the supernatant was made 0.07 M with respect to $MgCl_2$ by adding 1 M $MgCl_2$ (pH 9.0). At 0°, the extract was brought to 40% ethanol by adding ethanol redistilled from KOH. After standing for 30 min the precipitate was collected by centrifugation at 5000*g* and extracted with 100 ml of Tris-HCl buffer (pH 8.1). This extraction together with the 40% supernatant (dialyzed free of ethanol) was concentrated by pressure dialysis and loaded onto a column of DE-52 cellulose (Whatman) (2.3 × 43 cm) at pH 8.1. This amounted to about 1 g of creatine kinase. The enzyme was eluted by a linear NaCl gradient, started immediately, beginning at an ionic strength of 10^{-2}, increasing to 10^{-1} over 2 l. of buffer. The flow rate was 50 ml/hr and 10-ml fractions were collected. These were assayed using the pH-Stat procedure (Hooton and Watts, 1966) except that 1 mM cysteine was incorporated into the assay in place of mercaptoacetate. The fractions showing the highest specific activity were pooled, concentrated, and if necessary rerun on a similar column with a shallower gradient until the specific activity approached or exceeded 200 μmoles of H^+/min per mg of protein in the standard assay conditions of ATP (4 mM), $MgSO_4$ (4 mM), creatine (40 mM), and cysteine, (1 mM) at 37° (pH 9.0). The preparation was stored at a concentration of 20–30 mg/ml at 2° without deterioration for several months; 600–700 mg of enzyme I can be obtained by this method. The enzyme behaved in many respects like rabbit and mouse enzyme I.

Enzyme III. Chicken hearts (2500 g) (obtained from Northumbrian Chicken Farms Co. Ltd., Ponteland, Northumberland) less aorta and fat tissue were finely chopped, homogenized in 2 l. of 0.01 M KCl–1 mM EDTA, and centrifuged at 2000*g*. The sediment was reextracted with 2 l. of solution and centrifuged. The combined supernatants were made 0.07 M $MgCl_2$ by the addition of 1 M $MgCl_2$ (pH 9.0) and brought to 36% (v/v) ethanol at 0°. After standing at 0° for 60 min the precipitate was centrifuged at 2000*g* and the supernatant was taken up to 68.5% ethanol. The 68.5% ethanol precipitate was centrifuged, thoroughly extracted twice with 500-ml lots of Tris-HCl buffer (pH 8.6), and centrifuged at 5000*g* for 10 min. The combined extracts were dialyzed against two changes of 5 l. of H_2O. The dialyzed protein solution was taken up to 36% ethanol, the precipitate was discarded, and the supernatant was taken up to 68.5% ethanol. The precipitate was centrifuged at 2000*g* and thoroughly extracted with Tris-HCl buffer (pH 8.6).

The extract of the 68.5% ethanol precipitate was concentrated by pressure dialysis and dialyzed against Tris-HCl buffer (pH 7.8), ionic strength 10^{-2}. This was loaded onto a column (2.3 × 43 cm) packed with DE-52 cellulose equilibrated at pH 7.8 with Tris-HCl buffer. The enzyme was eluted with a linear NaCl gradient

from 0.01 to 0.3 ionic strength over 2 l. of eluent with a flow rate of 40 ml/hr. This procedure gives two enzyme activity peaks (Figure 1) corresponding to two close-running bands of activity on starch gel analysis; the peak or shoulder appearing before the main peak of activity was pooled separately and proved to be essentially free of the faster band. About 100 mg of this enzyme was obtained with a specific activity of 180 μmoles of H^+/min per mg; the preparation is denoted as enzyme IIIa. The main activity peak was pooled and yielded about 500 mg of enzyme with a specific activity of 230 μmoles of H^+/min per mg.

No requirement for added mercaptoethanol as a protection agent was found during the isolation, in contrast to earlier work (Eppenberger *et al.*, 1967a,b). The purified enzymes III and IIIa were stable over periods exceeding 6 months at concentrations around 7 mg/ml at 2° in pH 7.8 Tris-HCl buffer, in contrast to previous experience for type III enzyme from ox brain (Wood, 1963). It should be mentioned that the hearts used in this work contained almost exclusively isoenzyme III with trace amounts of enzyme I, with no intermediate form in evidence. The chickens were 60 days old when killed and the unusual enzyme pattern may be a result of the manner in which the birds are reared. Although enzyme III from heart muscle coincided in electrophoretic mobility with that from chicken brain, except in the double-band nature of the heart enzyme, there is no evidence proving that these enzymes are in fact identical and not the product of different cistrons. The differences in stability could be due to a slight structural dissimilarity.

Purity and Properties. Protein concentrations were measured by the biuret procedure (Gornall *et al.*, 1949). Enzyme preparations I, III, and IIIa migrated as well-defined protein bands on starch gel electrophoresis at pH 8.5 (Poulik, 1957) and 7.0 (Fine *er al.*, 1964); enzyme III appeared as two protein bands, close together after staining with Amido Black. No impurities were detected. In the ultracentrifuge (Model E) enzyme III sedimented as a single, sharp peak at a concentration of 7.5 mg/ml in pH 7.75, 0.078 M phosphate buffer containing 0.15 M KCl. The sedimentation coefficient extrapolated to 5.6 S at zero protein concentration. The molecular weight of enzyme III was measured in the presence and absence of 8 M urea using short-column (0.8 mm) equilibrium sedimentation. In pH 7.75, 0.078 M phosphate–0.15 M KCl a figure of 8.4×10^4 was obtained at zero concentration using method III of Van Holde and Baldwin (1958). After prolonged dialysis against 8 M urea–0.05 M cysteine (pH 8.0) the molecular weight was 3.9×10^4, in agreement with previous work (Dawson *et al.*, 1967; Yue *et al.*, 1967), indicating a dissociation into two subunits of equal molecular weight.

