THE FUNCTION OF COENZYME A IN LUMINESCENCE

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INTRODUCTION

The crystallization of firefly luciferase and luciferin has allowed for the first time an extensive study of the interaction of these two substances in the process of light emission. Previous evidence has indicated that the active site on luciferin ($C_{13}H_{12}N_2S_2O_3$) which reacts with adenosine triphosphate (ATP) is a carboxyl group[1]. The initial reaction leads to the reversible formation of adenyl-luciferin (active luciferin) and pyrophosphate[2]. Active luciferin reacts in some unknown way with molecular oxygen to give light emission and adenyl-oxyluciferin. Oxyluciferin is a potent inhibitor of the light reaction. The addition of coenzyme A (CoA) to a reaction mixture will stimulate luminescence, due to the removal of this inhibitor from the enzyme surface. Evidence presented in the present paper demonstrates that adenyl-oxyluciferin reacts with CoA to form oxyluciferyl-CoA. In the presence of cysteine, the CoA derivative is converted into stable N-oxyluciferyl-cysteine. The net effect is to remove the oxyluciferin from the enzyme surface, allowing luminescence to proceed. In a reaction analogous to acetate activation, the exchange of AMP in ATP during light emission can be shown to depend upon CoA. The data presented below have a direct bearing on the mechanism of action of luciferin in light emission and in electron transport processes.

METHODS

Crystalline luciferin and oxyluciferin were prepared by the method of BITLER AND MCELROY[1]. Crystalline luciferase was prepared according to the method of GREEN AND MCELROY[3]. Unless otherwise specified, three times recrystallized luciferase was used. The luciferase preparations contained approximately 20 mg of protein per ml. It was necessary to recrystallize luciferase four times in order to remove strongly bound oxyluciferin. Highly purified yeast inorganic pyrophosphatase was prepared according to the method of HEPPEL AND HILMOE[4]. The light reactions were carried out in a reaction mixture similar to that described by GREEN AND MCELROY[3], except that 0.025 *M* glycylglycine pH 7.6 was used as a buffer. The light measurements were made by a photomultiplier apparatus with automatic recording on an Esterline-Angus. The spectral changes were recorded by a Beckman Spectrophotometer DK 2 while the fluorescent emission and excitation spectra were made with an American Instrument Company spectrophotofluorometer. Ascending paper chromatography was used to separate luciferin and the various derivatives on Whatman No. 3 chromatographic paper.

The chromatographic solvent used throughout was a 3 to 7 (v/v) mixture of molar ammonium acetate pH 7.5 and 95% ethyl alcohol. The fluorescent spots on the paper were detected by using a Keese ultraviolet lamp.

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RESULTS

A. The enzymic formation of oxyluciferin from luciferin

One of the interesting characteristics of the luciferase-catalyzed light reaction is the progressive inhibition of the light emission as the reaction is allowed to proceed. Previous results indicated that luciferase is inhibited by the product of the reaction[5]. Chromatographic evidence indicates that in such a reaction mixture most of the luciferin remains unreacted even though light emission has essentially ceased. If such a reaction mixture is allowed to proceed for one hour and then stopped by heating at 100°C for two minutes one finds after chromatography four fluorescent products other than luciferin. The major product has an R_F of 0.41 while minor amounts of other fluorescent products with the following R_F values are obtained: 0.93, 0.91 and 0.85. That the R_F 0.41 compound should be a major reaction product is of interest since a compound of similar spectral and chromatographic properties is obtained by refluxing luciferin under alkaline conditions at 100°C[1]. The R_F 0.41 compound has been isolated and crystallized and has been identified as an oxidation product of luciferin. Oxyluciferin ($C_{13}H_{10}N_2S_2O_3$) will react with ATP in the presence of luciferase to form pyrophosphate in a reaction similar to that observed for luciferin[2]. That oxyluciferin is the primary product concerned with the inhibition of the light reaction is indicated by the results in Fig. 1. It is evident from the data that oxyluciferin is a potent inhibitor of the light reaction. If oxyluciferin is allowed to react with ATP and the enzyme for a few minutes prior to luciferin addition essentially no light emission occurs. These observations indicate that oxyluciferin must react with ATP in order to inhibit the subsequent function of the enzyme for light emission. The luciferase–oxyluciferin complex is still capable however of functioning catalytically in the breakdown of ATP into pyrophosphate and adenylic acid[2]. Since no light is emitted under these conditions even in the presence of luciferin, it is apparent that oxyluciferin does not dissociate readily from the enzyme surface.

B. The stimulation of luminescence by coenzyme A

When CoA is added to a normal reaction mixture which has been allowed to proceed until inhibition is prominent, there is an immediate rise in the light intensity which is directly proportional to the CoA concentration[6]. After the initial flash due to CoA addition, the luminescence will proceed at this high level of light emission and finally return to the normal base-line level. The duration of this stimulated light emission is directly proportional to the CoA concentration. The results in Fig. 2 indicate that the secondary flash height is directly proportional to the CoA concentration. If CoA is added initially to the reaction mixture there is no effect on the initial light intensity, but as the luminescence decays, the reaction mixture containing CoA always remains at a higher value. Under these conditions, therefore, using a limited amount of luciferin it is possible to show that the total light emitted is greater in the presence of CoA than in its absence. Under appropriate conditions it is possible to stimulate the complete utilization of luciferin in such a reaction mixture. Data supporting this will be presented in a later section.

