A Study of Uterine Actin*

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ABSTRACT: Smooth muscle actin was isolated from human uterus and from pregnant sheep uterus. The actin preparations were shown to be of high purity by starch-gel electrophoresis and sedimentation in the ultracentrifuge. The human and the sheep uterine actin appeared to be identical by the above-mentioned methods as well as by amino acid analysis and peptide mapping of their tryptic digests. They also resembled very closely preparations of mammalian skeletal and cardiac actin.

The uterine actin preparations contained approximately 1 mole of bound adenine (adenosine 5′-tri- or diphosphate) and stimulated the adenosine triphosphatase activity of rabbit skeletal myosin under appropriate conditions. The demonstration of actin in mammalian smooth muscle suggests that the mechanism of contraction in smooth muscle is very similar to that in skeletal muscle.

Chemical differences in the contractile proteins of smooth and skeletal muscle may account at least partly for the differences in their physiological properties, such as the elastic viscous behavior of smooth muscle and its rather ill-defined length-tension relationship. Yet it has been generally assumed that the proteins of the contractile apparatus are similar in skeletal and in smooth muscle. This assumption is based on ultracentrifugal and electrophoretic analyses of extracts from uterine muscle which were shown to contain actomyosin, myosin, tropomyosin, and other proteins but not free actin (Csapo, 1960; Kominz and Saad, 1956); and on measurement of viscosity changes upon addition of ATP[1] to various extracts (Needham, 1962; Csapo, 1950). As long as the individual proteins have not been isolated in pure form, there is no convincing evidence yet to show that they are different or identical in skeletal and in smooth muscle.

The preparation and purification of the uterine contractile proteins has proved to be notoriously difficult; in particular, actin was obtained in only 60% purity (Needham and Williams, 1963c). In the present communication the preparation of actin from human and from pregnant sheep uterus follows the methods previously used for preparation of skeletal actin (Carsten and Mommaerts, 1963). It will be shown that the actin preparations were homogeneous (by ultracentrifugation and starch-gel electrophoresis), and similar to mammalian skeletal actin (by amino acid analysis, peptide maps, and enhancement of skeletal myosin ATPase activity). Slight differences in some physicochemical properties will also be discussed.

Materials and Methods

Surgical specimens of premenopausal human uterus and pregnant sheep uterus were obtained. The human uteri were removed because of uterine prolapse and had no definite pathology. The sheep uteri were obtained after the fetus had been removed, but with the placenta still attached to the walls. The placental cotyledons were then resected, and the uterine mass was cut into pieces of approximately 5 cm^2 and frozen. Acetone-dried muscle powder was prepared by the method of Bárány *et al.* (1957). Actin was extracted in the cold and purified by ultracentrifugation after polymerization as previously described for skeletal actin (Carsten and Mommaerts, 1963) with slight modifications. The *p*H of the solution was kept at 7.5 or below at all times; the extracts were allowed to polymerize overnight and Sephadex treatment after final dialysis was carried out batchwise. Protein concentration was determined by the method of Lowry as before (Lowry *et al.*, 1951).

Starch-gel electrophoresis in Veronal buffer, *p*H 7.4 (in the presence of 0.4 mM ATP, 0.2 mM ascorbic acid), and amino acid and tryptophan analyses have also been described (Carsten, 1963; Carsten and Mommaerts, 1963). Actin was alkylated with iodoacetate prior to amino acid analysis (Carsten, 1963; Katz and Carsten, 1963), and the alkylated actin was precipitated at *p*H 4.6 and washed repeatedly (Carsten and Katz, 1964). Tryptic digestion of the alkylated actin preparations and two-dimensional chromatography and high-voltage elec-

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† Recipient of a U.S. Public Health Service research career development award (1-K3-GM-4547-01) from the National Institute of General Medical Sciences, National Institutes of Health, Bethesda, Md.

[1] Abbreviations used in this work: ATP, adenosine 5′-triphosphate; ATPase, adenosine triphosphatase; ADP, adenosine 5′-diphosphate.