Electrophoresis Patterns and Hybridization. Creatine kinase activity bands were revealed after starch gel electrophoresis by a modification of the staining technique of Fine *et al.* (1963) as adapted for creatine kinase by Dawson *et al.* (1965). This involved slicing the gel, run at pH 8.5 or 7.0 in the usual manner, carefully blotting the surface free of excess liquid, and placing it in close contact with a piece of blotted Whatman No. 3MM paper previously soaked in the reaction mixture (0.2 ml of 0.1 M glucose, 0.2 ml of 0.1 M $MgCl_2$, 2 ml of Tris-HCl buffer (0.1 M pH 7.5), 1 mg of hexokinase, 1 mg of glucose 6-phosphate dehydrogenase, 3.6 mg of phosphocreatine disodium salt hydrate, 2.4 mg of ADP, 2.8 mg of NADP, 3 mg of nitro blue tetrazolium chloride, and 0.01 mg of phenazine methosulfate); the gel and paper, between two Perspex sheets, were then incubated at 37° for 15 min in the dark. This procedure gave sharper bands than those given by incubating the gel in a bath of reaction mixture, needed smaller quantities of reagents, and gave, after drying of the paper, a permanent record of the run. Myokinase is detected by omitting phosphocreatine from the incubation mixture and staining the other half of the gel.

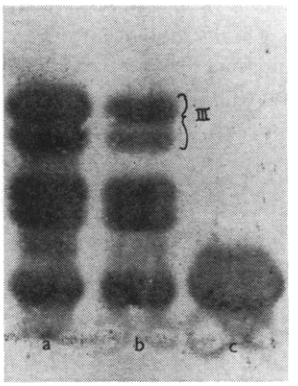

PLATE 1: Hybridization of chicken creatine kinase I and III. (a) Solution (0.3 ml) containing 2.8 mg of enzyme I and 1.5 mg of III dialyzed against 25 ml of 5 M guanidine hydrochloride–0.05 M cysteine (pH 8.7) for 3 hr at 2° and followed by dialysis overnight against 110 ml of 0.05 M cysteine (pH 8.7). (b) Conditions as for part a but native enzyme III replaced by enzyme which had been blocked by a 25-fold M excess of iodoacetamide for 2.5 hr at 25° when 26% of the initial activity remained. (c) Enzyme I. The gel was run at pH 7.0 for 6 hr at 12 mA.

Rat and pig tissues exhibited the three characteristic activity bands of creatine kinase, but only enzymes I and III were found in chicken heart, muscle, and brain. However, by hybridization enzyme II could be formed (Plate la). In calf heart enzymes I, II, and III had higher mobilities than the chicken enzymes, and, in addition, two extra bands were apparent, one moving 1 cm toward the negative electrode and another close to, and ahead of, isoenzyme I.

Enzyme III from chicken heart always appeared as two bands of activity corresponding to two protein bands of the purified enzyme (Plate la). The pattern was independent of whether the hearts were extracted immediately after death or after freezing and thawing. The same enzyme from fresh chicken brain appeared, however, as one band equal in mobility to the faster of the two bands from heart muscle. During a purification of enzyme III from chicken heart the slower moving of the two bands was isolated in an almost pure form from the other (Figure 1). With storage over several months there was no tendency for two bands to appear, and on dissociation and reassociation in guanidine hydrochloride only one band was evident.

The dissociation of enzyme IIIa was investigated on

TABLE I: The Thiol Content of Chicken Enzymes I and III and Apparent Second-Order Velocity Constants for the Reaction with $IAcNH_2$.[a]

Expt	Type of Measurement	SH Content: Enzyme I	SH Content: Enzyme III
1a	Reactive SH by DTNB		1.9
1a	Total SH by DTNB in 5 M guanidine-HCl		8.7
1b	Reactive SH by DTNB	1.6	1.6
1b	Total SH by DTNB in 5 M guanidine-HCl	6.9	8.7
1b	Reactive SH by Ag-AgI electrode	1.6	1.4
		App Second-Order Velocity Constant ($l./mole^{-1}\ min^{-1}$)	
1b	By Ag-AgI electrode	600	590

[a] For the DTNB estimation of reactive thiols the conditions were: 0.1 ml of Tris-HCl buffer (pH 8.0) (1.65 M Tris), between 1 and 3 mg of enzyme, and 0.05 ml of a 4-mg/ml DTNB solution (pH 7.0), all in a volume of 3 ml. The reaction was started by adding the DTNB last and the absorption at 420 mμ read after approximately 1 min when the reaction had ceased. To measure the total thiol content, 2.1 ml of 7.14 M guanidine-HCl was added to the above. Conditions for silver–silver iodide estimation of reactive thiols were 0.02% Tween-80, KI 10^{-6} M, Tris-HCl buffer (pH 8.0), ionic strength 10^{-2}, and iodoacetamide 4.44×10^{-4} M. The reaction was started by adding iodoacetamide; enzyme I, 4.44×10^{-6} M, and enzyme III, 4.63×10^{-6} M. For the determination of the velocity constants iodoacetamide was 4.44×10^{-5} M and the reaction was followed for 10% of the reaction when only 2% of the iodoacetamide will have been used.

