THE DISSOCIATION OF MYOSIN BY HEAT COAGULATION

by

R. H. LOCKER

Dominion Laboratory, Department of Scientific and Industrial Research, Wellington (New Zealand)

The particle weight of myosin determined from sedimentation-diffusion and osmotic measurements by PORTZEHL, SCHRAMM AND WEBER[1] gave a value of 850,000, although more recent results suggest it may be as low as 500,000[2,3]. There is evidence from a number of sources that the fundamental particles are smaller than this. Following earlier workers who showed that myosin in urea solutions suffered dissociation into smaller units, TSAO[4] found that prolonged treatment of myosin with strong urea solution caused the splitting of 8% of the protein as low-molecular-weight fragments, the remainder retaining a molecular weight of above 300,000. At pH 10.7 myosin showed a particle weight of 170,000. ENGELHARDT, LJUBIMOVA, VENKSTERN, TIMOFEEVA AND BABSKAYA[5] recently reported a separation of adenylic deaminase from myosin. On heat coagulation of a myosin solution most of the enzymic activity remained in solution although the bulk of the protein had been precipitated. It seemed likely to the author that this soluble fraction might correspond to the low-molecular-weight fragments of TSAO. In a previous study (LOCKER[6]) it was found that myosin contained only a small amount of C-terminal *iso*leucine. The present work was undertaken to establish whether this small amount of end-group might belong to the heat-dissociable material of ENGELHARDT *et al.* or the urea-dissociable material of TSAO and possibly establish a relation between the two. It has led to an examination of the composition and enzymic activity of ENGELHARDT's deaminase.

EXPERIMENTAL

Myosin was prepared from rabbit muscle by the original method of SZENT-GYÖRGYI[7], omitting the stirring at 20° to remove actomyosin. The myosin was redissolved in 0.5 *M* KCl, centrifuged, and further purified by one or two reprecipitations (two or three times precipitated myosin). Ammonium sulphate fractionation was carried out on twice-precipitated myosin using saturated ammonium sulphate solution adjusted to pH 6.5, the 39–45% saturation fraction being used as myosin.

In the experiments of ENGELHARDT *et al.*[5] the ionic conditions are not mentioned and the pH used in deaminase preparations is not made clear, although pH 5.8 is indicated. Heating was carried out for 5 minutes at 53°, and it would seem from their observations at higher temperatures that this should not be exceeded if enzymic activity is to be preserved. Of the original deaminase activity, 60–90% remained with the 6–10% of protein still in solution. In the present investigation, heat coagulations were carried out in Pyrex tubes of not more than 3 cm diameter, immersed in a water bath at the specified temperature and stirred constantly. Most of the work on a preparative scale was done with myosin solutions of pH 6.2 in 0.5 *M* KCl heated for 5 minutes at 53°. Under different conditions the results varied only quantitatively. After a sudden rise in viscosity, flocculation occurred at 2–3 minutes with rapid shrinkage to a leathery gel. Subsequent

References p. 521.

precipitation was very gradual. After centrifuging, the ionic strength of the supernatant (S) was reduced approximately ten-fold by dialysis on a rocking dialyser for two hours against cold distilled water. The precipitate (P) which formed was washed twice with 0.05 *M* KCl. Following the removal of (P), the dialysed solution (D) was concentrated in a cellophane sac using an air-blower in the cold-room. The sample used in the ultracentrifuge was freeze-dried.

Electrophoresis was carried out in a Perkin-Elmer Model 38 apparatus.

Adenylic deaminase assays by the KALCKAR[8] method were carried out in a Unicam Spectrophotometer using 1-cm cells. To 3 ml buffer (0.1 *M* succinate + 0.5 *M* KCl + 0.01 *M* $CaCl_2$, pH 5.9) was added 0.2 ml adenylic acid (0.3 mg/ml in the buffer) and 0.1 ml enzyme solution. The fall in optical density (O.D.) at 265 mμ was read at one-minute intervals. Activity was calculated from the early linear portion of the O.D.-time plot. The addition of 0.5 *M* KCl to KALCKAR's medium was found necessary to prevent precipitation of myosin or P. Q_{NH_3} values are expressed as μl NH_3/mg protein/h.

