The Nature of Inactivation of Rat Muscle 5'-Adenylate Aminohydrolase by Fluorodinitrobenzene

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1. The inactivation of rat skeletal-muscle AMP deaminase by Dnp-F (1-fluoro-2,4-dinitrobenzene) is accompanied by the arylation of thiol, amino and phenolic hydroxyl groups. 2. The number of thiol groups that react with Dnp-F is about 12; this is the number that reacts with Nbs₂ [5,5'-dithiobis-(2-nitrobenzoic acid)] and *N*-ethylmaleimide without loss of enzyme activity, and it appears to be the same thiol groups that all three reagents attack. 3. Dinitrophenylation of these reactive SH groups is not the cause of inactivation, since active *N*-ethylmaleimide-substituted enzyme is also inactivated by Dnp-F. 4. Complete inactivation of the *N*-ethylmaleimide-treated AMP deaminase occurs when about six tyrosine and two lysine residues are dinitrophenylated. 5. Since the treatment of Dnp-enzyme with 2-mercaptoethanol restores much of the enzyme activity, inactivation of AMP deaminase by Dnp-F is probably largely due to modification of tyrosine residues. 6. The kinetic properties of the Dnp-enzyme indicate that a marked decrease in *V* occurs only after extensive enzyme modification. The decreased activity after slight inactivation results from modification of *K_m*.

Arylation with Dnp-F* has been a fruitful means of investigating the active-centre residues of several enzymes. Labelling with a radioactive reagent, followed by degradation of the labelled enzyme, has made it possible to identify the sites of the dinitrophenylation responsible for decreased activity (Cohen, 1968). We have previously reported that AMP deaminase (AMP aminohydrolase, EC 3.5.4.6) from skeletal muscle of various species is inactivated by Dnp-F (Ronca-Testoni *et al.*, 1970). The reaction of Dnp-F with AMP deaminase from rat muscle causes inactivation which is strongly enhanced at alkaline pH values (Raggi *et al.*, 1970). At pH 7.5 *P_i* partly protects the enzyme from inactivation, but other effectors of AMP deaminase, such as nucleoside di- and tri-phosphates, are without effect.

Raggi *et al.* (1972) showed that the 12 thiol groups that react with Nbs₂ in the native holoenzyme of rat muscle AMP deaminase are not essential for enzymic activity, but titration of the additional thiol groups that become reactive in the apoenzyme inhibits the capacity of Zn²⁺ to reconstitute an active holoenzyme. Ronca-Testoni & Ronca (1974) found that substitution of six to seven lysines in rat muscle AMP deaminase treated with pyridoxal 5-phosphate and NaBH₄ modifies the kinetic and regulatory properties of the enzyme without an appreciable decrease in *V*. These observations

stimulated our interest in the nature of the inactivation of rat muscle AMP deaminase by Dnp-F. It will be shown that about ten cysteines, six tyrosine and two lysines are dinitrophenylated. Thiolytic with 2-mercaptoethanol of Dnp-enzyme restores most of the enzyme activity. These results, together with the analysis of the kinetic behaviour of the modified enzyme, suggest that the inactivation is essentially due to modification of tyrosine residues.

Experimental

Enzyme

AMP deaminase was prepared from freshly frozen rat skeletal muscles, as previously described (Ronca-Testoni *et al.*, 1970). The purified enzyme deaminated 17–20 μmol of AMP/s per mg of protein at 20°C in 50 mM-imidazole-HCl (pH 6.5)–100 mM-KCl–2 mM-AMP (Sigma Chemical Co., St. Louis, Mo., U.S.A.). The molecular weight of the enzyme was taken as 290 000 (Ronca-Testoni *et al.*, 1970). Protein was determined from *E*₂₈₀ measurements assuming an extinction coefficient (*E*_{280nm}^{1%}) of 9.8 (Ronca-Testoni *et al.*, 1970). The enzyme activity was assayed spectrophotometrically at 265 or 285 nm. Δ*E*_{mM} values of 8.86 and 0.23 were used at 265 and 285 nm respectively for the calculation of the μmol of substrate deaminated (Ronca *et al.*, 1968).

