Nicol and Smith, 1960; Wilson and Dixon, 1961; Kotaki, 1961).

ACKNOWLEDGMENTS

We are indebted to Dr. E. L. Grinnan of Eli Lilly and Company for generous supplies of insulin and for arranging for the biological assays. We would also like to thank Mrs. Bertha L. Martin for aid with the amino acid analyses and Miss Sharon L. Hayes for aid with the partition column chromatography.

ADDED IN PROOF

Sundby (1962) has recently reported on the acid-catalyzed transformation of insulin in which the transformation products were detected by paper electrophoresis in urea-containing buffers. His results indicated that the acid-transformation involves a progressive liberation of ammonia from the 6 amides of insulin. From a comparison of the rates of travel on paper electrophoresis of the various components in the mixture formed in the early stages of the transformation both before and after treatment of the mixture with carboxypeptidase, Sundy concluded, in agreement with our finding (Carpenter and Slobin, 1962), that the first amide was lost from the carboxyl-terminal asparagine. In view of Sundby's report, it appears probable that the desamido-insulin described in the present paper is a mixture of desamido-forms in which the major portion (85–90%) contains carboxyl-terminal aspartic acid instead of asparagine, and the minor portion (10–15%) has lost an amide from positions other than the carboxyl-terminal asparagine.

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A Study of Actin by Means of Starch Gel Electrophoresis*

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The properties of globular actin prepared by ultracentrifugal isolation of F-actin followed by reversible depolymerization in the presence of ATP have been investigated. By ammonium sulfate fractionation and by starch gel electrophoresis the actin preparations were found to be homogeneous and free of tropomyosin. In the starch gel electrophoresis under a variety of conditions the actin preparations revealed diverse characteristic patterns. Four or five new bands appeared in the starch gel electrophoresis on standing, on removal of free ATP, on substitution of the sulfhydryl groups, or on exposure to *p*H 2, *p*H 10, or 7.2 M urea.

The purification of actin by ultracentrifugal isolation of its polymer (Mommaerts, 1952, 1958) followed by depolymerization has become widely accepted. However, the judgment as to the purity of the product rests on somewhat limited criteria: the complete polymerizability of the product upon the addition of salt, and the homogeneous appearance of the sedimenting boundary of the monomer in the ultracentrifuge. The first criterion is most valuable, but it might not detect moderate amounts of impurity associated with the

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sedimenting polymer; the second criterion was not used quantitatively since the sedimentation in a medium without electrolyte other than a small excess of ATP might be a complex theoretical problem (Mommaerts, 1952).

In this investigation, additional criteria have been explored, such as solubility in ammonium sulfate and zone electrophoresis in starch gels. The latter method, besides giving information concerning the homogeneity of the protein, also revealed profound alterations caused by mild chemical influences. These changes will be presented descriptively but have not been explained.

METHODS

Purification of Actin.—In view of the extensive additional experiences since our last publications on this matter (Mommaerts, 1952, 1958), we shall describe in full our present methods of preparation.

The pretreatment of the ground rabbit muscle is based upon the procedure of Barany *et al.* (1957), which differs from the original Straub (1943) procedure in that the extraction of the myosin is omitted. In the cold room, the mince was extracted first with 3 volumes of 0.1 M KCl and second with 3 volumes of 0.05 M $NaHCO_3$. Each extraction, with gentle stirring, lasted 15 minutes. The mince was then washed with 10 volumes of 0.001 M Versene and twice with 10 volumes of water. At room temperature the residue was then extracted twice with 2 volumes of acetone precooled to 0° and once with one volume of acetone (0°) for a few seconds in the Waring blendor. All extractions or washings were followed by filtration through several layers of gauze. The fibrous mass was air-dried overnight, then stored in the deep freeze at −20° and extracted within 2 weeks.

