Biochemical Pharmacology, Vol. 32, No. 15, pp. 2333–2337, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00

EVIDENCE RELATING THE INHIBITORY EFFECT OF COBALT ON THE ACTIVITY OF δ-AMINOLEVULINATE SYNTHASE TO THE INTRACELLULAR CONCENTRATION OF PORPHYRINS

NILI SCHOENFELD*, YEHUDIT GREENBLAT*, ORIT EPSTEIN*, DONALD P. TSCHUDY† and ABRAHAM ATSMON*‡

*Laboratory of Biochemical Pharmacology, Department of Internal Medicine B, The Beilinson Medical Center, Petah Tiqva, Israel; and The Sackler School of Medicine, Tel Aviv University, Ramat Aviv, Israel; and † The National Institute of Health, Bethesda, Maryland, U.S.A.

(*Received* 20 *December* 1982; *accepted* 15 *March* 1983)

Abstract—This study shows that the inhibition of ALAS activity caused by cobalt is directly correlated with the intracellular porphyrin concentration, thus indicating that cobalt exerts its inhibitory effect on ALAS activity as a result of the formation of cobalt-protoporphyrin.

Cobalt is known to have complex effects on enzymes and chemical intermediates related to heme and hemoproteins and to their synthesis and degradation [1–9]. Special attention has been focused on the inhibition of δ-aminolevulinate synthase (ALAS) activity by $CoCl_2$, variously ascribed to a direct action of the metal ion [8, 10] or to the enzymatic formation of cobalt-protoporphyrin (CoP) [3, 6, 11]. Recently, the formation of CoP has been confirmed in several experimental systems [12–16].

The aim of this investigation was mainly to clarify the relation between the concentration of intracellular porphyrins and the inhibitory effect of $CoCl_2$ on the activity of ALAS.

MATERIALS AND METHODS

Metalloporphyrins and porphyrins were obtained from Porphyrin Products, Logan, UT, and from Sigma Chemicals, St. Louis, MO. Collagenase and hyaluronidase were purchased from Grand Island Biological Co., Grand Island, NY. Allyl-isopropyl-acetamide (AIA) was a gift from Hoffman La-Roche, Nutley, NJ. 1-4-Diethoxycarbonyl-3,5-dihydrocollidine (DDC) was obtained from Aldrich Chemicals, Milwaukee, WI and succinylacetone (SA) from Omega Organics, Longmont, CO. All other chemicals were of reagent grade. Modified Ham's F-12 medium [17] was purchased from the Central Biology Laboratory, Haim Sheba Medical Center, Tel Hashomer, Israel. Monolayers of chick embryo liver cells were prepared according to Sassa and Kappas [17]. Metalloporphyrin and $CoCl_2$ solutions were prepared according to the method of Burnham (personal communication) with slight modifications (submitted for publication).

‡ Correspondence should be addressed to: Professor A. Atsmon, Internal Medicine Department B, Beilinson Medical Center, Petah Tiqva, Israel.

For determination of ALAS activity two different methods were employed, both according to Sinclair and Granick [18]. In the indirect method the amount of aminolevulinic acid (ALA) accumulating in the medium and the cells during 18 hr of incubation in the presence of 25 mM levulinate and 5 mM glycine was measured. In the direct method the amount of ALA formed by a cell homogenate during 30 min of incubation at 37° was determined.

Activity of tyrosine-aminotransferase was determined as described previously [19].

Porphyrins were extracted and measured by the method of Granick [20]. Heme was determined by the pyridine hemochromogen method [21].

Incorporation of [^{14}C]leucine into protein and [^{14}C]thymidine and [^{14}C]uridine into DNA and RNA, respectively, was determined as described previously [22, 23]. Proteins were measured according to Lowry *et al.* [24].

The statistical tests used were the following: *t*-paired comparison test, Student's *t*-test, a single classification analysis of variance followed by a Sum of Squares Stimulation Test Procedure (SS-STP) and computation of Pearson's Product-Moment Correlation Coefficient [25].

RESULTS

In the experiments carried out during these investigations, both a direct and an indirect method for measuring ALAS activity were used (see Materials and Methods). The comparability of these methods, in the presence of the various substances used, was ascertained by determination of ALAS activity of batches of cultures, prepared and incubated simultaneously. The results are shown in Table 1.