The results in Table I indicate that CoA is very specific in its stimulatory effect. Neither dephospho-CoA nor phosphopantethine are effective in this respect. Results have been presented previously indicating that the rephosphorylation of dephospho-

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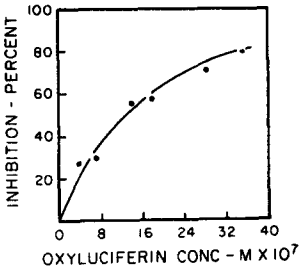

Fig. 1. The inhibition of light emission by oxyluciferin. The reaction mixture contained the following: glycyl-glycine 0.025 *M*, pH 7.6, 1.5 ml; $MgSO_4$, 10 μmoles; ATP, 1.7 μmoles; luciferin 6 mμ moles; luciferase 0.04 ml; varying oxyluciferin concentration as indicated and water to give a final volume of 2.5 ml.

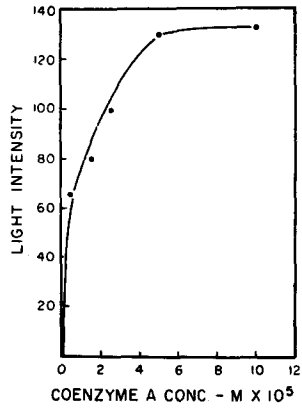

Fig. 2. The stimulation of light emission by coenzyme A. The reaction mixture contained the following: glycyl-glycine 0.25 *M*, pH 7.6, 0.25 ml; $MgSO_4$, 10 μmoles; ATP, 34 μmoles; luciferin, 0.25 μmoles; luciferase, 0.05 ml; varying amounts of CoA were added three minutes after the reaction was initiated with ATP. Water was added to give a total volume of 2.5 ml. The initial increase in light emission above the baseline intensity is shown on the ordinate.

TABLE I

EFFECT OF COENZYME A AND VARIOUS DERIVATIVES ON LIGHT EMISSION

Compound	*Amount added* μg	*Light response per cent*
CoA	100	100
Desamino CoA	100	3
Phosphopantethine	100	2
Dephospho CoA	100	1
3,5-diphosphoadenosine	90	0
2,5-diphosphoadenosine	70	0
3′-adenylic acid	50	0
2′-adenylic acid	50	0
5′-adenylic acid	50	0

The various compounds were added to a luminescent reaction three minutes after light emission was initiated with ATP. The increase in light intensity above the baseline light emission was compared to the response obtained from CoA which was taken as 100%. See text for details.

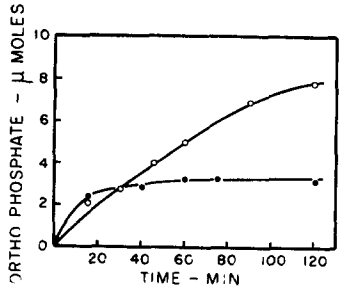

Fig. 3. The effect of coenzyme A on pyrophosphate release. The reaction mixtures contain the following: glycyl-glycine buffer, 0.25 *M*, pH 7.6, 0.5 ml; $MgSO_4$, 60 μmoles; ATP, 16 μmoles; oxyluciferin, 0.44 μmoles; luciferase, 0.3 ml; pyrophosphatase 0.10 ml and water to a total volume volume of 5.0 ml. In the curve with the solid points 0.5 μmoles of CoA were added.

CoA will restore the stimulatory properties[6]. In recent experiments it has been found that thioethanolamine is slightly stimulatory.

The inhibitory effect of oxyluciferin added initially can be removed by the addition of CoA. We have suggested previously that the stimulatory effect of CoA is due to the fact that it reacts with oxyluciferin and effectively removes it from the enzyme surface[6]. If this were correct then CoA should effectively inhibit pyrophosphate production which normally occurs in the presence of oxyluciferin. The results presented in Fig. 3 indicate that CoA is an effective inhibitor of pyrophosphate liberation and that time is necessary before this effect expresses itself. Apparently CoA is reacting with some component of the reaction mixture which is essential for pyrophosphate production. The evidence presented later indicates the formation of oxyluciferyl-CoA.

TABLE II

FLUORESCENCE PRODUCTS OF THE LIGHT REACTION IN THE PRESENCE AND ABSENCE OF COENZYME A

R_F	*Control*	*— CoA*	*+ CoA*
	Fluorescence intensity	*Fluorescence intensity*	*Fluorescence intensity*
0.66	43,600	13,600	10,900
0.41	134	1,163	3,916
0.31	—	—	1,579
0.14	—	—	1,589

The reaction mixtures contained the following: glycyl-glycine, 0.25 *M*, pH 7.6, 0.50 ml; $MgSO_4$, 30 μmoles; cysteine, 5 μmoles; luciferin, 0.25 μmoles; luciferase, 0.5 ml; CoA, 2 μmoles; ATP, 13 μmoles and water to give a total volume of 5.0 ml. In the control the luciferase was inactivated by heat before it was added to the reaction mixture. See text for details on fluorescence measurements.