The extraction was usually done with 35 g of dried muscle; the following description applies to this amount. The material, moved gently with a polyethylene spatula, was extracted in the cold (3°) for 30 minutes with 400 ml of 0.2 mM ATP and 0.2 mM ascorbate at *p*H 7.5, filtered by suction, and reextracted with 300 ml of the same solution. The filtered extracts were centrifuged in the no. 30 rotor of the Spinco model L ultracentrifuge at 30,000 rpm for 1 hour. KCl and $MgCl_2$ were added to 0.1 M and 0.1 mM concentration respectively. The solution was placed in a water bath at 18° for 1 hour for polymerization and was then placed in the cold-room (3°) for several hours or overnight. The first centrifugation was done at 30,000 rpm for 3 hours (Spinco rotor no. 30) or at 15,000 rpm overnight (Spinco rotor no. 21). The supernatant solutions were decanted, the tubes rinsed with cold ATP-ascorbate solution, and the pellets suspended in 100 ml cold 0.2 mM ATP–0.2 mM ascorbate solution of *p*H 7.5 by means of a hand-operated test tube homogenizer. This yielded a solution of G-actin, which was clarified by high-speed centrifugation (30,000 rpm for 1 hour). KCl was added to 0.1 M, and after repolymerization the ultracentrifugal purification cycle was repeated. The pellets were homogenized in approximately 40 ml of ATP-ascorbate and dialyzed overnight against 500 ml ATP-ascorbate solution at 3°. At times the depolymerization step was omitted, and the pellets were homogenized in ATP-ascorbate solutions in the presence of 0.1 M KCl and again submitted to ultracentrifugation. In this case, after dialysis it was sometimes necessary to clarify the solutions by high-speed centrifugation (30,000 rpm for 1 hour). This procedure did not seem to change the end-product significantly. After dialysis, the protein was largely depolymerized, although some remnants of flow birefringence were sometimes still present. The velocity of depolymerization was somewhat unpredictable, and may have depended on the amount of remaining salt, divalent cation in particular, and upon the protein concentration, which was kept high.

The solution of G-actin (40 ml) was then filtered through a column of Sephadex G-25, 3 × 40 cm, which had been equilibrated with ATP-ascorbate solution, all at 3°. The progress of filtration was followed by spectrophotometric readings. The pooled effluent solutions had a concentration of 1–2 mg actin/ml; they were concentrated to 8–10 mg/ml in a rotary evaporator at low temperature. Alternatively, batchwise treatment with Sephadex G-25, equilibrated with ATP-ascorbate, was used. The solutions were now devoid of birefringence, but would repolymerize upon addition of salt.

Among the necessary precautions, we mention the importance of not exceeding a *p*H of 8.0 at any step in the preparation. While higher alkalinity is sometimes tolerated, it does cause greater lability of the protein. The need for working at low temperature *throughout* the entire procedure was not explicitly stated in the original papers (Mommaerts, 1951, 1952), but was stressed in a later description (Mommaerts, 1958). The use of ascorbic acid, as in the original work by Straub and Feuer (1950), supersedes the need to work in the absence of oxygen (Mommaerts, 1958), which is impractical and not completely successful; mercaptoethanol would probably do equally well, but it has not been tested to the same extent.

To remove excess ATP, a 0.9 × 7.5 cm column of Dowex-1 was used (Asakura, 1961); the resin was equilibrated with 0.005 M glycine at *p*H 8.0. The actin solutions were concentrated in a rotary evaporator at low temperature and about 20 mg of actin in 2.5 ml was applied to the column at 3°, followed by the same buffer for elution. The effluent was again concentrated in a rotary evaporator to a protein content of 8–10 mg/ml.

Protein concentrations were determined colorimetrically according to Lowry *et al.* (1951), with lyophilized actin preparations used as standards.

Zone Electrophoresis in Starch Gel.—Starch gel electrophoresis was done according to the method of Smithies (1955), with some modifications (Carsten and Pierce, 1960). The buffer ordinarily used was glycine at *p*H 8.0, ionic strength 0.012; other media were glycine at *p*H 2, *p*H 8.5, and *p*H 10, Veronal at *p*H 7.5 and 8.6, and glycine at *p*H 8.5 containing 7.2 M urea. In the latter case, 80 ml of glycine buffer containing 9 M urea (33.3 ml buffer, ionic strength 0.036, and 46.7 ml water) was added to 15 g starch (Starch hydrolyzed, Connaught) suspended in 20 ml of water. The suspension was heated carefully, and the poured gels were left overnight in the cold-room (3°) to solidify. The urea was deionized and recrystallized before use. When ATP and ascorbic acid were used in the electrophoretic experiments, appropriate amounts (0.4 mM and 0.2 mM respectively) were added to the buffers just before use, so that ATP and ascorbate were present in the gel and in the buffer vessels. This caused a lowering of the *p*H by 0.1 unit.

