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STUDIES ON THE MECHANISM OF ACTION OF SALICYLATE: EFFECTS ON ORTHOPHOSPHATE-EXCHANGE REACTIONS ASSOCIATED WITH OXIDATIVE PHOSPHORYLATION

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SUMMARY

The action of salicylate on various reactions associated with the oxidative phosphorylation system of the respiratory chain of mitochondria has been investigated. The reactions studied were: (a) the $[^{32}P]P_i$–ATP exchange reaction, (b) the P_i–$H_2{}^{18}O$ exchange, and (c) ATPase activity.

Addition of salicylate to mitochondrial suspensions resulted in inhibition of both exchange reactions accompanied by a stimulation of ATPase. 2,4-Dinitrophenol had a qualitatively similar action but on a quantitative basis was found to be approx. 100 times as potent as salicylate with regard to inhibition of exchange reactions, and at a given level of inhibition of the $[^{32}P]P_i$–ATP exchange reaction resulted in greater stimulation of ATPase activity.

The effects of other compounds chemically or pharmacologically similar to salicylate were investigated. None of the compounds approached salicylate or 2,4-dinitrophenol in their ability to depress $[^{32}P]P_i$–ATP exchange or stimulate ATPase activity.

Under appropriate conditions human erythrocyte hemolysates or the 105400 × *g* supernatant fraction of rat-liver homogenates were found to catalyze a $[^{32}P]P_i$–ATP exchange reaction. These exchange reactions were found to be insensitive to salicylate or 2,4-dinitrophenol.

INTRODUCTION

Previous studies by BRODY[1], PENNIALL[2] and PACKER *et al.*[3] have demonstrated that salicylates are capable of uncoupling phosphorylation from oxidation in the respiratory chain of mitochondrial systems.

Mitochondria from various sources have been found to catalyze a rapid reversible incorporation of $[^{32}P]P_i$ into ATP, and an oxygen-exchange reaction between water and P_i under conditions of no net electron flux in the respiratory chain[4]. The latter reaction may be measured conveniently by use of ^{18}O initially in water or P_i. These reactions, termed respectively the $[^{32}P]P_i$–ATP and the P_i–$H_2{}^{18}O$ exchange reactions, have been attributed to the transphosphorylation reactions associated with oxidative

phosphorylation in the respiratory chain and have been useful parameters in various investigations concerned with oxidative phosphorylation.

The purpose of the investigations described in this paper was to explore the action of salicylate on the aforementioned exchange reactions.

METHODS

Rat-liver mitochondria were prepared from fasted rats of the Holtzman strain essentially as described by LARDY AND WELLMAN[5]. The final mitochondrial pellet was suspended in a volume of 0.25 M sucrose equivalent in ml to the number of grams of fresh liver employed. The resulting mitochondrial suspension contained 3–4 mg of nitrogen/ml. Identical procedures were employed for the preparation of rat-kidney mitochondria. The 105400 × *g* supernatant fraction from rat-liver homogenates was prepared by centrifuging for 60 min at 40000 rev./min the supernatant fraction which remained after separation of the rat-liver mitochondria, centrifugation was performed with a No. 40 rotor in the Spinco Ultracentrifuge Model L. In Table IV this fraction is referred to as the supernatant fraction. Human erythrocyte hemolysates were prepared by centrifuging 10 ml of heparinized freshly drawn blood at 700 × *g* for 10 min, washing the separated packed red blood cells three times by suspending with 5 volumes of 0.15 M NaCl at 0° and centrifuging at 700 × *g* for 10 min, and finally hemolyzing the washed packed cells by three alternate freezing and thawing cycles at dry ice–acetone temperatures. All of the various tissue preparations employed in this study were used within 60 min of their preparation.

Before use, commercially obtained [^{32}P]orthophosphate was purified by the following procedure: the radioactive orthophosphate was initially heated for 20 min at 100° in 1 N HCl, next precipitated as $MgNH_4PO_4$, then washed with 1.5 M NH_4OH at 0°, and finally passed through a Dowex-50 column.