A *t*-paired comparison test showed no significant difference between the results obtained with the two methods ($P > 0.05$). In view of these results and since the indirect method has many advantages compared to the direct method, ALAS activity was

Table 1. Changes in ALAS activity, measured by two different methods

Treatment	ALAS activity (% decrease)	
	Indirect method	Direct method
Protoporphyrin, 10^{-5} M	59, 61	61, 62
Iron-protoporphyrin, 10^{-5} M	61, 63	59, 62
$CoCl_2$, 10^{-5} M	40, 45	38, 40
Cobalt-protoporphyrin, 10^{-5} M	96, 97	91, 96

Monolayers of chick embryo liver cells were prepared and incubated for 24 hr as outlined in the Materials and Methods. AIA, 60 μg/ml medium, was added to all the dishes and further additions were made as outlined. After 18 hr ALAS activity was determined according to the indirect and direct methods (Materials and Methods). Each value shown is the average of two determinations. The absolute values of the AIA induced controls were 24.0 ± 1.8 nmoles ALA/mg protein · 18 hr according to the indirect method and 2.3 ± 0.2 nmoles ALA/mg protein · 30 min according to the direct method.

determined by the indirect method in most of the experiments.

Since cobalt is thought to depress ALAS activity through formation of CoP the effects of cobalt and porphyrins, separately and combined, were measured (see Table 2).

The results indicate that the various substances added to the medium had a dose-dependent inhibitory effect on the induction of ALAS activity. The inhibition caused by porphyrins was in the same range as that of heme. The addition of desferrioxamine, 1.5 mM, completely obviated this effect (not shown), indicating that it is probably caused by the porphyrins being converted into iron-porphyrins.

The effects of the various cobalt-porphyrins were compared to those of cobalt and the relevant porphyrin, each group at 10^{-7}, 10^{-6} and 10^{-5} M separately. A single classification analysis of variance was performed on the numerical values of ALAS activity obtained in the control and the test cultures. An SS-STP based on the variance within groups was employed to test whether the effects of the treatments indicated above are significantly different from each other. The evaluation showed that, at all concentrations examined, the inhibition of ALAS activity resulting from the simultaneous addition of cobalt and porphyrin did not differ significantly from that obtained by cobalt-porphyrin ($P > 0.05$).

It is of interest that CoP at the three concentrations examined depressed induced ALAS activity more than the purported physiological repressor heme.

The time related effects of the various combinations of cobalt and protoporphyrin on ALAS activity are shown in Fig. 1.

Figure 1 shows that, at all time points indicated, the inhibition of ALAS, caused by the addition of CoP, was similar to that obtained with cobalt and protoporphyrin added separately and constituted the approximate additive effects of cobalt and protoporphyrin. These results indicate that neither the transport of cobalt or of protoporphyrin from the medium to the location of the chelatase, nor the

Table 2. The percent inhibition of induced ALAS activity caused by cobalt, porphyrins and cobalt-porphyrins in monolayers of chick embryo liver cells

Additions	% inhibition of ALAS activity		
	10^{-5} M	10^{-6} M	10^{-7} M
$CoCl_2$	40 ± 5	16 ± 2	0 ± 2
Protoporphyrin	61 ± 6	38 ± 3	22 ± 3
Mesoporphyrin	69 ± 5	53 ± 4	28 ± 3
Deuteroporphyrin	58 ± 5	43 ± 5	25 ± 4
Cobalt-protoporphyrin	98 ± 1	65 ± 5	38 ± 3
$CoCl_2$ + protoporphyrin	95 ± 3	66 ± 1	37 ± 3
Cobalt-mesoporphyrin	92 ± 3	64 ± 2	25 ± 3
$CoCl_2$ + mesoporphyrin	90 ± 5	66 ± 3	20 ± 4
Cobalt-deuteroporphyrin	96 ± 4	65 ± 2	35 ± 4
$CoCl_2$ + deuteroporphyrin	90 ± 6	68 ± 5	28 ± 4
Iron-protoporphyrin	61 ± 2	32 ± 3	21 ± 2

Monolayers of chick embryo liver cells were prepared and incubated for 24 hr as outlined in the Materials and Methods. The medium was replaced and various substances, together with AIA, 60 μg/ml medium, were added as shown. After a further 18 hr of incubation ALAS activity was determined by the indirect method. Each value is the average and S.D. of 4–6 dishes.

