On the Heterogeneity of Gramicidin

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Gramicidin from *Bacillus brevis* contains at least four components, of which the new fourth component has been designated gramicidin D. Isolated by countercurrent distribution, gramicidin D is resolved at 2000 transfers from the main distribution peak hitherto designated "gramicidin A." It is distinguished from the other three peak materials by its unique content of one residue of isoleucine and by its having one residue less of valine. Available evidence indicates that the main distribution peak, having a partition coefficient of 0.70, for which the designation gramicidin A has been retained, is heterogeneous, pointing to the presence of a fifth component in gramicidin.

Gramicidin, the polypeptide antibiotic isolated from *Bacillus brevis* (Hotchkiss and Dubos, 1941), has been shown to be a mixture of at least three related substances (Gregory and Craig, 1948; Craig *et al.*, 1949) by countercurrent distribution. In order of increasing partition coefficient (K) the three fractions are known as the B (K = 0.31), A (K = 0.61), and C (K = 1.32) components (Craig *et al.*, 1949). Besides glycine, alanine, leucine, valine, phenylalanine, tyrosine, tryptophan, and ethanolamine, a new amino acid constituent, isoleucine, has recently been recognized to be present in nonstoichiometric amounts both in unfractionated gramicidin (Okuda *et al.*, 1962) and in "gramicidin A" (Ishii *et al.*, 1962; Gross *et al.*, 1963). The suggestion has been made that "gramicidin A" is heterogeneous and that it probably consists of two components, one containing one or two residues of isoleucine which replace one or two out of four residues of valine present in the second component (Ishii and Witkop, 1963). We report in this communication conclusive evidence on the heterogeneity of "gramicidin A" and the resolution and isolation therefrom, by countercurrent distribution, of a new component. Besides this constituent there appears to be present yet a third component. The amino acid content of all fractions of gramicidin obtained in this investigation has been determined.

Experimental and Results

Countercurrent Distribution.—A solvent system composed of methanol, water, benzene, and chloroform, mixed in the proportion 23:7:15:15 (Craig *et al.*, 1949), was used. The sample of gramicidin (2.73 g, Nutritional Biochemicals Corporation, Cleveland) was dissolved and loaded into the first ten tubes of a 500-tube all-glass countercurrent distribution machine (Craig *et al.*, 1951). The tubes had a capacity of 10 ml for each of the two solvent phases. Measurement of optical density at 280 mμ was used to locate material present in different tubes after distribution. Since most of the components had a partition coefficient of less than one, 0.2-ml aliquots of the lower phase from every fifth tube were withdrawn after distribution, dried, and then dissolved in 5 ml 95% ethanol for measurement of optical density.

The results of the distribution are depicted in Figures 1–3. Recorded optical densities are plotted against the theoretical tube numbers rather than the actual tube numbers on the machine. The distribution of the various components after 500 transfers is shown in Figure 1. At this stage, the presence of three major components with K's of 0.27, 0.58, and 1.36 could be noticed and also a marked skewness in the K 0.58 peak. The last tube in the machine was connected to the first, and 500 more transfers effected by recycling. The pattern of distribution at this stage is shown in Figure 2. Components which originally had K's of 0.27, 0.58, and 1.36 were now well separated and exhibited K's of 0.32, 0.65, and 1.41, respectively. The major peak ("gramicidin A," K 0.65) showed evidence of separation of a lesser component with a lower K. The contents of tubes 550–630 (K 1.41) and of tubes 210–280 (K 0.32) were removed and pooled separately. The solvent in tubes 281–310 was discarded, and fresh solvent was introduced into the empty tubes in the machine. The material with K 0.65 was now distributed through 1000 transfers by recycling. The distribution pattern is shown in Figure 3. The presence of a new component with a K of 0.59 was clearly indicated. The major peak with a K of 0.70 still showed a marked skewness on the side of higher K, and it appeared possible that this region contained yet another component. The distribution was, however, discontinued at this point. The contents of tubes 712–760 (K 0.59), 761–790 (K 0.59 + 0.70), 791–840 (K 0.70), and 841–960 (K 0.70 *tail*) were pooled separately. All the pooled fractions were evaporated to small volumes in a rotary evaporator at a temperature below 30° and were finally dried in a desiccator over P_2O_5 and NaOH. Samples with K's of 0.32 and 1.41 could not be completely dried, owing either to retention of solvent used for distribution or to impurities therefrom. The actual weight recoveries are indicated in Table I.