Water enriched with ^{18}O was obtained from the Weizmann Institute of Science, Rehovoth (Israel). Orthophosphate containing 1.85 atom per cent excess ^{18}O was prepared as described by COHN AND DRYSDALE[6] by reaction of P_2O_5 with $H_2{}^{18}O$.

Orthophosphate was determined by the procedure outlined by SUMNER[7].

The amount of [^{32}P]P_i–ATP exchange and the extent of exchange of ^{18}O between P_i and water were determined according to the procedures of BOYER, LUCHSINGER AND FALCONE[4] except that the ^{18}O content of H_2O derived from the pyrolysis of $KH_2P^{18}O_4$ was determined by a method which employed a high voltage discharge to catalyze the equilibration of oxygen between CO_2 and H_2O (see ref. 8).

All samples for ^{32}P analysis were counted for a total of 10000 counts.

The conditions under which the various exchange reactions were studied are given in the tables.

All reactions were terminated by addition of a volume of perchloric acid at 0° calculated to give final concentration of 0.25 M.

RESULTS

Comparison of the effect of various concentrations of salicylate and 2,4-dinitrophenol on inhibition of the [^{32}P]P_i–ATP exchange reaction and stimulation of ATPase activity

Fig. 1 graphically depicts the effects of various concentrations of salicylate on

the [^{32}P]P_i–ATP exchange reaction and the amount of ATP hydrolyzed during the period of incubation (ATPase activity). For comparative purposes, the effects of various concentrations of 2,4-dinitrophenol on these activities in the same mitochondrial preparation are presented. These data for 2,4-dinitrophenol are in accord with those previously reported[4]. The contents of the incubation mixtures and conditions of incubation are stated in the accompanying legend. The ordinates on the

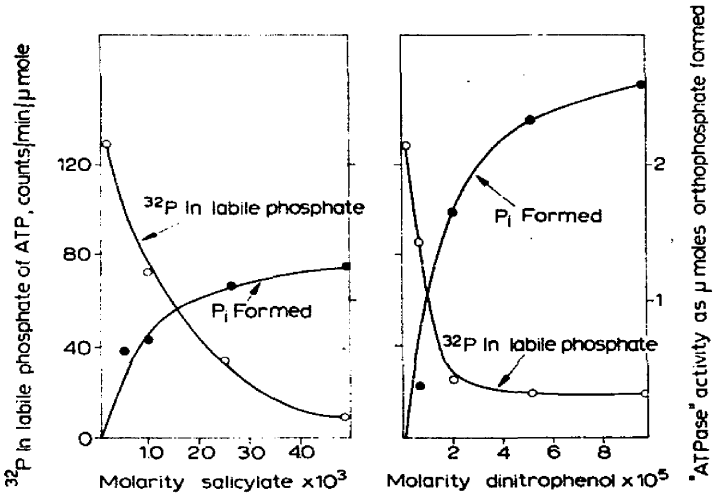

Fig. 1. Comparison of the effect of various concentrations of salicylate and 2,4-dinitrophenol on inhibition of the [^{32}P]P_i–ATP exchange reaction and stimulation of ATPase activity. Incubated at 20° for 15 min at pH 7. Contents: 10 μmoles ATP, 75 μmoles Tris, 10 μmoles P_i, 25 μmoles sucrose and concentrations of salicylate and 2,4-dinitrophenol as indicated. The reaction was initiated by addition of an amount of rat-liver mitochondria containing 0.37 mg of nitrogen. Final volume, 1.0 ml. Total radioactivity in P_i was equivalent to 14000 counts/min.

