The Protective Effect of Spermine and Other Polyamines Against Heat Denaturation of Deoxyribonucleic Acid

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The temperature (T_m) necessary for the denaturation of calf thymus DNA was measured spectrophotometrically and was shown to be markedly increased by the addition of various polyamines and diamines. The most effective amines were spermine and spermidine. In 0.03 M NaCl–0.001 M potassium dimethylglutarate, *p*H 6.2, spermine was effective in concentrations of 10^{-6} to 10^{-5} M, spermidine was effective at concentrations of 10^{-5} to 10^{-4} M, and the diamines were effective at concentrations of 2×10^{-4} to 10^{-2} M. The higher concentrations of these amines increased the T_m from 76° to 85–90°. The protective effect of the polyamines could also be demonstrated in solutions of higher ionic strength (0.18 M NaCl), but increased concentrations of the amines were necessary. During these studies evidence was also obtained indicating complex formation between spermine and citrate ions. Spermine also increased the T_m for *Bacillus subtilis* DNA; 1.33×10^{-5} M spermine increased the T_m from 78 to 86°.

The polyamines spermine[1] and spermidine have been shown to stabilize a number of biological systems. These experiments have included the protection of certain micro-organisms (Mager, 1955, 1959a,b) and protoplasts (Mager, 1959b; Tabor, C. W., 1960) against osmotic lysis, a decrease in the rate of mitochondrial "swelling" in certain hypotonic media (Tabor, C. W., 1960; Herbst and Witherspoon, 1960), prevention of the inactivation of certain bacteriophages (Fraser and Mahler, 1958; Tabor, H., 1960) resulting from heating or dilution, and effects on the structure and functional activity of ribosomes (Cohen and Lichtenstein, 1960; Hershko *et al.*, 1961; Colbourn *et al.*, 1961). More recently we have demonstrated (Tabor, 1961) that, even in very low concentrations, these polyamines have a marked protective action against the loss of transforming ability observed upon the heat denaturation of DNA from *Bacillus subtilis*.

In this paper we will describe the ability of spermine and other polyamines to protect calf thymus and *B. subtilis* DNA from heat denaturation. DNA denaturation was measured by the spectrophotometric techniques described by Marmur and Lane (1960), Doty (1959–60), and others, *i.e.*, by the increase in the optical density at 260 mμ observed upon denaturation. The temperature at which this denaturation occurred (the "melting temperature") was markedly increased by the addition of low concentrations of spermine or spermidine. Protective effects were also observed with a number of other compounds, but considerably higher concentrations were necessary.

[1] A more complete bibliography on spermine and other polyamines has been presented in Tabor *et al.* (1961).

Materials

"Highly polymerized" calf thymus sodium deoxyribonucleate (DNA) was obtained from the Sigma Chemical Company. Stock solutions, containing 2.8 mg of DNA per ml of 0.3 M NaCl, were stored frozen. No polyamines could be detected in these solutions (< 0.05 μmole per μmole of DNA base) by a dinitrofluorobenzene analysis (Rosenthal and Tabor, 1956) carried out by C. W. Tabor.

For the isolation of DNA from *B. subtilis*,[2] cells were grown on a glucose-salts medium (Vogel and Bonner, 1956) containing threonine (200 μg per ml), and frozen until used. The cells were suspended in 0.15 M NaCl containing disodium ethylenediaminetetraacetate, and treated with lysozyme, as described by Fox (1960). The DNA was then isolated from the lysed cells according to the procedure of Kornberg (1960) and stored frozen in 0.15 M NaCl ($O.D._{260}$ = 2.8). This preparation was active when tested for transforming ability by methods previously described (Fox, 1960; Anagnostopoulos and Spizizen, 1961).

The various amines used were commercial preparations and were recrystallized twice as the hydrochloride salts. All other compounds were obtained from commercial sources.