Amino Acid Analyses.—Samples were hydrolyzed in a 1:1 mixture of glacial acetic acid and concentrated HCl in sealed, evacuated tubes for 72 hours and another set of samples for the shorter duration of 20 hours. Aliquots of the 72-hour hydrolyzates were chromatographed on paper using the *sec* butyl alcohol–3% ammonia (3:1) system (Roland and Grove, 1954). All fractions were found to contain ethanolamine, glycine, alanine, valine, leucine, and tryptophan, but the K 0.59, 1.41, and 0.32 fractions contained, in addition, isoleucine, tyrosine, and phenylalanine, respectively.

Tryptophan was determined spectrophotometrically on the unhydrolyzed fractions using *N*-bromosuccinimide (Patchornik *et al.*, 1958; Ramachandran, 1962), with acetic acid as the solvent for gramicidin; and colorimetrically using the dimethylaminobenzaldehyde procedure K of Spies and Chambers (1949). With the colorimetric method, values could not be obtained on two of the fractions, since partial precipitation of pigment material was encountered. The xanthydrol

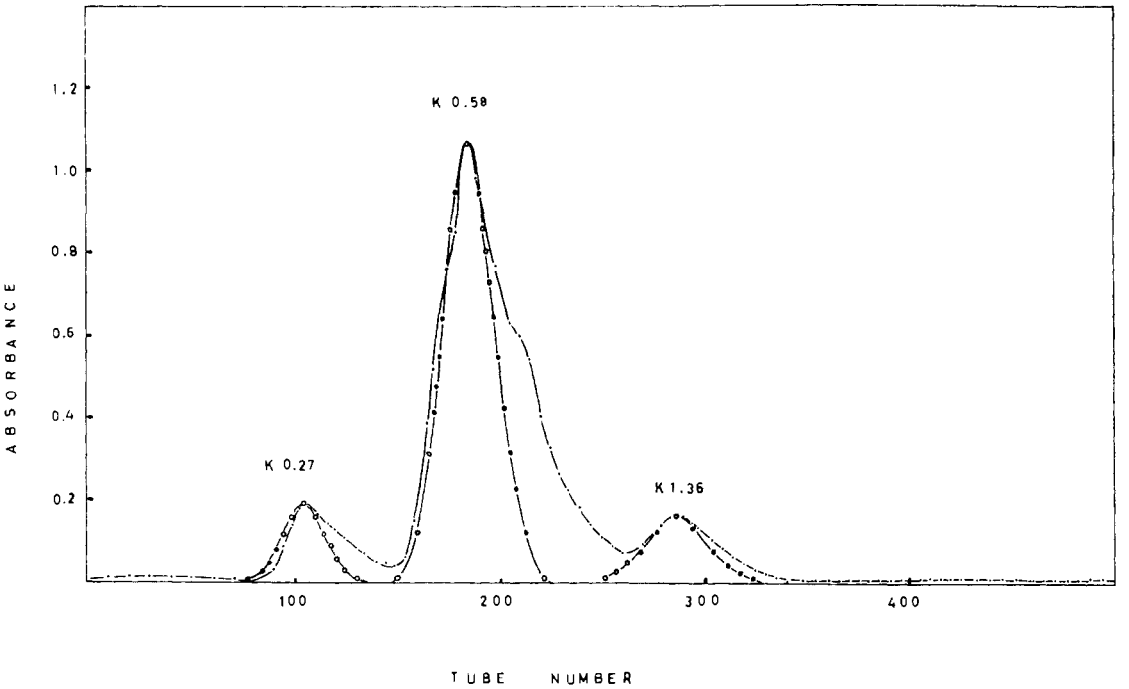

FIG. 1.—Distribution pattern of gramicidin at 500 transfers: experimental (—·—); calculated (—O—).