C. The formation of oxyluciferyl-coenzyme A

The results presented in Table II demonstrate the effect of CoA on the formation of fluorescent products from luciferin. Under the special chromatographic conditions indicated, luciferin has an R_F of 0.66. As shown in the second column, in a reaction in the absence of CoA, the primary product which is formed is oxyluciferin having an R_F of 0.41. The actual fluorescent figures are not a quantitative expression of the amount of the product formed since the absolute molar fluorescence of these substances is not known. In addition, oxyluciferin is slowly converted into non-fluorescent products by non-luminescent reactions. The data of Table III indicate this fact. In the

TABLE III

FLUORESCENCE PRODUCTS OBTAINED FROM OXYLUCIFERIN IN THE PRESENCE AND ABSENCE OF COENZYME A

R_F	*Control*	*— CoA*	*+ CoA*
	Fluorescence intensity	*Fluorescence intensity*	*Fluorescence intensity*
0.41	32,600	24,600	790
0.31	—	—	4,040
0.14	—	—	2,112

The reaction mixtures are the same as those described for Table II except 0.28 μmoles of oxyluciferin were used instead of luciferin.

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reaction containing CoA, considerably more luciferin is utilized with the production of a larger amount of oxyluciferin. In addition to residual luciferin and oxyluciferin, two new fluorescence spots appear in the reaction containing CoA, one having an R_F of 0.31 and the second having an R_F of 0.14. These compounds were also produced in a reaction mixture consisting of oxyluciferin, CoA, cysteine, ATP and luciferase as indicated in Table III. If the reaction of oxyluciferin with CoA is allowed to proceed in the absence of cysteine, only the R_F 0.14 compound is produced. The reaction can be studied quantitatively by measuring the change in fluorescence on the spectrophotofluorometer since the R_F 0.14 compound has a greatly reduced fluorescence compared to the oxyluciferin. Chromatographic and fluorescence evidence suggest that the amount of R_F 0.14 compound formed is proportional to the CoA added. Spectrophotofluorometric measurement of the effect of varying amounts of CoA on the final fluorescent intensity of a reaction mixture containing oxyluciferin, ATP, and luciferase is shown in Fig. 4. For several reasons we believe that the R_F 0.14 is oxyluciferyl-CoA. Because of the extremely small amounts of material used in these experiments it has not been possible to demonstrate the disappearance of a free SH group. However, a large amount of indirect evidence indicates the formation of a thioacyl compound.

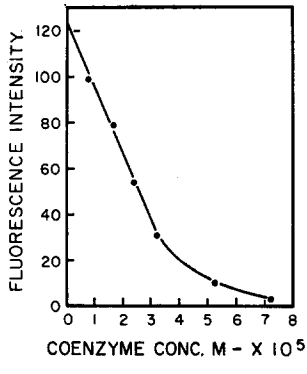

Fig. 4. The effect of coenzyme A on oxyluciferin fluorescence. Same conditions as described for Fig. 2 except oxyluciferin replaced luciferin. The oxyluciferin fluorescence was measured after the reaction was completed. The exciting wavelength was 350 mμ and the emission intensity at 540 mμ was measured in the spectrophotofluorometer.

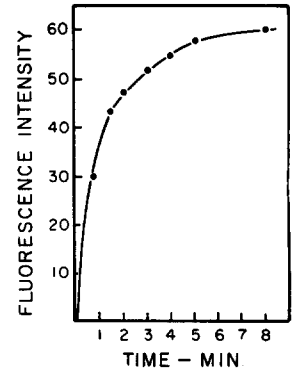

Fig. 5. Fluorescence change on alkaline hydrolysis of oxyluciferyl-CoA. The initial reaction mixture is the same as described for Fig. 4. 0.5 μmoles of CoA was added. After the fluorescence intensity had decreased to a minimum, the reaction mixture was heated in order to inactivate luciferase. The insoluble protein was removed by centrifugation and the supernatant was placed in a cuvette in the fluorometer. NaOH was added to give a final concentration of 0.01*N*. The increase in fluorescence with time was measured. Exciting wavelength 350 mμ, emission intensity measured at 540 mμ.

The increased solubility of luciferin in ethyl acetate as the pH of an aqueous solution is lowered to 3, is due to the decrease of the dissociation of a carboxyl group[1]. If CoA were reacting with the active oxyluciferin (adenyl-oxyluciferin) to form the oxyluciferyl-CoA then one would expect that such a compound would be insoluble in ethyl acetate even at acid pH's. We have made such an observation on the material having an $R_F$of 0.14. However, the fluorescent substance becomes ethyl acetate-soluble after treatment with sodium hydroxide, indicating the liberation of oxyluciferin. If this represents the splitting of oxyluciferyl-CoA into free oxyluciferin and CoA one would expect the fluorescence intensity to increase. The results of such an experiment

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on alkaline hydrolysis shown in Fig. 5, indicate that the ester linkage is extremely labile to alkali. Chromatographic evidence also indicates that oxyluciferin is reformed. The data presented earlier on the inhibition of pyrophosphate liberation by CoA would also support the conclusion that the carboxyl group of luciferin has reacted with the CoA to form an acyl compound.

