TABLE II.
"P" Values and Body Weight Gains of Treated Groups Compared to Untreated Groups.

$$S.D. = \sqrt{V\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}$$

Series	Group	Avg body wt gain (g) Control	Treated	"P" value*
I.	Adult spayed females	71	34	<0.01
II.	" intact "	53	34	0.03
III.	Castrated adult males	14	4	0.60
IV.	Immature intact "	65	36	0.04
"	Adult " "	23	12	0.40

* <0.05 is significant.

hormone, ethinyl estradiol, in an alcoholic solution was absorbed through the skin of albino rats very effectively by cutaneous application without massage and in this vehicle appeared to give a complete substitution for the natural estrogenic internal secretion from the ovary. This would seem to indicate a very simple and reliable means of cutaneous application. Effective absorption of this estrogen through the skin was evidenced by the pronounced influence similar to that generally known of other estrogens on the pituitaries, ovaries, uteri, vagina, testes, seminal vesicles and ventral prostates as determined by gross weights (Table I) and histological studies. 2. The potency of an alcoholic solution of ethinyl estradiol applied to the skin was further substantiated by the fact that estrus was induced in both spayed and intact females in 24 hours and that the daily dosage of 0.1 γ was sufficient to substitute for the loss of the ovaries in castrates as evidenced by uterine weights (Table I). 3. Some retardation of body growth gain (Table II) was caused by the estrogenic treatment in the adult females (spayed and intact) and in immature males. Whether the retardation of body growth was due to some specific toxicity or to some other factor was not determined, however, no visible toxic effects were witnessed in daily observations on the eating habits, general skin and hair condition.

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Effect of Sulfhydryl Reagents on Adenosinetriphosphatase Activity of Myosin.

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Myosin, the main constituent of contractile muscle fibers, has been identified by Engelhardt and Ljubimova[1,2] with adenosinetriphosphatase, the enzyme which catalyzes the breakdown of adenosinetriphosphate (ATP) to adenosinediphosphate and inorganic phosphate. Needham[3] and Bailey[4] have confirmed this discovery.

Needham[3] reported that iodoacetate had no effect on the adenosinetriphosphatase activity of myosin, while glycine and ammonium chloride (reagents known to abolish the nitroprusside test of myosin) actually increased enzyme activity. Needham came to the con-

1 Engelhardt, V. A., and Ljubimova, M. N., *Nature*, 1939, **144**, 513.

2 Ljubimova, M. N., and Engelhardt, V. A., *Biochimiya*, 1939, **4**, 716.

3 Needham, D. D., *Biochem. J.*, 1942, **36**, 113.

4 Bailey, K., *Biochem. J.*, 1942, **36**, 121.

clusion that the -SH groups of myosin were not essential for adenosinetriphosphatase activity.

In a preliminary note[5] on sulfhydryl enzymes data were presented showing that the adenosinetriphosphatase activity of myosin was abolished on addition of *p*-chloromercuribenzoic acid (*p*-ClHg benzoate) and was restored on addition of glutathione (GSH). It was concluded from this inhibition and reactivation that adenosinetriphosphatase was an -SH enzyme.* The -SH groups of myosin have been determined by a number of investigators.[6,7,8] Evidence for the identity of myosin with adenosinetriphosphatase could be obtained by studying the relation between the –SH groups of myosin and enzyme activity in a manner similar to that followed by Hellerman *et al.*[9] in their studies on urease. If the gradual abolition of the –SH groups of myosin by mercaptide forming reagents is followed by a gradual abolition of enzyme activity, this may be considered as a strong argument for the identity of myosin with adenosinetriphosphatase.

Experimental. Myosin was prepared according to Bailey;[4] it had an initial adenosinetriphosphatase activity of Q_P = 1150-1300 in carbonate buffer, pH 8.52 at 38° (Q_P = cmm P liberated per mg dry weight per h.); the activity in 0.1 *M* glycine buffer-0.1 *M* NaCl, pH 8.78 was Q_P = 2350-2400 at 38°. The –SH groups of myosin were estimated by the ferricyanide method of Anson,[10] the iodosobenzoate method of Hellerman *et al.*,[11] and by porphyrindin titration with nitroprusside as an internal indicator.[8] The "freely reacting" sulfhydryl groups (those –SH groups in the *native* protein which give a positive nitroprusside test and react rapidly with mild oxidizing agents), and the total sulfhydryl content of the protein (–SH groups determined in the presence of 1 g guanidine HCl per cc of protein solution) were separately determined. Adenosinetriphosphatase activity was measured by making the enzyme concentration the rate determining step in the reaction: Adenosinetriphosphate $\rightleftharpoons$ adenosinediphosphate + PO_4H_3, and estimating the inorganic phosphate liberated.