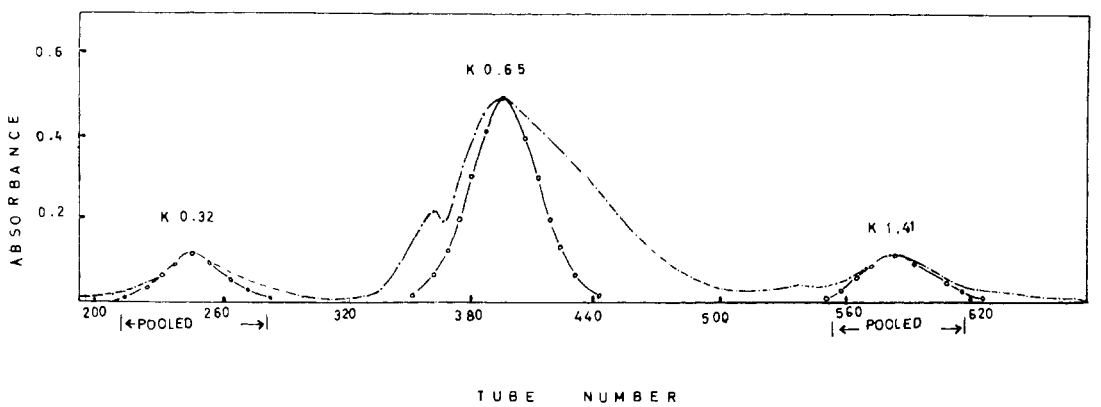

FIG. 2.—Distribution pattern of gramicidin at 1000 transfers: experimental (—·—); calculated (—O—).

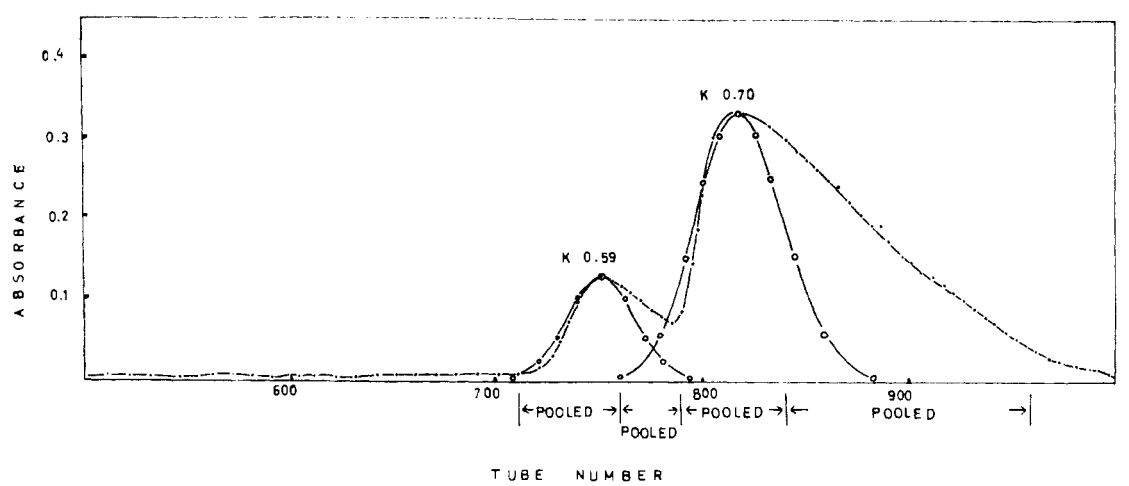

FIG. 3.—Resolution of the components of "gramicidin A" at 2000 transfers: experimental (—·—); calculated (—O—).