Methods

Just before use the stock DNA solutions were diluted with suitable cold diluting fluids to contain 15 to 35 μg of DNA per ml, in (A) 0.03 M NaCl–0.001 M potassium dimethylglutarate, *p*H 6.2, or (B) 0.18 M NaCl – 0.001 M potassium di-

[2] This organism was a threonine auxotroph and was obtained from Dr. Maurice Fox.

methylglutarate, *p*H 6.2, or (C) 0.15 M NaCl–0.015 M sodium citrate.

The diluted DNA solution (3 ml) was mixed in a glass-stoppered silica cuvet (1-cm light path) with either 10 μl of the solution being tested ("experimental") or 10 μl of water ("no-addition control"). The cuvets were gradually heated in a Beckman DU spectrophotometer by circulating heated water through the walls of the cell compartment. The optical density of each DNA solution was measured frequently against that of a reference cuvet containing water. The temperature of another reference cuvet was measured by a thermometer inserted through a hole in the cell-compartment cover. The rate of heating varied in different experiments, but usually approximately ½ hour was required for the 25°–60° range, and approximately 1 hour for the 60°–95° range. Essentially no differences were observed in the curves obtained in duplicate experiments when the heating time (for the 60°–95° interval) was varied from 20 to 85 minutes.

The maximum increase in optical density averaged 40%. The T_m (melting temperature) was defined as the temperature corresponding to a 20% increase in optical density, as determined from a plot of optical density versus temperature.

In many of the experiments the optical density at 320 mμ was also measured in order to be certain that no turbidity had developed. Any absorption at this wave length indicated turbidity, since otherwise the DNA solutions had essentially no absorption at this wave length. This was particularly important for those solutions containing compounds, such as spermine and protamine, that are known to precipitate nucleic acids (Razin and Rozansky, 1959; Cantoni, 1960; Huang and Felsenfeld, 1960; Alexander, 1953). We wish to thank Dr. Bernard Horecker for suggesting these measurements at 320 mμ.

Results

The Effect of Spermine and Other Additions on the "Melting" Temperature of Calf Thymus DNA in 0.03 M NaCl.—In the absence of any additions, the T_m value for calf thymus DNA was 76° in 0.03 M NaCl–0.001 M potassium dimethylglutarate, *p*H 6.2. In the presence of added spermine the T_m value was increased; some effect was noted with as little as 6.7×10^{-7} M spermine. The T_m value was increased to 84.5° by 1.67×10^{-5} M spermine hydrochloride (Fig. 1). Higher concentrations of spermine could not be tested, since turbidity was observed with 3.3×10^{-5} M spermine.

The T_m value was also increased by the addition of spermidine hydrochloride, but the concentrations required were ten times greater than those used in the experiments with spermine (Fig. 2). $MgCl_2$ and 1,4-diaminobutane also resulted in protection, but still higher concentrations were needed. With sufficiently high concentrations (0.01 M–0.2 M) even NaCl had a protective influence. The increase in T_m was closely proportional to the logarithm of the concentration of added amine or salt, with the exception of high concentrations of $MgCl_2$. The effects of NaCl and $MgCl_2$ on the T_m of DNA and RNA have been reported previously by Doty *et al.* (1959) and by Boedtker (1960).