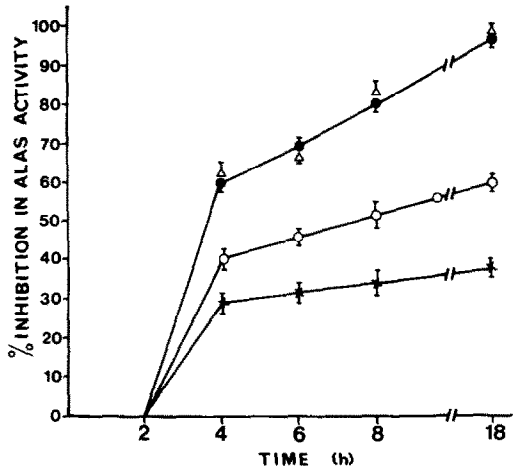

Fig. 1. Monolayers were prepared and incubated for 24 hr (see Materials and Methods). The medium was replaced by fresh medium and the various substances together with AIA, 60 μg/ml, were added (time 0). ALAS activity was determined by incubation of cell homogenates (direct method). All the data are averages of four determinations. ALAS activity of cultures treated with AIA only (controls) was determined at the time points indicated and was regarded as 100%. (×) Cobalt, (○) protoporphyrin, (●) cobalt-protoporphyrin and (△) cobalt + protoporphyrin. All these additions were 10^{-5} M, final concentration.

chelatase reaction are rate-limiting for the inhibitory effect on ALAS activity.

The inhibition of ALAS activity caused by cobalt was considerably less than that of CoP. Assuming that cobalt inhibits ALAS activity through formation of CoP it was surmised that the intracellular concentration of porphyrins might be rate-limiting for the formation of CoP. In order to test this assumption, the intracellular porphyrin concentrations were manipulated by various drugs (Table 3, Nos. 1–7) and by adding protoporphyrin to the medium (Table 3, Nos. 8–10).

The results of these experiments (Table 3) clearly show a significant correlation (Product-Moment Correlation Coefficient = 0.95, $P < 0.001$) between the concentration of porphyrins in the cells of the control cultures and the inhibition of ALAS activity caused by cobalt. In the cultures treated with the drugs which endogenously raise or lower intracellular porphyrins (Table 3, Nos. 1–7), no significant correlation was found between either the cellular content of heme and the inhibition caused by cobalt or the activity of ALAS and this inhibition ($r = 0.54$ and 0.16, respectively). On the other hand, in the cultures treated with various concentrations of protoporphyrin, the inhibition caused by cobalt was positively correlated with the intracellular heme levels ($r = 0.99$ $P < 0.001$), and negatively with ALAS activity ($r = 0.92$, $P < 0.01$).

CoP, 10^{-5} M, inhibited ALAS by 93–97% with all the various additions outlined in Table 3 (not shown).

The presence of cobalt during 18 hr of incubation would considerably reduce the concentration of cellular porphyrins [15], thus influencing the amount of CoP formed and the inhibition of ALAS. In order to investigate this point, the experiments shown in Fig. 2 were carried out. The results show that when cobalt is added 12 hr after addition of AIA, the ALAS activity at 16 hr is indeed lower than when cobalt is added simultaneously with AIA (A) ($P < 0.001$). This is in accordance with the higher level of porphyrins at 12 hr incubation with AIA only,

Table 3. The effect of $CoCl_2$ on ALAS activity related to the intracellular concentration of porphyrins and heme

		Without $CoCl_2$ (controls)			With $CoCl_2$ 10^{-5} M	
No.	Additions	ALAS activity (nmoles ALA/mg protein · 18 hr)	Heme content (pmoles/mg protein)	Total porphyrins (pmoles/mg protein)	% decrease in ALAS activity	P
1	None	5.0 ± 0.2	244 ± 17	126 ± 13	20 ± 3	<0.01
2	SA	40.5 ± 1.2	218 ± 12	91 ± 3	15 ± 5	<0.05
3	AIA + SA	175.0 ± 13.0	252 ± 15	91 ± 2	10 ± 3	N.S.
4	DES	5.2 ± 0.2	205 ± 17	227 ± 6	28 ± 1	<0.001
5	DDC	22.5 ± 1.9	344 ± 16	584 ± 58	35 ± 2	<0.001
6	AIA	24.0 ± 2.1	412 ± 10	635 ± 33	37 ± 5	<0.001
7	AIA + DES	65.1 ± 7.1	314 ± 16	4400 ± 132	80 ± 7	<0.001
8	AIA + PROTO 10^{-7} M	18.7 ± 1.7	390 ± 8	536 ± 19	35 ± 1	<0.001
9	AIA + PROTO 10^{-6} M	14.9 ± 1.2	512 ± 17	1291 ± 209	58 ± 3	<0.001
10	AIA + PROTO 10^{-5} M	10.2 ± 1.3	875 ± 26	16453 ± 737	94 ± 2	<0.001