AMP deaminase was dinitrophenylated at 20°C, pH 7.5. Inactivation was initiated by the addition of 10 μl of a solution of Dnp-F in benzene to 0.4 ml of

* Abbreviations: Dnp-F, 1-fluoro-2,4-dinitrobenzene; Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid).

the incubation mixture. At different incubation times samples were diluted into a solution containing 50 mM-imidazole-HCl (pH 6.5), 100 mM-KCl and 0.1 mM-AMP to assay for residual enzyme activity. Dnp-enzymes at various degrees of inactivation were isolated by gel filtration on a column (100 mm $\times$ 10 mm) of Sephadex G-25 equilibrated with 0.1 M-potassium phosphate buffer (pH 7.0). The enzyme was treated with *N*-ethylmaleimide (Mann Research Laboratories, New York, N.Y., U.S.A.) at pH 7.0, as previously described (Raggi *et al.*, 1972). The reaction took place for approximately 2 h until no further decrease in E_{305} was observed.

Reactivation of dinitrophenylated AMP deaminase by 2-mercaptoethanol was performed under various conditions with the enzyme previously inactivated by Dnp-F to give 25% of the original activity. The incubation mixture (pH 6.8, or 7.8, or 8.5) contained, in a volume of 0.4 ml: 0.4 mg of Dnp-enzyme/ml, 75 mM-potassium phosphate, 0.1 M-Tris-HCl and various concentrations of 2-mercaptoethanol.

Analytical measurements

Titration of AMP deaminase thiol groups with Nbs₂ (Sigma Chemical Co.) were carried out at room temperature as described by Ellman (1959). The reaction mixture contained 0.4 mM-Nbs₂, 0.1 M-potassium phosphate buffer, pH 7.8, and 0.6–1.1 μ M-AMP deaminase. Uptake of Nbs₂ was calculated by using a molar extinction coefficient (ϵ_{412}) of 13300 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹ (Raggi *et al.*, 1972). To establish the number of groups dinitrophenylated, AMP deaminase was allowed to react with [³H]Dnp-F (The Radiochemical Centre, Amersham, Bucks., U.K.). The resulting solution was passed, at various times of reaction, through Sephadex G-25 and the protein fractions were collected. Protein concentration was determined by the method described by Lowry *et al.* (1951) with native AMP deaminase as standard. The number of Dnp groups incorporated into the protein was calculated from the specific radioactivity of Dnp-protein and the starting Dnp-F solution. For identification of the groups that had been modified, the Dnp-enzyme was hydrolysed with 5.7 M-HCl in a sealed and evacuated tube for 17 h at 106°C. The hydrolysate was repeatedly extracted with ether. The aqueous layer was evaporated under N₂ and the residue was dissolved in 1 M-HCl. Samples were chromatographed on Whatman no. 4 paper in butan-1-ol-acetic acid-water (4:1:5, by vol., upper phase). Radioisotope measurements were taken in a Packard model 2002 Tri-Carb liquid-scintillation spectrometer in the solvent system described by Bray (1960).

Sucrose-density-gradient centrifugation was carried out as described by Martin & Ames (1961) in an SW 39 rotor in a Spinco ultracentrifuge model L. The $s_{20,w}$ was calculated by using bovine liver catalase and rabbit muscle aldolase as molecular-weight markers.

Spectrometric determinations were carried out in a Zeiss PMQ II spectrophotometer equipped with a Zeiss TE converter and with a Varian G-2000 recorder. All the reagents used were of analytical grade; urea was recrystallized from aqueous ethanol.

Results

Identification of amino acids involved

Raggi *et al.* (1972) reported that native rat muscle AMP deaminase reacts with about 12 mol of thiol reagents such as Nbs₂ and *N*-ethylmaleimide without loss of activity, whereas 16 to 18 additional thiol groups are available for reaction in the urea-denatured enzyme. In order to exclude the possibility that the arylation of the more reactive SH groups might be involved in the inactivation of AMP deaminase by Dnp-F, we dinitrophenylated AMP deaminase previously treated with *N*-ethylmaleimide. Substitution of the reactive SH groups with *N*-ethylmaleimide does not protect the enzyme from dinitrophenylation (Fig. 1); on the contrary, in the first phase of the reaction, a faster rate of inactivation is observed. This proves that inactivation by Dnp-F is independent of the state of the thiol groups that are

Fig. 1. Progress of the inactivation of rat muscle AMP deaminase by Dnp-F

The reaction mixtures contained 0.1 M-triethanolamine-HCl (pH 7.5), 0.5 M-KCl, 1.7 μ M-native (●) or *N*-ethylmaleimide-treated (△) AMP deaminase and 8 mM-Dnp-F added as the last reagent. Control samples (○) were incubated without Dnp-F and assayed as standards of activity.