TABLE I

WEIGHT RECOVERY OF FRACTIONS FROM THE DISTRIBUTION OF 2.73 G OF GRAMICIDIN

Distribution Coefficient of Fraction, K	Weight Recovered (g)	Recovery (% of sample taken)
0.32[a]	—	—
0.59	0.397	14.7
0.59 + 0.70 (mixed)	0.185	6.9
0.70	0.7500	27.8
0.70 *tail*	1.051	38.9
1.41[a]	—	—

[a] Fractions with K's of 0.32 and 1.41 would, by difference, together account for 11.7% of the weight of gramicidin.

color reaction, developed by Dickman and Crockett (1956), was also applied to the gramicidin fractions. The original reaction medium of 6 N HCl could not be used owing to the insolubility of the samples, and it was replaced by a 1:1 mixture of glacial acetic acid and concentrated HCl. Both samples and tryptophan standards were heated with the xanthydrol reagent for 1 hour at 100°. The extraction of excess reagent with benzene was omitted, and absorbances were measured at 500 mμ with a Beckman DU spectrophotometer. There was little difference in the absorbances recorded at 500 mμ and at 510 mμ. Standard (10–160 μg) and samples were run at the same time, and such measurements were reproducible to within 2%.

Aliquots of the 72-hour hydrolyzates were analyzed using the Spinco automatic amino acid analyzer according to the method of Spackman *et al.* (1958). Aliquots of the 20-hour hydrolyzates were analyzed for basic constituents, such as ethanolamine and ammonia, on a 15-cm column. The results of the analyses are shown in Table II.

DISCUSSION

The distribution pattern obtained at 500 transfers for gramicidin in this investigation (Fig. 1) is very similar to that found after a few hundred transfers by Craig *et al.* (1949). However, the effectiveness of countercurrent distribution as a separation tool is exemplified by the patterns obtained at 1000 and 2000 transfers (Figs. 2 and 3) which reveal the physical separation of a new component of gramicidin, hereafter designated gramicidin D (K 0.59), from that fraction of gramicidin hitherto known as "gramicidin A." The designation gramicidin A is retained for the distribution peak with K = 0.70. With increasing number of transfers the distribution coefficient of every fraction is increased somewhat, a situation which has been observed earlier (Gregory and Craig, 1948; Craig *et al.*, 1949). A direct examination of the shape of the K 0.70 peak at 2000 transfers would lead one to conclude either that there is intrinsic inhomogeneity in the material found in this peak or, alternately, that the K 0.70 material shows a concentration-dependent change in partition coefficient.

That gramicidin D is chemically distinct from the bulk of the material in "gramicidin A" is shown by the amino acid composition (Table II). Gramicidin D differs from all other components of gramicidin in its unique content of one residue of isoleucine, and by its having one residue less of valine. Since all the isoleucine, alanine, and tryptophan residues in gramicidin A are shown to be of the L-configuration (Ishii and Witkop, 1963), it may be inferred that all residues of these amino acids found in gramicidin D are also of the L-configuration. While it is known that valine is present in "gramicidin A" equally as L- and D-residues it would remain to be determined whether the L-isoleucine present in gramicidin D replaces L- or D-valine present in gramicidin A. So far isoleucine has been encountered as a new amino acid component in several commercial samples of gramicidin (Wallerstein Co., S. B. Penick & Co., and Nutritional Biochemicals Corporation), and it would appear therefore that gramicidin D is a normal product of biosynthesis by cultures of *Bacillus brevis*.