Nesslerisation experiments were carried out by the addition of 0.2 ml enzyme and 0.1 ml adenylic acid (15 mg/ml adjusted to pH 5.9) to the buffer (0.7 ml). To samples at different reaction times was added 2 ml 2% trichloroacetic acid. The filtrate (2 ml) was made up to 10 ml with 1 ml Nessler's reagent and the O.D. read at 480 mμ. Linear O.D.-time plots were obtained.

Carboxypeptidase experiments were carried out by the procedures described in an earlier paper[6]. The resultant amino acid spots were evaluated only by visual comparison with standard spots. The results given are the mean of a number of experiments in each case.

RESULTS

Properties of the dialysis precipitate (P)

This material dissolved readily in potassium chloride solutions only if the strength exceeded 0.3 *M*. At pH 2.1 the material migrated electrophoretically as a single very sharp peak. At pH 8.0 (Fig. 1b) both boundaries showed a large and very sharp peak followed in the case of the ascending boundaries by a small slower peak (6–10%). In the ultracentrifuge at pH 8.0, P (Fig. 2a) resolved into a fast-sedimenting disperse fraction carrying with it the turbidity of the solution and a single sharp peak P_1 ($S_{20,w} = 7.8 \cdot 10^{-13}$, 19°). The small slow peak is probably due to a slight contamination with D. Attempts to concentrate S in a dialysis sac with a current of cold air led

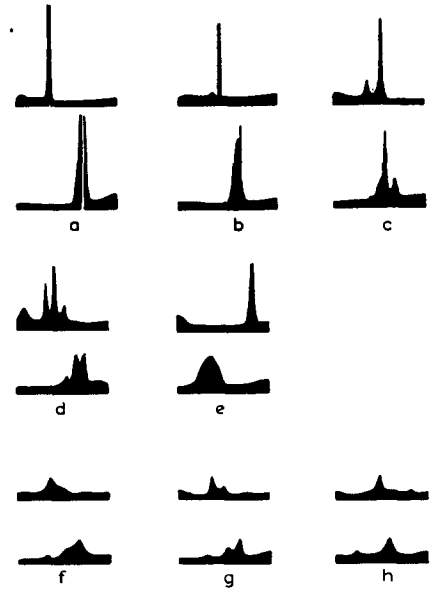

Fig. 1. Electrophoresis diagrams. The top member of each pair is the ascending boundary (left to right) and the bottom is the descending (right to left).

(a) Myosin (40–45% saturation ammonium sulphate fraction) in buffer (2).
(b) P, coagulation at 53° and pH 6.2 for 5 min. Buffer (2).
(c) S (freeze-dried), coagulation as in (b). Buffer (2).
(d) D (concentrated), coagulation as in (b). Buffer (1).
(e) Same as (d) in Buffer (3).
(f) D (concentrated), coagulation at 53° and pH 5.8 for 5 min. Buffer (1).
(g) D (concentrated), coagulation at 45° and pH 6.2 for 12 min. Buffer (1).
(h) D (concentrated), coagulation at 45° and pH 5.8 for 6 min. Buffer (1).

Buffers: (1) 0.05 *M* KH_2PO_4 adjusted to pH 7.0; (2) 0.05 *M* KH_2PO_4 + 0.25 *M* KCl adjusted to pH 8.0; (3) 0.01 *N* HCl + 0.09 *M* KCl, pH 2.1.

References p. 521.

to precipitation of denatured protein, apparently P, since D could be concentrated quite satisfactorily in the same manner. A sample of S was freeze-dried and redissolved. Some insoluble material, probably P, remained, but on electrophoresis of the soluble portion at pH 8.0 (Fig. 1c) the sharp peak of P could be seen superimposed on that of D_2 (see below). Fraction P resembles myosin rather strongly in its salt solubility, ease of denaturation, maintenance of a sharp boundary during electrophoresis, and the viscosity of its strong solutions.

Properties of the dialysis supernatant (D)

Electrophoresis of a concentrated solution of D at pH 7.0 or 8.0 showed the presence of 3 different proteins, a slow peak D_1, a faster peak D_2, and a minor peak D_3 of higher mobility (Fig. 1d). At pH 2.1 these components travelled as a single peak. In the ultracentrifuge (Fig. 2b), D sedimented at pH 7.0 as a single slow peak, which rapidly diffused ($S_{20,w} = 2.2 \cdot 10^{-13}$, 19°). For heat coagulations at 53° (pH 6.2) the relative amounts of the three constituents varied somewhat.

