Molecular Aspects of the Regulation of Rat Kidney Alkaline Phosphatase

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The mechanisms by which phosphate regulates the activity of alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) in rat kidney were investigated. Measurements of incorporation of [^{14}C]leucine into kidney alkaline phosphatase in rats fed on complete or phosphate-free diet provide evidence of a twofold increase in the rate of synthesis of the enzyme in diet-treated animals. Cycloheximide experiments indicated that control and diet-adapted enzyme decreases in activity according to first-order kinetics with a calculated half-life of 10.3 and 6.5 h after complete and phosphate-free diet administration respectively. Basal and diet-adapted enzymes exhibit similar K_m values for several phosphomonoesters and an identical degree of inhibition is produced by cysteine. In addition, the enzyme from both sources is the same with regard to heat inactivation at 45, 56 or 64°C, to the profile of elution from Sephadex and to electrophoretic properties on polyacrylamide gel. A failure of rat kidney alkaline phosphatase to respond to cortisol (hydrocortisone) was also observed.

Molecular mechanisms that regulate enzyme activity in animal tissues are not well understood, and several possibilities of regulation have been suggested (Civen & Knox, 1959; Greengard, Smith & Acs, 1963; Schimke, 1964; Schimke, Sweeney & Berlin, 1965; Cline & Bock, 1966; Griffin & Cox, 1966).

Melani, Guerriore & Baccari (1964) have shown that changes in the concentration of P_i in rat kidney produced by a diet lacking in phosphate are followed by a twofold increase in alkaline phosphatase activity. Subsequently other results obtained with inhibitors of protein or nucleic acid synthesis led to the conclusion that the increase of the alkaline phosphatase activity is due to the formation of a new enzyme (Melani, Ramponi, Farnararo, Cocucci & Guerriore, 1967).

The present paper is concerned with the molecular mechanism by which P_i regulates kidney alkaline phosphatase. The effect of phosphate-free diet on the rate of alkaline phosphatase degradation, on the rate of its synthesis and on some physicochemical properties of the enzyme from animals on normal and phosphate-free diet were investigated. The dependence of the activity of this enzyme in rat kidney on the action of glucocorticoids, which are well-known inducers of this enzyme during development in amphibian, chicken and mouse intestine (Moog, 1959; Chieffi & Carfagna, 1960), was also studied.

The results presented in this paper indicate regulation of alkaline phosphatase at the level of

genetic transcription, and support the conclusion that the activity of this enzyme in rat kidney is regulated by a repressible system on which P_i acts as a co-repressor, and that, as de-repression occurs, there is an increase in the number of identical enzymic molecules. In addition, as diet-adapted alkaline phosphatase is degraded more rapidly than basal enzyme, undoubtedly the rate of degradation, as well as the rate of synthesis, may be important in controlling the activity of this enzyme.

EXPERIMENTAL

Materials. L-[U- ^{14}C]Leucine and L-[G- ^3H]leucine were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). Actinomycin D and riboflavin were a generous gift from Merck, Sharpe and Dohme Inc. (Rahway, N.J., U.S.A.). Cycloheximide was obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.). Acrylamide and 3-dimethylaminopropionitrile were purchased from Eastman Organic Chemicals Dept. (Rochester 3, N.Y., U.S.A.); NN' -methylenebisacrylamide and NNN' -tetramethylethylenediamine were from Schuchardt (Munich, Germany); Fast Blue RR salt was from E. Gurr Ltd. (London S.W.14, U.K.); cortisol (hydrocortisone) hemisuccinate was from Richter (Milan, Italy); Sephadex G-200 and DEAE-Sephadex A-25 were from Pharmacia Fine Chemicals (Uppsala, Sweden); purified pancreatic lipase was from E. Merck A.-G. (Darmstadt, Germany); Amido-Schwartz 10B was from E. Merck A.-G.; 1-naphthyl phosphate and *p*-nitrophenyl phosphate were from Fluka A.-G. (Buchs, Switzerland). All other chemicals were reagent grade.

