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Interaction of Antimycin A with Biological Systems*

Minocher Reporter

ABSTRACT: A soluble protein (CAAF) obtained from washed chicken liver mitochondria mitigates the inhibition of respiration caused by antimycin A in isolated, intact mitochondria. The protein is active in differentiating chick muscle cultures and in explanted chick embryos treated with antimycin A. Bovine serum albumin (BSA) is one of several proteins which are inactive. A system of CAAF, antimycin A, and BSA has been used to investigate the mode of action of antimycin A. Antimycin A fluoresces at 421 mμ when excited at 348 mμ. The fluorescence of antimycin A at pH 7.5 is enhanced fivefold by an equimolar concentration of BSA. Addition of CAAF to the BSA-bound antimycin quenches this fluorescence. The BSA-bound antimycin also exhibits a characteristic fluorescence on excitation of the protein at 278 mμ, due probably to dipole–dipole coupling. This fluorescence is also quenched by CAAF. These data are interpreted to mean that antimycin A is capable of forming a ternary complex. The mechanism of complex formation is the likely cause of the effects of antimycin A in intact mitochondrial systems.

Antimycin A causes a pronounced specific effect(s) on tissues undergoing differentiation in addition to its action at the subcellular level. When explanted chick embryos are exposed to antimycin A heart formation is prevented. In muscle culture monolayers antimycin prevents maintenance and further formation of muscle fibrils. Single cells do not appear to be affected morphologically at concentrations of antimycin which destroy myofibrils. The effects of antimycin on these systems can be prevented by a purified protein (CAAF)[1] prepared from liver mitochondria of adult chickens (Reporter and Ebert, 1965). The present report describes a restorative effect of CAAF on isolated mitochondria treated with antimycin A and the interactions among BSA, antimycin A, and CAAF.

Experimental Section

Materials. Antimycin A_3 was obtained from Wisconsin Alumni Research Laboratories (lot order no. 12,243)

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[1] Abbreviations used: CAAF, the protein factor from chicken liver mitochondria which alleviates effects of antimycin A on chicken embryo explants and mitochondria; BSA, bovine serum albumin; ADP, adenosine diphosphate.

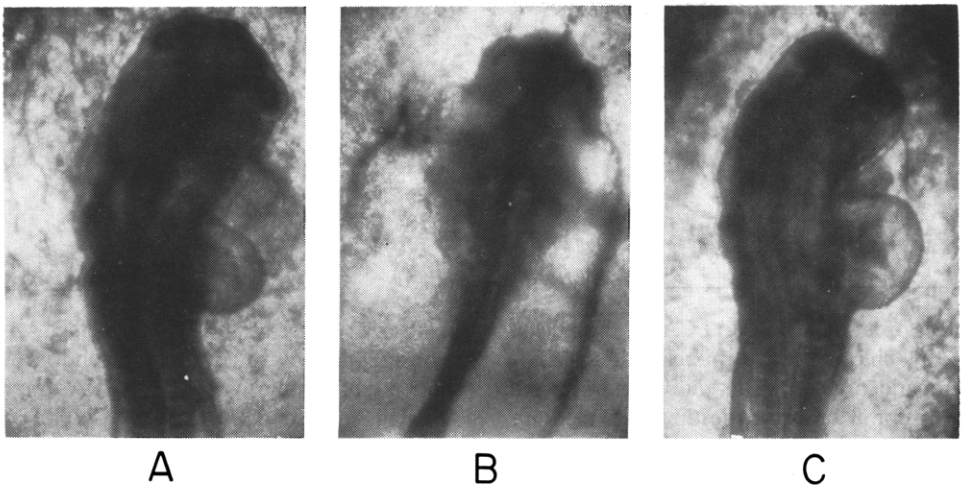

FIGURE 1: Effect of mitochondrial protein on explanted chick embryos treated with antimycin A. (A) Control embryo; (B) embryo exposed to 5×10^{-8} M antimycin A in the medium; (C) embryo exposed to 5×10^{-8} M antimycin A together with mitochondrial protein estimated at 6×10^{-8} M.

or from Sigma Chemical Co., St. Louis, Mo. (type III lot no. 54B1716). The antibiotic was used without further purification.

All other biochemicals were obtained from Sigma Chemical Co. or Worthington Biochemical Corp., Freehold, N. J. Reagents for iron determination were obtained from G. F. Smith Chemical Co., Columbus, Ohio. All other common chemicals used were Fisher reagent grade.