TABLE II: Extent of Inhibition of Chicken Enzymes I and III by a Variety of Inhibitors.[a]

Inhibitor	Creatine Kinase Act. Remaining (% of initial): Enzyme I	Enzyme IIIa	Enzyme III
$IAcNH_2$ (after 21 hr)			25 (25)
$IAcNH_2$	1.3 (100)	20 (81)	24.7 (100)
Iodoacetate		22 (100)	
DTNB	10 (50)		23 (50)
Iodomethane	8 (500)		32 (500)

[a] For the iodoacetamide and iodoacetate inhibition enzyme III (7 mg/ml), enzyme IIIa (4 mg/ml), and enzyme I (10 mg/ml) were incubated at pH 8.45 Tris-HCl buffer with varying concentrations of inhibitor at 25° (molar ratios of inhibitor to protein concentration are given in parentheses after the figure for percentage activity remaining). The reaction was begun by addition of the inhibitor and 0.02-ml samples were removed at the times indicated (Figure 2), diluted (50–100 times) in 4 mM cysteine (pH 8.5) at 0°, and assayed immediately under standard conditions (see text). The values given are those observed after 120-min incubation which did not decrease after much longer inhibition times. For DTNB inhibition 3×10^{-6} M enzyme was incubated under the conditions described (Table I) for 10 min at 25°. The conditions for iodomethane inhibition are given under Figure 3.

8 M urea–starch gels. The gels were made up in 0.06 M glycine buffer (pH 8.85) and 8 M urea using 12.5 g of starch/60 ml of buffer. After setting, the gel was equilibrated overnight with a urea–glycine solution containing 0.1 M mercaptoethanol. The protein solutions previously equilibrated with the urea–glycine–mercaptoethanol solution were loaded onto the gel with No. 3MM paper and run for 4 hr at 10-mA constant current with a gel of dimensions $20 \times 5 \times 0.7$ cm. Protein bands were stained with Amido Black. Under these conditions two bands were clearly apparent for isoenzyme III moving closely together, and for enzyme IIIa only one protein species could be detected. Enzyme I also moved as a single band.

Sixty per cent of enzyme III activity was usually recovered after dissociation by dialyzing 0.2 ml of enzyme solution (7.5 mg/ml) against 5 M guanidine-HCl or 8 M urea, containing 0.5 M cysteine (pH 8.7), for 3 hr at 2° followed by reassociation by dialysis against 500 ml of 0.05 M cysteine (pH 8.7) overnight at 2°. By mixing enzymes I and III and following the above procedure, an intermediate electrophoretic band (enzyme II) appeared on the gel stain (Plate Ia), always in the form of two bands similar to enzyme III. This intermediate form of creatine kinase was never found in fresh chicken tissues.

Thiol Content of Enzymes I and III. The thiol content of enzymes I and III was measured in the presence and absence of 5 M guanidine hydrochloride using DTNB (Ellman, 1959; Hooton and Watts, 1966). In addition, the reactive thiols associated with catalytic activity (Benesch *et al.*, 1955; Mahowald and Kuby, 1960) were measured by reaction with iodoacetamide using the silver–silver iodide electrode technique (Watts *et al.*, 1961) involving continuous recording of the production of iodide ions by monitoring the change in potential of the electrode with an EIL Vibron electrometer (Model 33B-2) with a type C33B-2 pH measuring unit coupled to a recorder. Table I lists the amounts found. Table I also includes apparent second-order velocity constants from the reaction with iodoacetamide which fall between the values obtained for rabbit and mouse enzyme I (Hooton and Watts, 1966). An attempt was made to utilize the silver–silver iodide setup for measuring iodide production in the presence of guanidine hydro-

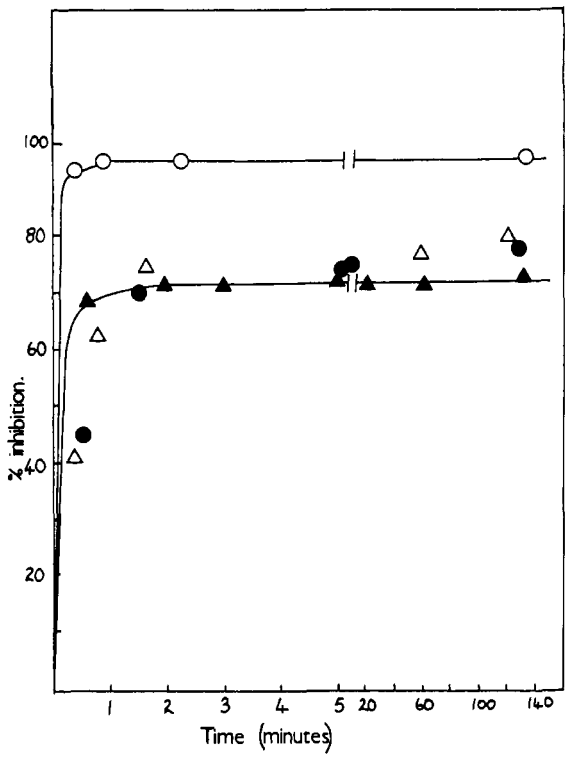

FIGURE 2: The inhibition by iodoacetamide and iodoacetate of enzymes III, IIIa, and I. The conditions and methods of measurement are described in the footnote to Table II. All curves are for a molar ratio of inhibitor to protein of 100. (○) Enzyme I with $IAcNH_2$. (△) Enzyme IIIa with $IAcNH_2$. (▲) Enzyme III with $IAcNH_2$. (●) Enzyme IIIa with iodoacetate.