Monolayers of chick embryo liver cells were prepared and incubated during 24 hr (see Materials and Methods). The medium was replaced and the various additions were made as outlined. After a further 18 hr of incubation the concentrations of cellular porphyrins and heme were measured. ALAS activity was determined by the indirect method and the heme content by the pyridine hemochromogen method. Percent inhibition of ALAS activity by cobalt was calculated separately for each treatment on the basis of its control cultures, to which the same additions had been made, but without cobalt. The data shown are the means ± S.D. of the results obtained in two or three separate experiments, i.e. 4–6 dishes. Final concentrations of the various additions were as follows: AIA, allyl-isopropylacetamide, 60 μg/ml; DES, desferrioxamine, 1.5 mM; DDC, diethoxycarbonyl dihydrocollidine, 15 μg/ml; SA, succinylacetone, 1 mM; PROTO, protoporphyrin, 10^{-7}–10^{-5} M. P was calculated on the basis of absolute values of ALAS according to Student's t test.

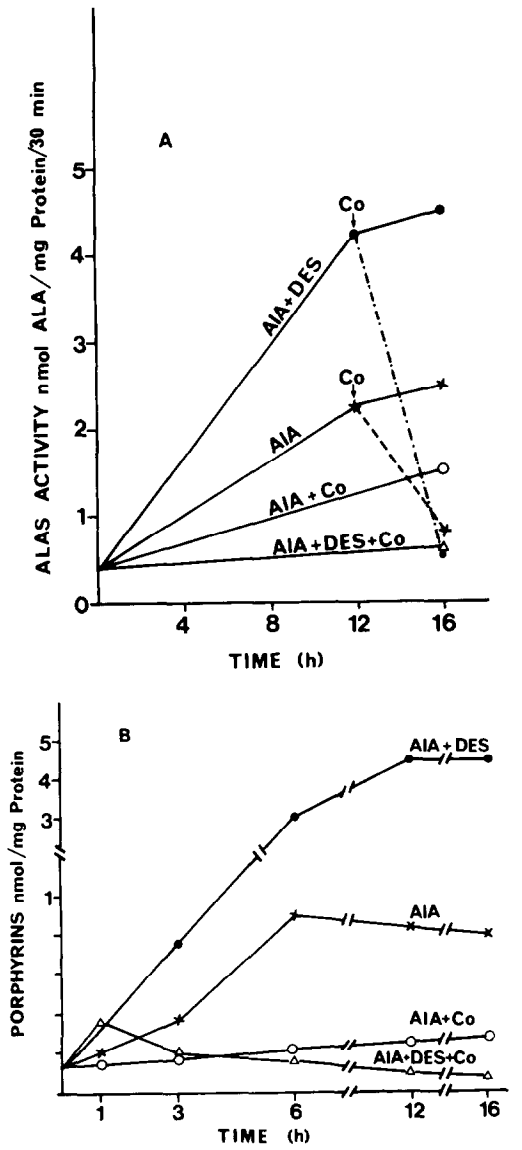

Fig. 2. AIA, 60 μg/ml, or AIA and desferrioxamine (DES), 1.5 mM, was added to monolayers of chick embryo liver cells. $CoCl_2$, 10^{-5} M, was added simultaneously or after 12 hr. ALAS activity, by the direct method (A) and porphyrins (B) were determined at the times indicated.

rather than with AIA + cobalt (B). This effect was not obtained in the presence of AIA and desferrioxamine. It should be pointed out that the ALAS values in the presence of AIA + DES and cobalt are very low and small differences might not have been detected.

$CoCl_2$, 10^{-5} M did not have any effect on ALAS induced activity in the presence of 2.5 mM EDTA, which inhibits the formation of CoP [26].