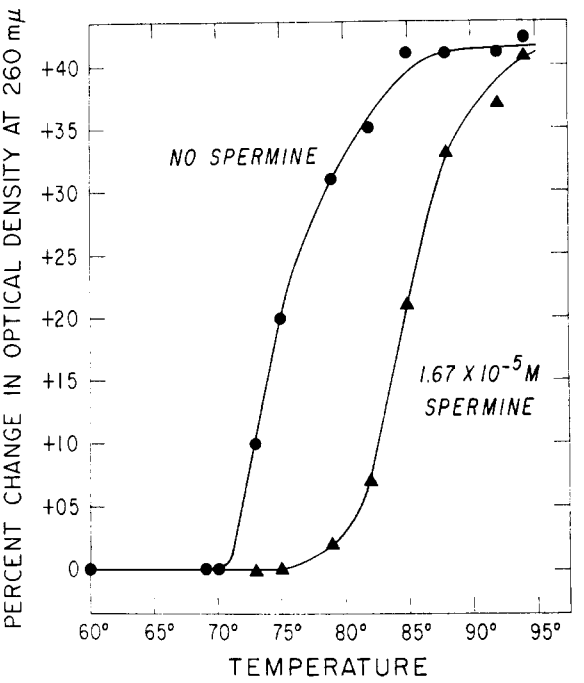

FIG. 1.—The effect of 1.67×10^{-5} M spermine on the optical density of calf thymus DNA solutions at various temperatures. The DNA was dissolved in 0.03 M NaCl–0.001 M potassium dimethylglutarate, *p*H 6.2. The initial optical density of the DNA in this experiment was 0.225 at 260 mμ, and was unaffected by the addition of spermine. The heating period for the 60° to 94° interval was 75 minutes. In this experiment there was no change in the optical density of the solutions during the period required to raise the temperature from 25° to 60°. In other experiments, however, a fall in optical density of 1 to 3% was occasionally observed during this heating interval. ▲, the experiment in which the final concentration of added spermine hydrochloride was 1.67×10^{-5} M; ●, the comparable control, without added spermine, that was heated at the same time.

The protective effect of a number of diamines was tested at a concentration of 10^{-3} M in 0.03 M NaCl–0.001 M potassium dimethylglutarate, *p*H 6.2. These results are shown in Table I, and confirm the previous report of Mahler *et al.* (1961) that diamines protect calf thymus DNA from heat denaturation. However, even though our results agree with those of Mahler *et al.* in indicating that 1,5-diaminopentane was the most effective diamine, the differences observed in the protective effect of the various amines under our conditions were considerably less than in their work. This discrepancy may be explained by the differences in experimental conditions; Mahler

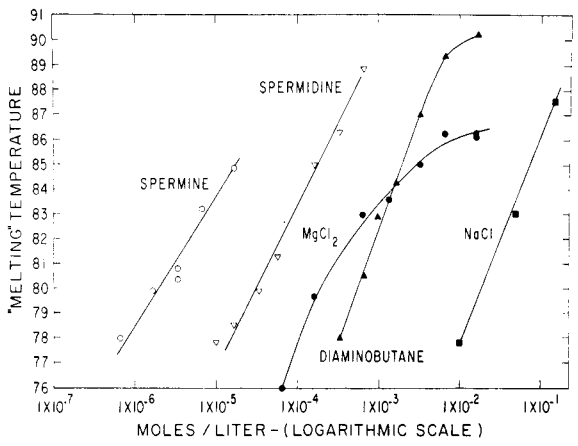

FIG. 2.—The effect of various concentrations of spermine, spermidine, 1,4-diaminobutane, $MgCl_2$, and NaCl on the "melting" temperature of calf-thymus DNA in 0.03 M NaCl–0.001 M potassium dimethylglutarate, *p*H 6.2. The initial optical density at 260 mμ in these experiments was approximately 0.25, indicating (Chargaff, 1955) approximately 0.038 μmole of nucleotide equivalents per ml (*i.e.*, 3.8×10^{-5} M). The T_m value for the DNA solution in 0.03 M NaCl–0.001 M potassium dimethylglutarate, *p*H 6.2, without any further additions, was 76.1 (average of 32 determinations).

et al. used 0.15 M NaCl–0.015 M sodium citrate, *p*H 7.2. As indicated below, the T_m was considerably higher under these conditions than in 0.03 M NaCl–0.001 M potassium dimethylglutarate, *p*H 6.2, which was used in our experiments. Furthermore, Mahler *et al.* added the diamines to a final concentration of 0.05 M, as compared to 0.001 M in the experiments reported in this paper.

We have also demonstrated protection by the addition of protamine sulfate to solutions of DNA in 0.03 M NaCl–0.001 M potassium dimethylglutarate, *p*H 6.2. The T_m was increased to 78.2°, 79.6°, and 84.0° in the presence of 1.67, 3.33, and 6.67 μg (per ml) of protamine sulfate, respectively. Higher concentrations of protamine could not be tested, since these resulted in precipitation of the DNA.