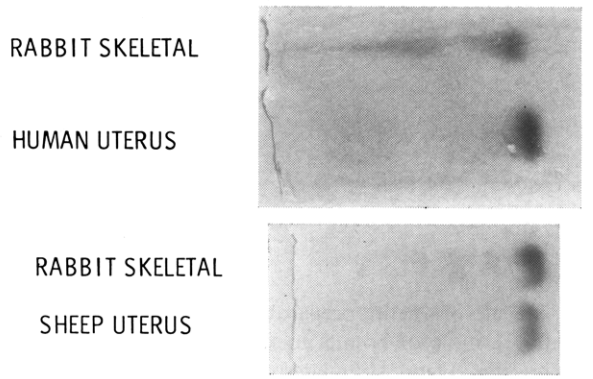

FIGURE 1: Starch-gel electrophoresis of human uterine actin and rabbit skeletal actin; sheep uterine actin and rabbit skeletal actin in Veronal buffer, *p*H 7.4, ionic strength 0.012, 0.4 mM ATP, and 0.2 mM ascorbic acid. At 6 v/cm of gel, 4 hours. Protein concentration 6 mg/ml.

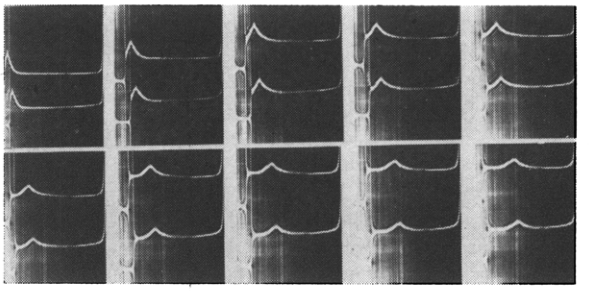

FIGURE 2: Ultracentrifugal sedimentation of human uterine actin. Protein (4 mg/ml, upper curve; 2 mg/ml, lower curve), Tris-nitrate buffer, *p*H 7.6, 0.001 M, 20°. Pictures were taken when top speed was reached and every 8 minutes thereafter. Sedimentation proceeds from left to right.

trophoresis were carried out as before (Carsten and Katz, 1964; Katz and Carsten, 1963). Sedimentation velocities of G-actin preparations in 1.0 mM Tris-nitrate buffer, 0.2 mM ascorbic acid, 0.2 mM ATP, *p*H 7.6 (Carsten and Katz, 1964; Katz and Carsten, 1963; Carten and Mommaerts, 1963), were measured in a Spinco Model E ultracentrifuge equipped with a R.T.I.C. temperature control. Viscosity was measured in an Ostwald-type viscometer with an outflow time for water of 76.1 seconds at 25 ± 0.02°.

To determine bound ATP (Ulbrecht *et al.*, 1960) 25-30 mg of actin, without prior dialysis, was treated first with Dowex 1-X10 (Cl^-) to remove free nucleotides. The protein concentration was determined and 1 volume 1 M perchloric acid was added to 4 volumes actin solution. The precipitate was washed twice with 0.3 M perchloric acid; the supernatant and washings were combined and made up to 10 ml, and adenine was determined spectrophotometrically. ATPase activity was measured on solutions containing 0.2 mg rabbit skeletal myosin or 0.25 mg actomyosin (reconstituted from one part skeletal or uterine actin plus four parts myosin). Assays were carried out in 0.145 M KCl, 3 mM ATP, 2 mM $MgCl_2$ or 20 mM $CaCl_2$, 0.01 M maleate buffer, *p*H 6.5, at room temperature (Mommaerts and Seraydarian, 1947). Inorganic phosphate was determined by the Fiske-Subbarow method (1925).