TABLE I

EFFECT OF VARIOUS COMPOUNDS ON [^{32}P]P_i–ATP EXCHANGE REACTION AND ATPASE ACTIVITY CATALYZED BY RAT-LIVER MITOCHONDRIA

*Addition to basic incubation mixture**	*Final concentration (M)*	*Specific activity of ^{32}P in ATP (counts/min/μmole)*	*Per cent inhibition of exchange*	*μmoles P_i formed*
None	0	648	0	0
Salicylic acid (*o*-hydroxybenzoic acid)	10^{-3}	332	49	1.40
m-Hydroxybenzoic acid	10^{-3}	560	14	0.10
p-Hydroxybenzoic acid	10^{-3}	517	20	0.12
2,4-Dihydroxybenzoic acid	10^{-3}	553	15	0
2,5-Dihydroxybenzoic acid	10^{-3}	571	12	0
2,5-Dihydroxybenzoic acid ethanolamide	10^{-3}	583	10	0
3,4-Dihydroxybenzoic acid	10^{-3}	564	13	0.08
Acetanilide	10^{-3}	609	6	0
Aminopyrine	10^{-3}	620	4	0
Antipyrine	10^{-3}	617	5	0
2,4-Dinitrophenol	10^{-4}	25	96	5.69

* Contents of basic incubation mixture and incubation conditions were identical to those stated in Fig. 1 with the following exceptions: Total radioactivity in P_i was equivalent to 20408 counts/min; reaction was initiated by addition of an amount of rat-liver mitochondria containing 0.39 mg of nitrogen.

left are expressed in terms of counts/min/μmole of ATP, those on the right express the μmoles of orthophosphate accumulating during the period of incubation as a result of ATPase activity. It is readily apparent that on a molar basis salicylate has approx. 0.01 the activity of 2,4-dinitrophenol in suppression of exchange activity and stimulation of ATP hydrolysis. At concentrations of $5 \cdot 10^{-3}$ M salicylate greatly inhibits exchange. Although both compounds have a qualitatively similar action on oxidative phosphorylation as noted by others[1,2], one important difference is shown by these data, namely, that salicylate appears to be somewhat less effective than 2,4-dinitrophenol with respect to stimulation of hydrolysis of ATP at concentrations where exchange is virtually abolished by both. It is noteworthy that at levels of salicylate corresponding to $1 \cdot 10^{-3}$ M (14 mg/100 ml) significant inhibition of exchange occurs. This concentration is well within the usual therapeutic levels obtained in blood. In other experiments it was found that inclusion of EDTA in the incubation mixture at a concentration of 10^{-3} M had no significant effect on the amount of exchange or ATPase activities in the presence of various concentrations of salicylate.

Effect of various compounds structurally or pharmacologically similar to salicylate on [^{32}P]P_i–ATP exchange reaction and ATPase activity

The influence of various compounds which are structurally or pharmacologically similar to salicylate on the [^{32}P]P_i–ATP exchange reaction and ATPase activity is seen in Table I. The data indicate that inhibition of exchange and stimulation of ATP hydrolysis are most marked with salicylate and 2,4-dinitrophenol. Other mono- and dihydroxy-substituted benzoic acid derivatives have much less inhibitory effect on the exchange and virtually no effect on stimulation of ATP hydrolysis. It is interesting that acetanilide, aminopyrine, and antipyrine, compounds which are in some respects pharmacologically similar to salicylate, are virtually inert in this system. The extent of inhibition of exchange and stimulation of ATPase noted with several of these compounds are in keeping with the observations of BRODY[1] which demonstrated that antipyrine, 2,5-dihydroxybenzoic acid, and 2,4-dihydroxybenzoate were weak or ineffective inhibitors of oxidative phosphorylation by rat-liver mitochondrial preparations. The latter investigator measured the overall process of oxidative phosphorylation by determining the P:O value.

The effect of salicylate on the [^{32}P]P_i–ATP exchange reaction and ATPase activity catalyzed by rat-kidney mitochondria

The data in Table II indicate that the effect of salicylate on the [^{32}P]P_i–ATP exchange reaction and ATPase activity catalyzed by rat-kidney mitochondria closely approximate the results obtained in rat-liver mitochondrial systems. We have observed findings similar to those in Table II when beef-heart mitochondria were employed.