Treatment of animals. The animals were young male

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rats of the Wistar strain weighing 100–150 g. When dietary treatment was not under experimental control, the rats were maintained on a standard laboratory diet (Zoofarm pellets; Zoofarm, Padua, Italy). For experiments on the turnover and on the physicochemical characteristics of basal and diet-adapted enzymes, the animals were divided into groups and each group received a phosphate-free diet or a complete synthetic diet as described by Melani *et al.* (1964). Actinomycin D was administered daily (50 µg/g body wt.) intraperitoneally, beginning at various intervals from the start of the dietary treatment. Cycloheximide was used as a 500 µg/ml solution; the total amount given was 250 µg/100 g body wt., injected intraperitoneally at the end of dietary treatment. The effect of cortisol on the enzyme activity was determined in intact and adrenalectomized rats. Cortisol hemisuccinate was used as a 10 mg/ml solution; the total amount given was 3 mg/100 g body wt., injected intraperitoneally. The animals were adrenalectomized and maintained for 4 days on a complete diet; drinking water was replaced with 1% NaCl. For determination of specific radioactivity of basal and diet-adapted enzymes, the animals were pulse-labelled *in vivo* with 30 µCi of [^{14}C]leucine (16 mCi/mol)/rat for a period of labelling of 1 h. For experiments on the molecular size, control animals were labelled with 30 µCi of [^3H]leucine (14.7 Ci/mmol)/rat for 1 h and diet-adapted animals were labelled as above.

Enzyme preparation. Melani, Farnararo & Sgaragli (1968) have described the procedure for purification of rat kidney alkaline phosphatase. In brief, alkaline phosphatase was extracted from rat kidney microsomal preparation by simultaneous treatment with deoxycholate and lipase, and was purified successively by chromatography on DEAE-Sephadex A-25 with elution with a linear NaCl gradient, and by rechromatography on DEAE-Sephadex A-25.

Assay procedure. Alkaline phosphatase activity was determined by continuous measurement of the extinction at 405 nm when *p*-nitrophenyl phosphate was used as substrate (Melani & Gueritore, 1964). With other substrates the generation of P_i was determined by the method of Fiske & Subbarow (1925). Assay for P_i was performed after 0, 3, 6 and 10 min to ensure linearity of the reaction with time. Total proteins were determined by the biuret method as described by Beisenhertz *et al.* (1953) except for the column fractions, in which the protein content was determined from the extinction at 280 nm as described by Warburg & Christian (1941).

Gel filtration. To compare the molecular size of basal alkaline phosphatase labelled with [^3H]leucine and of diet-adapted enzyme labelled with [^{14}C]leucine, chromatography on Sephadex G-200 was used. Elution of mixed purified enzymes labelled as above from a column (1 cm $\times$ 25 cm) was accomplished with 50 mM-tris-HCl buffer, pH 8.1. The radioactivity of each fraction (0.5 ml) was measured as follows: after addition of 0.1 ml of 10% (w/v) albumin solution as a carrier and 0.6 ml of 10% (w/v) trichloroacetic acid, each tube was stored at 0°C overnight; the precipitate was then filtered through a Whatman CFC fibre-glass filter, washed with ethanol and ethanol-ether and then dried in an oven. ^3H and ^{14}C radioactivities were measured together by the channels-ratio method with a toluene scintillation system composed

of 0.5% 2,5-diphenyloxazole and 0.05% 1,4-bis-(5-phenyloxazol-2-yl)benzene. Radioactivities were measured at an efficiency of about 40% for ^{14}C and 10% for ^3H .

Disc electrophoresis. The conditions for electrophoresis on polyacrylamide gel were essentially those described by Ornstein & Davis (1959) for the anionic system at pH 8.5. The concentration of the gel was 7.5%, the size 5 cm and the use of urea was avoided to prevent enzyme denaturation. Split-gel electrophoresis was performed as described by Low & Wool (1967).

For the location of the phosphatase activity the gels were placed in a freshly mixed solution containing 50 mg of α -naphthyl phosphate, 50 mg of Fast Blue RR salt, 1 ml of 10 mM-MgCl₂, 15 ml of 50 mM-sodium barbital buffer, pH 9.4, and water to a volume of 100 ml. After 15–20 min the gels were washed in 50% (v/v) ethanol.