Results

The successful preparation of actin depends on its polymerization to F-actin in the presence of 0.1 M KCl and of small amounts of divalent cation. When the method of Straub (1943) was used to prepare muscle powder, no polymerization took place, but the method of Bárány *et al.* (1957) as previously used in this laboratory (Carsten and Mommaerts, 1963) invariably led to polymerization, though polymerization was slower than for skeletal muscle actin. However, while 12 g muscle powder and approximately 75 mg actin were obtained regularly from 100 g skeletal muscle, the yield from pregnant sheep uterus muscle was often only 5.5 g muscle powder and 14 mg actin, and from human uterus 17 g powder and 35 mg actin were obtained. In the first attempts to isolate actin from uterus, some tropomyosin was seen in the preparations on starch-gel electrophoresis. Since high concentration of Mg^{2+} has been reported to increase the amount of tropomyosin in actin preparations (Drabikowski and Gergely, 1962), polymerization was later on routinely carried out without adding Mg^{2+}. This procedure did not seem to affect the yield or the time required for polymerization. The extracts were allowed to polymerize overnight in the cold room after an initial 1-hour polymerization at 18°. Similarly in the final step depolymerization by dialysis was slow and often took as much as 40 hours or more.

Starch-gel electrophoresis patterns of human and sheep uterine actin are shown in Figure 1. In both preparations only one band was seen which moved the same distance as rabbit skeletal actin, shown also in Figure 1. There was no tropomyosin detectable. Analysis for tropomyosin by ammonium sulfate precipitation (Bailey, 1948; Drabikowski and Gergely, 1962) revealed 2.5–3.0% tropomyosin in the preparations, a value considered to demonstrate high purity even for skeletal actin preparations (Drabikowski and Gergely, 1962).

Sedimentation in the ultracentrifuge revealed at times hypersharp peaks and a rather pronounced concentration dependence of the sedimentation coefficient. In these instances the extrapolated value for the sedimentation constant arrived at by averaging the data from three experiments using the method of least squares was 3.72 for the human and 3.85 for the sheep, slightly higher than the values for dog skeletal and cardiac actin (Katz and Carsten, 1963). It seemed unlikely that the trace of tropomyosin present could cause this pronounced concentration anomaly. Furthermore, actin prepared after extraction of the muscle powder with butanol (Tsao and Bailey, 1953), a procedure designed to remove tropomyosin, did not improve these results.

The hypersharp peaks, however, were no longer seen after prolonged dialysis (40–64 hours) of our uterine actin solutions, prepared routinely as described above. These preparations showed the ultracentrifugal characteristics of skeletal and cardiac actin, such as the shape of the sedimenting boundary (Figure 2), and the only slight dependence of the sedimentation velocity on protein concentration, as shown in Figure 3. The extrapolated value for the sedimentation constant, calculated by the method of least squares, was 3.67 for the human and 3.78 for the sheep, slightly higher than that of skeletal and heart actin which was 3.44 (Katz and Carsten, 1963; Katz and Hall, 1963).

Viscosity measurements showed a slight concentration dependence of the reduced viscosity (η_{sp}/c) with values for η_{sp}/c of 0.36, 0.45, 0.55, and 0.75 dl/g at 0.07, 0.1, 0.15, and 0.2 g actin per 100 ml. From these data the extrapolated value for the intrinsic viscosity is about 0.12 dl/g. This is in reasonable agreement with intrinsic viscosities of 0.104 dl/g (Lewis *et al.*, 1963) and 0.14 dl/g (Kay, 1960) obtained for skeletal actin, in view of its low viscosity and the high error in measuring. The concentration dependence indicates the presence of some polymers or possibly a trace of tropomyosin (Lewis *et al.*, 1963; Martonosi, 1962).

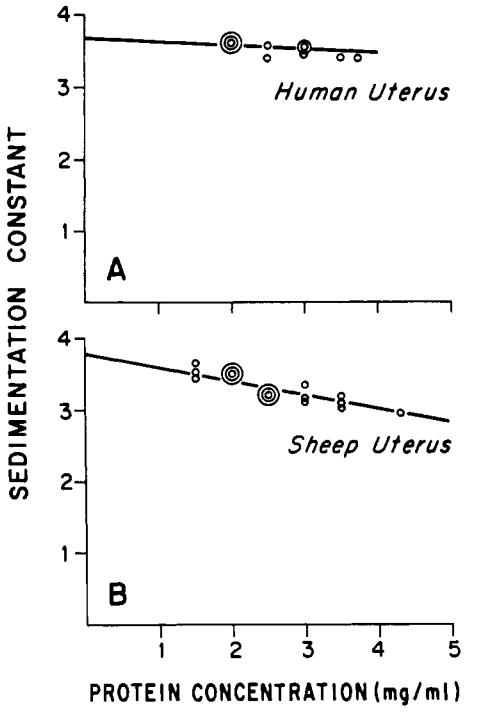

FIGURE 3: Sedimentation velocity of (A) human and (B) sheep uterine actin as a function of protein concentration.