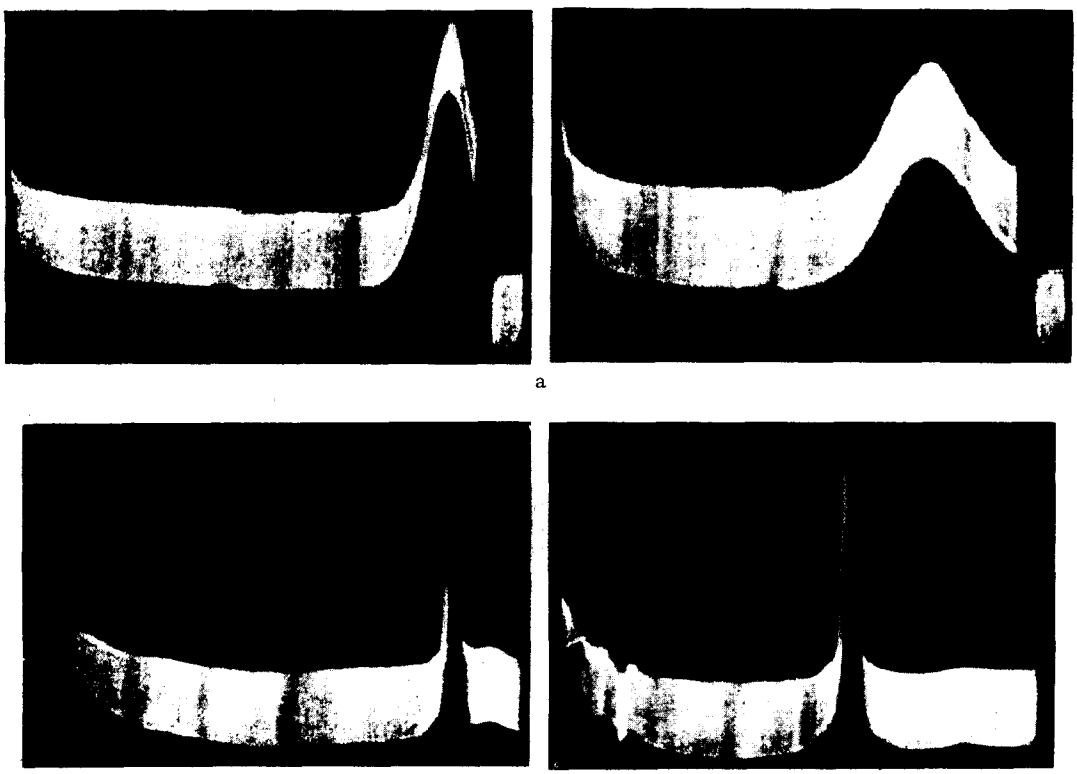

Fig. 2. (Ultracentrifuge runs on P and D (60,000 r.p.m., 19°)
(a) P in (0.25 *M* KCl + 0.05 *M* phosphate) pH 8.0 (left) first photograph at speed (right) after 30 min.
(b) D in (0.1 *M* KCl + 0.05 *M* phosphate) pH 7.0 (left) after 15 min at speed (right) after 60 min at speed.

References p. 521.

				Mean
D_1	46%	37%	37%	40%
D_2	51	48	54	51
D_3	5	15	9	9

In an attempted fractionation of concentrated D using saturated ammonium sulphate adjusted to pH 6.5, the material was approximately equally distributed between the 40–50% and 50–60% saturation fractions. There was a small enrichment of D_2 in the former and D_1 in the latter fraction, the amount of D_3 (9%) being the same in each.

Prolonged dialysis of D against distilled water caused partial precipitation of the proteins, but electrophoresis of both this precipitate and its concentrated supernatant gave the characteristic pattern of three peaks.

Effect of method of preparation

It may be seen from Table I that whether the myosin was prepared by two or three precipitations or by ammonium sulphate fractionation the results were comparable. In preparation (i) almost identical results were obtained with the 0–40% and 40–45% saturation fractions. Electrophoresis diagrams of D from two or three times precipitated myosin, the 0–40% or 40–45% saturation fractions all showed the same pattern of peaks. The proteins dissociated from myosin do not appear to depend on the degree of purification of the myosin, and cannot therefore be regarded as due to absorbed impurities. Most of the work has been done with three times precipitated myosin, this method of preparation being more economical in yield and time than ammonium sulphate fractionation, and less damaging to enzymic activity. The turbidity of such myosin solutions is carried down with the coagulum.