Table 1. Reaction with Nbs_2 of AMP deaminase at different degrees of enzyme inactivation by Dnp-F

Thiol groups were titrated on the enzyme previously treated with Dnp-F, as described in Fig. 1, and then, after inactivation to various degrees, chromatographed on a column of Sephadex G-25 equilibrated and eluted with 0.1M-potassium phosphate buffer, pH7.8. (a) Titration of the thiol groups of 1.0–1.1 μM -enzyme under non-denaturing conditions. (b) Titration of the thiol groups of 0.6–0.7 μM -enzyme in the presence of 4M-urea. Each value is the mean of two determinations.

Percentage of AMP deaminase activity	Residues per enzyme molecule (mol.wt. 290000)		
	(a) Non-denatured	(b) Denatured	(b)–(a)
100	12.0	29.0	17.0
50	6.0	23.0	17.0
39	5.6	20.7	15.1
30	5.3	21.4	16.1
21	2.8	17.0	14.2
1	2.6	16.0	13.4

reactive towards specific reagents in non-denaturing conditions. In order to ascertain whether the additional thiol groups may be sites of dinitrophenylation, we assessed the reactivity to Nbs_2 of the thiol groups after inactivation of AMP deaminase to different extents with Dnp-F (Table 1). The second and third columns of Table 1 show the number of thiol groups titrated with Nbs_2 in the absence and in the presence of 4M-urea respectively. With the untreated enzyme average values of 12 and 29 SH groups were obtained in the two conditions. Titration of the modified enzymes revealed fewer 'fast'-reacting thiol groups; six to seven were no longer available to react with Nbs_2 in the 50–70% inactivated enzyme and nine to ten in the 80–100% inactivated enzyme. The 'total' thiol groups titratable in the 50–70% inactivated Dnp-enzyme differed from the 'total' thiol content of the native enzyme by an amount close to the difference observed in the 'fast' thiols. A greater decrease in 'total' rather than in 'fast' thiols takes place when inactivation is greater. The fourth column of Table 1 provides a better evaluation of the results and shows that the 'buried' thiols are markedly decreased in number by dinitrophenylation only when inactivation is almost complete. These results also show that the high number of non-essential thiol groups involved in the reaction with Dnp-F can interfere with the investigation into the number and types of the essential amino acid residues modified. In order to define the Dnp-F reaction more specifically, dinitrophenylation of the *N*-ethylmaleimide-treated enzyme was carried out with $[^3\text{H}]\text{Dnp-F}$. The *N*-

Fig. 2. Dnp-F inactivation of *N*-ethylmaleimide-treated AMP deaminase: plot of percentage of initial activity versus mol of $[^3\text{H}]\text{Dnp}$ -residues per mol of enzyme

The reaction mixture contained 50mM-triethanolamine-HCl (pH7.5), 0.5M-KCl, 4mM- $[^3\text{H}]\text{Dnp-F}$ and 14 μM -AMP deaminase treated with *N*-ethylmaleimide as described in the Experimental section. The radioactivity was determined on the protein previously chromatographed after various degrees of inactivation on a column of Sephadex G-25, equilibrated with 0.1M-potassium phosphate buffer, pH6.5, to remove the extra reagent. Calculations are based on a specific radioactivity of $[^3\text{H}]\text{Dnp-F}$ of 3.0×10^7 c.p.m./ μmol and a molecular weight for AMP deaminase of 290000.

ethylmaleimide-treated enzyme was precipitated with 75% $(\text{NH}_4)_2\text{SO}_4$ and then dissolved to a concentration of 4mg/ml in 0.05M-triethanolamine-HCl buffer, pH7.5, containing 0.5M-KCl. Dinitrophenylation was started by addition of the labelled reagent. The ratios of $[^3\text{H}]\text{Dnp}$ to the enzyme at various degrees of inactivation are plotted in Fig. 2. Extrapolation of the line to the horizontal axis gives 10.5mol of $[^3\text{H}]\text{Dnp}$ /mol of enzyme. Identification of the Dnp-labelled amino acids involved hydrolysis of the ^3H -labelled Dnp-enzyme isolated at 70–90% inactivation by gel filtration on a column (100mm $\times$ 10mm) of Sephadex G-25 equilibrated with 0.05M-ammonium acetate in degassed water. The hydrolysed enzyme contained about 80% of the radioactivity detected in the AMP deaminase after dinitrophenylation (Table 2). On subsequent ether extraction about 90% of recovered radioactivity was found in the aqueous layer. Paper chromatography showed the presence of a major radioactive spot migrating identically with a marker of *O*-Dnp-tyrosine. By measurement of the radioactivity localized in this spot an average value of 5.9 ± 1.2 $[^3\text{H}]\text{Dnp}$ derivatives/mol of AMP deaminase was calculated in four experiments with different AMP

Table 2. Dinitrophenylation with [^3H]Dnp-F of AMP deaminase

Procedure was as described in the Experimental section.