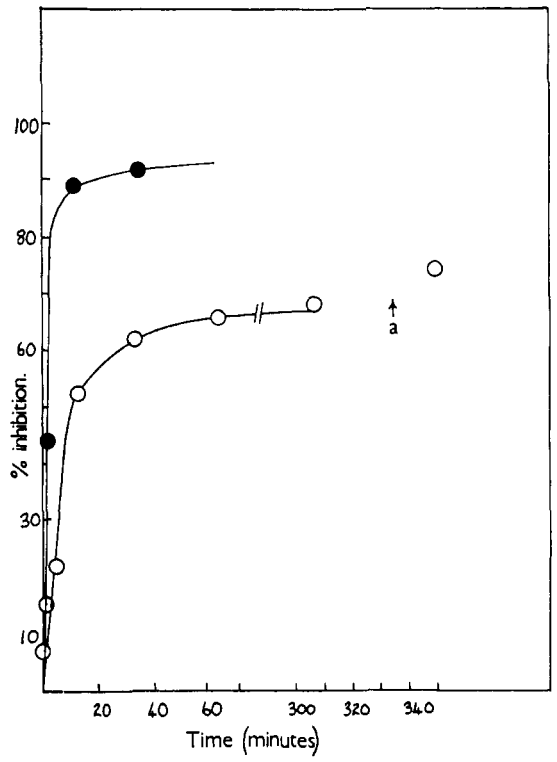

FIGURE 3: The inhibition of enzymes I and III by iodomethane. For enzyme I (●), the conditions were: 1.1 ml of incubation mixture containing 2.6 mg of protein/ml, Tris-HCl buffer (pH 8.45), ionic strength 2×10^{-2}, and a 560 M excess of inhibitor. The reaction was started by the addition of inhibitor and 0.02-ml samples were taken at intervals and diluted 100 times in 50 mM cysteine (pH 8.5); the remaining activity was assayed immediately. For enzyme III (○), the conditions were as above but for protein, 0.83 mg/ml, and 500-fold M excess of inhibitor. For enzyme III (○), after 335 min (a) a 100-fold M excess of iodoacetamide was added.

chloride on reaction of iodoacetamide with protein thiols. This was found to be impracticable due to rapid drifting in electrode potential and slow response to iodide concentration changes at concentrations of guanidine-HCl as low as 0.2 M.

Inhibition by Reaction of the Active-Site Thiols. The inactivation of enzymes I and III by a variety of thiol reagents was measured by following the loss of enzyme activity. The reaction with iodoacetamide and iodoacetate has been well investigated (Watts *et al.*, 1961; Mahowald *et al.*, 1962a), and the results obtained here are shown in Table II and Figure 2. The activity of enzyme III which was stable to iodoacetamide had no ATPase activity, and the percentage of the forward reaction (synthesis of phosphocreatine) occurring at pH 7.0 compared with that at pH 9.0 was 64.5% for the native enzyme and 48% for the inhibited enzyme. Michaelis constants were measured for the inhibited and native enzymes under conditions previously employed (Hooton and Watts, 1966). This involved consideration only of the total nucleotide concentration and neglecting the small error due to the difference between the concentration of the real substrate, metal–nucleotide complex, and the total nucleotide concentration. Since constants were the same for both enzymes, the error involved will be equal in both. For creatine the K_m was 8.6×10^{-3} M for both activities and for ATP values of 0.98×10^{-4} M (native) and 1.02×10^{-4} M (inhibited) were obtained.

Iodomethane, which to the author's knowledge has not been used as an inhibitor of creatine kinase, was found to be an effective thiol reagent, although higher concentrations were necessary corresponding to the lower reactivity of iodomethane. The extent of inhibition was much the same as for that with iodoacetamide (Table II, Figure 3).

Enzymes I and III were also treated with DTNB and assayed (Table II). Although the assay mixture contains 1 mM cysteine there was no tendency for the rate of acid production to increase with time, indicating the reduction of the disulfide bond between the protein and the *p*-nitrobenzoic acid moiety which does occur with 0.1 M cysteine at pH 8.6, giving full recovery of activity.

Inhibition in Guanidine-HCl. The reaction of DTNB with enzymes I and III was studied in increasing concentrations of guanidine hydrochloride (Figure 4), final readings being taken at 420 mμ after 20 min when the reaction was complete. Above 0.5 M guanidine all the thiols of enzyme III were reactive, but 1 M guanidine was required to reveal all the thiols of enzyme I.

Since approximately seven thiols appear to be buried within the protein of the folded enzyme III, the fully reacted protein may be incapable of forming its native tertiary structure sufficiently to allow the two subunits to combine as guanidine-HCl is dialyzed away. To test

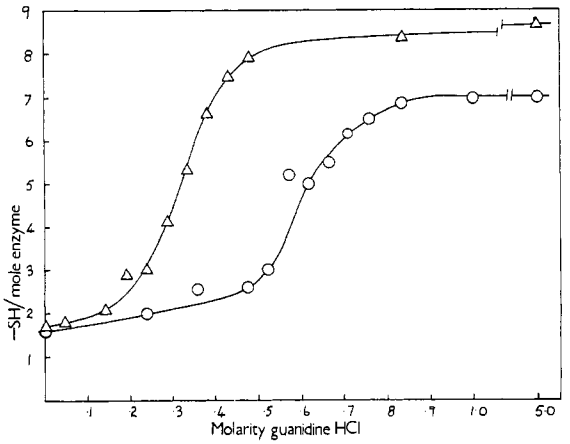

FIGURE 4: The unfolding of enzymes I and III. The conditions for the DTNB estimation were those used in Table I. The guanidine-HCl concentration was varied with 7.14 M guanidine-HCl and the absorption at 420 mμ was read 20 min after adding the DTNB. Enzyme concentrations were: III, 0.172 mg/ml (△), and I, 0.192 mg/ml (○).