TABLE I: Amino Acid Composition of Uterine Actin Preparations.

Amino Acid	Number of Amino Acid Residues[a] per 60,000 g of Protein	
	Human Uterus	Sheep Uterus
Lysine	26.8 ± 0.7[b]	26.7 ± 0.7
Histidine	10.1 ± 0.2	10.5 ± 0.4
Arginine	25.0 ± 0.9	26.0 ± 0.8
SCM-cysteine[c,d]	7.0 ± 0.6	7.3 ± 0.3
Aspartic acid	46.6 ± 0.4	46.4 ± 0.4
Threonine[d]	37.2 ± 0.8	36.7 ± 0.4
Serine[d]	35.9 ± 1.3	37.7 ± 0.6
Glutamic acid	56.1 ± 1.5	55.9 ± 1.3
Proline	25.8 ± 0.6	25.9 ± 0.4
Glycine	38.9 ± 1.0	39.8 ± 0.5
Alanine	42.1 ± 0.2	41.9 ± 0.5
Valine	25.4 ± 0.9	25.8 ± 1.4
Methionine	16.1 ± 1.0	16.3 ± 1.4
Isoleucine[e]	36.2 ± 0.9	37.6 ± 0.9
Leucine	38.3 ± 1.0	38.6 ± 0.7
Tyrosine	18.8 ± 0.4	19.6 ± 0.9
Phenylalanine	16.5 ± 0.5	16.9 ± 0.4
Tryptophan	4.5 ± 0.2	5.4 ± 0.5
Total	507.3 ± 0.7	515.0 ± 0.7

[a] The values were corrected to a recovery of 95% in order to facilitate comparison (see text, and Carsten, 1963). [b] The variation is expressed as the average of the deviations from the mean. [c] SCM, *S*-carboxymethyl. [d] Corrected for losses during hydrolysis (Carsten, 1963). [e] Corrected for incomplete hydrolysis (Carsten, 1963).

Amino acid analysis for alkylated actin preparations from uterus are shown in Table I. The results were averaged for three preparations of actin from each source. Recoveries were corrected to 95% in order to facilitate comparison with previous analyses of skeletal actin in which 95% of the dry weight was recovered (Carsten, 1963). The amino acid compositions of human and sheep uterine actin seem to be identical and showed no major differences from skeletal (Carsten, 1963; Carsten and Katz, 1964) and cardiac actin (Katz and Carsten, 1963) with the exception of serine, which was higher than in the other types of muscle, and of methionine, which was lower. The latter may be due to oxidation during hydrolysis. The differences in the value for serine may have been caused by experimental error as serine is labile during hydrolysis.

Peptide maps of tryptic digests are shown in Figure 4. Human and sheep uterine actin resembled each other closely and both were quite similar to the peptide patterns obtained from skeletal actin of various origins (Carsten and Katz, 1964).

The adenine content of the uterine actin preparations was found to be 14.0 and 13.4 μmoles/g actin for the sheep and the human, respectively. Assuming a molecular weight of 60,000, the bound adenine would be 0.84 and 0.80 mole/mole actin.

Figure 5 shows the activation of rabbit skeletal myosin with human uterine actin in the presence of Mg^{2+}. This