D. Reactions of oxyluciferyl coenzyme A

Several different experiments have indicated that the second fluorescent substance having an R_F of 0.31 is due to the interaction of the CoA derivative with cysteine. In addition it has been shown that the conversion of the CoA derivative to the 0.31 compound by treatment with cysteine will occur non-enzymically. The results presented in Fig. 6 represent a spectrofluorometric study of such a reaction using 350 mμ as the exciting wavelength and measuring the light intensity which is emitted at 540 mμ. In this experiment, the initial rapid decrease in fluorescence is due to the formation of the CoA derivative from oxyluciferin and CoA. After the reaction had been allowed to proceed to completion, the mixture was heated at a 100°C for 2 min.

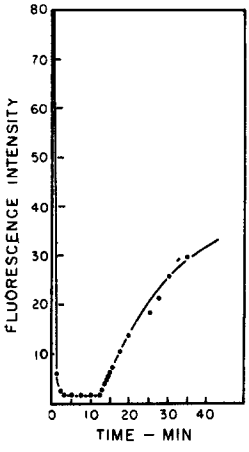
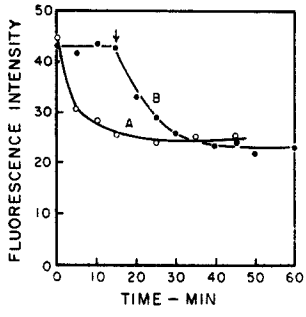


Fig. 6. Effect of cysteine on oxyluciferyl-CoA fluorescence. The initial reaction mixture is the same as described for Fig. 5. At 12 min 0.1 mmole of cyteine was added.

Fig. 7. The fluorescence change on the conversion of oxyluciferin into N-oxyluciferyl-cysteine. Reaction mixture was the same as for Fig. 2 except 0.32 μmole of oxyluciferin replaced the luciferin. In reaction A 0.5 μmole of CoA and 0.1 mmole cysteine were added initially. In reaction B the CoA and cysteine were added at 15 min.



After centrifugation the supernatant material was returned to the photofluorometer cuvette and cysteine was added. The increase in fluorescence upon the addition of cysteine is due to the formation of the R_F 0.31 compound as indicated by paper chromatographic evidence. A detailed analysis of the reaction mixture indicates that the fluorescence intensity of the R_F 0.31 compound is approximately one-half of oxyluciferin. Under these circumstances when a reaction is performed with oxyluciferin and ATP in the presence of CoA and cysteine the fluorescence intensity decreases to approximately 40% of the starting value. The results of such an experiment are shown in Fig. 7. When CoA and cysteine are withheld from the reaction mixture, the fluorescence remains essentially constant. After 15 min CoA and cysteine were added and the fluorescence intensity rapidly decreased to the level indicated. This is to be compared with the second curve in which CoA and cysteine were added initially. Chromatography of the reaction mixture after 50 min indicated the presence of only one substance having an R_F of 0.31.

Since the two fluorescent compounds resulting from CoA and cysteine interaction with oxyluciferin could be produced in relatively pure form in a reaction mixture, it

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was possible to study some of their chemical characteristics in detail. Inasmuch as oxyluciferin was obtained by alkaline hydrolysis of the CoA derivative, the possibility that the latter is a thiol ester which would react with hydroxylamine was considered. The interaction of hydroxylamine with the CoA derivative and the formation of the hydroxamic acid derivative of oxyluciferin was followed spectrophotofluorometrically. The results of such an experiment are shown in Fig. 8. Chromatography of the above reaction mixture indicated that either oxyluciferin or its hydroxamic acid derivative was formed. A fluorescent spot from such a reaction mixture was observed near R_F 0.4.

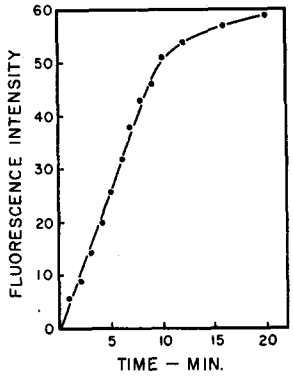

Fig. 8. Effect of hydroxylamine on oxyluciferyl-CoA fluorescence. Same conditions as in Fig. 5 except 0.6 mmole of hydroxylamine was added instead of NaOH.

The fact that this latter spot always has an R_F value slightly less than authentic oxyluciferin, is additional evidence indicating the formation of a hydroxamic acid derivative.

Since the CoA derivative reacts with cysteine to form a new substance its reactivity with other SH reagents was tested. When glutathione was mixed with the CoA derivative, the latter disappeared and a new fluorescent spot having an R_F of 0.24 was formed. When the glutathione derivative was hydrolyzed with alkali, the fluorescence intensity increased and paper chromatography indicated the formation of oxyluciferin. The alkaline lability as well as the specific molar fluorescence of the glutathione derivative appears to be similar to the CoA derivative. Consequently there is no apparent fluorescence change in the reaction mixture during the conversion of the CoA derivative into the glutathione derivative. However, the following experiment demonstrates that reduced glutathione is capable of stimulating the removal of oxyluciferin from the reaction mixture using the amount of CoA which would normally produce only a slight decrease in fluorescence. It was found that the addition of an excess of glutathione to such a reaction mixture lead to a rapid decrease in fluorescence. Chromatographic evidence also indicated the complete utilization of oxyluciferin. This experiment demonstrates that CoA is acting in a catalytic manner in the conversion of oxyluciferin into the glutathione derivative.