Comparison of the effect of salicylate and 2,4-dinitrophenol on [^{32}P]P_i–ATP exchange reactions and ATPase activity catalyzed by various fractions of rat-liver homogenates and erythrocyte hemolysates

Under appropriate conditions erythrocyte hemolysates and the 105400 $\times$ g supernatant fraction from rat-liver homogenates catalyze appreciable [^{32}P]P_i–ATP exchange. The data in Tables III and IV illustrate this point. In contrast to the mitochondrial-catalyzed exchange which is closely associated with oxidative phosphory-

lation, the former exchange reactions require addition of magnesium ions, diphosphopyridine nucleotide and a phosphorylated glycolytic intermediate such as glucose 6-phosphate. The aforementioned requirements strongly indicate that the $[^{32}P]P_i$–ATP exchange reaction observed in these preparations is a result of the coupled action of triose phosphate dehydrogenase and phosphorylglyceryl kinase.

The data in Tables III and IV indicate that salicylate and 2,4-dinitrophenol do not inhibit the $[^{32}P]P_i$–ATP exchange reactions catalyzed by human erythrocyte hemolysates and the 105400 $\times$ g supernatant fraction from rat-liver homogenates. In addition, there is no stimulation of ATPase activity with these preparations. These findings are in marked contrast to those noted in mitochondrial systems.

TABLE II

EFFECT OF SALICYLATE ON THE $[^{32}P]P_i$–ATP EXCHANGE REACTION AND ATPASE ACTIVITY CATALYZED BY RAT-KIDNEY MITOCHONDRIA

*Molarity salicylate in incubation mixture**	*Specific activity of ^{32}P in ATP (counts/min/μmole)*	*Per cent inhibition of exchange*	*μmoles P_i formed*
0	83.1	0	0
$1 \cdot 10^{-3}$	30.0	64	0.75
$5 \cdot 10^{-3}$	3.7	96	1.08

* Contents of basic incubation mixture and incubation conditions were indentical to those stated in Fig. 1 with the following exceptions: Total radioactivity in P_i was equivalent to 8403 counts/min; reaction was initiated by addition of an amount of rat-kidney mitochondria containing 0.16 mg of nitrogen.

TABLE III

COMPARISON OF EFFECT OF SALICYLATE AND 2,4-DINITROPHENOL ON $[^{32}P]P_i$–ATP EXCHANGE REACTIONS AND ATPASE ACTIVITY CATALYZED BY RAT-LIVER MITOCHONDRIA AND HUMAN ERYTHROCYTE HEMOLYSATE

Enzyme preparation	*Final concentration 2,4-dinitrophenol (M)*	*Final concentration salicylate (M)*	*Specific activity of ^{32}P in ATP* (counts/min/μmole)*	*μmoles P_i formed*
Mitochondria**	0	0	479	0
Mitochondria**	0	10^{-3}	327	1.43
Mitochondria**	10^{-4}	0	14	5.87
Hemolysate***	0	0	110	0.61
Hemolysate***	0	$5 \cdot 10^{-3}$	100	0.41
Hemolysate***	10^{-4}	0	110	0.41
Hemolysate§	0	0	4	0.41

* Total radioactivity in P_i in all experiments was equivalent to 18180 counts/min.

** Contents of basic incubation mixture and incubation conditions were identical to those stated in Fig. 1. The reaction was initiated by addition of an amount of rat-liver mitochondria containing 0.44 mg of nitrogen.

*** Contents of basic incubation mixture and incubation conditions were identical to those stated in Fig. 1 except that 5 μmoles of $MgCl_2$, 10 μmoles of glucose 6-phosphate, and 2.5 μmoles of diphosphopyridine nucleotide were added. The reaction was initiated by addition of an amount erythrocyte hemolysate containing 6.8 mg nitrogen. This amount was used in all subsequent experiments in this table.