For determination of the radioactivity/enzyme activity ratio of the basal and the diet-adapted enzymes, they were labelled *in vivo* at the end of dietary treatment and then purified as described above. A 150 µg sample of each labelled protein was applied to polyacrylamide gel and subjected to electrophoresis. The gels were then cut in sections and each of them was divided into two equal parts. One part was homogenized in 50 mM-carbonate-bicarbonate buffer, pH 9, and assayed for enzyme activity, the other was homogenized in Bray's (1960) solution and assayed for radioactivity by liquid-scintillation counting. The recovery of radioactivity (c.p.m.) was about 50%; counting efficiency for ^{14}C was about 45% in these conditions.

RESULTS

Effect of cycloheximide on the rate of degradation of alkaline phosphatase. To establish whether a different steady state may require changes in the rate of degradation of this enzyme, the effects of cycloheximide on the rate of degradation of the basal and diet-adapted enzyme were studied. This antibiotic is a well-known inhibitor of protein synthesis at the level of the transfer of aminoacyl-tRNA to the nascent polypeptide chain (Siegel & Sisler, 1963). The results (Fig. 1) show that the decay of the specific activity of alkaline phosphatase follows first-order kinetics with half-times of 10.3 h for the basal enzyme and of 6.5 h for the diet-adapted enzyme.

Effect of phosphate-free diet on the rate of synthesis of alkaline phosphatase. The first attempt to obtain information on the rate of synthesis of alkaline phosphatase in control and diet-adapted rats was made by determining the extinction at 280 nm, enzyme activity and radioactivity of the column fractions at the last step of the purification procedure. The extinction elution pattern (Fig. 2) is the same for both enzymes, whereas specific enzyme activity and radioactivity of the diet-adapted alkaline phosphatase are enhanced by comparison with those of the basal enzyme. As this finding could be vitiated by contamination by foreign proteins, the fractions corresponding to the enzyme

Fig. 1. Rate of degradation of rat kidney alkaline phosphatase after 5 days of feeding on complete diet (○) or phosphate-free diet (●) as described in the Experimental section. At the end of dietary treatment the animals were injected intraperitoneally with cycloheximide (250 μ g/100 g body wt.). At the indicated times the animals were killed and kidneys were removed and pooled. The alkaline phosphatase activity was determined on fractions (supernatant from 4000g at 15 min) of 10% kidney homogenate in a 300mM-sucrose-2mM-EDTA-10mM-triethanolamine medium as outlined in the Experimental section. The results are given as means for four animals at each point.

peaks were pooled, concentrated and then subjected to electrophoretic analysis on polyacrylamide gel. With this technique a very sharp separation was found, since the enzyme activity in both cases was restricted to a very narrow band distinct from contaminating proteins. Through the region where alkaline phosphatase was expected to be, the gel was cut in sections approx. 1 mm thick. Each was divided into halves: one of these was used for assay of enzyme activity and the other for radioactivity. As shown in Fig. 3 the radioactivity/enzyme activity ratio in animals fed with phosphate-free diet is about double that in control rats.

Experiments with actinomycin D. The dietary adaptation of rat kidney alkaline phosphatase is sensitive to the action of actinomycin D (Melani *et al.* 1967). In fact, as is shown in Fig. 4, drug administration at the start of dietary treatment with a phosphate-free diet and after 1, 2 and 3 days blocks the increase of the enzyme activity, although if the antibiotic administration is delayed until the fourth day after the start of feeding the inhibitory effect is less and not uniform in the group of rats; administration at the fifth day is completely ineffective. These results indicate that after a feeding period of 4 days the adaptation is no longer dependent on a concomitant synthesis of RNA.

Physicochemical properties of the basal and the diet-adapted alkaline phosphatase. (a) Michaelis

Fig. 2. Incorporation of [14 C]leucine into the basal (a) and the diet-adapted (b) alkaline phosphatase determined by DEAE-Sephadex chromatography. After maintenance of rats for 5 days on complete diet or phosphate-free diet as described in the Experimental section each rat was given a single intraperitoneal injection of 30 μ Ci of [14 C]leucine. After 1 h four animals from each group were killed and their kidneys were pooled. Alkaline phosphatase was prepared from kidney microsomal preparation as outlined in the Experimental section and adsorbed on a column (20 cm $\times$ 2 cm) of DEAE-Sephadex A-25. The specific radioactivity of microsomal preparation was in the range of 4000-5000 c.p.m./mg of protein and portions corresponding to 30 mg of protein were employed. Elution was achieved with a linear gradient formed from 100 ml of 50 mM-tris-HCl buffer, pH 8.1, in the mixing chamber and the same buffer + 1 M-NaCl in the reservoir flask. The eluate fractions (1.8 ml) were assayed for radioactivity ($\blacktriangle$), E_{280} (—) and alkaline phosphatase activity (○) as described in the Experimental section.