Fractions	Radioactivity recovered (c.p.m.)	
	Aqueous layer	Ether extract
Dinitrophenylated enzyme	669 100	—
After hydrolysis	517 000	—
Hydrolysate after ether extraction	359 000	36 300
Hydrolysate after dinitrophenylation*	10 300	46 000

* Data obtained after ether extraction of a sample of the [^3H]Dnp-enzyme hydrolysate submitted to a second dinitrophenylation procedure with unlabelled Dnp-F.

Fig. 3. Reactivation of Dnp-enzyme by 2-mercaptoethanol at pH 7.8

Inactivated enzyme was incubated at 20°C with 2-mercaptoethanol to a final concentration of 1.5 (○), 6.5 (●) and 50 mM (△). □, Untreated AMP deaminase. Samples were removed at the indicated times and assayed for activity.

deaminase preparations and assuming 10.5 dinitrophenylated amino acid residues at 100% inactivation (see Fig. 2). A second minor spot migrated similarly to an N^6 -Dnp-lysine marker, but its R_F was not clearly distinguishable from that for N^2 -Dnp-arginine. The average value of the radioactivity of this spot corresponded to 1.8 ± 1.3 [^3H]Dnp groups/mol of enzyme. Two other spots containing respectively 0.97 ± 0.45 and 1.2 ± 0.3 [^3H]Dnp groups/mol of enzyme migrated

Fig. 4. Reactivation of Dnp-enzyme by 2-mercaptoethanol at pH 8.5

Inactivated enzyme was incubated with mercaptoethanol, 2 mM (○) and 10 mM (●), at 20°C. □, Untreated AMP deaminase. Samples were removed at the indicated times and assayed for activity.

identically with S -Dnp-cysteine and Dnp-OH. In order to establish the nature of the second spot more precisely we treated a sample of the hydrolysed Dnp-enzyme with unlabelled 0.2 M-Dnp-F at pH 10 (1 M- Na_2CO_3 at 40°C for 3 h), to transform the N^6 -Dnp-lysine and the O -Dnp-tyrosine into bis-Dnp derivatives. On subsequent ether extraction more than 80% of the recovered radioactivity was found in the ether layer (Table 2). Paper chromatography of a sample in benzene-1% acetic acid (1:1, v/v, upper phase) showed the presence of two major completely resolved spots with R_F values identical with those of bis-Dnp-lysine and bis-Dnp-tyrosine, which contained the radioactivity corresponding respectively to 2.1 and 5.75 mol of [^3H]Dnp groups/mol of enzyme.

Reactivation of Dnp-enzyme by 2-mercaptoethanol

Treatment of dinitrophenylated AMP deaminase with 2-mercaptoethanol produces a pH-dependent restoration of enzyme activity. Incubation for 2 h at pH 6.8 increased the enzyme activity by less than 10%. However, 54 and 70% regain of the original activity is observed when reactivation is carried out at pH 7.8 and 8.5 respectively (Figs. 3 and 4). It can be seen from Fig. 3 that the rate of reactivation at pH 7.8 strongly depends on the

Fig. 5. Lineweaver-Burk plots of native, *N*-ethylmaleimide- and Dnp-F-treated AMP deaminase activities

The reaction mixture contained 50mM-imidazole-HCl (pH 6.5) and 100mM-KCl. Temperature 20°C. Dnp-F-treated enzymes at 10 (■), 25 (□), 50% (▲) of the original activity; native (○) and *N*-ethylmaleimide-treated (●) enzymes. With the 90% inactivated Dnp-enzyme 0.022, 0.024 and 0.038 reciprocal velocity values were respectively obtained at 0.30, 0.15 and 0.10mM-AMP.

2-mercaptoethanol concentration: after 15 min of incubation, 28, 46 and 54% of the original activity is restored respectively in the presence of 1.5, 6.5 and 50mM-2-mercaptoethanol. However, reactivation at this pH value would seem to be limited since the enzyme shows 54% of the original activity after the incubation with either 6.5 or 50mM-2-mercaptoethanol. A similar pattern is observed on reactivation at pH 8.5 (Fig. 4), when the incompleteness of recovery would seem to be a consequence of the instability of the AMP deaminase at this pH value. The activity of the controls decreases with time below the values attained by the reactivated enzyme.