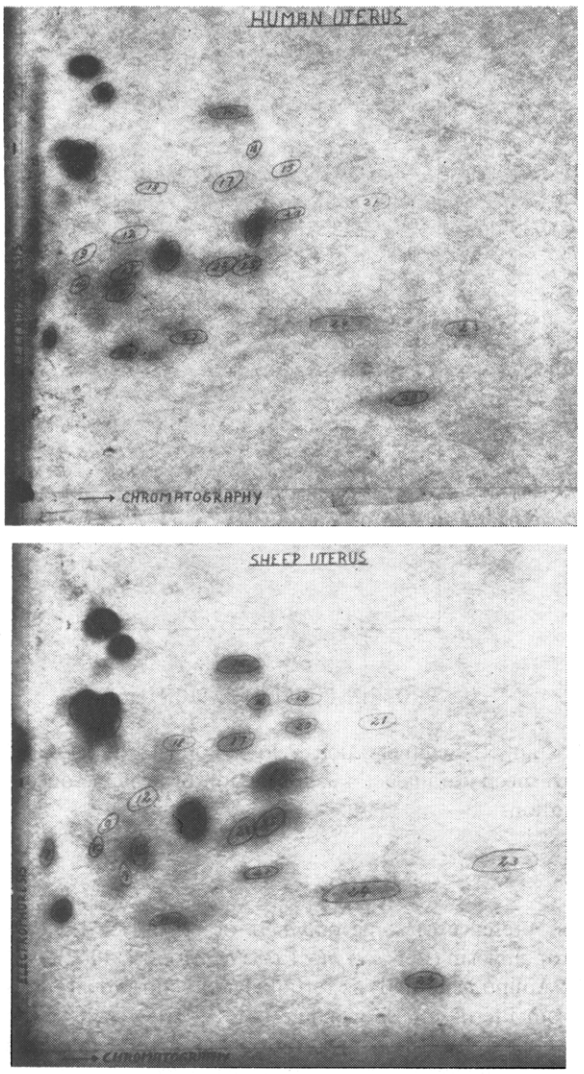

FIGURE 4: Peptide maps of tryptic digests of human and sheep uterine actin. Descending chromatography was from left to right, electrophoresis was in the vertical direction with the cathode at the top of the paper, 1 hour at 2000 v.

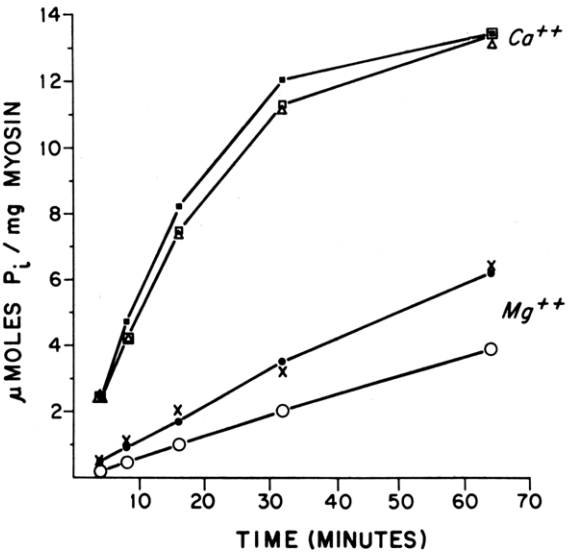

FIGURE 5: ATPase activity of rabbit skeletal myosin, ○; rabbit skeletal myosin and human uterine actin, ●; rabbit skeletal myosin and rabbit skeletal actin, ×; in the presence of 3 mM $MgCl_2$: ATPase activity of rabbit skeletal myosin, □; rabbit skeletal myosin and human uterine actin, ■; rabbit skeletal myosin and rabbit skeletal actin, △; in the presence of 20 mM $CaCl_2$.

activation was quantitatively identical with that obtained for skeletal actin, also shown in Figure 5. When Mg^{2+} was replaced by Ca^{2+} neither uterine nor skeletal actin activated the ATPase, as was to be expected. Sheep uterine actin gave identical results.

