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A Nonphosphorylative Function of Adenosine 5′-Diphosphate in the Maintenance of Mitochondrial Integrity*

Jerald L. Connelly and Curtis H. Hallstrom†

With the technical assistance of Mrs. Charles Basom

ABSTRACT: The swelling of rat liver mitochondria subjected to a phosphate-containing environment is known to be delayed by a number of agents including respiratory substrate, adenosine diphosphate (ADP), and adenosine triphosphate (ATP). Furthermore, the inhibition of oxidative phosphorylation by oligomycin A is known to enhance the "protective" ability of substrate. The relative effectiveness of these compounds, singly and in combination, in this capacity has been investigated in an attempt to determine whether the phosphorylation of ADP accounts for the effects of substrate and ADP. Although time of onset (TO) of swelling is delayed in proportion to the concentrations of succinate and ADP, oligomycin acts not to reverse but to enhance this effect. Thus, contrary to general belief, the influence of substrate plus ADP on TO is not manifest through the production of ATP by oxidative phosphorylation. Whereas oligomycin blocks the ATP-induced increase in TO, the addition of AMP, which allows the production of ADP by oligomycin-insensitive adenylate kinase, results in marked delays of swelling. ADP is effective at concentrations of 10^{-5} M. These results support the possibility that the metabolism of succinate (and other oxidizable substrates) is responsible for the resistance of mitochondrial structure toward the swelling influence of phosphate-containing media and further that this action of substrate is positively modified by ADP. It is suggested that the role of ADP may be coenzymatic or allosteric.

A number of singular observations have been reported which relate to the "protection" of mitochondria against the process of swelling (Lehninger, 1964). Such agents as ATP,[1] EDTA, Mg^{2+}, respiratory substrates, anaerobiosis, and respiratory inhibitors have been shown to delay the onset of swelling. To date, no satisfactory explanation has been provided which accounts for the similarity of action of this variety of compounds or conditions. With the idea that at least some of these agents owe their influence to a common mechanism and under the proposition that swelling is opposed by an active process, efforts in this laboratory have been directed toward the elucidation of the chemistry involved in maintaining (or controlling) mitochondrial integrity against swelling influences. Attempts to describe a metabolic machinery whose function is to oppose those forces or conditions which encourage water and ion uptake have disclosed primary roles for ADP and respiratory substrate under conditions in which oxidative phosphorylation is prevented. Furthermore, this report indicates that the function of ATP as a protective agent is dependent upon its conversion to ADP.

Experimental Section

The preparation of rat liver mitochondria and the conduct of swelling studies were essentially the same as

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† U. S. Public Health Service predoctoral fellow.

[1] Abbreviations used: AMP, ADP, and ATP, adenosine 5′-mono-, di-, and triphosphates; TO, time of onset of swelling (Connelly and Lardy, 1964b,c).

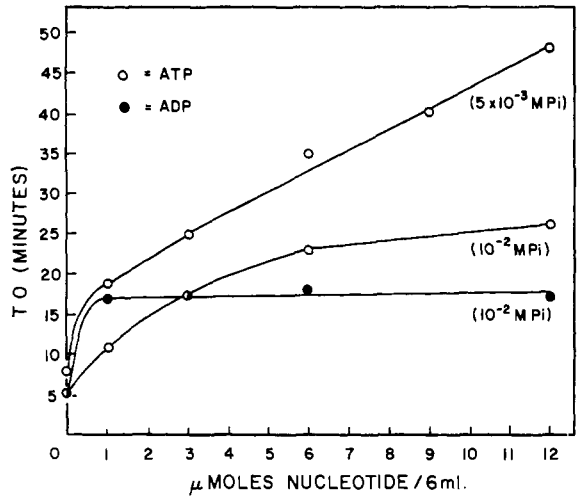

FIGURE 1: The effect of ADP and ATP on TO. Media contained 0.139 M sucrose and 1.67×10^{-2} M histidine (pH 7.5). Orthophosphate and nucleotides, at the concentrations noted, were present prior to the addition of mitochondria (0.1 ml) (2.5 mg of protein), in a final volume of 6 ml. (◑) Indicates coincident data.