Measurements and Analysis. pH measurements were made with a DELTA-matic expanded-scale meter from Instrumentation Laboratories, Inc., Boston, Mass. Absorption spectra were obtained with a Bausch and Lomb 505 ratio recording spectrophotometer. Other measurements were made with a Beckmann DU spectrophotometer, equipped with a Gilford Laboratories optical density converter.

An Aminco-Bowman spectrophotofluorometer was used to record fluorescence spectra. Fluorescence is given in relative intensity units obtained with a meter multiplier setting of 0.1.

Oxygen uptake was measured with a bare platinum vibrating-reed electrode polarized at 0.66 v. The platinum electrode was dipped into a cylindrical chamber, 0.9 cm in diameter, and was connected to a reference electrode *via* the floor of the cylindrical chamber, which was made from a silver sheet. The reference electrode was Ag–AgCl. Temperature was regulated by circulating water around both electrode chambers. The circulating water was passed through a Haake Thermoregulator water pump. Calibrations for linearity of oxygen consumption were obtained by using suitable mixtures of glucose, glucose oxidase, and catalase. Iron was determined with *o*-phenanthroline using the method of Beinert as outlined by Rieske *et al.* (1964a). Protein was determined by the method of Lowry *et al.* (1951).

The technique of Spratt was used for explanting chick embryos. Explanted chick embryos were used for confirmation of antimycin A and CAAF potency (Reporter and Ebert, 1965). Photographs of representative embryos during such a test are presented in Figure 1.

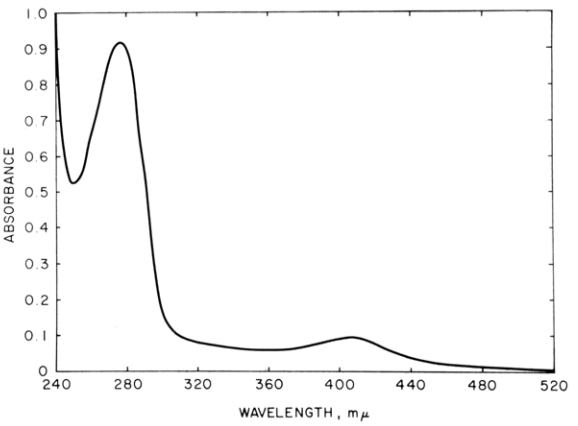

FIGURE 2: Absorption spectrum of protein factor (CAAF) isolated from a preparation of washed chicken liver mitochondria.

Preparation of Mitochondria. Mitochondria were isolated in a medium consisting of 0.3 M sucrose, 10^{-3} M EDTA, and 0.05 M Tris–HCl (pH 7.7). Tissues were homogenized with either a Polytron machine made by Kinematica GmgH. Lucerne, or a Teflon and glass Potter–Elvehjem homogenizer. All operations were carried out at 0–4°.

Preparation of CAAF. The preparation of CAAF has been published (Reporter and Ebert, 1965). All but one of the experiments reported below were conducted with a single preparation. The yield of CAAF from 1 kg of chicken livers was 50 mg. The CAAF

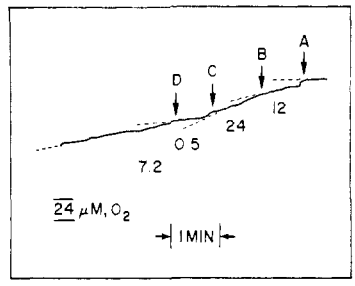

FIGURE 3: Effect of antimycin A and CAAF on oxygen consumption by chicken heart mitochondria. Mitochondria were added at A. The mitochondria were incubated at 4° for 8 hr before testing in a medium which contained 50 μg of BSA, 100 μM succinate, 100 μM P_i, 20 μM $MgSO_4$, 0.25 M sucrose, and Tris-HCl 0.01 M, pH 7.2. At B–D, 40 μM ADP, 0.2 μM antimycin A, and 70 μg of CAAF, respectively, were added. The numerals after each addition refer to oxygen consumption in micromoles per minute per milligram of protein. Mitochondria protein (900 μg) was used in a final volume of 1 ml. Incubation temperature, 25°.