Efforts have been made to form the cysteine and glutathione derivatives of oxyluciferin in the absence of CoA. However, all the results have been essentially negative. It would appear that neither glutathione nor cysteine is capable of reacting with adenyl-oxyluciferin, even under enzymic conditions, to form the corresponding derivatives. This is not too surprising since CoA is highly specific in its effect on the stimulation of luminescence. The secondary addition of either cysteine or glutathione

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to a light reaction does not stimulate light emission. Recently, however, it has been found that the secondary addition of hydroxylamine will give some stimulation of light emission indicating the possibility that this compound is capable of reacting directly with adenyl-oxyluciferin, releasing luciferase for further reaction with luciferin. The secondary light stimulation due to hydroxylamine addition is similar to the CoA stimulation in that the light intensity slowly returns to the normal base-line level.

Although the glutathione and CoA derivative of oxyluciferin are quite labile to alkali, this is in contrast to the cysteine derivative. When the latter compound is treated with alkali, only small amounts of oxyluciferin are obtained. The possibility existed, therefore, that cysteine was converting the CoA compound into some other product without actually forming an oxyluciferyl-cysteine compound. Another possibility existed, however, namely that the S-oxyluciferyl compound was cyclizing and was being converted into a stable N-oxyluciferyl-cysteine. To test the possibility that cysteine is actually incorporated into the fluorescent derivative, a reaction was carried out in the presence of ^{35}S-labelled cysteine. The ^{35}S-cysteine was prepared from ^{35}S-cystine by exchange in acid solution (0.1 ml of $2.5 \cdot 10^{-3} M$ cystine, containing 1 μc ^{35}S in 0.06 M HCl was added to 0.1 ml of $2.5 \cdot 10^{-2} M$ cysteine and allowed to incubate for approximately 5 h). Complete equilibration of ^{35}S was assumed. The CoA derivative was produced in a reaction mixture consisting of ATP, luciferase, oxyluciferin, and CoA. After incubation for 15 min the mixture was heated at 100°C for two minutes and the denatured protein was removed by centrifugation. A control was heated prior to the addition of ATP. Both supernatants were added to the sulphur-labelled cysteine (each tube containing 1 μc) and the mixtures were incubated at room temperature for 1 ½ h. One-half ml aliquots of each solution were then placed on chromatographic paper and developed with the alcohol solvent. One difficulty was that cysteine itself has an R_F very close to that of the R_F 0.31 compound to be counted. However, it was found that when cysteine was applied to the paper under a stream of hot air, most of the cysteine was converted to cystine, which has an R_F of 0.05. After chromatography linear strips of 2.5 cm width were cut from the paper and each strip was scanned for its ^{35}S content. The results of such an experiment are presented in Table IV. Calculations of the theoretical incorporation into the R_F 0.31 compound were based on the following assumptions: (1) complete equilibration of ^{35}S between cysteine and cystine and (2) complete conversion of oxyluciferin to the R_F 0.31 compound. Such calculations

TABLE IV

INCORPORATION OF ^{35}S-CYSTEINE INTO OXYLUCIFERIN DERIVATIVE

	Control c.p.m.	*Experimental* c.p.m.	*Percent incorporation*	
			expected	*observed*
Total	28,369	27,615		
R_F 0.31	3,512	5,493	9.6	7.8
Blank	200	200		

The reaction mixtures contained the following: glycyl-glycine 0.25M, pH 7.6, 0.30 ml; $MgSO_4$, 10 μmoles; oxyluciferin, 0.26 μmoles; CoA, 2 μmoles; luciferase, 0.20 ml; ATP, 10 μmoles and water to give a total volume of 2.5 ml. In the control the luciferase was inactivated by heat before it was added to the reaction mixture. ^{35}S cysteine was added secondarily.

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give a theoretical incorporation of 9.6% of the total counts. The data of Table IV indicate that 7.8% of the counts above the control were incorporated. Thus, it may be calculated that about 80% of the theoretically possible incorporation had taken place. It can be concluded therefore that cysteine is incorporated into the new fluorescent spot. This experiment indicates the extreme sensitivity of the fluorometric method for the analysis of enzyme reactions as compared to the isotopic method.

To demonstrate the importance of the SH group of cysteine in its reaction with oxyluciferyl-CoA, the following reactions were carried out. Oxyluciferyl-CoA was reacted with homocysteine in one case and methionine in the other. In the case of the former compound an increase in fluorescence was observed, a new chromatographic spot at R_F 0.50 appeared, while the R_F 0.14 spot disappeared. Methionine did not change the oxyluciferyl-CoA fluorescence and chromatography indicated no reaction. The product of the homocysteine reaction is relatively stable to alkali and unreactive to hydroxylamine. The rate of reaction of cysteine and homocysteine with oxyluciferyl-CoA, as judged by fluorescence, is about the same.

The evidence indicates clearly, therefore, that cysteine reacts with the CoA compound to form an oxyluciferyl-cysteine derivative. Judging from its alkaline stability and the evidence of other workers on the lability of thiol esters compared to the nitrogen esters, we may conclude that the cysteine derivative is an N-oxyluciferyl-cysteine.