§ Conditions were identical to the preceding three experiments in this table except that 1 μmole of iodoacetate was added.

TABLE IV

COMPARISON OF EFFECT OF SALICYLATE AND 2,4-DINITROPHENOL ON [^{32}P]P_i–ATP EXCHANGE REACTIONS AND ATPASE ACTIVITY CATALYZED BY VARIOUS FRACTIONS OF RAT-LIVER HOMOGENATE

Homogenate fraction	*Final concentration 2,4-dinitrophenol (M)*	*Final concentration salicylate (M)*	*Specific activity of ^{32}P in ATP* (counts/min/μmole)*	*μmoles P_i formed*
Mitochondria**	0	0	440	0
Mitochondria**	0	10^{-3}	272	0.96
Mitochondria**	10^{-4}	0	11.5	4.75
Supernatant fraction***	0	0	0	0.23
Supernatant fraction§	0	0	124	0.45
Supernatant fraction§	0	$5 \cdot 10^{-3}$	125	0.14
Supernatant fraction§	10^{-4}	0	114	0.14
Supernatant fraction§§	0	0	2	0.14

* Total radioactivity in P_i in all experiments was equivalent to 20000 counts/min.

** Contents of basic incubation mixture and incubation conditions were identical to those stated in Fig. 1. The reaction was initiated by addition of an amount of rat-liver mitochondria containing 0.37 mg of nitrogen.

*** Contents of basic incubation mixture and incubation conditions were identical to those stated in Fig. 1. The reaction was initiated by addition of an amount of supernatant fraction containing 0.12 mg nitrogen. This amount was used in all subsequent experiments in this table.

§ Contents of basic incubation mixture and incubation conditions were identical to those stated in Fig. 1 except that 5 μmoles $MgCl_2$, 10 μmoles glucose 6-phosphate, and 2.5 μmoles of diphosphopyridine nucleotide were added.

§§ Conditions were identical to preceding three experiments in this table except that 1 μmole of iodoacetate was added.

Demonstration that the effect of salicylate on [^{32}P]P_i–ATP exchange reaction and ATPase activity catalyzed by rat-liver mitochondria is removable by washing the mitochondria

The experiment described by Table V demonstrates that salicylate inhibition of the [^{32}P]P_i–ATP exchange reaction and stimulation of ATPase activity may be largely removed from a mitochondrial suspension preincubated with salicylate by rewashing the mitochondria in 0.25 M sucrose. Thus, it appears that salicylate exerts its effect on the mechanism of oxidative phosphorylation without firm binding to a component of the mechanism. In this respect the action of salicylate is similar to the findings of LOOMIS AND LIPMANN[9] for 2,4-dinitrophenol.

Salicylate inhibition of the P_i–$H_2{}^{18}O$ exchange reaction catalyzed by rat-liver mitochondria

The P_i–$H_2{}^{18}O$ exchange reaction catalyzed by mitochondria in the absence of net electron transport in the respiratory chain appears to constitute another partial reaction of oxidative phosphorylation[4]. Accordingly, it was of interest to investigate the effect of salicylate on this activity. The data set forth in Table VI conclusively demonstrate that addition of salicylate in concentrations of 10^{-3} M results in a pronounced inhibition of this exchange reaction as evidenced by the increased retention of ^{18}O in orthophosphate. For comparative purposes the extent of [^{32}P]P_i–ATP exchange and ATPase activity were determined simultaneously in this experiment. The results indicate that all three activities are progressively affected by increasing concentrations of salicyl...