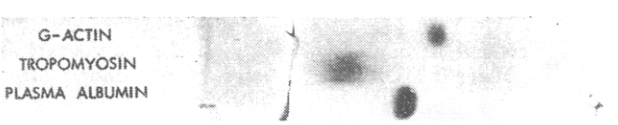

FIG. 1.—Photograph of starch gel electrophoresis pattern of G-actin, tropomyosin, and plasma albumin in Veronal buffer, *p*H 7.4, ionic strength 0.012, 0.4 mM ATP, and 0.2 mM ascorbic acid. 8 volts per cm of gel, 4 hours.

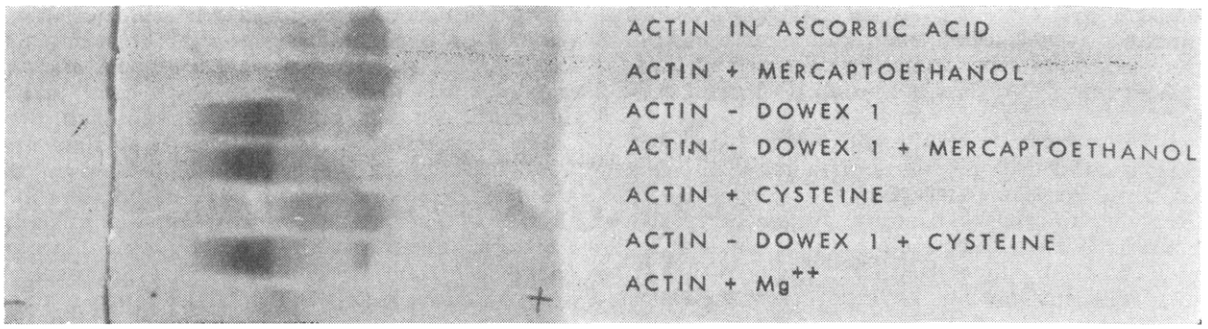

FIG. 2.—Photograph of starch gel electrophoresis pattern of G-actin in the presence of reducing agents (*A*,*B*); after Dowex-1 treatment (*C*); addition of mercaptoethanol after Dowex-1 treatment (*D*); addition of cysteine before and after Dowex-1 treatment (*E*,*F*); addition of KCl and $MgCl_2$ to G-actin (*G*). Protein concentration 8–10 mg/ml. Glycine buffer, *p*H 8.5, ionic strength 0.012, 7 volts per cm of gel, 5 hours.

Ultracentrifugal Sedimentation.—The Spinco model E ultracentrifuge was used at 59,780 rpm to observe the sedimentation behavior of actin solutions. The buffers used were 0.2 M glycine *p*H 8.5 or 0.01 M Tris nitrate at *p*H 8.0 or 8.3.

RESULTS

Homogeneity of the Protein.—The actin preparations used in this work were ultracentrifugally homogeneous according to the criteria available for this case as previously reported (Mommaerts, 1952). The fact that on fractionation with ammonium sulfate no protein precipitated between 0.45 and 0.70 saturation indicated the complete absence of tropomyosin (Bailey, 1948; Martonosi, 1962).

Starch Gel Electrophoresis.—Most of the experiments were done in glycine buffer, which caused no polymerization; experiments in Veronal gave similar results. In Veronal buffer at *p*H 7.4 in the presence of 0.4 mM ATP, 0.2 mM ascorbic acid G-actin formed one band (Fig. 1). Lyophilized actin preparations, redissolved in ATP, also showed only one band on starch gel electrophoresis in the presence of ATP and polymerized on addition of KCl and $MgCl_2$. Tropomyosin, prepared by the method of Bailey (1948), had only one third of the mobility of G-actin (Fig. 1) in the starch gel electrophoresis, and no tropomyosin could be detected in the electrophoretic pattern of our actin preparations. At *p*H 8.0 and 8.5 and without the addition of sufficient ATP at *p*H 7.5, G-actin formed one band, but a small amount of trailing material was seen (Fig. 2*A*, *B*) and a mixture of actin and tropomyosin, containing 5 to 10 times as much actin as tropomyosin, separated into the two constituents upon electrophoresis in starch gel, each taking its characteristic position.