F. The enzymic breakdown of oxyluciferyl-CoA by adenylic acid

The enzymic activation of oxyluciferin by ATP in the presence of CoA leads to the formation of pyrophosphate and adenylic acid as well as oxyluciferyl-CoA. This suggested the possibility that the latter might react with adenylic acid and pyrophosphate in a reversible manner to form free CoA, oxyluciferin and ATP. The results

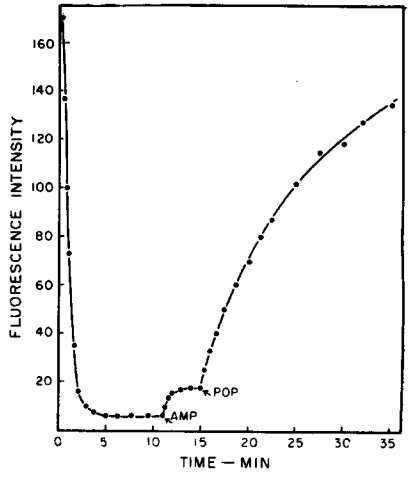

Fig. 9. Effect of adenylic acid and pyrophosphate on oxyluciferyl-CoA decomposition. The reaction mixture contained the following: glycyl-glycine, 0.25*M*, pH 7.6, 0.35 ml; $MgSO_4$, 8 μmoles; ATP, 4 μmoles; oxyluciferin, 0.16 μmole; luciferase, 0.06 ml; CoA, 0.6 μmole and water to give a final volume of 1.5 ml. At 11 min 1.0 μmole of adenylic acid was added and at 15 min 20 μmoles of inorganic pyrophosphate was added. Fluorescence excitation at 340 mμ. Emission intensity was measured at 540 mμ.

presented in Fig. 9 indicate that this reaction does occur. Oxyluciferyl-CoA was produced in a reaction mixture consisting of oxyluciferin, CoA, and ATP. This is shown on the graph by the initial decrease in fluorescence. Upon the addition of adenylic acid after 11 min, there was a small increase in fluorescence. When high concentrations of pyrophosphate were added there was a large increase in fluorescence

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due to the formation of free oxyluciferin. ATP was also formed in the reaction and was measured by the firefly system. The high concentration of pyrophosphate required to obtain an increase in fluorescence indicates that the equilibrium is in favor of oxyluciferyl-CoA formation. In this reaction luciferase must be present. Adenylic acid is incapable of splitting oxyluciferyl-CoA non-enzymically in contrast to the reactions with cysteine, hydroxylamine, or glutathione.

It has shown previously that if the reaction of oxyluciferin, ATP, and luciferase is carried out in the presence of labelled pyrophosphate and the reaction is stopped before all ATP is broken down, there is an incorporation of pyrophosphate into the residual ATP[2]. The results were interpreted to mean that adenyl-oxyluciferin and pyrophosphate were the immediate products of the enzyme-catalyzed reaction. Likewise, one should be able to show a CoA-dependent incorporation of labelled adenylic acid into ATP. Reaction mixtures consisting of oxyluciferin, luciferase, CoA, ^{14}C-labelled adenylic acid and pyrophosphate were incubated for the times specified in Table V. The reactions were stopped by heating at 100°C for two minutes. The mixtures were then placed on a Dowex-1 chloride column and the ATP and adenylic acid were separated by gradient elution according to the method of COHN AND CARTER[7].

TABLE V

INCORPORATION OF ^{14}C-AMP INTO ATP

Reaction	*Specific activity*	
	Counts/min/mg ATP	
	I	*II*
Complete	86	2,731
— CoA	—	918
— L	7	550
Heated	0	—

The complete reaction mixtures contained the following: glycyl-glycine 0.25*M*, pH 7.6, 1.7 ml; $MgSO_4$, 40 μmoles; CoA, 3 μmoles; ^{14}C-AMP, 5.2 μmoles, inorganic pyrophosphate, 100 μmoles; ATP, 20 μmoles and water to give a final volume of 10 ml. Reaction I contained 0.75 μmole oxyluciferin and 0.2 ml luciferase. Incubation time was for one hour. Reaction II contained 0.80 μmole oxyluciferin and 0.3 ml luciferase. Incubation time was for two hours. Reactions without CoA and oxyluciferin as well as with heated enzyme are shown in the corresponding rows.

The ATP was precipitated with barium acetate, filtered into sinterglass crucibles and counted with a methane-flow proportional counter. Data from two such experiments are presented in Table V. In both experiments there was a significant incorporation above the controls. One difficulty with this experiment was due to the contamination of the luciferase with a small amount of myokinase which also catalyzes the incorporation of adenylic acid into ATP. The myokinase was decreased to a considerable extent by recrystallizing the enzyme two additional times. The control in which oxyluciferin was omitted shows the level of myokinase activity. Numerous controls in which the reaction mixture was heated prior to the addition of ATP have eliminated the possibility of chemical exchange and shown that there is good separation of the adenylic acid and ATP. The fact that the incorporation of adenylic acid into ATP is dependent upon the presence of CoA supports the spectrophotofluorometric evidence that the reactions leading to the formation of oxyluciferyl-CoA are reversible.

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G. The spectrophotometric and fluorescent characteristics of luciferin and its derivatives

The reactions of oxyluciferin are summarized in Fig. 10 and the various fluorescence and spectral characteristics of luciferin, oxyluciferin and the glutathione, cysteine and CoA derivatives are shown in Table VI.