The nonideal distribution behavior of the peak at K 0.70, indicative of possible heterogeneity, had warranted separate removal and pooling of the first and second halves of the peak, and these have been designated as the K 0.70 and K 0.70 *tail* fractions. These two materials show a great similarity in amino acid composition, but a significant difference exists in the content of tryptophan (Table II), the K 0.70 *tail* fraction containing two less residues. Further attempts to resolve this major peak from gramicidin into its two constituents, by countercurrent distribution or other means, would be justified on the basis of the above observations. In this context, it is interesting to recall that Gregory and Craig (1948) had suggested that gramicidin perhaps contains as many as three constituents with a K greater than that of gramicidin

TABLE II

AMINO ACID CONTENT OF GRAMICIDIN FRACTIONS[a]

Amino Acid	K 0.32	K 0.59	K 0.70	K 0.70 *tail*	K 1.41
Glycine	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)
Alanine	1.97 (2)	2.11 (2)	1.93 (2)	1.98 (2)	2.02 (2)
Valine	3.80 (4)	2.98 (3)	3.89 (4)	3.79 (4)	3.78 (4)
Isoleucine	*0.13* (−)	0.92 (1)	—	—	—
Leucine	3.76 (4)	4.35 (4)	4.14 (4)	3.89 (4)	4.08 (4)
Tyrosine	—	—	—	—	0.77 (1)
Phenylalanine	0.89 (1)	—	—	—	—
Ethanolamine[b]	0.64 (1)	0.60 (1)	0.77 (1)	0.84 (1)	1.24 (1)
Ammonia[b]	1.87	2.69	2.81	1.85	3.49
Tryptophan[c]					
A	3.44 (3–4)	5.78 (6)	5.53 (6)	4.24 (4)	6.18 (6)
B	(*d*)	5.40 (5–6)	5.88 (6)	4.10 (4)	(*d*)
C	2.86 (3)	3.94 (4)	4.44 (4–5)	3.17 (3)	5.00 (5)

[a] All values are expressed as the number of moles present, based on glycine as 1. [b] Contents found in 20-hour hydrolyzates; ethanolamine values not corrected. [c] Determined on the unhydrolyzed fractions: A, by *N*-bromosuccinimide oxidation; B, by colorimetry with *p*-dimethylaminobenzaldehyde; C, by colorimetry with xanthydrol. [d] Values obtained not reliable, owing to precipitation of pigment matter after color development.

A (including gramicidin A), and that the various fractions also differ in tryptophan content.

The analyses on gramicidin B (*K* 0.32) reveals the presence of glycine, alanine, valine, leucine, phenylalanine, tryptophan, and ethanolamine, as observed earlier (Stein and Moore, 1949–50), while the analytical data on gramicidin C (*K* 1.41) would indicate the presence of all constituents in gramicidin B except phenylalanine, which is replaced by tyrosine. However, the two differ markedly in tryptophan content. Besides, gramicidin B contains a fractional amount of isoleucine (0.13 moles), and it is not clear whether this is an indication of heterogeneity in the peak or whether the material chromatographing like isoleucine is in reality a small amount of some unhydrolyzed resistant peptide. However, the chances of the presence of unhydrolyzed peptide material in 72-hour hydrolyzates are remote.

Some of the recent molecular weight determinations on "gramicidin A" by isothermal distillation in methanol, sedimentation equilibrium in dimethylformamide, and amino acid composition (Ishii *et al.*, 1962; Gross *et al.*, 1963) have yielded values approaching 2000, while higher values have been recorded in the earlier literature for gramicidin. If a molecular weight of the order of 2000 is assumed for all gramicidin fractions, then our analytical data would yield the following composition for the different gramicidins:

Gramicidin A, *K* 0.70
(Gly_1, Ala_2, Val_4, Leu_4, Try_6, $ethanolamine_1$).

Gramicidin A, *K* 0.70 *tail*
(Gly_1, Ala_2, Val_4, Leu_4, Try_4, $ethanolamine_1$).

Gramicidin B, *K* 0.32
(Gly_1, Ala_2, Val_4, Leu_4, Phe_1, Try_{3-4}, $ethanolamine_1$).