The Effect of Spermine, 1,4-Diaminobutane, and $MgCl_2$ on the "Melting" Temperatures of Calf Thymus DNA in the presence of 0.18 M NaCl.—As indicated in Figure 2, when the DNA was dissolved in 0.18 M NaCl–0.001 M potassium dimethylglutarate, *p*H 6.2, the T_m was markedly increased (87.5°) compared to the value (76°) in 0.03 M NaCl–0.001 M potassium dimethylglutarate. The addition of spermine or 1,4-diaminobutane (Table II) to this mixture increased the T_m value further but the concentrations of amines needed were considerably higher than those needed for protection at the lower ionic strength. The effect of spermine was essentially the same in other experiments carried out under essentially the same conditions except for a fourfold variation in the DNA concentration ($O.D._{260}$ = 0.25 to 1.15).

Under these conditions $MgCl_2$ had no significant effect, which is in striking contrast to the protective effect of $MgCl_2$ at lower ionic strengths. The lack of protective effect with $MgCl_2$ at the higher ionic strength is consistent with the

TABLE I

THE EFFECT OF VARIOUS DIAMINES ON THE T_m VALUES FOR SOLUTIONS OF CALF THYMUS DNA

The T_m value was obtained as described in the text in the presence and absence of the various diamines. The final concentration of the added diamines was 0.001 M. The initial optical density of the DNA solution was 0.27 at 260 mμ, and was not affected by the addition of the diamines. The results represent the average of two separate determinations. The diamines were added as the hydrochloride salts.

Diamine	T_m[a]
None	77.4
1,2-Diaminoethane	81.9
1,3-Diaminopropane	83.8
1,4-Diaminobutane	83.4[b]
1,5-Diaminopentane	84.2
1,6-Diaminohexane	83.4
1,8-Diaminooctane	82.6

[a] Under these conditions the highest concentration of spermine tested (*cf.* Fig. 2) was 1.67×10^{-5} M with a corresponding T_m of 85°. The highest concentration of spermidine tested was 6.7×10^{-4} M, with a T_m of 89°. [b] In other experiments (*cf.* Fig. 2) a tenfold higher concentration of 1,4-diaminobutane (0.01 M) was added, and the T_m was increased to 90°.

TABLE II

THE EFFECT OF SPERMINE, $MgCl_2$, AND 1,4-DIAMINOBUTANE ON THE "MELTING" TEMPERATURE OF CALF THYMUS DNA IN 0.18 M NaCl–0.001 M POTASSIUM DIMETHYLGLUTARATE, pH 6.2

The T_m for calf thymus DNA in 0.18 M NaCl–0.001 M potassium dimethylglutarate, *p*H 6.2, was determined as indicated in the text. The initial optical density of the DNA solutions used in these experiments was 0.35 at 260 mμ. Spermine, Mg^{++}, and 1,4-diaminobutane were added as the chloride salts. The data on the same compounds in 0.03 M NaCl–0.001 M potassium dimethylglutarate, *p*H 6.2, are included for comparison in the lower half of the table, and are taken from Figure 2.

NaCl[a]	Other Additions		T_m
0.18 M	None	—	87.5°
	Spermine	6.67×10^{-4} M	89.4°
	Spermine	6.67×10^{-5} M	88.4°
	Spermine	1.67×10^{-5} M	87.8°
	Mg^{++}	1.67×10^{-2} M	87.5°
	1,4-Diaminobutane	1.67×10^{-2} M	89.7°
0.03 M	None	—	76.1°
	Spermine	1.67×10^{-5} M	84.8°
	Mg^{++}	1.67×10^{-3} M	84.2°
	1,4-Diaminobutane	1.67×10^{-3} M	84.3°

[a] All the mixtures also contained 0.001 M potassium dimethylglutarate, *p*H 6.2 (final concentration).

results of Mahler *et al.* (1961), who showed that 0.05 M $MgCl_2$ had essentially no protective action when added to DNA in 0.15 M NaCl–0.015 M Na citrate, *p*H 7.2.