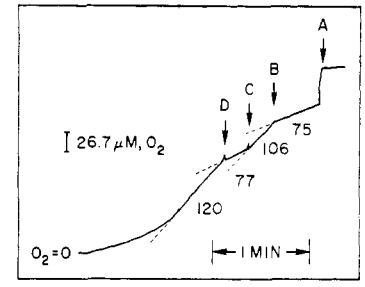

FIGURE 4: Effect of antimycin A and CAAF on oxygen consumption by rat liver mitochondria. Mitochondria (6.8 mg of protein) were added at A to a medium containing sucrose 0.3 M; Tris–HCl 0.05 M, pH 7.4; EDTA 10 μM; P_i 250 μM; and succinate 600 μM. At B–D were added 600 μM ADP, 0.2 μM antimycin A, and 50 μg of CAAF, respectively. The numerals after each addition refer to oxygen consumption in μmoles per minute per milligram of protein. Final volume of the mixture was 1.5 ml. Incubation temperature, 25°.

was frozen at −70° in small aliquots and used as needed after absorption and reisolation from hydroxyapatite with 0.06 M phosphate buffer, pH 7.1.

Results

Observations on CAAF. The ability of CAAF to counter the effects of antimycin A on chick embryos was unaffected by ribonuclease, deoxyribonuclease, or trypsin under a variety of conditions. CAAF was destroyed after heating at 60° for 10 min or after repeated freezing and thawing (five cycles). It was also inactivated in presence of 0.1 M phosphate (pH 7.1) or in the presence of 0.002 M phosphate (pH 7.1) and 0.2 M NaCl.

CAAF is dissociated into at least two precipitin bands by these inactivation treatments. The precipitin bands were demonstrated by Ouchterlony (1949) technique with rabbit antiserum prepared *vs.* purified CAAF and also by immunoelectrophoresis (Grabar and Williams, 1953). The absorption spectrum of the CAAF used is shown in Figure 2. After treatment of CAAF with alkaline pyridine and borohydride, a spectrum similar to a protoheme was obtained with peaks at 561, 416, and 390 mμ (Lemberg and Legge, 1949). The heme is regarded as adventitious because the CAAF has been isolated in a few cases with no indication of a 405–410-mμ peak. In such cases, CAAF was quite capable of reversing the effects of antimycin on embryos. One such sample contained 5 mg of protein/ml and gave a single precipitin band. The iron content of this sample was 0.8–0.9 mole/mole of protein. (The molecular weight of CAAF is assumed to be 100,000 from its exclusion pattern in Sephadex G-200 and from preliminary runs in the analytical ultracentrifuge.)[2] This iron is not involved in the action of CAAF *vs.* antimycin A as shown in tests with explanted chick embryos. For one such test, 615 μg of CAAF was treated with 2 mg of sodium borohydride followed by 1 mmole of *o*-phenanthroline/ml of histidine buffer (0.002 M, pH 7.5). The mixture was placed in a dialysis bag that had been previously treated with EDTA and dialyzed twice *vs.* 4 l. of the histidine buffer. The treated CAAF was capable of reversing the effects of antimycin in explanted chick embryos as shown in Table I. The dialyzed CAAF was active after a second such treatment of the same sample in which dithionite was substituted for borohydride. (The iron content of the treated CAAF was not determined.)

In explanted chick embryos no effects were noted when up to 20 μg of CAAF was added per ml of the control medium. Upon increasing the concentration of CAAF to 30 μg or higher/ml of medium, embryo growth slowed. Embryos failed to develop at concentrations of CAAF higher than 50 μg. Oxygen consumption by isolated mitochondria was also inhibited by CAAF if the protein was added to mitochondria in large amounts [CAAF protein (500 μg) added to mitochondria containing 2–3 mg of protein/ml of incubation medium]. CAAF inhibited up to 90% of oxygen consumption by mitochondrial preparations which failed to exhibit acceptor control.

A second effect of CAAF was the relief of inhibition of oxygen consumption in antimycin A treated mitochondria. To observe this reversal it was necessary

[2] A weight-average molecular weight was estimated from a plot of log c_n *vs.* x^2 or the concentration change across the solution column relative to the initial concentration (Young *et al.*, 1965). The help of Miss Sylvia Himmelfarb for these measurements is greatly appreciated.