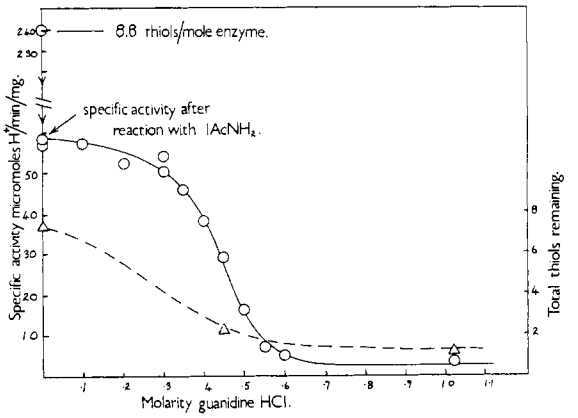

FIGURE 5: The inhibition of enzyme III in guanidine hydrochloride. The inhibition mixture comprised 3.8 mg/ml of enzyme, 4.63×10^{-3} M iodoacetamide, Tris-HCl buffer, ionic strength 0.02, and varying concentrations of guanidine-HCl in a final volume of 0.11 ml. Controls consisted of the above without the iodoacetamide plus 10 mM cysteine. The reaction was started by adding iodoacetamide and stopped after 20 min by adding 2 ml of 2.5 mM cysteine (pH 8.5) at 0°. The activity remaining was assayed immediately. (○) Specific activity of inhibited enzyme. The total number of thiols remaining were measured in 5 M guanidine-HCl (see text and Table I) (△).

this possibility, which might prove useful in separating nonidentical subunits, 0.4 ml of enzyme III (7 mg/ml) containing DTNB (0.33 mg/ml) was dialyzed against a solution of guanidine-containing DTNB (0.33 mg/ml) and phosphate buffer (pH 8.0) for 3 hr followed by dialysis against 120 ml of DTNB–phosphate buffer solution overnight. In the ultracentrifuge the reacted protein behaved as a diffuse peak spreading rapidly with some evidence of a high molecular weight aggregate (less than 1%). It is concluded that the reacted protein is not present as a distinct species either of mol wt 40,000 or 80,000.

The susceptibility of the creatine kinase activity remaining after inhibition by a 100-fold excess of inhibitor was examined in increasing concentrations of guanidine-HCl; at all concentrations of guanidine-HCl a 100-fold excess of iodoacetamide was employed (Figure 5). Early controls with no iodoacetamide and 1 M guanidine-HCl showed that inhibition occurred which could be prevented by the addition of cysteine and was presumably due to oxidation which should not occur with iodoacetamide present. In the presence of iodoacetamide, dilution of the enzyme 20 times in 2.5 mM cysteine (pH 8.5) 0° gave noticeable precipitates above 0.6 M guanidine-HCl which became very marked at 1.2 M, indicating that the extent of thiol reaction was preventing correct folding of the protein. It was of interest to see what extent of thiol reaction was occurring to give complete inhibition of enzyme III (Figure 5). The enzyme was incubated with excess iodoacetamide in varying guanidine-HCl concentrations for 20 min and the remaining thiols were estimated by DTNB in 5 M guanidine-HCl. It had been previously found that low concentrations of iodocacetamide did not interfere with the DTNB reaction or react with the free thionitrobenzoic acid moiety.

Electrophoretic Mobility of Inhibited Enzymes. Inhibition of rabbit creatine kinase (I) by iodoacetamide gives rise to a protein band on starch gel electrophoresis with a slightly lower mobility toward the anode (Watts, 1964). This may arise by virtue of a negative charge on the essential thiol groups compatible with the reactivity of these toward electrophilic inhibitors over a wide range of pH (Watts and Rabin, 1962). Thus it was of interest to see how the residual activity of enzyme III migrated after inhibition by iodoacetamide and iodoacetate (Plate 2).

Hybridization of the native enzyme I with iodoacetamide-inhibited enzyme III gave the pattern shown (Plate 1b). If the residual activity of enzyme III is due to protein with all its active-site thiols reacted then a new hybrid has formed consisting of an enzyme with one normal subunit I and a subunit from enzyme III with no active-site thiol.

High-Voltage Electrophoresis of Labeled Tryptic Peptides. Enzymes I and III (20 mg each) were incubated with 2.49 μmoles of [α-^{14}C]iodoacetamide (0.5 mCi/mmole) in a volume of 3 ml at 25° (pH 8.45), Tris-HCl buffer, ionic strength 10^{-3}, for 20 min. Enzymes I and III (10 mg each) were also incubated under the same conditions except for the presence of 0.55 M guanidine-HCl. After 20 min all solutions were cooled to 0° and dialyzed for 72 hr against three changes of 3 l. of H_2O. Before dialysis the solutions were assayed for creatine kinase activity; enzymes I and III with no guanidine present during inhibition had 3 and 26% activity remaining, respectively; in the presence of 0.55 M guanidine-HCl, enzyme III showed 16% of its initial activity.