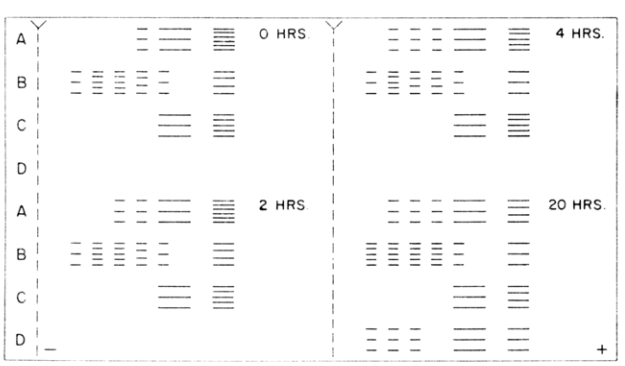

FIG. 3.—Tracing of starch gel pattern obtained after electrophoresis of actin samples exposed to room temperature for 0, 2, 4, and 20 hours after Dowex treatment. (*A*) Control not through Dowex-1; (*B*) ATP added after specified time; (*C*) ATP, KCl, and $MgCl_2$ added after specified time and dialyzed against ATP-ascorbic acid solution; (*D*) same as *C*, but not dialyzed. Protein concentration 8–10 mg/ml. Glycine buffer, *p*H 8.5, ionic strength 0.012, 8 volts per cm of gel, 5 hours. The number of lines in each band indicates the relative intensity of staining.

F-actin (Fig. 2*G*) obtained by addition of KCl (0.1 M) and $MgCl_2$ (0.1 mM) stays at the origin, probably because of its size (a heavily stained area can be seen in the incision of the starch gel at the point of application of the sample).

Removal of Excess Nucleotide.—Treatment with Dowex-1 leads primarily to a removal of the free nucleotide in the solution (Asakura, 1961) but in the long run causes a dissociation of the bound nucleotide. Such preparations (Fig. 2*C*) showed several distinct bands of lower mobility, while the band of native protein had almost disappeared. Similar changes were also observed after other forms of unfavorable treatment, such as warming during handling. In these cases ascorbate, mercaptoethanol, or cysteine (Fig. 2*A*,*B*,*E*) seemed to act protectively but did not reverse the reaction once the multiple components had formed (Fig. 2*D*,*F*). In the ultracentrifuge, a peak moving faster than that of G-actin and showing some spreading of the boundary was observed after the removal of free nucleotide.

Under certain conditions, however, the changes may be reversible. Thus, Dowex-treated actin was kept at room temperature, and 0.2 mM ATP was restored to the aliquots of the solutions at zero time and after 1, 2, 4, 6, or 20 hours. Other aliquots received 0.1 M KCl and 0.1 mM $MgCl_2$ together with the ATP, and one-half of each of these was dialyzed against 0.2 mM ATP and ascorbate for depolymerization. It is shown in Figure 3 that, during standing, after removal of free nucleotide, the aberrant bands progressively formed at the expense of the native protein, but that after polymerization and depolymerization the original native pattern was restored. Thus, the modifications are in principle reversible, although after 20 hours of standing polymerization is incomplete.

Denaturing Agents.—Electrophoresis at *p*H 8.5 in the presence of urea led to complete disappearance of the band of native actin, and, as shown in Figure 4*A*, to the appearance of four or five bands of low mobility. At *p*H 10, without urea, three or four of the slowly moving bands were found besides greatly diminished native protein (Fig. 4*B*), while at *p*H 2 there were five narrow bands moving to the cathode (Fig. 4*C*).

Effect of Sulfhydryl Reagents.—As shown in Figure 5, the band of native protein was completely replaced by four or five bands of lower mobility after exhaustive reaction of the sulfhydryl groups with iodoacetate.

Similarly, this change was observed in actin treated with the full stoichiometric amount of *p*-chloromercuric benzoate (Katz and Mommaerts, in press), in which case early treatment with cysteine brought about a partial reversal. Actin reacted with iodoacetate (*S*-carboxymethylactin) showed one boundary which sedimented more rapidly than that of native actin in the ultracentrifuge (Fig. 6).

Discussion

The results will be discussed first in relation to the purity of actin. This is timely, since there have been several reports recently to the effect that actin prepared according to our methods (Mommaerts, 1952, 1958) is contaminated with sizable amounts of tropomyosin (Martonosi, 1962; Drabikowski and Gergely, 1962), but that this contamination is largely suppressed if the preparation is performed in the cold (Drabikowski and Gergely, 1962). Inasmuch as our procedures (Mommaerts, 1952, 1958) were meant to be carried out at 3° in the first place, there should be no question as to the homogeneity in the ultracentrifuge of the resulting actin preparations and this was confirmed by experiment. Nevertheless, it would be valuable to apply additional criteria of purity. Zone electrophoresis has been found to be a very critical method with some proteins (Smithies, 1959). Our findings with this method show that actin is free of tropomyosin; also, no tropomyosin could be precipitated with ammonium sulfate. The small amount of trailing material seen in the electrophoresis at *p*H 7.5, 8.0, and 8.5 was not present upon addition of ATP to the starch gel at *p*H 7.5 and therefore seems to be due to some denaturation caused by the loss of ATP during the electrophoretic run rather than to an inherent impurity.