The inhibitory effect of cobalt, protoporphyrin or CoP, 10^{-5} M, on ALAS activity was a specific one since no inhibition of incorporation of [^{14}C]leucine, [^{14}C]thymidine and [^{14}C]uridine into proteins, DNA and RNA, respectively, was observed (not shown). Moreover, induction of tyrosine aminotransferase with dexamethasone was stimulated by 20%, $P < 0.005$, by this concentration of $CoCl_2$. Protoporphyrin, 10^{-5} M, had no effect and CoP, 10^{-5} M, decreased the induced activity of TAT by about 20% (not shown).

DISCUSSION

Since the mid-1970s cobalt has been known to inhibit ALAS in rat [6, 8, 27, 28] and in chick embryo liver cells [29]. The mechanism of this effect was variously attributed to a direct action of cobalt on the synthesis of ALAS [8] or to the formation of CoP [3, 6, 11]. During the last few years it was conclusively shown that small amounts of CoP are formed in rat and chick embryo liver cells, after injection of $CoCl_2$ or addition of $CoCl_2$ to the medium of monolayers [12–16], and it was implied that this CoP acts like heme on the synthesis of ALAS. It is of interest that, also similar to heme, CoP stimulates heme synthesis in murine erythroleukemia cells, though it does not form cobalt-globin [30].

Preliminary investigations with cobalt and protoporphyrin seemed to indicate that the inhibition caused by cobalt is limited by the concentration of intracellular protoporphyrin. Additional indications to this effect were published by De Matteis and Gibbs [28] and Sinclair *et al.* [11]. If the inhibition of ALAS activity by cobalt were shown to be dependent, within limits, on the intracellular concentration of protoporphyrin, this would constitute strong additional evidence indicating that the effect of cobalt is indeed caused by the formation of CoP and that the latter is the inhibiting agent.

The experiments in which the intracellular porphyrins were raised or lowered (Table 3) convincingly show that the inhibition of ALAS activity caused by cobalt is directly correlated with the intracellular porphyrin concentration. No correlation was found between this inhibition and the cellular concentration of heme, except under conditions where heme was positively correlated with the cellular concentrations of porphyrins.

In additional experiments, it was shown that cobalt-proto-, cobalt-meso- and cobalt-deuteroporphyrins inhibit induced ALAS activity to approximately the same extent, indicating that the inhibitory effect is not specific for the protoporphyrin moiety. We were unable to find comparable experiments in the literature. The inhibition caused by the cobalt-porphyrins was found to be considerably more effective than that caused by heme, $P < 0.001$, as compared on a molar basis according to their concentration in the medium. The same effect was observed by Maines [31] *in vivo* in rat liver. However, Drummond and Kappas [32] reported that, in rats, iron-protoporphyrin was a stronger inhibitor than CoP.

The mechanism of inhibition of ALAS activity by CoP is probably similar to that of heme. CoP might, in addition, affect ALAS activity by competitive inhibition of the heme oxygenase system. This could result in an increase in the concentration of heme in the regulatory heme pool. However, this direct competitive inhibition was proven *in vitro* [33] at a concentration which was higher by several orders of magnitude from that found intracellularly by Sinclair *et al.* [16] in the system of chick embryo liver cells.

The formation of CoP is thought to be mediated by chelatase. It is well-known that the chelatase enzyme is not entirely specific for either Fe^{2+} or protoporphyrin [21, 34–38]. Several lines of evidence indicate the existence of more than one species of chelatase [35, 38]. Recently, Canepa and Llambias described differential effects of various drugs on the formation of ferro-mesoporphyrin and cobalt-mesoporphyrin indicating that, possibly, different enzymes are involved [26]. Our results seem to confirm this assumption since desferrioxamine did not obviate the inhibition caused by cobalt whereas EDTA, which chelates both iron and cobalt [26], prevented the decrease in ALAS activity caused by cobalt.

These observations extend those of De Matteis and Gibbs [28] and Sinclair *et al.* [11, 15, 16] and strongly indicate that cobalt exerts its inhibitory effect on ALAS activity as a result of CoP formation.