Discussion

The structural and enzymatic studies presented in this paper have made possible the characterization of actin from a mammalian smooth muscle and allow us to compare the uterine actin preparations with those from skeletal and heart muscle. Actin of high purity has not previously been prepared from smooth muscle. The difficulties encountered in its preparation have several reasons. The myometrium contains less actin than skeletal muscle and contains a relatively large amount of proteins associated with the muscle cells other than those of the contractile apparatus. Noteworthy is the presence of collagen which has been estimated to be three times as much as in skeletal muscle (Cretius, 1959; Needham and Williams, 1963a,b). In view of the presence of collagen and other unknown proteins (Needham and Williams, 1963a) it becomes more difficult to extract the relatively small amounts of actin, and purification presents a greater problem. The low yields obtained, compared with skeletal muscle, may merely reflect the lower actin content of the myometrium or difficulties in extraction and polymerization. An alternative interpretation is that the very striking differences in the amounts of actin which can be extracted from pregnant sheep uterus and human uterus (5.5 mg and 14 mg, respectively), compared to 75 mg actin from 100 g of skeletal muscle, may at least partly account for some of the physiological properties of smooth muscle such as the sluggishness in response to a stimulus. The low content of actin in pregnant sheep uterus might also account for its relatively poor contractile force. Since increases in actomyosin content during pregnancy have been reported (Needham and Williams, 1963a), still smaller amounts in the nonpregnant uterus would have to be expected, a problem which will be further studied.

The observations reported in this paper demonstrate that smooth muscle actin preparations from human and from sheep resemble each other very closely. This is in accordance with previously reported results on the

absence of species differences in mammalian skeletal actin preparations (Carsten and Katz, 1964).

In comparing the smooth muscle preparations with skeletal and heart muscle actin from various species (Carsten, 1963; Carsten and Katz, 1964; Katz and Carsten, 1963) the similarities found are also striking, particularly in the starch-gel electrophoresis, amino acid composition, and peptide maps. The difference in one amino acid, serine, is probably owing to variable losses during hydrolysis and cannot be taken as sufficient evidence for difference in structure. Furthermore, comparison with amino acid analysis of cardiac actin from the same species[2] showed no significant differences. Smooth muscle actin also showed identical behavior with skeletal actin in its activation of myosin ATPase; thus reported differences in ATPase activity of smooth and skeletal muscle actomyosin (Needham and Williams, 1963c) must be sought in the uterine myosin. The adenine content was a little lower than that found in skeletal actin, 14 μmoles/g actin as compared to 16 in skeletal muscle (Ulbrecht *et al.*, 1960). From the latter result a molecular weight of 62,000 has been calculated on the basis of 1 mole ATP or ADP bound per mole actin. Our results are probably due to a small loss of bound nucleotide in the preparative procedure, but could reflect a higher molecular weight.

Whether the small differences in the sedimentation constants for the sheep and human uterine actin preparations from those obtained for skeletal and cardiac actin are significant cannot be ascertained at present. A higher sedimentation constant could result if there were some aggregated or partially polymerized actin present, as has been observed in some preparations of skeletal and cardiac actin (Carsten and Mommaerts, 1963; Katz and Hall, 1963). This interpretation is compatible with the viscosity measurements. On the other hand, uterine actin may be slightly different in its physicochemical properties, as reflected in the low rate of polymerization and depolymerization. The former could be explained by the low concentration of actin in the extracts. The reason for the low rate of depolymerization is not entirely clear at present. The cause for the hypersharp peaks and the great concentration dependence of the sedimentation coefficient, obtained at times, may have to be sought in small differences from skeletal actin in its secondary and tertiary structure. The latter may favor a tendency for partial polymerization or some linear alignment of the molecules of G-actin. Differences in secondary and tertiary structure may seem doubtful in view of the similarities in primary structure of skeletal and smooth muscle actin; yet they may be based on minor differences in the primary structure, detectable only with methods more sensitive than the ones used in the present investigation.

The great similarity of the actin preparations from the different types of muscle is so much more striking in view of the anatomical differences in their structure, i.e., the irregular alignment of the filaments or of the sarcomeres in the myofibril (Lowy and Hanson, 1962). Indeed, the presence of actin in uterine smooth muscle and the demonstration of its great resemblance with skeletal and cardiac actin suggest that the contractile event in smooth muscle may be similar to that in other types of muscle. While the observed differences in the amounts of actin extractable from various types of muscle may have some bearing on the physiological performance of the respective muscles, it appears that the chemical properties of uterine actin do not account for the differences in physiological behavior of skeletal and smooth muscle such as slowness of contraction and changes in length-tension relationships in smooth muscle.