Effect of dinitrophenylation on kinetic properties and aggregation state

In order to establish whether the loss of activity on dinitrophenylation was at least partly due to the decreased ability of the enzyme to bind substrate, we investigated the kinetic properties of AMP deaminase after various degrees of Dnp-F inactivation, determined by assaying in the presence of 0.1mM

AMP the isolated Dnp-enzymes. The activity of native, *N*-ethylmaleimide-treated and Dnp-F-treated AMP deaminases as a function of substrate concentrations were compared in the presence of 100mM-KCl. At this salt concentration the native as well as the modified enzymes were completely activated also at low substrate concentrations; as a consequence all the substrate-velocity curves looked hyperbolic. The double-reciprocal plots (Fig. 5) corresponding to the Dnp-F-treated enzymes show a concave downward deviation so that the kinetic data can be fitted with more than one K_m value. The K_m at the higher substrate concentrations is about the same (1.0–1.2mM) for the Dnp-enzymes with 10–50% residual activity. The K_m values obtained with the native and the *N*-ethylmaleimide-treated enzymes are 0.5 and 0.3mM respectively. The V value of the 50% inactivated Dnp-enzyme does not noticeably differ from that of native and *N*-ethylmaleimide-treated enzymes. A marked decrease in V is observed in the 75 and 90% inactivated Dnp-enzymes. Since polymerization has been suggested to play a role in the regulation of AMP deaminase (Ashman & Atwell, 1972) these kinetic properties of the Dnp-enzymes may be interpreted in terms of protein structure. We examined the aggregation state of native and modified AMP deaminase by sucrose-density-gradient centrifugation. The Dnp-enzyme (40% of the original activity) showed an $s_{20,w}$ value of 12.2 identical with that of the native enzyme.

Discussion

The data presented in this paper show that the inactivation of rat muscle AMP deaminase by Dnp-F cannot be explained solely in terms of modifications of the catalytic site(s) of the enzyme. The kinetic properties of the partially inactivated enzyme indicate that dinitrophenylation also elicits a more than twofold increase of the enzyme K_m , that can be ascribed to the modification of amino acid residues more reactive towards Dnp-F than those involved in the inactivation process. About the same K_m value (1.0–1.2mM) was actually obtained for the Dnp-enzymes with 10–50% residual activity at 0.1mM-AMP, whereas V was markedly decreased only in inactivation to an extent greater than 50%.

The observed changes in kinetic properties of AMP deaminase deserve consideration in establishing the nature of the responsible functional groups. The Dnp-F reaction is not highly specific and dinitrophenylation of thiol, amino and phenolic hydroxyl groups is observed. However, by coupling the results of this paper with those obtained with specific modification of SH groups, we can rule out the involvement of the reactive thiols in the

inactivation process. It was demonstrated that 12 of the 32 groups of rat muscle AMP deaminase, reactive with Nbs₂ and *N*-ethylmaleimide in the absence of denaturing agents, are not essential for the enzyme activity (Raggi *et al.*, 1972). The number of thiols that react with Dnp-F is limited to 10–13, which is in agreement with the value of 12 groups which are titrated with the thiol-specific reagents without any loss of the enzyme activity. Further, AMP deaminase is also inactivated by Dnp-F when the 12 reactive SH groups are replaced by *N*-ethylmaleimide, in this condition no dinitrophenylation of the 'buried' thiols being observed. It can also be excluded that the higher K_m of Dnp-enzyme depends on the modification of thiol groups, since the *N*-ethylmaleimide-treated enzyme exhibits a K_m (0.3 mM) lower than that of the native enzyme (0.5 mM).

Since complete inactivation of the *N*-ethylmaleimide-treated enzyme occurs when about six tyrosine and two lysine residues are dinitrophenylated, the role of these groups must be examined. From the data reported in this paper on the reactivation of Dnp-enzyme by 2-mercaptoethanol, the obligatory participation in the catalysis of the two lysines reactive with Dnp-F seems to be excluded. Treatment with 2-mercaptoethanol at pH 8.0 has been shown to displace the Dnp groups from histidine, tyrosine and cysteine side chains, whereas *N*⁶-Dnp-lysine was unaffected by this treatment (Shaltiel, 1967). Moreover AMP deaminase is inactivated by pyridoxal 5-phosphate and NaBH₄ only after six to seven lysines that are not essential for the enzyme activity have been modified, and complete inactivation is obtained when approximately 16 equivalents of reagent are bound (Ronca-Testoni & Ronca, 1974).