Effect of protein concentration

The considerable variability of the results at first concealed the importance of protein concentration. However, a plot of 23 values for S obtained under otherwise similar conditions, against myosin concentrations, showed a correlation coefficient of −0.62, significant at the 1% level. There was no corresponding correlation for D. Thus a low myosin concentration favours the yield of P. A further experiment in which the same preparation (k) was heated at different concentrations showed a marked increase in S with decreasing myosin concentration, reaching 18.6% at the lowest concentration used.

Effect of pI

Below pH 5.6 myosin came out of solution. Above pH 6.6 there was difficulty in obtaining coagulation. From preparations (c, e, f) it can be seen that the protein content of S increased with pH owing to increase in both P and D, particularly between 5.8 and 6.2. Electrophoresis (Fig. 1f) showed that the major middle peak D_2 (coagulation at pH 6.2) was much diminished in experiments at pH 5.8.

Ionic conditions

Nearly all experiments were carried out in 0.5*M* KCl using the protein itself as buffer. With preparation (j), heating in 2*M* KCl caused a considerable reduction in the P content of the supernatant with a small drop in D content.

References p. 521.

TABLE I

Prepn.	*Method*	*Myosin concn. mg/ml*	*pH*	*Time of heating (min)*	*Protein in supernatant as % of original myosin (S)*	*Protein in dialysed supernatant as % of original myosin (D)*	
a	2 ×	11.4	5.8	3	7.6		
b	2 ×	12.8	6.2	4	14.4	4.5	
				24	9.6		
c	3 ×	13.8	6.2	5	9.9	4.0	
				15	7.2		
				35	6.8		
		13.6	5.8	5	5.9	2.4	
				15	5.2		
				35	4.6		
d	2 ×	11.5	6.2	3	15.3	4.9	
				5	13.8	5.6	
				30	12.3	5.0	
e	3 ×	16.8	5.8	5	7.3	2.3	
		17.6	6.2	5	9.9	4.5	
				35	9.3		
		17.4	6.4	5	11.7	5.5	
				35	12.1		
f	3 ×	13.4	6.2	5	10.5	3.6	
			6.4	5	12.2	4.5	
			6.6	5	15.8	4.9	
			6.2	10	15.4	2.6	(45°)
g	3 ×	11.8	6.2	12	17.3	2.4	(45°)
h	40–45	5.9	6.2	3	16.4		
				11	12.9		
				30	12.2		
				7	11.0	5.3	
i	0–40	9.0	6.2	5	16.1	3.8	
	40–45	14.7	6.2	5	15.7	3.9	
j	39–45	12.8	6.2	5	10.4	4.1	
		14.5	6.2	5	6.3	4.1	(2 *M* KCl)
k	2 ×	6.0	6.2	5	18.6		
		11.9	6.2	5	11.1		
		17.9	6.2	5	7.8		

2 × = twice precipitated; 3 × = three times precipitated; 0–40, 40–45 etc. refer to ammonium sulphate saturation fractions.

Effect of temperature

A bath temperature of 53° has been used in most experiments. This is higher than the actual coagulation temperature. In a 3 cm-diameter pyrex tube with constant stirring, the internal temperature was 38° at 1 min, 47° at 2 min, 51° at 3 min when coagulation occurred (pH 6.2). With a bath temperature of 45°, five minutes were needed to reach 44°, and coagulation occurred in 8 minutes. At 40° no coagulation occurred within 30 min, the solution merely becoming viscous. Heating at 45° caused a small increase in P and a pronounced decrease in D compared with 53°. Electrophoresis (Fig. 1g) showed that lowering the coagulation temperature has the same effect as lowering the pH, *i.e.* a suppression of D_2. By heating at pH 5.8 and 45° it was possible to virtually eliminate D_2 (Fig. 1h).

Effect of time of heating

Following flocculation of the myosin there was only a slow decrease in the protein

References p. 521.

content of the supernatant (c, d, e, h). From preparation (d) it can be seen that after 5 minutes both P & D decline slowly. In the case of D there appeared to be a small initial rise immediately following coagulation.