REFERENCES

1. P. L. Scholnick, L. E. Hammaker and H. S. Marver, *J. biol. Chem.* **247**, 4132 (1973).
2. N. Hayashi, Y. Kurashima and G. Kikuchi, *Archs. biochem. Biophys.* **148**, 10 (1972).
3. T. R. Tephly, C. Webb, P. Trussler, F. Kniffen, E. Hasegawa and W. Piper, *Drug Met. Dispos.* **1**, 259 (1973).
4. Y. Yasukochi, M. Nakamura and S. Minakami, *Biochem. J.* **144**, 455 (1974).
5. M. D. Maines and A. Kappas, *J. biol. Chem.* **250**, 4171 (1975).
6. F. De Matteis and A. H. Gibbs, *Ann. clin. Res.* **8**, suppl 17, 193 (1976).
7. M. D. Maines and A. Kappas, *Biochem. J.* **154**, 125 (1976).
8. M. D. Maines, V. Janousek, J. M. Tomio and A. Kappas, *Proc. natn. Acad. Sci. U.S.A.* **73**, 1499 (1976).
9. T. R. Tephly, G. Wagner, R. Sedman and W. Piper, *Fedn Proc. Fedn Am. Socs. exp. Biol.* **37**, 35 (1978).
10. M. D. Maines and A. Kappas, *Science N.Y.* **198**, 1215 (1977).
11. P. Sinclair, A. H. Gibbs, J. F. Sinclair and F. De Matteis, *Biochem. J.* **178**, 529 (1979).
12. J. Igarashi, N. Hayashi and G. Kikuchi, *J. Biochem.* **84**, 997 (1978).
13. S. Watkins, J. Baron and T. R. Tephly, *Biochem. Pharmac.* **29**, 2319 (1980).
14. A. Lodola, *FEBS Lett.* **123**, 137 (1981).
15. J. F. Sinclair, P. R. Sinclair, J. F. Healey, E. L. Smith and H. L. Bonkowsky, *Biochem. J.* **204**, 103 (1982).
16. P. R. Sinclair, J. F. Sinclair, H. L. Bonkowsky, A. H. Gibbs and F. De Matteis, *Biochem. Pharmac.* **31**, 993 (1982).
17. S. Sassa and A. Kappas, *J. biol. Chem.* **252**, 2428 (1977).
18. P. R. Sinclair and S. Granick, *Analyt. Biochem.* **79**, 380 (1977).
19. O. Epstein, N. Schoenfeld and A. Atsmon, *FEBS Lett.* **73**, 251 (1977).
20. S. Granick, *J. biol. Chem.* **241**, 1359 (1966).
21. R. J. Porra and O. T. G. Jones, *Biochem. J.* **87**, 181 (1963).
22. N. Schoenfeld and A. Atsmon, *Biochem. Pharmac.* **26**, 867 (1977).
23. N. Schoenfeld, O. Epstein and A. Atsmon, *Biochem. biophys. Res. Commun.* **76**, 460 (1977).
24. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
25. R. R. Sokal and F. J. Rohlf (Eds), *Biometry*, 2nd Ed. W. H. Freeman, San Francisco (1981).
26. E. T. Canepa and E. B. C. Llambias, *Enzyme* **26**, 288 (1981).
27. M. Nakamura, Y. Yasukochi and S. Minakami, *J. Biochem.* **78**, 373 (1975).
28. F. De Matteis and A. H. Gibbs, *Biochem. J.* **162**, 213 (1977).
29. M. D. Maines and P. Sinclair, *J. biol. Chem.* **252**, 219 (1977).
30. C. S. Chang and S. Sassa, *J. biol. Chem.* **257**, 3650 (1982).
31. M. D. Maines, *Biochim. biophys. Acta* **673**, 339 (1981).
32. G. S. Drummond and A. Kappas, *Proc. natn. Acad. Sci. U.S.A.* **78**, 6466 (1981).
33. T. Yoshinaga, S. Sassa and A. Kappas, *J. biol. Chem.* **257**, 7778 (1982).
34. R. J. Porra and O. T. G. Jones, *Biochem. J.* **87**, 186 (1963).
35. E. Hasegawa, C. Smith and T. R. Tephly, *Biochem. biophys. Res. Commun.* **40**, 517 (1970).
36. R. F. Labbe and N. Hubbard, *Biochim. biophys. Acta* **52**, 130 (1961).
37. A. Johnson and O. T. G. Jones, *Biochim. biophys. Acta* **171**, 321 (1969).
38. A. M. Mazanowska, A. Neuberger and G. H. Tait, *Biochem. J.* **98**, 117 (1966).