After dialysis the proteins were denatured by heating for 2 min at 90° (0.5 mg/ml) and then digested at the same concentration by trypsin (2% w/w) at pH 8.5, keeping the pH constant by using a Radiometer titrator (Hooton and Watts, 1966). The trypsin (Worthington Biochemicals) had been previously treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (Kostka and Carpenter, 1964) to remove any chymotryptic activity and produce a more highly specific proteolytic

attack. The peptides were freeze dried after a digestion of 70-min duration. Protein–tryptic peptides (3 mg equiv) were separated by high-voltage electrophoresis under Isopar-L light oil (Esso Chemical Co. Ltd.) on Whatman No. 3MM paper (8 × 57 cm) at 3 kV for 40 min; the papers were then dried and stained (Hooton and Watts, 1966). Alternatively, radioactive peptides were located by scanning the strips in a chromatogram scanner.

Enzymes inhibited in the absence of guanidine-HCl yielded only one major labeled peptide in both cases (enzymes I and III) at pH 6.4 (pyridine–acetic acid–H_2O, 10:0.4:90). The labeled peptide was neutral at this pH. After inhibition in the presence of guanidine-HCl five main labeled peptides appeared on electrophoresis at pH 6.4; there were only slight differences between the patterns obtained with enzymes I and III.

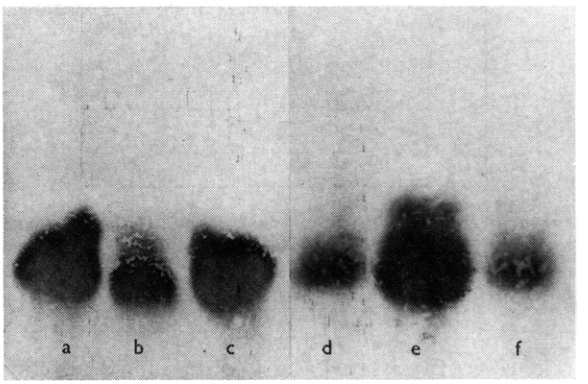

PLATE 2: The mobility of enzyme activity after inhibition by iodoacetamide and iodoacetate. Enzyme IIIa was incubated at 25° in the presence of 100-fold M excess of iodoacetamide or iodoacetate at concentrations of 5 mg/ml. After 2-hr incubation the enzymes were immediately run on starch gel at pH 7.0 overnight at 10 mA for 18 hr before staining (see text). a, c, e are native enzyme; b is inhibited with iodoacetamide, and d and f are iodoacetate inhibited enzyme.

Discussion

Chicken creatine kinase isoenzyme III was found to dissociate in 8 M urea into equal subunits of approximately 40,000 molecular weight in agreement with earlier work (Dawson *et al.*, 1967; Yue *et al.*, 1967). The idea of a simple model explaining the origin of three creatine kinase isoenzymes on the lines of that proposed for lactic dehydrogenase (Apella and Markert, 1961) has been put forward (Dawson *et al.*, 1965). However the existence of two different subunits giving rise to three possible dimers does not explain the five bands observed here in calf heart or the two bands characteristic of isoenzyme III. It has been reported (Eppenberger *et al.*, 1967b) that the two brain bands (enzyme III) are interconvertible, and isolated single bands from sparrow brain can be converted into the other by freezing and thawing on guanidine hydrochloride. This was not observed in the present work, and the purified single band (enzyme IIIa) remained stable over 6 months. Two protein bands were observed on starch gel for enzyme III in 8 M urea, in which conditions the enzyme exists in a subunit form. This argues that there are in fact two forms of closely similar subunits in enzyme III.

For enzyme I the number of reactive and total thiols (Table I) is close to the values obtained for rabbit and mouse enzyme I (Bayley and Thomson, 1967; Hooton and Watts, 1966), as are the apparent second-order rate constants. Enzyme III shows a higher number of unreactive thiols, probably two more than in enzyme I. The figure of 8.7 thiols/mole of enzyme may represent a real value of 10 in view of the difficulty of obtaining agreement between the number detected by DTNB and the total cysteine recovered as cysteic acid. Approximately seven thiols can be detected by the DTNB procedure in rabbit enzyme I (Thomson *et al.*, 1964), whereas eight can be obtained by amino acid analysis (Mahowald *et al.*, 1962b). Thus the likely value of five thiols per molecular weight of 40,000 is the same as that for arginine kinase (Virden and Watts, 1966; Blethen and Kaplan, 1967) to which enzyme III is related by a similar electrophoretic mobility. Since creatine kinase may have developed from arginine kinase during evolution, these two further points of similarity are interesting (Virden and Watts, 1966).

Figure 4 shows an interesting difference between enzymes I and III. Enzyme III is more susceptible to unfolding by guanidine-HCl (0.3 M) than enzyme I (0.6 M), indicating perhaps a less rigid structure in enzyme III in which the hydrophobic interior is exposed more readily to the surrounding solution. This lower stability corresponds to a greater sensitivity to heat denaturation (Dawson *et al.*, 1967).