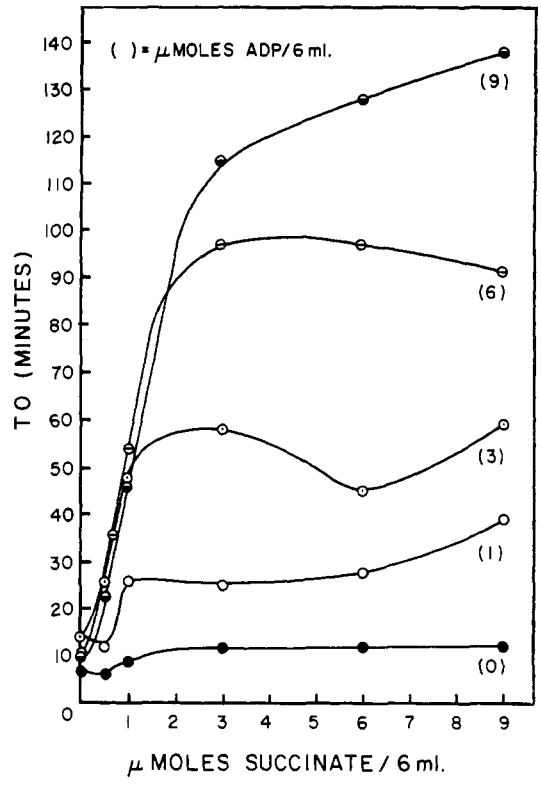

FIGURE 2: The effect of ADP plus succinate on TO. Media are the same as in Figure 1 with 5×10^{-3} M orthophosphate. Succinate and ADP were present from the beginning at the concentrations noted.

previously described (Connelly and Lardy, 1964a; Connelly and Hallstrom, 1966a). Nucleotides and substrate were obtained from the Sigma Chemical Co. and oligomycin A was a gift of Dr. H. A. Lardy, University of Wisconsin.

Results

Of the factors which act to increase the time of onset (TO) of mitochondrial swelling, ATP has been the most readily accepted as a primary agent. Although ATP is recognized as an energy source for active processes, the chemistry of its activity in delaying swelling is not known and furthermore the delay of swelling is not generally recognized as an active process (except, see Emmelot *et al.*, 1960). A major complication in this area has been the observation by Hunter *et al.* (1959) and others (Connelly and Hallstrom, 1966a; Corwin and Lipsett, 1959) that ADP acts to delay swelling.

The patterns and relative effectiveness of ADP and ATP protection against phosphate-induced swelling has been investigated by titrating TO with these compounds. The results indicated in Figure 1 show an increasing TO with increasing concentrations of ATP. It can also be seen that at any one nucleotide concentration the influence of ATP is inversely related to the concentration of a swelling agent. This is consistent with the inverse relationship between swelling agent concentration and TO previously shown (Connelly and Hallstrom, 1966a). In contrast to the picture seen with ATP, ADP increases TO at concentrations from zero to about 1 μmole/6 ml (1.67×10^{-4} M) after which increasing amounts of ADP have no effect. It is significant that while the TO does not continue to increase with increasing ADP concentration, ADP reaches its maximal effectiveness at a concentration much lower than that of ATP. The data in Figure 1 suggest that ATP is rate limiting while ADP is dependent upon a second factor or factors provided by the mitochondria. This idea is further substantiated by the observation that TO's obtained with ATP are relatively comparable with each mitochondrial preparation whereas the ADP-governed TO's are much more variable.