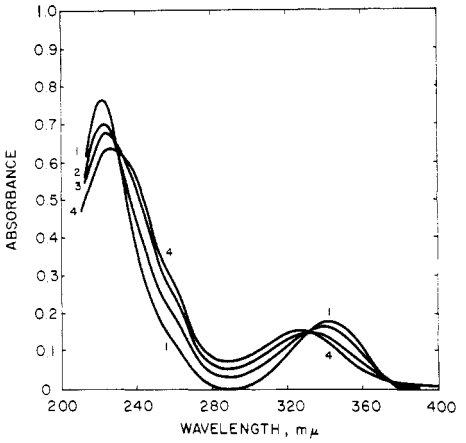

FIGURE 5: Absorption spectra of antimycin A at different pH values. The final concentration of antimycin A was 2.38 × 10^{-5} M and was obtained by adding 0.05 ml of a stock solution in 90% alcohol to 1 ml each of 0.05 M phosphate buffers. The pH values of buffers in curves labeled 1-4 are, respectively, 7.75, 7.34, 6.80, and 6.38.

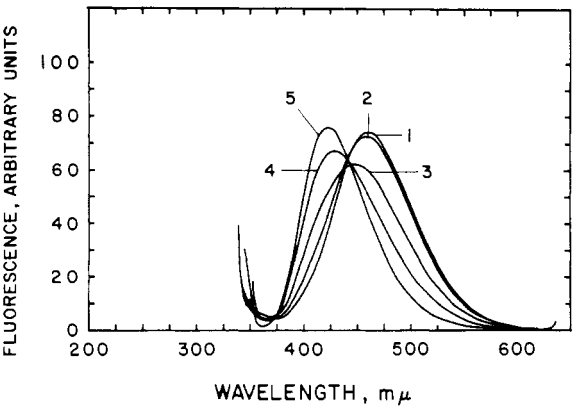

FIGURE 6: Fluorescence spectra of antimycin A. The final concentration of antimycin A was 2.38 × 10^{-5} M and was obtained by adding 0.05 ml of a stock solution in 90% alcohol to 1 ml each of 0.05 M phosphate buffers. Curves are uncorrected for detector response.

TABLE I: Effect of Treating CAAF with Reducing Agents and *o*-Phenanthroline.

	Average No. of Somite Pairs			
Group[a]	No. of Embryos	Initial	Final	Heart
A	3	6	24	Present
B	4	6.1	11	Absent
C	4	5.2	22	Present
D	4	4.5	26	Present

[a] A, control group, no additions; B, 0.027 μg of antimycin A/ml of medium; C, 0.027 μg of antimycin + 10 μg of untreated CAAF/ml of medium; D, 0.027 μg of antimycin + 10 μg of CAAF/ml of medium (factor treated with $NaBH_4$ and *o*-phenanthroline).

to use mitochondrial preparations which exhibited some degree of acceptor control, *i.e.*, presumably preparations in which cytochrome *b* was functional in electron transport. CAAF was used in moderate amounts (up to 200 μg of CAAF/2–3 mg of mitochondrial protein per ml of medium) to exhibit this reversal. Relief by CAAF of antimycin-inhibited oxygen uptake was obtained with preparations of mitochondria from chick heart, rat heart, chick and rat livers, as well as whole homogenates of normal chick embryos harvested near the time of heart formation. Figure 3 shows relief of antimycin inhibition by CAAF on a preparation of mitochondria isolated from hearts of 12-day-old chicks. The small degree of recovery with addition of the factor after severe inhibition of oxygen consumption by antimycin is usual. If an amount of antimycin A which would give less than a 50% inhibition of oxygen consumption was added to a mitochondrial preparation in Chance's (1958) state 3, the inhibition was fully reversed by CAAF. Figure 4 shows this type of reversal with rat liver mitochondria. In all cases further addition of antimycin inhibited uptake of oxygen, indicating a functional respiratory chain. Because of irregular and unpredictable response it cannot be ascertained if acceptor control was present in CAAF-reversed mitochondrial respiration.

Observations on Antimycin A. The pH-dependent changes in the absorption spectrum of antimycin A are shown in Figure 5. The absorption changes at 320 and 345 mμ were proportional to the pH, between pH values of 6 and 9. The concentration of phosphate buffers did not affect the absorption curve at pH 7.4. When pH was changed from acidic (pH 6.05) to basic (pH 7.63) and back with phosphate buffers, the spectrum between 400 and 450 mμ could be recovered. The antimycin therefore could not have suffered much damage. Three experiments were performed to determine ionization constants by spectrometry (Albert and Serjeant, 1962). pH was controlled by the use of 0.05 M phosphate (pH 5–8), 0.05 M Tris–HCl (pH 8–11.5), or 0.05 M acetate (pH 3.5–5) buffers. The alcohol concentration was kept below 5%. Under these conditions, the pK_a for antimycin was calculated to be 7.3 ± 0.07. At alcohol concentrations greater than 30%, pK_a values were estimated between 5.5 and 6.