Dawson *et al.* (1967), whilst studying the inhibition of several types of creatine kinases, showed that chicken brain enzyme (III) was anomalous in its behavor toward iodoacetate, whereas chicken enzyme I and rabbit enzymes I and III behaved normally. By taking theoretical lines, which their experimental points approximated, values of two thiols per mole of enzyme were obtained for all but chicken enzyme III. In this case only 70% inhibition was observed with a molar excess of iodoacetate of 4, incubated over 2.5 hr. They considered the possibility that only one thiol per mole was being alkylated. However, apart from this leading to 50% inhibition and not 70%, from their data it was not possible to decide whether or not inhibition levels approaching 100% could be obtained by longer incubation or higher concentrations of inhibitor. In any case the data described here (Table I) show conclusively that in fact chicken enzyme III has approximately two thiols per mole which react quite normally. It should be mentioned that the conditions for complete reaction used in this work fulfill those required for complete inhibition of rabbit enzyme I (Mahowald *et al.*, 1962a). Figure 2 shows that with a molar excess of 100 of iodoacetamide and iodoacetate chicken enzyme I is completely inhibited in 3 min but enzyme III and IIIa rapidly reach a level of 70–75% inhibition which remains constant thereafter. Indeed, after 21 hr at 25° in 100-fold M excess of iodoacetamide no further inhibition takes place. This is a remarkable stability and any essential thiols involved directly in the enzymic mechanism would need to be completely shielded. The

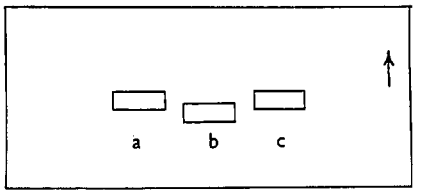

FIGURE 6: The mobility of enzyme protein after reaction with iodoacetate. The drawing represents a stain of the starch gel runs, depicted in Plate 2. (a) Native enzyme; (b) iodoacetamide-treated enzyme; (c) iodoacetate-treated enzyme.

residual activity assayed as a rate of proton production was shown to be definitely creatine kinase activity and there was no ATPase detectable, ruling out any activation of the intrinsic ATPase of creatine kinase which occurs in the case of myosin when specific thiols are blocked (Kielley and Bradley, 1956).

Such behavior could result if a conformational rearrangement occurred as one thiol was reacted leading to a burial of the other active-site thiol. The complete inaccessibility of this thiol to a number of reagents (Table II) suggests that substrates would also not approach it without difficulty, leading surely to altered enzymic characteristics. The perfectly normal K_m's measured argue against this, and in any case nearly two thiols react with DTNB (1.6–1.9 thiols/mole, Table I), so that any folding taking place can only affect a small fraction of the protein population.

However, there is some evidence that substrates can induce conformational changes in creatine kinase (James and Morrison, 1966; Lui and Cunningham, 1966), so it is possible that although the thiol is inaccessible to inhibitors, substrates may induce analogous conformational changes in the alkylated enzyme, reexposing the buried essential thiol so that it can participate in catalysis. This may leave the K_m's unaffected, but if the conformational changes are obligatory to substrate binding, then the anomalous rearrangements, caused by partial alkylation which must involve large free-energy changes, in view of the total unreactivity of this thiol, may have to be overcome on binding, leading, possibly, to increased K_m values. The conditions used for K_m determination (Hooton and Watts, 1966) would probably miss any effect on ATP binding in view of the high saturating conditions used, but any significant effect on creatine binding would probably have been detected. Additional isotopic and kinetic experiments are planned to investigate this point.

A further possibility is that preparations of enzyme III contain 25% of a species of enzyme which is totally unreactive to the inhibitors tried. If such an enzyme has thiols involved directly in the enzyme mechanism (Watts and Rabin, 1962), they would presumably be near the surface. Inhibition was carried out in the presence of low, increasing concentrations of guanidine-HCl in an attempt to reveal such thiols and achieve 100% inhibition. It transpires that the inhibition of the remaining 25% activity (Figure 5) follows closely the complete reaction of all thiols by DTNB in the presence of guanidine-HCl (Figure 4). Indeed, measuring the thiols remaining after a 20-min incubation with a large excess of iodoacetamide in the presence of increasing guanidine-HCl indicates that when half of the stable activity still remains, only 2.5 thiols out of a total 9 are left unreacted. Figures 4 and 5 also show that loss of stable activity in guanidine-HCl follows closely the reaction of the buried thiols. To achieve maximum inhibition nearly all thiols must be reacted (Figure 5), by which stage the enzyme precipitates and is presumably no longer able to fold into a native conformation. It is difficult to see how, in view of this, thiol groups are essential to the catalytic mechanism.

By reaction in 0.55 M guanidine-HCl with a tenfold molar excess of [α-^{14}C]iodoacetamide for 20 min at 25° the 25% activity normally stable to iodoacetamide is reduced to 16%. On analysis of the labeled tryptic peptides by paper electrophoresis, five major peptides were already in evidence representing sequences of the enzyme containing thiols normally buried below the surface. Thus to increase only partly the level of inhibition beyond 75% extensive blocking of the buried thiols must occur. If the explanation for this behavior is that the so-called essential thiols are in fact not part of the enzymic mechanism (Watts and Rabin, 1962), the chicken enzyme III gives clear indication of this, confirming an earlier report that even for rabbit enzyme I all activity is not lost until 6 ± 1 thiols have been blocked (Noda *et al.*, 1961). It is interesting to note that with chicken enzyme I complete inhibition is never actually achieved (Table II), and Mahowald *et al.* (1962a) state that 2% of the activity of rabbit enzyme remains after complete reaction of the thiols.