The adenylic deaminase activity of the heat supernatants

The deaminase activities of the myosin preparations and supernatant components have been measured in most cases by the KALCKAR[8] spectrophotometric technique, observing $-\Delta$ O.D. (265 mμ) as adenylic acid is converted to inosinic acid. It has been found, however, that Q_{NH_3} values determined by this method are about a hundred times lower than those determined by Nesslerisation (*e.g.* myosin having a Q_{NH_3} of 5000 by Nesslerisation gave a Q_{NH_3} of 56 by the KALCKAR method). In the present experiments the enzyme concentration was seven times and the substrate concentration 80 times higher for Nesslerisation than for the KALCKAR method, where the high absorption of adenylic acid is a limiting factor. Indeed it was found in the KALCKAR method that halving the standard substrate concentration lowered the Q_{NH_3} of a myosin sample from 46 to 28. It is clear that the method is working far below the substrate-saturation level of the enzyme. Provided the enzyme concentration was not too high, linear O.D. -time plots were obtained, the gradient being reproducible and proportional to enzyme concentration. The method, which permits a rapid and simultaneous assay of three samples, was therefore used at a standard substrate concentration as a fair comparison of activities, although no significance was attached to the absolute Q_{NH_3} values.

The distribution of activity among the ammonium sulphate fractions of myosin is of interest (Table II). The 0–39% saturation fraction, which contains a good proportion of the myosin yield and some actomyosin, may have an activity higher than that of the twice precipitated myosin from which it is derived. The activity of the 39–45% saturation fraction is, however, considerably lower. The ammonium sulphate treatment and subsequent long dialysis might be expected to cause some inactivation of both. No activity was found in the 45% saturation supernatant.

TABLE II

Prepn.	*Assay method*	*Assay temp. °C*	*Deaminase activity (Q_{NH_3})*		
			2×	*0–39*	*39–45*
p	Kalckar	17	28	42	17
q	Nessler	25	5100	2900	640
r	Nessler	23	9100	9300	3300

Abbreviations as in Table I.

The enzymic results of the coagulations are recorded in Table III. The supernatant S from all heat coagulations is seen to possess an enhanced specific activity, usually 2–3 times that of the original myosin. This activity does not fall sharply into the P or D fractions, but is concentrated in P, while a variable but sometimes appreciable amount remains in the D fraction. This residual activity is not due to incomplete precipitation of P. The fact that it persists on coagulation at 45°, pH 5.8 (preparation d) indicates that it is not connected with D_2, which is suppressed under these conditions. From (e) it appears that pH 6.2 is optimal for activity of S but not

References p. 521.

critical. Coagulation at 45° (g, d) gives results enzymically comparable to coagulation at 53°.

TABLE III

Prepn.	Method	pH	Temp. °C	Time of heating (min)	Deaminase activity (Q_{NH_3})				Assay temp. °C
					Myosin	S	P	D	
d	2 ×	6.2	53	5	32	73	98	21	21
		5.8	45	6	25	71	88	13	21
e	3 ×	5.8	53	5		112			21
		6.2	53	5		118			21
		6.4	53	5		74			21
g	3 ×	6.2	45	12	72	86	131	0	25
l	3 ×	6.2	53	5	36	94	113	49	18
m	3 ×	6.2	53	5	68	174	128	30	21
n	2 ×	6.2	53	3	27	90	104	7	22
o	2 ×	6.2	53	4	40	43	75	0	18

C-terminal groups in the heat supernatant proteins

In a previous paper[8] myosin was found by the carboxypeptidase method to contain small amounts of C-terminal *iso*leucine (*ca.* one end-group in a particle weight of 300,000), together with lesser amounts of alanine, valine, and leucine. The present experiments began with the observation that treatment of S with carboxypeptidase liberated a pattern of amino acids similar to but much stronger than that of myosin itself. The amount of *iso*leucine liberated was eight times higher for the same amount of protein. The source of the C-terminal *iso*leucine has been sought among the P and D fractions.