Results. The freely reacting –SH content of native myosin was 0.33-0.35 micromole –SH per 10 mg protein by both the porphyrindin and the *p*-ClHg benzoate methods. This amount of native myosin reduced 0.34 micromole of ferricyanide. The total –SH content of guanidine HCl-denatured myosin gave 1.0 micromole –SH per 10 mg protein by the porphyrindin method and 1.08 micromoles by the iodosobenzoate method. The values are in good agreement with the values reported by Greenstein and Edsall.[8] Titration of the total –SH content by *p*-ClHg benzoate gave a lower figure: 0.73 micromole per 10 mg (in 10 *M* guanidine HCl). The reason for this discrepancy is not clear.

The evidence that sulfhydryl groups are necessary for adenosinetriphosphatase activity is summarized in Table I. *p*-ClHg benzoate (1.3×10^{-5} *M*) inhibited the enzyme completely, while 3-NH_2, 4-OH phenyldichloroarsine HCl (6×10^{-4} *M*) produced 48% inhibition. Where mercaptide-forming reagents were used, the inhibitions were completely reversed by 10 equivalents of GSH added 20 minutes after the addition of the inhibitor. As a matter of fact, GSH (5×10^{-4} *M*) raised the activity of older preparations 20-40% above the level of the controls, probably by reducing some of the –SH groups that had been oxidized on standing. The inhibition with porphyrindin (1.3×10^{-5} *M*) was 32%. These data are taken as evidence for the sulfhydryl nature of the enzyme, as in our experience organic mercurials and arsenicals are the most reliable reagents for the detection

[5] Barron, E. S. Guzman, and Singer, T. P., *Science*, 1943, **97**, 356.

* After this paper was submitted for publication, M. Ziff (*J. Biol. Chem.*, 1944, **153**, 25) and J. W. Mehl (*Science*, 1944, **99**, 518) have confirmed our experiments on the reversible inhibition of adenosinetriphosphatase by –SH reagents.

[6] Mirsky, A. E., *J. Gen. Physiol.*, 1936, **19**, 559.

[7] Todrick, A., and Walker, E., *Biochem. J.*, 1937, **31**, 292.

[8] Greenstein, J. P., and Edsall, J. T., *J. Biol. Chem.*, 1940, **133**, 397.

[9] Hellerman, L., Chinard, F. P., and Deitz, V. R., *J. Biol. Chem.*, 1943, **147**, 443.

[10] Anson, M. L., *J. Gen. Phys.*, 1941, **24**, 399.

[11] Hellerman, L., Chinard, F. P., and Ramsdell, P. A., *J. Am. Chem. Soc.*, 1941, **63**, 2551.

TABLE I.
Effect of Sulfhydryl Reagents on Adenosinetriphosphatase Activity of Myosin.

Exp.	Inhibitor	Concentration of inhibitor	Inorganic phosphate liberated (γ P) Control	Inhibitor	Inhibition (%)
1*	3-NH_2, 4-OH phenyldichloroarsine HCl	6x10-4 *M*	25.6	13.1	48
2†	*p*-ClHg benzoate	1.3x10-5 *M*	27.8	0	100
3†	Porphyrindin	1.3x10-5 *M*	27.8	18.8	32
4†	Iodoacetamide	3x10-3 *M*	27.8	27.8	0

* Enzyme, 1.0 cc (≅ 0.17 mg N) in 0.5 *M* KCl—0.05 *M* veronal, pH 7.4. ATP, 0.5 cc (≅ 0.14 mg) 7 min. P (P hydrolyzed in 7 min. in 1 *N* HCl at 100°). $CaCl_2$, 0.05 cc, 0.18 *M*. Temperature, 38°. Duration of experiment, 10 min.

† Same as above, but 1.0 cc enzyme 0.34 mg N; duration of experiment, 5 min.

of –SH-enzymes.

Concerning the failure of inhibition by iodoacetamide, the reason may be either that those groups which alkylating agents attack are not essential for adenosinetriphosphatase activity (this is the case with urease)[11] or that under the experimental conditions iodoacetamide does not combine with any of the –SH groups of the enzyme. When only the freely reacting sulfhydryl groups of myosin were destroyed by stoichiometric amounts of *p*-ClHg benzoate or excess ferricyanide, porphyrindin, or iodosobenzoate,* an inhibition of 11 to 16% occurred (Table II). If iodoacetamide had combined with these reactive –SH groups, this much inhibition would have been evident. Evidence that the freely reacting –SH groups of the protein are not attacked by iodoacetamide comes from the observation that myosin, after treatment with iodoacetamide as in Table II gave a strong nitroprusside test both in the native state and after denaturation with guanidine HCl. Furthermore, myosin treated with iodoacetamide reduced the same amount of ferricyanide as the untreated control. Under these conditions, then, iodoacetamide did not react with the –SH groups of myosin, and if the enzyme were identical with myosin, this would explain the lack of inhibition by alkylating agents.