The excitation and fluorescence maxima of antimycin A also changed with pH. Figure 6 shows changes in fluorescence maxima at various pH values between 6.5 and 7.5 (see Table II). Fluorescence was not in-

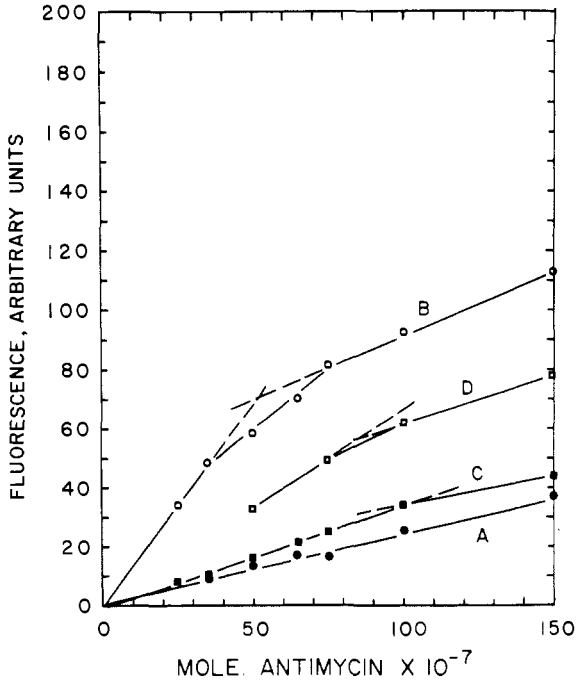

FIGURE 7: Plot of fluorescence of increasing concentrations of antimycin A at 421 mμ under excitation by 348 mμ of light in 0.05 M phosphate buffer, pH 7.5. Curve A, antimycin alone; curve B, in presence of 33 × 10^{-7} M BSA; curve C, in presence of 100 × 10^{-7} M CAAF; and curve D, 1:1 mixture of solutions of curves B and C. Fluorescence values are uncorrected for detector response.

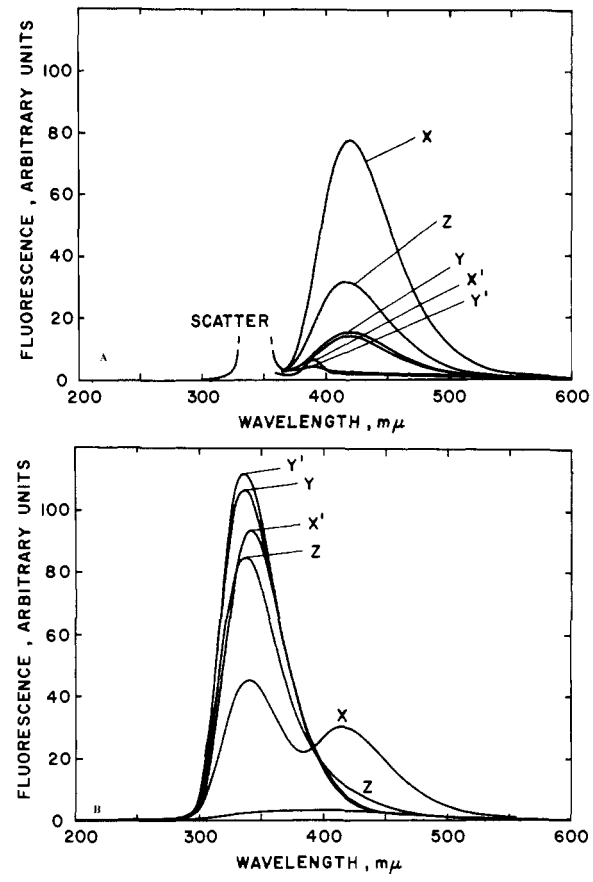

FIGURE 8: Fluorescence spectra of antimycin A under excitation by light at 348 (A) and 278 mμ (B). Unlabeled curve, antimycin A alone; curve X, antimycin A in presence of BSA; curve Y, antimycin A in presence of CAAF; curve Z, antimycin A in presence of both proteins. X′ and Y′ are spectra of the respective proteins without antimycin A. (B) Letters labeling curves similar to Figure 8A. Spectra uncorrected for detector response. The concentration of antimycin A, CAAF, and BSA were 5.7, 5, and 5 × 10^{-9} M, respectively.