The Influence of 0.015 M Sodium Citrate on the Effect of Spermine in Stabilizing DNA.—Since many of the earlier experiments (Marmur and Lane, 1960; Doty, 1959–60) concerned with the heat denaturation of DNA have been carried out in solutions containing 0.015 M sodium citrate in addition to 0.15 M NaCl, the effect of spermine on the melting curve for DNA was studied in this mixture. The data presented below indicate that the protective effect of spermine was decreased by the presence of the sodium citrate.

In 0.15 M NaCl–0.015 M sodium citrate the T_m value for calf thymus DNA was 87.4; this was approximately the same as the T_m in 0.18 M NaCl–0.001 M potassium dimethylglutarate, *p*H 6.2, without sodium citrate. The protective effect of spermine was markedly decreased, however, by the addition of the sodium citrate. In 0.18 M NaCl–0.001 M potassium dimethylglutarate, *p*H 6.2, a spermine concentration of 6.67×10^{-4} M increased the T_m to 89.4° (Table II); in 0.15 M NaCl–0.015 M sodium citrate the T_m was increased to only 87.9° by this concentration of spermine. Higher concentrations of spermine (1.67×10^{-3} M) could not be tested in DNA dissolved in 0.18 M NaCl–0.001 M potassium dimethylglutarate, *p*H 6.2, as turbidity resulted upon addition of this quantity of spermine. In a similar solution containing the DNA in 0.15 M NaCl–0.015 M sodium citrate, however, no turbidity could be detected with this concentration of spermine; under these conditions the T_m was increased to 89.7°. Thus, the citrate increased both the concentration of spermine that was necessary for a given increase in the T_m value, and the concentration of spermine that caused turbidity and precipitation of the DNA. Control experiments, with a 0.015 M citric acid–sodium citrate buffer at *p*H 6.2, demonstrated that these effects were due to the citrate and not to a change in *p*H.

The above data suggested that part of the spermine might be bound in an undissociated form with the citrate, and thus not be available for either stabilization or precipitation of DNA. This was confirmed by a titration experiment (Fig. 3) in which 0.001 M citric acid was titrated with 6 N KOH in the presence (A) and absence (B) of 0.01 M spermine tetrahydrochloride. A third titration (C) was also carried out in 0.1 M NaCl as a control for the effect of ionic strength. The decrease in cH observed in 0.01 M spermine hydrochloride, pompared with the values in 0.1 M NaCl, is indicative of the formation of a complex between spermine and citrate. The titration curve (D) for the 0.01 M spermine hydrochloride solution in this *p*H range is also included in Figure 3; relatively little KOH is required to bring the *p*H to 6.0, and thus the titration of spermine could not account for the large decreases in *p*H observed in

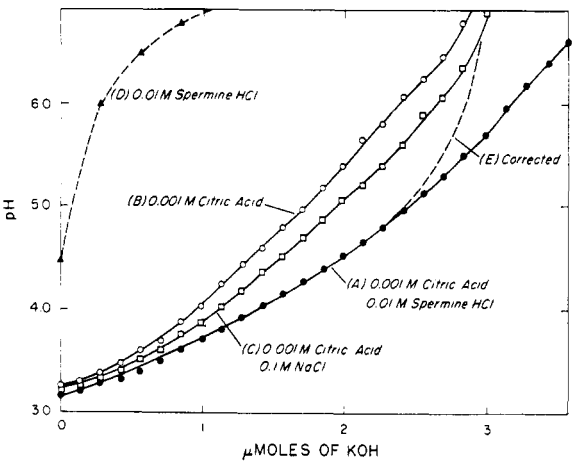

FIG. 3.—Titration of (A) 0.001 M citric acid–0.01 M spermine tetrahydrochloride, (B) 0.001 M citric acid, (C) 0.001 M citric acid–0.1 M NaCl, and (D) 0.01 M spermine tetrahydrochloride with 6 N KOH. The abscissa is listed as the number of μmoles of KOH added per ml. The dotted line (E) was obtained by subtracting the μmoles of KOH used to titrate 0.01 M spermine hydrochloride (curve D) from curve A. Below *p*H 5 curves E and A were identical.

curve A. The dotted curve (E) represents curve (A) after correction for the titration curve of 0.01 M spermine hydrochloride.