Gramicidin C, *K* 1.41
(Gly_1, Ala_2, Val_4, Leu_4, Tyr_1, Try_6, $ethanolamine_1$).

Gramicidin D, *K* 0.59
(Gly_1, Ala_2, Val_3, $Ileu_1$, Leu_4, Try_6, $ethanolamine_1$).

Tryptophan analyses in proteins and polypeptides have often posed problems peculiar to this amino acid. This is evident also in the data recorded in Table II. For the three fractions where the colorimetric method of Spies and Chambers (1949) could be used, the values showed fair agreement with those obtained by the spectrophotometric method involving oxidation with *N*-bromosuccinimide. The difference in content noted for the *K* 0.70 and *K* 0.70 *tail* fractions was also apparent in the difference in absorption at 280 mμ shown by the two materials in glacial acetic acid. Also, of the two only the *K* 0.70 *tail* fraction developed a strong purple tinge on brief exposure to light of solutions in acetic acid. In general, the xanthydrol method, little used until now, has yielded values for tryptophan which are lower by one mole, compared to those from the other two methods, for four of the fractions, and in one instance lower by two moles. While reproducible data are obtained by this method, the exact relation of values obtained to the true results remains to be elucidated. In view of these observations, it is worth determining whether tryptophan in gramicidin fractions exhibits the same molar extinction coefficient and chromogenicity as it does in simple derivatives. Pertinent in this connection is the suggestion that ethanolamine in gramicidin is probably linked to a peptide bond close to tryptophan, through its hydroxyl group, and joined by its amino group with either the indole group of tryptophan or with an enolized peptide bond (James and Synge, 1951). Such modified structures may be expected to show a behavior different from that of tryptophan in normal peptide linkage. That there are differences in tryptophan content in several of the gramicidin fractions is indicated by all the analyses, and the differing yields of ammonia (Table II), which presumably arise from the decomposition of tryptophan, seem to reflect this variation. Reported literature values for the tryptophan content of unfractionated gramicidin fall in the range 39.3–45% (Edwards, 1949; Lewis *et al.*, 1950; Ramachandran and Witkop, 1959), and gramicidin A and gramicidin B are reported as containing four moles of tryptophan per mole of glycine (Stein and Moore, 1949–50; Ishii *et al.*, 1962). A final statement on the actual tryptophan content of the different gramicidins would be made possible by direct identification of the different tryptophanyl sequences in each molecule during structural investigations.

Previous investigators (Ishii *et al.*, 1962) have found that silica gel chromatography of "gramicidin A" yielded three fractions, all of which contained isoleucine, whereas thin-layer chromatography gave three spots whose yields were dependent on the polarity of the solvent used to dissolve the sample. On these grounds it has been suggested that, besides evident structural heterogeneity, there may also be present in "gramicidin A" configurational or transannular tautomers involving the stereochemistry of the points of attachment of the ethanolamine bridge. The successful resolution of at least one of the components of gramicidin A, namely, gramicidin D, from the rest should now permit detailed investigation of configurational isomerism, uncomplicated by sample heterogeneity, in this component.

Acknowledgment

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Uncoupling of Oxidative Phosphorylation by Carbonyl Cyanide Phenylhydrazones. II. Effects of Carbonyl Cyanide *m*-Chlorophenylhydrazone on Mitochondrial Respiration

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The effects of carbonyl cyanide *m*-chlorophenylhydrazone (*m*-Cl-CCP) on respiration in rat liver mitochondria has been studied polarographically and manometrically. Experiments with the oxygen electrode have shown that 2 μM *m*-Cl-CCP abolishes respiratory control when β-hydroxybutyrate, a glutamate-malate mixture, or succinate is used as a substrate. A similar concentration of *m*-Cl-CCP reverses the inhibition of respiration by oligomycin. The depression by *m*-Cl-CCP of succinate oxidation has been shown to be prevented by addition of either cysteine sulfinic acid or lipoic acid. However, neither of these agents protects against uncoupling by *m*-Cl-CCP. The ability of a fixed amount of *m*-Cl-CCP to abolish respiratory control and lower the P/O ratio is dependent on the amount of mitochondrial protein in the system. However, respiratory control is somewhat more sensitive to *m*-Cl-CCP than is phosphate esterification. The ATP-P_i^{32} exchange reaction is also inhibited by *m*-Cl-CCP. The possible site of carbonyl cyanide phenylhydrazone action is discussed in relation to these data.