[2] M. E. Carsten, unpublished.

Acknowledgments

The author wishes to thank Dr. Nicholas S. Assali and Dr. Dean Moyer for kindly supplying the sheep and human uterus material, respectively. The technical assistance of Miss Doris Repke is gratefully acknowledged.

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Distinct Subunits for the Regulation and Catalytic Activity of Aspartate Transcarbamylase*

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ABSTRACT: Kinetic investigations of regulatory enzymes have led to the current view that such enzymes bind their regulatory metabolites at sites distinct from the active sites, and that inhibition (or activation) requires the cooperative interaction of subunits within the enzyme molecules. This communication presents direct physicochemical studies concerning one such enzyme, aspartate transcarbamylase (ATCase), in terms of its subunit structure and binding of the specific inhibitory metabolite, cytidine triphosphate (CTP). As judged from sedimentation and viscosity studies, ATCase from *Escherichia coli* is a compact, globular protein with a sedimentation coefficient ($s_{20,w}$) of 11.7 S, an intrinsic viscosity of 0.045 dl/g, and a molecular weight of 3.1×10^5. Ultracentrifugal analysis of the binding of the CTP analog, 5-bromocytidine triphosphate (BrCTP), reveals the existence of eight receptor sites on the enzyme. Upon the addition of the mercurial, *p*-mercuribenzoate, native ATCase dissociates into two types of subunits which are easily separable by zone centrifugation or column chromatography. The larger, with $s_{20,w} = 5.8$ S and molecular weight of 9.6×10^4, possesses the entire catalytic activity of the native enzyme and is completely insensitive to the inhibitor, CTP. This protein is termed the *catalytic subunit*. The second protein, with $s_{20,w} = 2.8$ S and molecular weight of 3×10^4, is enzymically inactive and is unnecessary for the activity of the catalytic subunit. This smaller protein, termed the *regulatory subunit*, bears the receptor sites for the inhibitor, BrCTP, and is required for the control of enzymic activity. Since the regulatory subunits comprise 37% of the total weight of the native enzyme, it can be calculated that there are four such subunits in each ATCase molecule. Similarly it can be concluded that the native enzyme contains two catalytic subunits.

The native enzyme can be reconstituted from the unfractionated subunits upon removal of the *p*-mercuribenzoate through the addition of mercaptoethanol. Also, upon mixing of the separated subunits from which the mercurial had been removed, aggregation occurs spontaneously to produce a complex having the catalytic and regulatory properties of the native enzyme.

Although the numerous metabolic pathways of a normal cell are biochemically independent, they are closely interconnected and coordinated with regard to the rates of synthesis of their products. Much of the coordination derives from a small, important group of regulatory enzymes which operate at critical steps in the sequences of metabolic reactions. These enzymes are equipped to interact specifically with metabolites in addition to their substrates and products. Characteristically, a regulatory enzyme of one pathway is strongly activated or inhibited by a specific metabolite arising from another pathway, or from another step of the same pathway. Thus, through these enzymes, a mechanism exists for the close coordination of metabolism.

Some of the better-known regulatory enzymes have been found in the biosynthetic pathways of bacteria (Umbarger, 1961; Wilson and Pardee, 1964; Monod *et al.*, 1963). Generally the first enzyme of a sequence is inhibited by the end product of the entire pathway. This pattern of control, known as feedback inhibition, couples the production of low molecular weight metabolites to their consumption for the synthesis of macromolecules. One such example is observed in pyrimidine biosynthesis in *Escherichia coli*. The first enzyme in that pathway is aspartate transcarbamylase (ATCase)[1]

* From the Department of Molecular Biology and Virus Laboratory, University of California, Berkeley. *Received April 7, 1965*. This research was supported in part by U.S. Public Health Service research grants from the National Cancer Institute (CA 07410) and from the National Institute of General Medical Sciences (GM 12159), by a National Science Foundation research grant (G-10795), and by a contract between the Office of Naval Research and the Regents of the University of California.

[1] Abbreviations used in this work: ATCase, aspartate transcarbamylase; CTP, cytidine triphosphate; BrCTP, 5-bromocytidine triphosphate.