Incubation of P with carboxypeptidase (6 hours) yielded *iso*leucine (*ca.* one group in 200,000), glycine, and valine, with lesser amounts of other amino acids. At 18 hours *iso*leucine (*ca.* one group in 80,000) exceeded the others. The slow rate of liberation, however, as in the case of myosin, made it difficult to distinguish between true liberation of an end-group and secondary action and hence to make a true estimate of C-terminal content. While P has a relatively small amount of C-terminal *iso*leucine, it has more than myosin.

In the case of the D fraction there was a very rapid and clear-cut liberation of *iso*leucine, which reached completion at a level of *ca.* one end-group in 40,000. Serine was the second most rapidly liberated amino acid, but in lesser amount. The three components of the D fraction have not yet been separated, but by coagulating the myosin at 45°, pH 5.8, it is possible to virtually eliminate D_2. In this case, although *iso*leucine was still the most rapidly liberated amino acid, the yield was reduced to a quarter of its former value. It may be assumed that most of the C-terminal *iso*leucine of the D fraction belongs to D_2. As D_2 comprises half of D, it must possess C-terminal *iso*leucine to the extent of one group in a particle weight of 20–30,000.

DISCUSSION

The above results indicate that at least four different sub-units of myosin are revealed on heat coagulation. Of these P_1 appears to be of considerable size, resembling myosin

References p. 521.

in many respects but distinguished from it by its greater thermal stability and enhanced adenylic deaminase activity. The highest yield obtained for P was 13% and it is possible that by further decreasing the myosin concentration this could be increased. At this level it must be considered an important component of the myosin. It is intended to isolate P_1 free from the heavy disperse material (probably partial denaturation products) revealed in the ultracentrifuge and further characterize it. This protein may have some relation to L-meromyosin, which also has the solubility properties of myosin combined with an enhanced deaminase activity[9,10].

Since the three components of the D fraction sediment at the same rate they must be of similar size, and the low sedimentation constant of 2.2 indicates a low molecular weight. This is borne out by the end-group results for D_2. It would appear, then, that the D proteins are of a size comparable with the urea fragments of TSAO[4]. Further discussion of the heat supernatant proteins will be reserved for a forthcoming paper, where the effect of inorganic polyphosphates on the heat coagulation will be considered.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. J. W. LYTTLETON of the Grasslands Division, Palmerston North, who kindly carried out the ultra-centrifuge runs, and the Director of the Dominion Laboratory for permission to publish this paper.

SUMMARY

When myosin solutions were coagulated at 53°, pH 6.2, 8–18% of the protein remained in solution. This material could be divided by dialysis into two parts: (a) a myosin-like fraction, P, comprising 4–13% of the myosin and containing a well defined major component P_1 together with some heavy disperse material; (b) A low-molecular-weight, water-soluble fraction, D, containing three components D_1, D_2, D_3 in the approximate proportions 4:5:1. The adenylic deaminase activity of P was 3–4 times greater than that of myosin while that of D was low. D_2 contained C-terminal *iso*leucine to the extent of one end-group in a particle weight of 20–30,000.

The effect on the coagulation products of variables such as myosin concentration, temperature, pH, etc., has been studied.

REFERENCES

1 H. PORTZEHL, G. SCHRAMM AND H. H. WEBER, *Z. Naturforsch.*, 5b (1950) 61.
H. PORTZEHL, *Z. Naturforsch.*, 5b (1950) 75.
2 R. G. PARRISH AND W. F. H. M. MOMMAERTS, *J. Biol. Chem.*, 209 (1954) 901.
3 K. LAKI AND W. R. CARROLL, *Nature*, 175 (1955) 389.
4 T. C. TSAO, *Biochim. Biophys. Acta*, 11 (1953) 368.
5 V. A. ENGELHARDT, M. LYUBIMOVA, T. V. VENKSTERN, M. Y. TIMOFEEVA AND Y. B. BABSKAYA, *Doklady Akad. Nauk. S.S.S.R.*, 85 (1952) 397.
6 R. H. LOCKER, *Biochim. Biophys. Acta*, 14 (1954) 533.
7 A. SZENT-GYÖRGYI, *Muscular Contraction*, Academic Press, New York, 1947, p. 136.
8 H. M. KALCKAR, *J. Biol. Chem.*, 167 (1947) 466.
9 J. NEKHOROCHEFF, J. DONDON AND Z. GOUSSEF, *Compt. rend.*, 238 (1945) 1450.
10 J. GERGELY, private communication.

Received September 15th, 1955