The phenomenon of respiratory control as defined and studied by Chance and his collaborators (Chance and Williams, 1956) has been of great value in the study of oxidative phosphorylation. Through studies of respiratory control, it is possible to discriminate between substances that inhibit oxidative phosphorylation, such as guanidines (Pressman, 1963; Hollunger, 1955), and true uncouplers of this process, i.e., 2,4-DNP. Polarographic observation of respiration provides useful information about the mode of action of inhibitory substances known to prevent phosphate esterification; e.g., Estabrook's (1961) studies with oligomycin.

With these considerations in mind, a study of the effects of *m*-Cl-CCP on mitochondrial respiration and respiratory control has been conducted and is reported here. In addition to studies of CCP effects on respiration, it was felt that useful information might be obtained by a study of the ATP-P_i^{32} exchange reaction described by Boyer *et. al.* (1956). An earlier paper (Heytler, 1963) reported the effect of *m*-Cl-CCP on several other aspects of mitochondrial oxidative phosphorylation.

Experimental

Mitochondria used in these studies were obtained from the livers of young adult male rats. The method of isolation is outlined in the previous paper (Heytler, 1963).

Oxidation of substrate was measured either by direct determination of oxygen uptake or in some cases by colorimetric measurements of acetoacetate (Walker, 1954). Direct measurements of oxygen uptake were obtained manometrically by standard Warburg technique, or polarographically with a modified Clark electrode (Yellow Springs Instrument Company, Yellow Springs, Ohio).

Determinations of phosphate uptake were made by measuring the disappearance of inorganic phosphate from the medium (Taussky and Schoor, 1953). Protein determinations were made according to the method of Lowry *et al.* (1951).

The ATP-P_i^{32} exchange was conducted under a nitrogen atmosphere at 20°. Incorporation of labeled phosphate into ATP was measured after removal of inorganic phosphate by solvent extraction (Nielson and Lehninger, 1955). Radioactivity determinations were performed by drying aliquots of P^{32}-containing solutions on aluminum planchets and counting with a thin-window GM tube.

The carbonyl cyanide *m*-chlorophenylhydrazone used in this study was synthesized by Dr. W. W. Prichard of this laboratory by the method described in a previous report (Heytler and Prichard, 1962). The oligomycin was the generous gift of Dr. H. A. Lardy. All other chemicals used in these investigations were obtained from commercial sources in the highest purity routinely available.

Results

Polarographic Studies of Coupled Mitochondria.— The polarographic tracing of Figure 1 shows that the mitochondria used in these studies exhibit respiratory control[1] with respect to both ADP and P_i. Figure 2 shows that the respiratory control of β-hydroxybutyrate oxidation by ADP is abolished by 2 μM *m*-Cl-CCP. As shown in Figure 3, the respiration of a coupled mitochondrial system deficient in ADP and P_i is markedly stimulated by 2 μM *m*-Cl-CCP. The stimulation of respiration is seen immediately (<2 seconds) after the introduction of *m*-Cl-CCP to the electrode chamber.

The antibiotic oligomycin A has been shown to block the respiration of coupled mitochondria (Lardy *et al.*, 1958). This block is not removed by arsenate (Estabrook, 1961), an uncoupler which acts at the level of phosphate. However, 2,4-DNP, which ap-

* Contribution No. 839.

[1] Respiratory control is defined as that physiological state in which ADP and P_i are rate limiting for mitochondrial respiration.