TABLE II: Spectral Changes of Antimycin A.

Curve	pH	Activn Max (mμ)	Fluorescence Max (mμ)	Fluorescence Units
1	6.50	328	460	75
2	6.82	330	458	71
3	7.02	334	448	62
4	7.20	342	432	66
5	7.48	348	421	76

fluenced by changing the molarity of the phosphate buffers used at pH 7.5. An ethanol solution of antimycin used for the above experiments was calculated to show an ϵ_{329} of 4787.

Binding Studies. The effects of BSA and CAAF on antimycin A fluorescence are shown in Figure 7. Under the conditions of measurement, neither protein exhibited fluorescence. The fluorescence of antimycin was greatly enhanced by BSA in contrast to CAAF as shown in curves A–C. When the antimycin fluorescence curve was repeated after mixing the solutions used for obtaining curves B and C, a quenching of the fluorescence was observed. The fluorescence values at low concentrations of antimycin (below 10^{-5} M) were lower than those expected by averaging of curves B and C. Equilibrium dialysis experiments were performed in order to obtain a possible explanation of this quenching. Table III shows the results of one experiment. No change was expected in the fluorescence of the protein adducted antimycin when the two dialyzed proteins were mixed as compared with the mixture of the dialysate and BSA–antimycin A. The assumption was that both proteins had bound sufficient quantities of the antibiotic after the 24-hr dialysis period. The BSA-induced fluorescence was quenched by 15 units. The degree of this quenching was proportional to the amount of factor used as determined by other experiments not shown here.

TABLE III: Fluorescence of Antimycin A After Equilibrium Dialysis.[a]

Sample	Fluorescence Units
Dialysate	24
BSA + Antimycin A	147
CAAF + Antimycin A	33
1 + 2 (1 :1 addition)	66
2 + 3 (1 :1 addition)	50

[a] 5×10^{-9} mole/ml of BSA and 5×10^{-9} mole/ml of mitochondrial factor were placed in separate dialysis bags and dialyzed for 24 hr at 4° *vs.* a solution of 9.85×10^{-9} mole/ml of antimycin A. The antimycin was dissolved in 100 ml of phosphate buffer, pH 7.5. Fluorescence was excited at 348 mμ and measured at 421 mμ. The fluorescence is designated in arbitrary units and uncorrected for detector response.

The protein–antimycin A fluorescence spectra were also examined by excitation of the tryptophan peak of the proteins. Excitation of antimycin A fluorescence could be expected due to dipole–dipole coupling or resonance energy transfer (Förster, 1959), because excitation of the proteins at 278 mμ yielded fluorescence at 348 mμ, a wavelength at which antimycin A, under these conditions, exhibited a maximum absorption peak. A check showed that *at the concentrations of antimycin used* there was negligible fluorescence at 348 mμ upon exciting the molecule at 278 mμ with the Aminco-Bowman instrument. (The absorbance of antimycin A alone in the buffer used was near 0.05.) Figure 8A and B show fluorescence spectra of the proteins alone, BSA plus antimycin, CAAF plus antimycin, and a mixture of BSA plus antimycin plus CAAF. Upon exciting a combination of BSA plus antimycin at 278 mμ there resulted two peaks with fluorescence maxima at 348 and 421 mμ (Figure 8B, curves X and X′). The value for 348-mμ fluorescence fell from 94 to 43 units. When interacting with antimycin alone, there was only a small decrease in the tryptophan fluorescence of CAAF from 117 to 102 units and only a slight increase in fluorescence at 421 mμ could be detected.

When the above solutions of BSA with antimycin and CAAF with antimycin were mixed in a ratio of 1:1 and excited at 278 mμ, there was negligible fluorescence at 421 mμ when compared to the BSA plus antimycin alone (Figure 8B, curve Z). Fluorescence at 348 mμ was 86 (an average of curves, X, Y, X′, and Y′) instead of the expected 98 units from fluorescence of proteins alone (an average of curves X′ and Y′).

To estimate the degree of interaction among CAAF, antimycin, and BSA, a series of experiments were performed with rabbit antiserum to the CAAF. The antiserum variations for these experiments consisted of whole antiserum, antisera from two different rabbits, crude globulin fractions isolated from the antiserum *vs.* CAAF, negative control serum from nonimmunized rabbits, and finally, as a positive control, anti-BSA rabbit serum from a commercial source (Mann Research Laboratories).