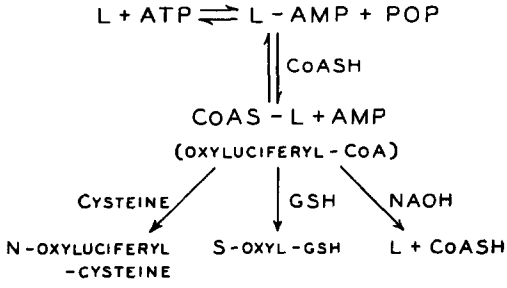

Fig. 10. The reactions of oxyluciferin.

TABLE VI

SOME CHARACTERISTICS OF LUCIFERIN, OXYLUCIFERIN AND DERIVATIVES

Compound	R_F	*Excitation Max mμ pH = 7.6*	*Emission Max mμ pH = 7.6*	*Relative fluorescence intensity*
Luciferin (LH_2)	0.66	327	530	100
Oxyluciferin (L)	0.41	347	540	100
Oxyluciferyl-CoA	0.14	367	450	5
N-oxyluciferyl-cysteine	0.31	360	545	40 (approx.)
S-oxyluciferyl glutathione	0.21	370	545	5

The excitation and emission peaks are not corrected for the photocell sensitivity and are therefore only relative values.

DISCUSSION

It has been proposed that luciferin reacts with ATP to form adenyl-luciferin (LH_2-AMP) and pyrophosphate. Adenyl-luciferin in the presence of O_2 gives off light and produces adenyl-oxyluciferin (L-AMP), a potent inhibitor of the light reaction. Additional evidence presented in the present paper supports this general scheme of the light reaction.

A compound of R_F 0.41 is one of the major products of the light reaction. Direct chemical evidence indicates that this is an oxidation product of luciferin. Therefore, this product has been designated as oxyluciferin. The inhibition of the light reaction by oxyluciferin depends first upon its reaction with ATP. Once oxyluciferin has reacted with ATP and luciferase, the latter is incapable of catalyzing the oxidation of luciferin with light emission. Isolation of luciferase also indicates that oxyluciferin is tightly bound to the enzyme and is removed with difficulty only after several recrystallizations of the enzyme. On the other hand, luciferin is completely removed during the first crystallization. The fact that luciferin can be isolated from a reaction mixture that has ceased to luminesce is indicative also that an inhibitor is formed during light emission.

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CoA and pyrophosphate have been shown to be capable of rapidly removing oxyluciferin from the enzyme surface. Adenyl-oxyluciferin apparently is the active form of the inhibitor which reacts with CoA.

The oxyluciferin–luciferase complex is capable of rapidly breaking down ATP into adenylic acid and pyrophosphate and the evidence indicates the formation of adenyl-oxyluciferin as an intermediate. Why luciferin cannot compete with oxyluciferin after the latter has reacted, is not clear. Luciferin can compete with oxyluciferin when both are initially mixed with the enzyme. The reasons why pyrophosphate stimulates light emission is not so readily apparent although it must in some way displace oxyluciferin from the enzyme surface. If pyrophosphate were to act by reversing the following reaction ATP + L $\longrightarrow$ L-AMP + POP why shouldn't the enzyme be equally free for luciferin utilization when L-AMP is hydrolyzed to give L and AMP? The evidence suggests that in the reaction of enzyme with oxyluciferin and ATP an intermediate is formed on the enzyme surface which can be removed by pyrophosphate. Subsequent rearrangements prior to the release of adenylic acid apparently leads to a stable complex between enzyme and oxyluciferin which is not removed by pyrophosphate.

The formation of the intermediate, adenyl-oxyluciferin, has not been directly demonstrated. Indirect evidence for such a compound is the release of pyrophosphate during the reaction of oxyluciferin, ATP, and luciferase. In addition, the exchange of pyrophosphate in ATP depends upon oxyluciferin. Further indication of the formation of active oxyluciferin is the ethyl acetate insolubility of the products of the reaction. Under acidic conditions (pH approx. 3.0) luciferin and oxyluciferin are soluble in ethyl acetate. However, the reaction product is ethyl acetate insoluble. Since the relative solubilities of luciferin and oxyluciferin in ethyl acetate and water depends upon the dissociation of a carboxyl group, the formation of an anhydride type linkage, as suggested for active luciferin, should decrease its solubility in ethyl acetate.

This ethyl acetate-insoluble fraction is rapidly hydrolized at neutral pH to give an increase in fluorescence with a peak emission at 540 mμ. These characteristics would be expected of adenyl-oxyluciferin. All other attempts to isolate the compound have proven unsuccessful, although the above observations are consistent with the postulation of such an intermediate. The fact that the product of light emission inhibits the initial reaction of ATP and luciferin further complicates the isolation of a reasonable quantity of this material. The similarities between the above reactions and those recently described by BERG[8] for acetate activation and DE MOSS AND NOVELLI[9] for amino acid activation are striking.

When CoA is added secondarily to a bioluminescent reaction, the extra light emission observed can be shown to be due to the removal of oxyluciferin from the enzyme surface. The formation of oxyluciferyl-CoA frees the enzyme for reaction with a luciferin molecule and ATP. That the above reaction takes place, has been demonstrated by reacting oxyluciferin, ATP, CoA and luciferase. In this reaction, the oxyluciferin chromatographic spot, R_F 0.41, disappears and a new spot with an R_F 0.14 appears. Several different experiments indicate that this latter spot is oxyluciferyl-CoA (CoA-L). The R_F 0.14 compound yields oxyluciferin upon alkaline hydrolysis and either oxyluciferin or its hydroxamic acid derivative upon treatment with NH_2OH. The extent of oxyluciferin utilization is dependent on the concentration of CoA and both oxyluciferin and CoA are required for the adenylic acid exchange with ATP.