constants. Michaelis constants for seven substrates were calculated by the double-reciprocal-plot method of Lineweaver & Burk (1934). As shown in Table 1 the K_m values are within a fairly narrow range (from 3×10^{-5} to 3.7×10^{-4} M) for different phosphorylated compounds and are the same for both the basal and the diet-adapted alkaline phosphatase. In addition, an investigation into the

Fig. 3. Incorporation of [^{14}C]leucine into the basal (a) and the diet-adapted (b) alkaline phosphatase determined by electrophoresis on polyacrylamide gel. Samples (150 μg of protein) of the basal and the diet-adapted enzyme labelled with [^{14}C]leucine and purified as described in the legend for Fig. 2 were subjected to electrophoresis in 7.5% (w/v) polyacrylamide gel at 3 mA/tube for 3 h. After electrophoresis the gels were cut into sections approx. 1 mm thick and each section was divided into halves for determination of enzymic activity (O) and radioactivity (Δ) as described in the Experimental section.

behaviour of K_m at various pH values was carried out with *p*-nitrophenyl phosphate as substrate. Similar pH-activity profiles were found with the diet-adapted and basal enzyme (Fig. 5).

(b) Inhibition by cysteine. Melani & Farnararo (1969) found that L-cysteine inhibits purified rat kidney alkaline phosphatase. This inhibition is reversible, pH-dependent and non-competitive. When cysteine concentration in the assay mixture

Fig. 4. Effect of actinomycin D on dietary adaptation of kidney alkaline phosphatase. The rats, kept on phosphate-free diet were divided into six groups: A, B, C, D, E and F. The animals were injected daily with actinomycin D (50 $\mu\text{g}/100\text{g}$ body wt.) intraperitoneally according to the following schedule: group A received the first injection at the start of dietary treatment and groups B, C, D, E and F were injected with the same drug after 1, 2, 3, 4 and 5 days of dietary treatment respectively. On day 6 all the animals were killed and alkaline phosphatase activity was determined on a fraction of kidney homogenate as described in the legend for Fig. 1. The enzyme activities are given as means $\pm$ S.E.M. for groups of four rats each.

is varied both enzymes are inhibited to the same extent (Fig. 6).

(c) Thermal inactivation. For these experiments 0.2 ml of enzyme solution eluted from DEAE-Sephadex during the purification procedure was incubated in a water bath at 45, 56 or 64°C, and 10 μl portions were removed at intervals and tested for enzyme activity with the specific substrate (Fig. 7). It is clear that the thermal-inactivation profiles at the three different temperatures in the absence of the substrate are the same for the basal and the diet-adapted enzyme.

(d) Elution from Sephadex G-200. To obtain the basal and the diet-adapted alkaline phosphatase labelled with ^3H and ^{14}C respectively, at the end of dietary treatment the rats were injected with [^{14}C]leucine and the controls were injected with [^3H]leucine. After an approx. 400-fold purification, labelled enzymes were pooled and then subjected to gel filtration on Sephadex G-200. Enzyme

Table 1. *Michaelis constants for the basal and the diet-adapted alkaline phosphatase with various substrates*

The incubation mixtures (1ml) contained 0.1M-carbonate-bicarbonate buffer, pH8.8, and substrates in concentrations ranging from 2mM to 25μM. Enzyme activity was determined by measuring the release of P_i. Basal and diet-adapted enzymes were both purified as outlined in the Experimental section.