TABLE V

DEMONSTRATION THAT THE EFFECT OF SALICYLATE ON [^{32}P]P_i–ATP EXCHANGE REACTION AND ATPASE ACTIVITY CATALYZED BY RAT-LIVER MITOCHONDRIA IS REMOVABLE BY WASHING THE MITOCHONDRIA

Treatment of mitochondria Expt.	*Final concentration salicylate (M)*	*Specific activity of ^{32}P in ATP per milligram of mitochondrial nitrogen* (counts/min/μmoles/mg)*	*μmoles P_i formed/mg mitochondrial nitrogen (μmoles/mg)*
1** (a)	0	5794	0
(b)	$5 \cdot 10^{-3}$	546	5.1
2*** (a)	0	5876	0
(b)	$5 \cdot 10^{-3}$	4053	0

* Total radioactivity in P_i in all experiments was equivalent to 68000 counts/min.

** An amount of mitochondria containing 0.30 mg of nitrogen was preincubated for 5 min at 0° with stirring in the following preincubation mixture: 75 μmoles Tris, 25 μmoles sucrose and in the case of (b) 5 μmoles of salicylic acid. The final volume was 0.8 ml (pH 7.0). The entire contents of each preincubation mixture was then incubated with 10 μmoles ATP and 10 μmoles P_i in a final volume of 1.0 ml at 20° for 15 min.

*** Mitochondria in Expts. 2 (a) and 2 (b) preincubated respectively as described in 1 (a) and (b). After preincubation the contents were centrifuged at 10000 × *g* for 10 min and the supernatant fractions discarded. Each of the pellets was suspended in 20 ml of 0.25 M sucrose and recentrifuged at 10000 × *g* for 10 min. The supernatant fractions were discarded. Incubation conditions for measurement of exchange and ATPase activity were identical to 1 (a) and 1 (b).

TABLE VI

SALICYLATE INHIBITION OF P_i–$H_2{}^{18}O$ AND [^{32}P]P_i–ATP EXCHANGE REACTIONS CATALYZED BY RAT-LIVER MITOCHONDRIA

*Molarity salicylate in incubation mixture***	*ΔP_i (μmoles)*	*Atom per cent excess ^{18}O in P_i**		*Specific activity of ^{32}P in ATP§ (counts/min/μmole)*
		Observed	*Decrease as a result of oxygen exchange****	
0	0	0.928	0.638	449
$1 \cdot 10^{-3}$	5.1	1.046	0.418	242
$5 \cdot 10^{-3}$	9.4	1.233	0.154	29

* The atom per cent excess ^{18}O in P_i of the zero-time control was 1.566.

** Incubated at 20° for 15 min at pH 7. Contents: 50 μmoles ATP, 375 μmoles Tris, 75 μmoles P_i, and 125 μmoles sucrose. The reaction was initiated by addition of an amount of rat-liver mitochondria containing 1.8 mg of nitrogen. Final volume, 5.0 ml.

*** Calculated as follows[4] for P_i formation as a result of ATPase activity:

$$\text{decrease as a result of oxygen exchange} = \left[\frac{\text{initial } \mu\text{moles } P_i \times 1.566}{\text{final } \mu\text{moles } P_i}\right] - \text{observed atom per cent excess } {}^{18}O \text{ in } P_i.$$

§ Total radioactivity in P_i was equivalent to 130000 counts/min.

DISCUSSION

The findings presented in this paper demonstrate that salicylate in mitochondrial systems inhibit the [^{32}P]P_i–ATP and P_i–$H_2{}^{18}O$ exchange reactions and that these actions are accompanied by stimulation of ATPase activity. Although the mechanism of oxidative phosphorylation in the respiratory chain is largely unknown, much evidence has been accumulated which has led to a consensus of opinion which regards

the aforementioned activities as partial reactions of the over-all process of oxidative phosphorylation[4,10,11]. In agreement with the conclusions of others[1,2], the data indicate that the actions of salicylate in this respect bear a qualitative similarity to the action of 2,4-dinitrophenol. On a quantitative basis, however, 2,4-dinitrophenol appears to be about 100 times as potent as salicylate with respect to suppression of $[^{32}P]P_i$–ATP exchange and at a given level of inhibition of exchange results in greater stimulation of ATPase activity.