The obvious explanation for these findings is that endogenous substrate, which varies among mitochondrial preparations, is used in conjunction with inorganic phosphate and the ADP to provide a limited amount of ATP which in turn functions in some unknown matter to extend TO. In support of this, the addition of increasing amounts of succinate to ADP at different concentrations increases the TO in a regular fashion (Figure 2). The data shown in Figure 2 also indicate that ADP readily becomes rate limiting. That ADP functions primarily in this manner requires that ATP acts by some mechanism other than the terminal steps of the oxidative phosphorylation machinery. That is, it is difficult to explain the effect of ATP and ADP on mitochondrial structure by their roles in the phosphorylation mechanism, since in that mechanism they act in opposite directions whereas their effects on mitochondrial structure are in the same direction. Nevertheless, that ATP

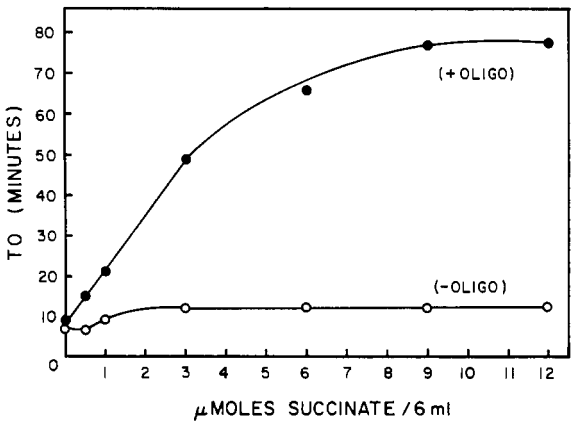

FIGURE 3: The effect of oligomycin on succinite-increased TO. Media are the same as in Figure 2 with oligomycin present at a concentration of 1.0 μg/ml, sufficient to block oxygen uptake.

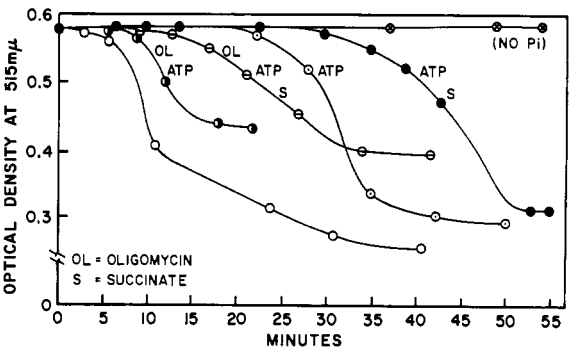

FIGURE 5: The effect of oligomycin on the ATP plus succinate-increased TO. Media are the same as in Figure 2 with ATP = 10^{-3} M, succinate = 8.3×10^{-5} M, and oligomycin = 2 μg/ml. Note that the succinate concentration was decreased from the use in Figure 4 in order to keep the TO of succinate plus ATP at a practical level.

does act through the terminal portion of the phosphorylation mechanism has been indicated by the inhibition of ATP increases in TO by oligomycin. Thus, it is unlikely that the capacity of ADP to increase TO is based on its conversion to ATP. It is also noteworthy that ADP in the presence of succinate is comparatively much more influential than ATP added directly (see Figure 5 of Connelly and Hallstrom, 1966a). Since it is reasonable to assume that one of the two nucleotides is the primary protective agent and that the activity of the second nucleotide is explained by its conversion to the first, the alternative possibility that ADP is the primary agent has been investigated further.

Because of its unique ability to inhibit the terminal portion of the phosphorylation mechanisms, oligomycin is a particularly useful tool for the elucidation of the relative importance of ADP and ATP. The minimal influence of substrate alone is markedly enhanced by oligomycin as shown in Figure 3 (see also Connelly and Lardy, 1964b; Chappell and Greville, 1961). It should be noted here that the curve for succinate alone is quite similar to that seen in Figure 1 for ADP alone, suggesting that succinate is not a rate-limiting factor although it does increase TO slightly up to 1 μmole/ml. The effect of oligomycin, inactive alone on TO, most certainly involves its ability to inhibit phosphorylation and further to block the use of respiratory-generated, high-energy intermediates for that purpose.