Substrate	K_m (M)	
	Basal enzyme	Diet-adapted enzyme
<i>p</i> -Nitrophenyl phosphate	3×10^{-5}	2.5×10^{-5}
Glycerol 1-phosphate	1.1×10^{-4}	1×10^{-4}
Glycerol 2-phosphate	2×10^{-4}	1.8×10^{-4}
Glucose 6-phosphate	2.4×10^{-4}	2×10^{-4}
Glucose 1-phosphate	3.7×10^{-4}	3.5×10^{-4}
Phosphorylcholine	2.1×10^{-4}	2.2×10^{-4}

Fig. 5. pH profiles of pK_m for alkaline phosphatase preparation from control and diet-adapted rats. The enzymes from basal (○) and diet-adapted (●) rats were prepared from kidney as described in the legend for Fig. 1. Michaelis constants were calculated from a series of Lineweaver-Burk plots obtained at various pH values.

Fig. 6. Effects of L-cysteine on the activities of the basal and diet-adapted alkaline phosphatase. The enzymes from basal (○) and diet-adapted (●) rats were prepared from kidney as specified in the legend for Fig. 1. The reaction mixtures contained 3mM-*p*-nitrophenyl phosphate buffered at pH10.4 with carbonate-bicarbonate buffer. The activities, measured by the release of *p*-nitrophenol, are given as percentages of the control with no added cysteine.

activity and radioactivity were measured on each fraction. As illustrated in Fig. 8, the alkaline phosphatase activity is eluted as a nearly symmetrical peak coincident with peaks of ¹⁴C and ³H radioactivity. Hence the molecular sizes of the basal and the diet-adapted enzymes are the same within experimental error.

(e) Electrophoretic properties on polyacrylamide gel. The electrophoretic properties of the basal and the diet-adapted enzyme were analysed by using the method of split polyacrylamide gel. A perfect correspondence of the bands of migration toward the anode was observed for the two enzymes by using the specific staining with α-naphthyl phosphate and Fast Blue RR.

(f) Effect of cortisol and adrenalectomy on alkaline phosphatase activity. The dependence on

glucocorticoids of alkaline phosphatase activity in cell cultures (Cox & MacLeod, 1961, 1962) and in some tissues of amphibian and chick during development has been reported (Moog, 1959; Chieffi & Carfagna, 1960). As shown in Table 2, the kidney alkaline phosphatase activity appears unaffected by adrenalectomy and by administration of cortisol in intact and in adrenalectomized rats.

DISCUSSION

The diet-adapted and the basal rat kidney alkaline phosphatase exhibit different rates of degradation, that of the former being higher (Fig. 1). A similar enhancement in the rate of degradation

was observed by Schimke (1964) for rat liver arginase induced with an arginine-rich diet and by Segal & Kim (1963) for glutamate-pyruvate transaminase induced with glucocorticoids. As the possibility of a decrease in the rate of breakdown can be ruled out in our case, an increase in the rate of synthesis seemed likely. Direct evidence of an increase in the rate of synthesis was obtained by measurements of the relative extent of incorporation of [^{14}C]leucine into the enzyme in normal and diet-adapted animals: an approximately twofold increase of labelling occurs in the adapted enzyme

(Figs. 2 and 3). The dietary adaptation of alkaline phosphatase was proved to be sensitive to the action of actinomycin D (Melani *et al.* 1967), but if administration of the antibiotic is delayed until the fifth day after the start of dietary treatment it is com-

Fig. 7. Thermal inactivation of alkaline phosphatase purified from control and diet-adapted rats. Column-purified enzymes from control (○) and diet-adapted (●) rats were preincubated in 50mM-tris-HCl buffer, pH 8.1, at the temperatures and times indicated. Samples of heated enzymes were then assayed by using the standard conditions outlined in the Experimental section.

Fig. 8. Elution from Sephadex G-200 of alkaline phosphatase preparations from kidneys of control and diet-adapted rats. Animals were divided into two groups: the first was maintained on complete diet and the second on phosphate-free diet. At day 5 of dietary treatment control rats were given a single intraperitoneal injection of 30 μCi of [^3H]leucine, whereas rats fed on phosphate-free diet were injected with 30 μCi of [^{14}C]leucine. Then 1 h later animals from each dietary group were killed and their pooled kidneys were subjected to purification of alkaline phosphatase as outlined in the Experimental section. Column-purified enzymes were pooled, concentrated and then applied to a Sephadex G-200 column (1cm $\times$ 15cm). Elution was achieved with 50mM-tris-HCl buffer, pH 8.1. The eluate fractions (0.5ml) were assayed for alkaline phosphatase activity (○) and for ^3H (▲) and ^{14}C (△) radioactivities as described in the Experimental section.