In order to determine the correlation between abolition of the –SH groups of myosin and the extent of inhibition of adenosinetriphosphatase activity, Hellerman's reagent, *p*-ClHg benzoate, was chosen as the -SH reagent. It was made up in 0.5 *M* KCl in a concentration of 1×10^{-3} *M* by weight. (By titration against standard GSH to nitroprusside end point, this solution was 1.06×10^{-3} *N*). One cc portions of 0.97% solution of myosin were treated with varying amounts of the standard *p*-ClHg benzoate. After 3 minutes the solutions were diluted with veronal buffer (0.05 *M*, pH 7.1, 0.5 *M* in KCl) to 25 cc. One cc aliquots of such solutions were analyzed for adenosinetriphosphatase activity, duplicate assays checking within 2% (Table II). The 1 cc portions of myosin solution contained 1.0 micromole total –SH as determined by porphyrindin titration and 0.28 micromole freely reacting –SH groups as determined by porphyrindin and *p*-ClHg benzoate titrations. Fig. 1 gives the results of a typical experiment. The abscissæ represent the freely reacting and total –SH content of 1 cc of myosin solution as well as the micromoles of *p*-ClHg benzoate added. The ordinate represents the degree of inhibition of enzyme activity. It is apparent that 0.1 micromole of *p*-ClHg benzoate caused no inhibition of enzyme activity although 30% of the freely reacting –SH and 10% of the total –SH groups were abolished. Abolition of all the freely reacting –SH groups (as checked by nitroprusside test) produced 11% inhibition of enzyme activity; as soon as all these –SH groups were destroyed, a steep rise in enzyme inhibition occurred, indicating that the sluggish –SH groups attacked from this point on were more essential for enzyme activity.

If the porphyrindin titer of the total –SH content is chosen as the correct value, 70%

* These oxidizing agents attack only freely reacting –SH groups in the native myosin.

TABLE II.
Relation of Amount of Myosin –SH to Adenosinetriphosphatase Activity.

Reagent	Amt. reagent added to 1 cc of myosin* (micromoles)	Myosin –SH groups destroyed by reagents†		Inhibition of adenosine-triphosphatase activity (%)
		Freely reacting –SH	Total –SH (%)	
p-ClHg benzoate	0.28	all‡	40	11
" "	0.42	all	60	53
" "	0.60	all	86	96
Porphyrindine	0.50§	all	40	14
o-iodosobenzoate	0.50§	all	40	16
Ferricyanide	50.0	all	40	11
Iodoacetamide	1.0	0	0	0

* One cc of 0.97% myosin in 0.5 *M* KCl; freely reacting –SH titer = 0.28 micromoles per cc (by ferricyanide and porphyrindin); total –SH titer = 1.0 micromoles per cc (by porphyrindin and iodosobenzoate); total –SH titer by *p*-ClHg benzoate to nitroprusside end point = 0.70 micromoles per cc. To 1 cc of this myosin the above reagents and then enough 0.05 *M* veronal (pH 7.1, 0.5 *M* in KCl) were added to bring the volume to 25 cc. After 15 minutes a 1 cc aliquot was tested for activity; conditions as in Table I.

† These values are calculated in terms of titrations with the respective –SH reagents. The oxidizing agents in the table react only with the freely reacting –SH groups.

‡ The amount of reagent added is just enough to abolish the nitroprusside test.

§ 0.50 = 1.0 micromoles –SH.

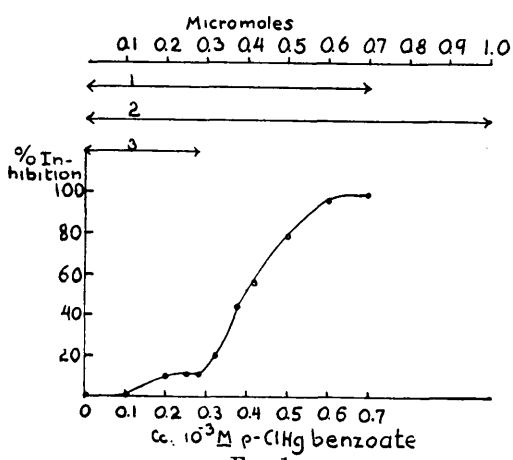

FIG. 1.

Correlation Between the –SH Groups of Myosin and Adenosinetriphosphatase Activity.

Upper abscissæ—

1 = "Total –SH" content by p-ClHg benzoate titration.

2 = "Total –SH" content by porphyrindin titration.

3 = "Freely reactive –SH" content by porphyrindin titration and by reduction of ferricyanide.

Lower abscissæ—

Amounts of ClHg benzoate added to produce gradual destruction of –SH groups of myosin.