The possibility of inactivation by Dnp-F may be, at least partly, due to a decreased ability of the enzyme to bind substrate by impairment of the phosphate group binding to protonated *N*⁶-amino groups (Hirs *et al.*, 1965) or indirectly by an allosteric mechanism similar to that postulated for the action of pyridoxal 5-phosphate on AMP deaminase (Ronca-Testoni & Ronca, 1974). In this case the *V* of the modified enzyme would be the same as the native enzyme. In the Dnp-enzyme a decrease in *V* is actually observed.

The above considerations exclude the thiol and amino groups that are dinitrophenylated from an obligatory role in catalysis. A plausible interpretation of the data of this paper may be presented as the hypothesis that the inactivation of rat muscle AMP deaminase by Dnp-F depends on the modification of tyrosine residues. However, the obligatory participation of some tyrosines in the constitution of the active centre does not represent

the only deduction of this paper. At salt concentrations which maximally activate the native as well as the modified enzymes, the Dnp-enzymes with 10–50% residual activity do not follow normal Michaelis–Menten kinetics, since the corresponding double-reciprocal plots show a concave downward deviation. This behaviour may be due either to the presence of a heterogeneous enzyme population of different K_m values or to negative co-operativity (Conway & Koshland, 1968) induced by chemical modification. Skeletal-muscle AMP deaminase shows a polymeric structure with four or more subunits (Zielke & Suelter, 1971; Ellis *et al.*, 1971); it shows positive homotropic co-operativity for AMP in the absence of univalent cations, or in the presence of inhibitors, or after treatment with pyridoxal 5'-phosphate (Smiley & Suelter, 1967; Ronca *et al.*, 1968; Ronca-Testoni & Ronca, 1974), but shows negative homotropic co-operativity in the presence of high salt concentrations (A. Raggi & M. Raggi-Ranieri, unpublished work). It seems therefore reasonable to suppose that a conformational alteration may be induced in AMP deaminase by dinitrophenylation, so that differences in the binding constants of AMP are produced, causing negative co-operativity. Regardless of the interpretation of the curved Lineweaver–Burk plots, the sucrose-density-gradient-centrifugation data show that this phenomenon is not caused by the dissociation of enzyme subunits. A preliminary study indicates that a negative homotropic effect is induced in the AMP deaminase partially inactivated by tetranitromethane, a more specific reagent for tyrosine residues, producing homogeneous classes of modified enzyme (A. Raggi & C. Bergamini, unpublished work).

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References

- Ashman, L. K. & Atwell, J. L. (1972) *Biochim. Biophys. Acta* **258**, 618–625
- Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–285
- Cohen, L. A. (1968) *Annu. Rev. Biochem.* **37**, 695–726
- Conway, A. & Koshland, D. E., Jr. (1968) *Biochemistry* **7**, 4011–4022
- Ellis, K. J., Kuntz, K. & Sturtevant, J. N. (1971) *J. Biol. Chem.* **246**, 6631–6637
- Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77
- Hirs, C. H. W., Halmann, M. & Kycia, J. H. (1965) *Arch. Biochem. Biophys.* **111**, 209–235
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Martin, R. G. & Ames, B. N. (1961) *J. Biol. Chem.* **236**, 1372–1379

- Raggi, A., Ranieri, M., Ronca-Testoni, S. & Ronca, G. (1970) *Boll. Soc. Ital. Biol. Sper.* **47**, 84–87
- Raggi, A., Ranieri, M., Ronca, G. & Rossi, C. A. (1972) *Biochim. Biophys. Acta* **271**, 102–113
- Ronca, G., Raggi, A. & Ronca-Testoni, S. (1968) *Biochim. Biophys. Acta* **167**, 626–629
- Ronca-Testoni, S. & Ronca, G. (1974) *J. Biol. Chem.* in the press
- Ronca-Testoni, S., Ranieri, M., Raggi, A. & Ronca, G. (1970) *Ital. J. Biochem.* **19**, 262–276
- Shaltiel, S. (1967) *Biochem. Biophys. Res. Commun.* **29**, 178–183
- Smiley, K. L. & Suelter, C. H. (1967) *J. Biol. Chem.* **242**, 1980–1981
- Zielke, C. L. & Suelter, C. H. (1971) *J. Biol. Chem.* **246**, 2179–2186