In another experiment titration curves were measured for solutions containing equimolar quantities of citric acid and spermine tetrahydrochloride, instead of the ten-fold excess of spermine hydrochloride in the experiments reported above. The changes observed were similar to those observed in the latter experiments, but were considerably smaller.

The Effect of Spermine on the "Melting" Temperature of B. subtilis *DNA.*—The T_m value for *B. subtilis* DNA in 0.037 M NaCl–0.001 M potassium dimethylglutarate, *p*H 6.2, was 79°. In the presence of 6.6×10^{-6} M spermine hydrochloride this increased to 83°; in the presence of 1.33×10^{-5} M spermine the T_m was 86°. The initial optical density at 260 mμ in these experiments was 0.26–0.27.

DISCUSSION

Several different experiments have now been carried out on the stabilization of DNA by polyamines and diamines. These include the current studies on the effect of polyamines and diamines on the temperature–optical density curves of DNA, comparable studies by Mahler *et al.* (1961) with diamines, our previous work (Tabor, H., 1960) on the effect of these amines on the heat inactivation of transforming DNA from *B. subtilis*, and recent studies on the stabilizing effects of spermine against mechanical shearing of DNA from bacteriophage λ (Kaiser, D., Tabor, H., Tabor, C. W., unpublished). As indicated in Figure 2, the polyamines were considerably more

effective than the diamines, and a stabilizing effect could be demonstrated with as little as 10^{-6} M spermine.

The most likely explanation for the stabilizing effects reported in this paper is the effective neutralization of the phosphate groups of the DNA by complex formation with the amines or, in the case of high NaCl concentrations, by shielding of the negatively charged phosphate groups; the consequent increase in the effective strength of the van der Waals' and hydrogen-bonding forces would lead to increased stabilization. The marked differences in the concentrations of amines that are effective might be attributed to the differences in their association constants with the phosphate groups of the DNA. Thus the effectiveness of spermine at a low concentration would probably be due to a high affinity of spermine for the DNA. This is consistent with the evidence from several laboratories (Razin and Rozansky, 1959; Felsenfeld and Huang, 1961) that spermine has a high affinity for nucleic acids although there are relatively few quantitative measurements of this affinity. It is also possible that spermine may be stabilizing the DNA by participating in cross-linkages, but the lack of effect of DNA concentration on the protective action of spermine makes this somewhat less likely.

In the presence of higher ionic strengths spermine was also effective in stabilizing the DNA, but higher concentrations were necessary. This is consistent with the above explanation since dissociation constants usually increase with increasing ionic strength (*cf.* dissociation constants of $MgHPO_4$, for example [Greenwald *et al.*, 1940; Tabor and Hastings, 1943]). A probable explanation for the decreased effect of spermine in the presence of citrate is the formation of an undissociated spermine-citrate complex.

In view of the relatively high concentration of polyamines in certain tissues and organisms and the high affinity of DNA for polyamines, it is of some interest to consider whether sufficient polyamines are present in the usual DNA preparations to affect the T_m values reported by others. In general, we do not consider that any significant error in the reported T_m values can be attributed to polyamines for the following reasons: (1) It seems unlikely that the polyamines would be present in the usual DNA preparations, since these were isolated by alcohol precipitation from solutions containing high salt concentrations (Marmur, 1961). Using C^{14}-spermine (unpublished experiments) we have found that under these circumstances relatively little spermine is precipitated with the DNA. (2) The media used for most of these heating studies (Marmur and Lane, 1960; Doty, 1959–1960) contained 0.15 M NaCl, and the results shown in the latter part of this paper indicate that in the presence of this concentration of NaCl the melting temperature would not be significantly affected by concentrations of spermine equivalent to the base-binding capacity of the DNA. (3) The most commonly used diluting medium (Marmur and Lane, 1960; Doty, 1959–1960) for DNA-heating experiments contains 0.015 M citrate, in addition to NaCl, and this would further decrease the effect noted with the low concentrations of spermidine or spermine that might be still left in the DNA preparations used.