The following observations are drawn from a number of representative experiments on the precipitin reaction. (a) Antimycin A changed the isolated mitochondrial protein because slightly larger precipitates were consistantly obtained from the controls with antimycin A than from the CAAF alone. (b) A still larger precipitate was formed when small amounts of BSA were added to antimycin. This may reflect an interaction between all components of the system. Interference with precipitin reaction was brought about by increasing the amount of BSA *vs.* anti-CAAF serum and *vice versa*. (c) Antimycin was qualitatively detected in precipitates digested with a combination of the proteinases, Nagarse and trypsin. The detection was made using the fluorescence spectrum of the digested mixture and the characteristic change in this spectrum with a change in pH of the mixtures. (If antimycin acts as a hapten with the antiserum to CAAF, the perturbation of the tryptophan residues of the antiserum can be studied.) (d) Precipitin bands of Ouchterlony plates after photographic enlargement showed bands at 0, 2.8, 3.0, 2.9, and 3.1 cm from the antiserum-containing center well of the plate. The respective wells contained BSA, CAAF, BSA plus CAAF, CAAF plus antimycin, BSA plus antimycin, and BSA plus CAAF plus antimycin. A small spur could be detected between precipitin bands of wells containing CAAF plus antimycin and CAAF plus BSA. The wells lacking the CAAF failed to show any bands. When antimycin was included with the factor in Ouchterlony plates, denser and and narrower precipitin bands were noted.

Discussion

A pH-dependent alteration of the antibiotic molecule has been proposed (Estabrook, 1962a,b) as one representative factor in the antimycin inhibition of succinoxidase activity of nonphosphorylating heart muscle mitochondrial particles. This proposal was based on interpretation of results from an analog computer simulation of a succinoxidase system. The inhibitor was also shown to prevent splitting by bile salts of coenzyme Q–cytochrome *c* reductase complex (Rieske *et al.*, 1964b). These workers suggested that the prevention of electron flow by the antibiotic was due to conformational changes in proteins involved in the linkage between cytochrome *b* and another component of the mitochondrial electron transport chain. However cytochrome *b* may not be an obligate electron transport carrier in nonphosphorylating mitochondrial particles (Chance, 1958). It has also been suggested that a nonheme iron protein may not be intimately involved at the "antimycin-sensitive" site (Rieske *et al.*, 1964a,b).

The observations with CAAF strengthen the possibility that the isolated protein can involve a part of the mitochondrial oxidative phosphorylation system.

TABLE IV: Precipitin Reaction.[a]

Expt	Anti-mycin Alone	BSA Alone	CAAF Alone	CAAF + BSA	CAAF + Anti-mycin	CAAF + BSA + Anti-mycin	BSA + Anti-mycin	
I, anti-CAAF serum B	50	10	100	96	137	192	...	20 μg of CAAF, 20 μg of BSA, antimycin 10^{-8} M, antiserum 0.1 ml
II, anti-CAAF serum B	20	26	100	...	110	100	37	30 μg of CAAF, 120 μg of BSA, antimycin 10^{-8} M, antiserum 0.12 ml
III, anti-BSA serum	2	100	−10	97	0	88	113	50 μg of CAAF, 50 μg of BSA, antimycin 10^{-8} M, antiserum 0.15 ml

[a] Experiments I and II were carried out with the same sample of rabbit antiserum *vs.* the mitochondrial factor, CAAF. Experiment III was performed with anti-BSA rabbit serum. The figures have been normalized for easy comparison. The antigen–antibody precipitates were analyzed for protein by the method of Lowry *et al.* (1951). In expt I and II, 0.167 and 0.189 mg of protein were precipitated respectively when the antiserum was reacted with the CAAF alone. Each of these values has been set at 100. In expt III, 0.597 mg of protein was precipitated when BSA alone was reacted with its corresponding antiserum and this value is set at 100. The amount of each ingredient used is given to the right. The precipitin reactions were carried out in conical centrifuge tubes. Each of the test tubes contained a volume of 1.2 ml. The volume was adjusted in each case with 0.05 M phosphate buffered at pH 7.4. The ratio of BSA:CAAF in expt I was 1; in expt II the ratio was 4.