Table 2. *Effects of cortisol and of adrenalectomy on the alkaline phosphatase activity*

Intact and adrenalectomized rats weighing 100–150g were treated with cortisol hemisuccinate (3mg) by a single intraperitoneal injection. After 4, 12 or 24h animals were killed and their kidneys were assayed for alkaline phosphatase activity as described in the legend to Fig. 1.

Treatment	No. of rats	Enzyme sp. activity ($\mu\text{mol/min per g of protein}$)
Intact rats		
Control	6	88 $\pm$ 16
Cortisol-treated		
4h	6	89 $\pm$ 14
12h	6	92 $\pm$ 13
24h	6	90 $\pm$ 12
Adrenalectomized rats		
Control	6	83 $\pm$ 14
Cortisol-treated		
4h	6	86 $\pm$ 15
12h	6	88 $\pm$ 17
24h	6	85 $\pm$ 14

pletely ineffective (Fig. 4). This finding may suggest the relative stability of the enzyme template.

It has been proposed that configurational changes that may occur during protein synthesis may alter the function of these proteins (Cline & Bock, 1966). Griffin & Cox (1966) reported that the induction of alkaline phosphatase in HeLa-cell cultures by prednisolone occurs at the level of protein synthesis as a result of steroid-induced changes in the conformational state of the enzyme. In contrast the properties of the basal and the diet-adapted rat kidney alkaline phosphatase are indistinguishable by the kinetic parameters studied. Thus the basal and the diet-adapted enzyme exhibit similar K_m values for several phosphomonoesters (Table 1), identical pH profile of pK_m with *p*-nitrophenyl phosphate as substrate (Fig. 5) and identical degree of inhibition produced by cysteine (Fig. 6). In addition, the enzymes from both sources do not differ with regard to thermal inactivation at 45, 56 or 64°C (Fig. 7), to speed of elution from Sephadex G-200 columns (Fig. 8) and to electrophoretic mobility on polyacrylamide gel. These findings on the whole do not suggest the possibility of a conformational change in enzyme leading to an increased activity of the individual enzyme molecules.

Another experimental result that requires mention is that rat kidney alkaline phosphatase does not respond to cortisol (Table 2), although this enzyme is very sensitive to glucocorticoids in human cell cultures (Cox & Macleod, 1961, 1962) and during development in amphibian, chick and mouse intestine (Moog, 1959; Chieffi & Carfagna, 1960). Hormonal regulation of alkaline phosphatase activity in human cell cultures has been extensively studied by De Carli, Santachiara Beneceretti & Cesari (1968). The finding of two enzymic fractions that are regulated in opposite fashion by prednisone leads these authors to the hypothesis that two alkaline phosphatase operons exist in these cells, one inducible and one repressible by prednisone. In addition, the physicochemical properties of the two enzymic fractions suggest the existence of multiple molecular forms, and therefore of a number of structural genes that code for their synthesis. In our case the existence in kidney cells of only one molecular form of alkaline phosphatase, firmly located in the microsomal fraction, the failure of cortisol to affect its activity and the specificity of the regulatory effect of P_i support the hypothesis that there is only one operon on which P_i acts. Such regulation of alkaline phosphatase at gene level has been widely accepted for *Escherichia coli* on the basis of much experimental support (Echols, Garen, Garen & Torriani, 1961; Garen & Echols, 1962; Rothman & Byrne, 1963; Garen &

Garen, 1963a,b; Gallant & Stapleton, 1964a,b). However, several differences in genetic organization between prokaryotes and eukaryotes (Ussing, 1941; Rotman & Speigelman, 1954; Hogness, Cohn & Monod, 1955; Koch & Levy, 1955; Thompson & Ballou, 1956; Swick, 1959; Buchanan, 1961; Tomkins *et al.* 1970) indicate the lack of a strict analogy between the changing of enzyme activity in bacteria and in animal tissues.