With respect to the modifications occurring in the actin molecule under a variety of conditions in the starch gel electrophoresis the results have been completely surprising. Up to five new bands appeared upon denaturation in urea, after removal of free ATP, and after complete substitution of the SH groups. The bands obtained in the latter case have mobilities almost, though not quite, identical with those obtained after removal of free ATP. With reference to sulfhydryl reagents it is known that they may dissociate bound ATP from G-actin (Martonosi and Gouvea, 1961; Strohman and Samorodin, 1962). In all cases, one might consider the possibility that bound ATP is removed concomitantly with the changes we observed in the electrophoretic patterns, possibly during the electrophoretic run, unless the electrophoresis is carried out in the presence of excess free ATP. It would require further examination to establish whether the direct effect of chemical alteration upon molecular charge would suffice to account for the new patterns, or whether they indicate the preexistence of subunits of different composition, or whether size differences due to secondary aggregation or to formation of partial low polymers play a role. The last possibility seems to be the most likely, since it is known that in starch gel electrophoresis molecular size is a factor determining mobility (Smithies, 1959). In particular, actin reacted with iodoacetate acquired several negative charges yet showed a lower mobility toward the positive pole. Furthermore, ultracentrifugal observations of actin preparations reacted with iodoacetate or devoid of free ATP revealed a boundary moving more rapidly than that of native G-actin, both observations suggesting aggregation or partial polymerization. The changes that occur in the starch gel electrophoretic patterns after the removal of the free nucleotide become slowly irreversible, probably coincident with the loss of direct polymerizability (Asakura, 1961). That a loss of nuleotide causes a configurational change in the molecule has been concluded before (Asakura, 1961; Barany *et al.*, 1961), but it has not been anticipated that a change of such magnitude occurs as is suggested by our observations.

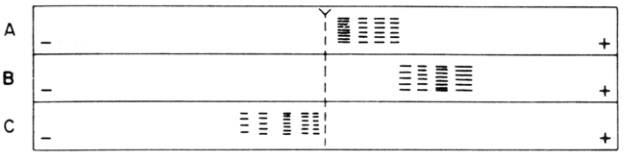

Fig. 4.—Tracings of starch gel patterns obtained after electrophoresis. Protein concentration 8–10 mg/ml. G-actin in (*A*) 7.2 M urea in glycine buffer, *p*H 8.5, ionic strength 0.012, 8–10 volts per cm of gel, 4 hours; (*B*) glycine buffer, *p*H 10.0, ionic strength 0.012, 8–10 volts per cm of gel, 3 hours; (*C*) glycine buffer, *p*H 2.0, ionic strength 0.012, 5–7 volts per cm of gel, 4 hours.

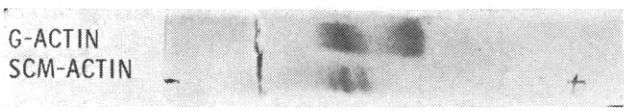

Fig. 5.—Photograph of starch gel electrophoresis patterns of G-actin and of actin reacted with iodoacetate. Protein concentration 10 mg/ml. Glycine buffer, *p*H 8.5, ionic strength 0.012, 8–10 volts per cm of gel, 3 hours.

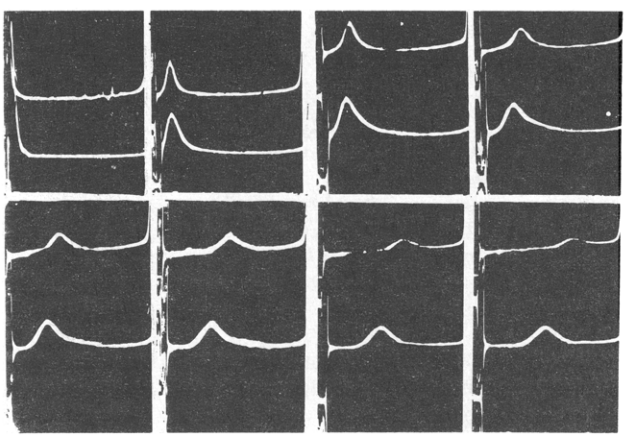

Fig. 6.—Sedimentation diagrams of *S*-carboxymethylactin (*upper curve*) and of a preparation of lyophilized G-actin (*lower curve*). 6 mg of protein per ml. Tris nitrate buffer, *p*H 8.0. Pictures were taken when top speed (59,780 rpm) was reached and every 16 minutes thereafter. Sedimentation proceeds from left to right.