It was shown that CAAF mitigated the inhibitory action of antimycin A in explanted chick embryos after treatment of this protein with *o*-phenanthroline. Under favorable conditions CAAF also relieved the inhibition of respiration in antimycin A treated mitochondria.

BSA enhanced the fluorescence of antimycin. The fluorescence of antimycin could also be demonstrated by exciting the tryptophan peak of BSA. This observation leads to the hypothesis that BSA bound antimycin close to one tryptophan residue. The addition of CAAF to the BSA–antimycin adduct quenched this fluorescence of the antibiotic. One explanation of the fluorescence changes (Figure 8) is that CAAF competed with BSA for the antibiotic.

A second interpretation of the fluorescence changes is that an interaction between antimycin and the proteins took place to give a ternary complex. This possibility is also suggested by immunochemical data. A hypothesis can be made that at physiological pH values two species of antimycin are present as shown in Figures 5 and 6. Antimycin can interact at two sites within the mitochondria. The two sites may differ either in conformation, charge, or lipophilic properties. In situations where mitochondria are uncoupled or where the usually functional cytochrome *b* site is not involved in the electron transport chain, antimycin obtains a better fit at one preferred site. The latter type of binding does not lend itself to easy relief for further electron transport. In coupled mitochondria the antimycin does not obtain a good fit and relief is possible. Thus the concentration and state of the mitochondria as well as their acceptor systems may play a role in reactions inhibited by antimycin.

Acknowledgments

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Activity of Glutamine Synthetase toward *threo*-γ-Methyl-L-glutamic Acid and the Isomers of γ-Hydroxyglumatic Acid

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ABSTRACT: Previous studies on the unusual optical specificity of glutamine synthetase led to the hypothesis that L-glutamic acid is oriented on the enzyme in an extended conformation in which the α-hydrogen atom is directed away from the active site of the enzyme. This hypothesis has been examined further by studies on several γ-substituted derivatives of glutamic acid. Only one of the four isomers of γ-methylglutamic acid (*threo*-γ-methyl-L-glutamic acid) is a substrate. Of the four γ-hydroxyglutamic acids, *threo*-γ-hydroxy-L-glutamic acid is the most active substrate, but appreciable activity was also observed with *erythro*-γ-hydroxy-L-glutamic acid and *erythro*-γ-hydroxy-D-glutamic acid. These observations and the finding that *threo*-γ-hydroxy-D-glutamic acid is only slightly active are consistent with the original hypothesis and provide additional clues to the spatial relationships between the substrate and the enzyme. The substrate specificity of glutamine synthetase from peas is substantially the same as that of the enzyme isolated from sheep brain.

Earlier studies in this laboratory have shown that highly purified glutamine synthetase from sheep brain exhibits unusual optical specificity (Table I). Thus, the enzyme acts on both optical isomers of glutamic acid, but exhibits absolute L specificity toward α-methylglutamic acid and absolute D specificity toward *threo*-β-methylglutamic acid. β-Glutamic acid [which possesses a *meso* carbon atom (Schwartz and Carter, 1954)], is converted only to D-β-glutamine. The findings with glutamic acid, β-glutamic acid, and α-methylglutamic acid led to an hypothesis concerning the conformation of the enzyme-bound substrates, according to which L-glutamic acid is oriented on the enzyme in an extended conformation in which the α-hydrogen atom is directed away from the active site of the enzyme. The amino and carboxyl groups of D-glutamic acid (also in an extended conformation) are bound to the same respective sites of the enzyme as the corresponding groups of L-glutamic acid; thus, the α-hydrogen atom of D-glutamic acid is oriented toward the enzyme. The proposed conformations of these substrates are shown in color stereophotographs of models given in a previous communication (Kagan and Meister, 1966). This hypothesis led to prediction of the results subsequently obtained with the isomers of β-methylglutamic acid.

The present studies represent an extension of this work to γ-substituted derivatives of glutamic acid. Examination of the models of the extended conformations of L- and D-glutamic acid (Figure 5, Kagan and Meister, 1966) indicates that the *erythro*-γ-hydrogen atoms of both L- and D-glutamic acids occupy about the same position in space and lie just between the γ-carboxyl and amino groups of these molecules. Study of the models suggests that introduction of an *erythro*-γ-methyl group might provide considerable steric hindrance to formation of an activated γ-carboxyl derivative (*e.g.*, γ-glutamyl phosphate) and also to reaction of the amino group with the enzyme or metal nucleotide complex. The *threo*-γ-hydrogen atom of D-

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