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REFERENCES

- Beisenhertz, G., Boltze, H. I., Bucher, Th., Czok, R., Garbade, R. K., Meyer-Arendt, E. & Pfeiderer, G. (1953). *Z. Naturf.* **8b**, 555.
- Bray, G. A. (1960). *Analyt. Biochem.* **1**, 279.
- Buchanan, D. L. (1961). *Archs Biochem. Biophys.* **94**, 501.
- Chieffi, G. & Carfagna, M. (1960). *Acta Embryol. Morph. exp.* **3**, 213.
- Civen, M. & Knox, W. E. (1959). *J. biol. Chem.* **234**, 1787.
- Cline, A. L. & Bock, R. M. (1966). *Cold Spring Harb. Symp. quant. Biol.* **31**, 321.
- Cox, R. P. & MacLeod, C. M. (1961). *Nature, Lond.*, **190**, 85.
- Cox, R. P. & MacLeod, C. M. (1962). *J. gen. Physiol.* **45**, 439.
- De Carli, L., Santachiara Beneceretti, A. S. & Cesari, I. (1968). *Ricerca scient.* **47**, 226.
- Echols, H., Garen, A., Garen, S. & Torriani, A. (1961). *J. molec. Biol.* **3**, 425.
- Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
- Gallant, J. & Stapleton, R. (1964a). *J. molec. Biol.* **8**, 431.
- Gallant, J. & Stapleton, R. (1964b). *J. molec. Biol.* **8**, 442.
- Garen, A. & Echols, H. (1962). *Proc. natn. Acad. Sci. U.S.A.* **48**, 1398.
- Garen, A. & Garen, S. (1963a). *J. molec. Biol.* **6**, 433.
- Garen, A. & Garen, S. (1963b). *J. molec. Biol.* **7**, 13.
- Greengard, O., Smith, M. A. & Acs, G. (1963). *J. biol. Chem.* **238**, 1584.
- Griffin, M. J. & Cox, R. P. (1966). *Proc. natn. Acad. Sci. U.S.A.* **56**, 946.
- Hogness, D. S., Cohn, M. & Monod, J. (1955). *Biochim. biophys. Acta*, **16**, 99.
- Koch, A. L. & Levy, H. R. (1955). *J. biol. Chem.* **217**, 947.
- Lineweaver, H. & Burk, D. (1934). *J. Am. chem. Soc.* **56**, 658.
- Low, R. B. & Wool, I. G. (1967). *Science, N.Y.*, **155**, 330.
- Melani, F. & Farnararo, M. (1969). *Biochim. biophys. Acta*, **178**, 93.
- Melani, F., Farnararo, M. & Sgaragli, G. (1968). *Experientia*, **24**, 114.
- Melani, F., Gueritore, A. (1964). *Experientia*, **20**, 474.
- Melani, F., Gueritore, A. & Baccari, V. (1964). *Nature, Lond.*, **201**, 710.
- Melani, F., Ramponi, G., Farnararo, M., Cocucci, E. & Gueritore, A. (1967). *Biochim. biophys. Acta*, **138**, 411.

- Moog, F. (1959). In *Cells, Organism and Milieu*, p. 121.
Ed. by Rudnich, D. New York: Roland Press Co.
- Ornstein, L. & Davis, B. J. (1959). *Proc. Soc. Study of Blood*. New York Academy of Medicine.
- Rothman, F. & Byrne, R. (1963). *J. molec. Biol.* **6**, 330.
- Rotman, B. & Spiegelman, S. (1954). *J. Bact.* **68**, 419.
- Schimke, R. T. (1964). *J. biol. Chem.* **239**, 3808.
- Schimke, R. T., Sweeney, E. W. & Berlin, C. M. (1965). *J. biol. Chem.* **240**, 322.
- Segal, H. L. & Kim, Y. S. (1963). *Proc. natn. Acad. Sci. U.S.A.* **50**, 912.
- Siegel, M. R. & Sisler, H. D. (1963). *Nature, Lond.*, **200**, 675.
- Swick, R. W. (1959). *J. biol. Chem.* **231**, 751.
- Thompson, R. G. & Ballou, J. R. (1956). *J. biol. Chem.* **223**, 795.
- Tomkins, G. M., Gelherter, T. D., Granner, T., Martin, D., Samuel, H. H. & Thomson, E. B. (1970). *Science, N.Y.*, **166**, 1474.
- Ussing, H. H. (1941). *Acta physiol. scand.* **2**, 209.
- Warburg, O. & Christian, W. (1941). *Biochem. Z.* **310**, 384.