Ordinate—

Inhibition of enzyme activity (%).

of the sulfhydryl content of the myosin is destroyed before complete inhibition of enzyme activity occurs; if the *p*-ClHg benzoate titer (to a nitroprusside end point) is selected as the basis of the calculation, then 85-90% of the sulfhydryl is destroyed by the time complete inactivation is found. With the technic just described, the effects of ferricyanide, of dilute porphyrindin, and of iodosobenzoate have also been investigated. In urease these reagents in dilute solutions attack only the "a" groups and leave the activity unaltered. In myosin they reacted with the freely reacting –SH groups and brought about 11-16% inhibition of adenosinetriphosphatase activity, in good agreement with the effect of *p*-ClHg benzoate (Table II). Thus, the freely reacting –SH content per cc of myosin was 0.28, 0.28, and 0.27 micromole, by the *p*-ClHg benzoate, ferricyanide, and porphyrindin methods, respectively. The inhibition obtained by this amount of *p*-ClHg benzoate was 11%; porphyrindin (3-fold excess) inhibited only 14%, iodosobenzoate 16%, and ferricyanide (150-fold excess) 11%. (At higher concentrations porphyrindin gave 32% inhibition, Table I.) The gradual inhibition of enzyme activity closely corresponding to the gradual abolition of the –SH groups of myosin is evidence that myosin and adenosinetriphosphatase are identical, as has been postulated by Engelhardt. These results could, however, also be explained on the basis that the enzyme is a small impurity adsorbed on the surface of myosin. It would have to be small enough to escape elec-

trophoretic detection. Furthermore, the properties of the –SH groups of myosin and enzyme would have to be closely similar to explain the experiments reported in Table II and Fig. 1.

The effect of arsenicals on the enzyme was studied as follows. Varying amounts of a 1×10^{-3} *M* solution of mapharsen (3-NH_2, 4-OH phenylarsine oxide)—standardized by KIO_3—were added to 1 cc of 0.97% myosin at pH 7.1, under the conditions outlined above. Five-tenths of a micromole of the arsenical gave no inhibition, and 10 micromoles of arsenoxide produced only 9% inhibition. (Five-tenths of a micromole of *p*-ClHg benzoate inhibited 80%.) Since in most other enzymes we have studied[8] such a marked difference in the effect of this arsenical and of *p*-ClHg benzoate was not found, the possibility arose that the substituents on the benzene nucleus of the compound interfered with the approach of the arsenical to the –SH groups of the enzyme. In agreement with this assumption, 0.5 micromole of the unsubstituted phenylarsine oxide per cc of myosin gave 22% inhibition. At higher concentrations the inhibition by phenylarsine oxide increased.

We are indebted to Dr. Harry Eagle and to Messrs. Parke, Davis and Co. for the generous samples of the arsenicals, and to Dr. L. Hellerman for the *p*-chloromercuribenzoic acid used in this study.

Summary and Conclusions. The inhibition of adenosinetriphosphatase by mercaptide forming compounds and by mild oxidizing agents, and the reactivation by glutathione can be considered as proof that adenosinetriphosphatase is a sulfhydryl enzyme. The close parallelism between the number of –SH groups of myosin attacked by *p*-ClHg benzoate and oxidizing agents and the degree of inhibition of adenosinetriphosphatase activity is evidence in favor of Engelhardt's assumption that myosin and adenosinetriphosphatase are identical.

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A New Method of Preventing Blood Coagulation.

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Many anticoagulant substances have been employed for preserving blood in the fluid state. Agents such as oxalates and fluorides act by combining chemically with the available calcium in the blood to form an insoluble calcium compound. Citrates have been employed as the sodium or potassium salt and act by forming calcium citrate, which does not ionize. Heparin has been used to preserve the blood in a fluid state; its action being that of an antithrombin which inhibits the prothrombin necessary for coagulation. Oxalates and fluorides may be employed effectively *in vitro* for maintaining the fluidity of the blood in order that it may be used for certain chemical determinations on either whole blood or plasma. The toxicity of these substances, however, precludes their use in transfusions.

Citrates, particularly sodium citrate, have long been used as anticoagulants in transfusion. Moreover, under present wartime conditions, their employment has increased manifold times, particularly in the preparation of plasma. No untoward effects have attended their clinical use. Heparin, although infrequently employed as anticoagulant in routine transfusions, may prove to be a more ideal agent, but this would be contingent on less expensive methods of manufacture. When blood was collected in paraffin-lined tubes it was found to remain fluid for some time due to the failure of the platelets to disintegrate and release thromboplastin. The same observation was subsequently made when methylmethacrylate tubes were employed.

A method was sought whereby the calcium could be removed by the physical phenomenon of adsorption. In a search for a suitable ion-