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Both adenylic acid and pyrophosphate are required for the formation of oxyluciferin and ATP from the CoA derivative. Finally the R_F 0.14 compound will react with other SH compounds non-enzymically to form the corresponding oxyluciferin derivatives. The subsequent nonenzymic reactions of oxyluciferyl-CoA with such sulfhydryl compounds as cysteine and glutathione are also similar to reactions found with acetyl-CoA[10].

The nonenzymic formation of oxyluciferyl-glutathione (GS-L) from oxyluciferyl-CoA has been indicated in several ways: (1) on reacting oxyluciferyl-CoA with glutathione the former compound disappears and a new chromatographic spot appears at R_F 0.24, (2) alkaline hydrolysis of the suspected intermediate yields oxyluciferin, (3) the amount of R_F 0.24 material formed is a function of the glutathione concentration and (4) treatment with NH_2OH produces oxyluciferin or its hydroxamic acid derivative. This latter point would suggest that S-oxyluciferyl-glutathione was formed.

When the oxyluciferin derivative of CoA or glutathione is reacted with cysteine a new chromatographic spot appears at R_F 0.31, and the former thiol ester spots disappear. Alkaline hydrolysis of the cysteine-dependent spot gives oxyluciferin, however, this compound is much more alkaline-stable than either the CoA or glutathione derivative of oxyluciferin. The experiment using ^{35}S-labelled cysteine indicates that the R_F 0.31 chromatographic spot is oxyluciferyl-cysteine. The fact that this compound does not give a NH_2OH test and is relatively alkaline-stable indicates that it is probably N-oxyluciferyl-cysteine. The SH group is important in the reaction however as indicated by the non-reactivity of oxyluciferyl-CoA and methionine. STADTMAN has shown that acetyl mercaptans having unsubstituted amino groups adjacent to the carbon to which sulfur is attached are very unstable and decompose readily in aqueous solution[10]. On the other hand, NOVELLI concludes that the disubstituted compound is more stable and in the case of diacetyl cysteine an immediate NH_2OH test is obtained[11]. The synthesis of N-acetyl-cysteine from S-acetyl-glutathione using thioesterase at pH 5.4 has been found by STRECKER[12]. This type of linkage in the case of oxyluciferyl-cysteine seems to be much more stable than either the S- or N-acetyl-cysteine and is under investigation.

The question of the biological significance of some of the above reactions is under consideration. The control of the enzymic oxidation of luciferin by the product of the reaction may be of general importance in electron transport processes. Chromatographic evidence indicates that oxyluciferin is present in the firefly lantern but the various derivatives of oxyluciferin have not been found. Firefly lanterns are also known to contain considerable quantities of CoA and consequently oxyluciferyl-CoA is very likely formed. Preliminary experiments indicate that there are enzymes in the lanterns which will hydrolyze oxyluciferyl-CoA, oxyluciferyl-cysteine and oxyluciferyl glutathione. This could possibly explain the absence of the oxyluciferyl derivatives in the firefly lantern.

The possibility that oxyluciferyl-CoA is reduced to produce luciferyl-CoA which would react with adenylic acid to form active luciferin and CoA, is under investigation. It seems more than likely that this latter reaction represents a pathway of electron transport which is only incidentally concerned with light emission.

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SUMMARY

Firefly luciferin ($C_{13}H_{12}N_2S_2O_3$) reacts with ATP to form active luciferin (apparently adenyl-luciferin) and pyrophosphate. The oxidation of active luciferin leads to light emission and adenyl-oxyluciferin, the latter compound eventually decomposes into adenylic acid and oxyluciferin ($C_{13}H_{10}N_2S_2O_3$). Oxyluciferin is a potent inhibitor of the light reaction and once it has reacted with ATP and luciferase, the latter is incapable of catalyzing the oxidation of luciferin. Coenzyme A stimulates light emission by removing oxyluciferin from the enzyme surface. The evidence indicates that oxyluciferyl-CoA is formed, which can react non-enzymically with cysteine, glutathione or hydroxylamine to form the corresponding oxyluciferyl derivatives. Chromatographic, isotopic and fluorometric data are presented to support the above conclusions. Oxyluciferyl-CoA in the presence of luciferase can be split by adenylic acid and when excess pyrophosphate is added ATP and free oxyluciferin are formed. The incorporation of ^{14}C-adenylic acid into ATP depends upon the presence of CoA in the reaction mixture. The importance of these various reactions for light emission and electron transport is discussed.

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THE PROTEOLYTIC ENZYME SYSTEM OF SKIN

IV. THE PURIFICATION OF PROTEINASE A*

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Aromatic amino acid esters and their substituted derivatives can be hydrolyzed by at least three enzymes, *viz.*, Proteinase A, the A_1 enzyme and the A_2 enzyme, present in extracts of rat skin acetone powder[1]. Both the A_1 enzyme and Proteinase A attack acylated amino acid esters and acylated and non-acylated dipeptide esters in which

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