It is tempting to speculate that the stabilizing effects presented in this paper indicate a corresponding significance for the role of the polyamines in the cell. There is no direct evidence for this at present, however, and evaluation of this problem is complicated by the presence in the cell of other materials that bind the polyamines, and of other compounds that have an affinity for the nucleic acids.

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Studies on the Metabolism of Adipose Tissue. XI. Activation of Phosphorylase by Agents Which Stimulate Lipolysis*

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A regime of fasting and refeeding of rats has been employed to produce adipose tissue with a high glycogen content. Incubation of this tissue in a bicarbonate-buffered medium under anaerobic conditions, 5% CO_2–95% N_2, results in the formation of lactic acid which can be quantitatively accounted for by glycogen disappearance. The formation of acid results in the liberation of CO_2 from bicarbonate of the medium and permits the time course of the reaction to be followed manometrically. The rate of the reaction decreases with time but is promptly increased by the addition of epinephrine, serotonin, ACTH,[1] glucagon, fat-mobilizing substance, thyrotropic hormone, or β-mercaptoethylamine. Assay of the tissue for phosphorylase indicates that such agents may produce this effect by increasing the activity of this enzyme. The aerobic esterification of fatty acids to triglycerides in adipose tissue from normally fed rats is inhibited by β-mercaptoethylamine. This substance has no effect on esterification by glycogen-rich tissue, incubated under either aerobic or anaerobic conditions, from fasted and re-fed rats. The results are discussed from the standpoint of energy requirements for the esterification process and the ability of certain hormones to stimulate simultaneously fatty acid release, oxygen consumption, and phosphorylase activity in adipose tissue.

The phosphorylase of many mammalian tissues shows some specificity with regard to the hormone (or hormones) which initiates its activation. For example, in the adrenal cortex, ACTH plays this role: in the liver, glucagon and epinephrine; and in muscle it is the catecholamines (*cf.* Sutherland and Rall, 1960). On the other hand the phosphorylase of white adipose tissue appears to be unique in that it may be activated by all of the mentioned substances (Vaughan, 1960a; Hagen, 1961). In addition these same substances have been shown to be capable of stimulating the release of fatty acids from adipose tissue (Engel and White, 1960; Vaughan, 1960b; Hagen, 1961). Thus in adipose tissue a dual mobilization of the two chief storage forms of foodstuffs, glycogen and fat, may be brought about simultaneously by any one of these substances. The question therefore arises whether any substance which stimulates fatty acid release from adipose tissue will also activate the phosphorylase of this tissue. We describe here a procedure whereby the time course of the activation of phosphorylase in intact adipose tissue may be followed and present data obtained thereby bearing upon this question.

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[1] The following abbreviations are used: ACTH, adrenocorticotropin; ATP, adenosine triphosphate; G-1-P, glucose-1-phosphate; AMP, adenosine-5′-phosphate; CoA, coenzyme A.

METHODS

The epididymal fat body of rats purchased from Holtzman Company has been used exclusively for these studies. The general care and precautions employed in handling of the animals as well as the procedure for removal of adipose tissue have been described previously (Ball and Merrill, 1961). In order to obtain tissue with a high glycogen content, rats which had been fed *ad libitum* for 1 week on Purina laboratory chow were fasted for 3 days and then re-fed *ad libitum* for 2 days. During the refeeding period the rats were given the so-called "fat-free" test diet supplied by Nutritional Biochemicals Corporation containing 21% casein, 58% sucrose, 16% cellulose, vitamins, and a salt mixture. Animals had access to water at all times. Such rats will be referred to as fasted and re-fed. The tissue obtained from them was found to contain 1.5–3.9 mg of glycogen per 100 mg wet weight. Rats of the same age which were maintained continuously on Purina chow were employed as controls. Adipose tissue