Perhaps the well-investigated inhibition of creatine kinase by iodoacetate and iodoacetamide is really due to steric interaction of the inhibitor grouping with incoming substrates. In this case, if iodomethane blocked these thiols, the inhibition should be less, since methyl groups are considerably smaller than say carboxymethyl groups. In fact, the results (Figure 3, Table II) do not bear this out, nor does inhibition by DTNB. The alternative may be that the two reactive thiols are involved, perhaps by hydrogen bonding, in maintaining a strict geometry of the active site. Thus, blockage of such groups would lead to alterations in conformation which may or may not lead to complete inhibition. In the case of creatine kinase III the thiols are either not so important in maintaining conformation or conformation is not so critical to enzymic function. Whether this reflects the physiological significance of creatine kinase isoenzymes is difficult to say. It would be of interest to know how enzyme II behaves.

The mobilities of enzyme activity after iodoacetamide and iodoacetate inhibition are shown in Plate 2. A lower mobility of enzyme activity due to the neutralization of the negative charge on the free thiols (Watts, 1964) would strongly suggest that the fully blocked enzyme was in fact partially active. The results do not allow for a definite conclusion, but a small difference in mobility is seen in the case of iodoacetamide-inhibited enzyme III which does not occur with iodoacetate inhibition which, of course, would not alter the negative charge on the thiol group. This small difference is mirrored by the protein bands (Figure 6).

Acknowledgments

The author wishes to thank Dr. R. H. Pain for carrying out the ultracentrifuge analysis. Discussions with Professor K. Burton, Dr. R. H. Pain, Dr. A. Allen, and Dr. R. Virden have been most useful.

References

Apella, E., and Markert, C. L. (1961), *Biochem. Biophys. Res. Commun. 6*, 171.

Bayley, P. M., and Thomson, A. R. (1967), *Biochem. J. 104*, 33c.

Benesch, R. E., Lardy, H. A., and Benesch, R. (1955), *J. Biol. Chem. 216*, 663.

Blethen, S. L., and Kaplan, N. O. (1967), *Biochemistry 6*, 1413.

Di Sabato, G., and Kaplan, N. O. (1964), *J. Biol. Chem. 239*, 438.

Dawson, D. M., Eppenberger, H. M., and Kaplan, N. O. (1965), *Biochem. Biophys. Res. Commun. 21*, 346.

Dawson, D. M., Eppenberger, H. M., and Kaplan, N. O. (1967), *J. Biol. Chem. 242*, 210.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.

Eppenberger, H. M., Dawson, D. M., and Kaplan, N. O. (1967a), *J. Biol. Chem. 242*, 204.

Eppenberger, M. E., Eppenberger, H. M., and Kaplan, N. O. (1967b), *Nature 214*, 239.

Eppenberger, H. M., Von Fellenberger, R. Richterich, R., and Abei, H. (1963), *Enzymol. Biol. Clin. 2*, 139.

Fine, I. H., Kaplan, N. O., and Kuftinec, D. (1963), *Biochemistry 2*, 116.

Gornall, A. G., Badawill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177*, 752.

Hooton, B. T., and Watts, D. C. (1966), *Biochem. J. 100*, 637.

James, E., and Morrison, J. F. (1966), *J. Biol. Chem. 241*, 4758.

Kielley, W. W., and Bradley, L. B. (1956), *J. Biol. Chem. 218*, 653.

Klotz, J. M. (1967), *Science 155*, 697.

Koshland, D. E. (1960), *Advan. Enzymol. 22*, 45.

Kostka, V., and Carpenter, F. H. (1964), *J. Biol. Chem. 239*, 1799.

Kuby, S. A., Noda, L., and Lardy, H. A. (1954), *J. Biol. Chem. 209*, 191.

Lui, N. S. T., and Cunningham, L. (1966), *Biochemistry 5*, 144.

Mahowald, T. A., and Kuby, S. A. (1960), *Federation Proc. 19*, 46.

Mahowald, T. A., Noltmann, E. A., and Kuby, S. A. (1962a), *J. Biol. Chem. 237*, 1535.

Mahowald, T. A., Noltmann, E. A., and Kuby, S. A. (1962b), *J. Biol. Chem. 237*, 1138.

Martin, C. J., and Frazier, A. R. (1963), *J. Biol. Chem. 238*, 3268.

Noda, L., Nihei, T., and Moore, E. (1961), *5th Intern. Congr. Biochem., Moscow*, 118.

Noltmann, E. A., Mahowald, T. A., and Kuby, S. A. (1962), *J. Biol. Chem. 237*, 1146.

Poulik, M. D. (1967), *Nature 180*, 1477.

Thomson, A. R., Eveleigh, J. W., and Miles, B. J. (1964), *Nature 203*, 267.

Van Holde, K. E., and Baldwin, R. L. (1958), *J. Phys. Chem. 62*, 734.

Virden, R., and Watts, D. C. (1966), *Biochem. J. 99*, 159.

Watts, D. C. (1964), *1st Meeting Federation European Biochem. Soc., London*, A13.

Watts, D. C., and Rabin, B. R. (1962), *Biochem. J. 85*, 507.

Watts, D. C., Rabin, B. R. and Crook, E. M. (1961), *Biochim. Biophys. Acta 48*, 380.

Wood, T. (1963), *Biochem. J. 87*, 453.

Yue, R., Palieri, R., Olson, O., and Kuby, S. A. (1967), *Federation Proc. 26*, 1929.