Acknowledgment

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Actin, Its Amino Acid Composition and Its Reaction with Iodoacetate*

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G-Actin was reacted with iodoacetate under conditions that permitted complete reaction of the sulfhydryl groups. All the sulfur was accounted for by cysteine and methionine. Amino acid analyses indicated that the actin preparations were free of tropomyosin.

Since we were able to obtain actin preparations of high purity (Carsten and Mommaerts, 1963), the amino acid composition of G-actin was reinvestigated. Laki and Standaert (1960), in a discussion of a previous analysis of performic acid-oxidized actin by Kominz *et al.* (1954), estimated that their preparations contained about 10% tropomyosin. The amino acid analyses of actin reacted with iodoacetate presented in this paper confirm this estimate.

The reaction with iodoacetate also permitted an accurate analytical determination of the sulfhydryl groups by determining the *S*-carboxymethylcysteine in the hydrolysate. The results are also presented in this paper.

METHODS

Actin.—G-Actin was prepared according to the ultracentrifugal purification method (Mommaerts, 1952a, 1958) as described in the preceding paper (Carsten and Mommaerts, 1963). Protein concentrations were determined by the colorimetric method of Lowry *et al.* (1951), with lyophilized actin preparations used as standards.

Reaction with Iodoacetate.—This was carried out according to Moore *et al.* (1958). Iodoacetic acid, recrystallized from deionized water, was dissolved in 8 M deionized urea, containing 0.02% Versene. About 40 mg of freshly prepared actin in 4 ml of solution at *p*H 8.5 was reacted with 20 mg of iodoacetic acid in 12 ml of urea solution. After mixing, the *p*H was readjusted to 8.5 by addition of a few drops of 1 N NaOH; the final *p*H adjustment was made with a *p*H stat (TTT 1 b, Radiometer, Copenhagen), the *p*H adjustment taking not more than 2 minutes (timed). The reaction was allowed to proceed for 20 minutes at 25°. The mixture was then filtered through a 3 × 40 cm column of Sephadex G-25 which had been equilibrated with 0.05 M ammonium acetate, *p*H 8.0, and was eluted with this same ammonium acetate solution The *S*-carboxymethylactin was recovered in the effluent well in advance of the urea and was lyophilized.

Hydrolysis and Analysis.—The protein was hydrolyzed according to the method of Hirs *et al.* (1954), with 1 ml of constant-boiling HCl (three-times redistilled in glass) in evacuated sealed tubes. Hydrolysis was conducted at 110° for 22 hours, and also for 70 hours in order to correct for losses. After the termination of hydrolysis, the solutions were evaporated to dryness at 40° in a stream of nitrogen.

Amino acid analyses on the hydrolysates were carried out with the Spinco automatic amino acid analyzer (Spackman *et al.*, 1958). The analyses were corrected for 4.9 to 6.4% moisture.

Tryptophan was determined spectrophotometrically in the unhydrolyzed protein according to Beaven and Holiday (1952).

RESULTS

The actin preparations used in this work were ultracentrifugally homogeneous according to the criteria available in this case (Mommaerts, 1952a). Their behavior on zone electrophoresis and on fractionation with ammonium sulfate have been described in the preceding paper (Carsten and Mommaerts, 1963) and indicate the complete absence of tropomyosin.

Reaction with Iodoacetate.—Under the conditions used, cysteine was the only constituent which reacted with iodoacetate, since chomatographic analysis revealed no derivatives originating from other amino acids. The analyses presented in Table I show that after correction for losses during hydrolysis there were 6.7 moles of *S*-carboxymethylcysteine per mole (60,000 g) of actin. The correction for decomposition was derived from the difference between the 22 and the 70 hour hydrolysates and, assuming linearity with time, was estimated to be 7% after 22 hours of hydrolysis. The chromatogram showed no indication of any cysteine or cystine that had not reacted with the iodoacetate.

Amino Acid Analysis.—Four different preparations of *S*-carboxymethylactin were analyzed, one of them in duplicate, with an average recovery of 95% of the dry weight. Corrections for decomposition of threonine

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