There have been many reported experimental findings suggesting that uncoupling of oxidative phosphorylation occurs as a result of salicylate administration in man and in experimental animals. The recent findings of AUSTEN *et al.*[12] have demonstrated a significant correlation between oxygen consumption and serum salicylate concentration in euthyroid patients. This has received firm experimental support from various studies *in vitro* which have shown an increase in oxygen uptake by whole tissue preparations from salicylate-treated rats[1], and in isolated whole tissue or mitochondrial systems to which salicylate has been added[1,13]. Studies with mitochondrial systems have demonstrated that the increase in oxygen uptake as a result of salicylate addition is accompanied by uncoupling of oxidative phosphorylation[1–3].

Review of the findings of others indicates that salicylate administration induces widespread alterations in carbohydrate metabolism. Thus, in intact experimental animals depletion of liver glycogen has been observed in the normal rat[14], and in the diabetic rat reduction in glycosuria and hyperglycemia has been reported[15]. Diminished glycogen synthesis has been noted in isolated rat-liver slices[16]. SMITH AND MOSES[17] have reported that salicylate and 2,4-dinitrophenol, a classical uncoupler of oxidative phosphorylation, produce similar qualitative effects on the extent of incorporation of radioactivity from labeled glucose or acetate into water-soluble metabolic intermediates of various isolated rat tissues. The latter authors concluded that the general effects of salicylate and 2,4-dinitrophenol could be explained in terms of their uncoupling action on oxidative phosphorylation in that synthetic reactions involving ATP were inhibited and catabolic reactions were not affected.

At the present level of knowledge the above considerations do not offer a simple explanation of various specific pharmacologic actions of salicylate such as analgesia, antipyresis and antirheumatic action. An uncoupling action mediating these varied effects, however, cannot be excluded. For example, salicylate penetration and distribution intracellularly may markedly differ with various cell types, and various metabolic mechanisms of different cells may have a wide divergency of dependence on the fundamental process of oxidative phosphorylation.

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REFERENCES

1 T. M. BRODY, *J. Pharmacol. Exptl. Therap.*, 117 (1956) 39.
2 R. PENNIALL, *Biochim. Biophys. Acta*, 30 (1958) 247.
3 L. PACKER, F. K. AUSTIN AND E. C. KNOBLOCK, *Proc. Soc. Exptl. Biol.*, 100 (1959) 239.
4 P. D. BOYER, W. W. LUCHSINGER AND A. B. FALCONE, *J. Biol. Chem.*, 223 (1956) 405.
5 H. A. LARDY AND H. WELLMAN, *J. Biol. Chem.*, 195 (1952) 215.
6 M. COHN AND G. R. DRYSDALE, *J. Biol. Chem.*, 216 (1955) 831.
7 J. B. SUMNER, *Science*, 100 (1944) 413.
8 A. B. FALCONE, *Anal. Biochem.*, 2 (1961) 147.
9 W. F. LOOMIS AND F. LIPMANN, *J. Biol. Chem.*, 173 (1948) 807.
10 A. L. LEHNINGER, C. L. WADKINS, C. COOPER, T. M. DEVLIN AND J. B. GAMBLE, *Science*, 128 (1958) 450.
11 E. C. SLATER, *Revs. Pure and Appl. Chem. (Australia)*, 8 (1958) 221.
12 F. K. AUSTEN, M. E. RUBINI, W. H. MERONEY AND J. WOLFF, *J. Clin. Invest.*, 37 (1958) 1131.
13 M. J. H. SMITH AND S. W. JEFFREY, *Biochem. J.*, 63 (1956) 524.
14 G. LUTWAK-MANN, *Biochem. J.*, 36 (1942) 706.
15 M. J. H. SMITH, B. W. MEADE AND J. BORNSTEIN, *Biochem. J.*, 50 (1952) 18.
16 M. J. H. SMITH, *Biochem. J.*, 59 (1955) 52.
17 M. J. H. SMITH AND V. MOSES, *Biochem. J.*, 76 (1960) 579.