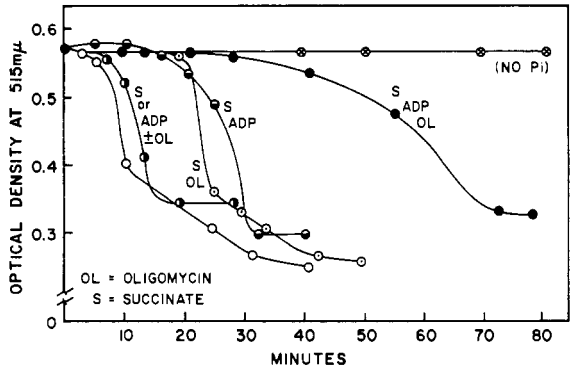

FIGURE 4: The effect of oligomycin on ADP plus succinate-increased TO. Media are the same as in Figure 2 with ADP = 10^{-3} M, succinate = 1.67×10^{-4} M, and oligomycin = 2 μg/ml. The control curve is shown by open circles. Additions made prior to adding mitochondria are noted on curves according to key.

If the phenomenon observed with ADP plus substrate is in fact due to phosphorylation, it should be inhibited by oligomycim. The effects on TO of various combinations of substrate, ADP, and oligomycin are shown in Figure 4. It is seen that the addition of either ADP or oligomycin substantially increases the TO over that provided by substrate alone. Most important, however, is the observation that oligomycin, when added to substrate plus ADP, markedly enhances the TO. This observation alone eliminates ATP, generated by oxidative phosphorylation, as a primary factor in the control of TO. It should be noted further that the addition of oligomycin to ADP alone is ineffective, thus underlining the importance of the substrate contribution. The effects of succinate in this capacity are not unique since glutamate, α-ketoglutarate, malate, and pyruvate act in the similar manner (C. H. Hallstrom and J. L. Connelly, unpublished data). In similar studies (Figure 5), it was noted that substrate was able to increase the TO of ATP-containing systems (see also Figure 5, Connelly and Hallstrom, 1966a). However, in contrast to the data in Figure 4, oligomycin was found to reverse the effects of ATP in the presence of both P_i and P_i plus substrate. The TO with the latter system was effectively reduced to that of succinate plus oligomycin A.

In an attempt to illustrate the nature of the role of ADP in this system, low levels of succinate were titrated

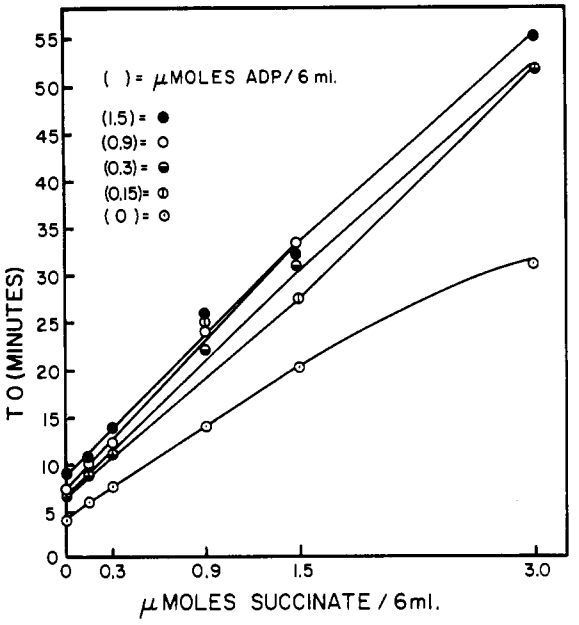

FIGURE 6: Titration of TO *vs.* succinate in the presence of ADP and oligomycin. Media are the same as in Figure 2 with oligomycin = 1 μg/ml. ADP and succinate concentrations are as indicated.

against minimal amounts of ADP in the presence of oligomycin at concentrations sufficient to block all phosphorylation (1μg/ml). These results are shown in Figure 6. In the absence of added ADP linearity was obtained to about 2 μmoles of succinate/6 ml. With added ADP increases in TO could be seen at levels as low as 10^{-5} M ADP. At the concentrations of substrate used, it was further noted that the resulting TO's at concentrations above 10^{-5} M ADP did not vary. It is concluded that, within the substrate range shown in the graph, succinate was a rate-limiting factor.

The availability of an active adenylate kinase in rat liver mitochondria made possible experiments designed to substantiate the singular importance of ADP in the control of mitochondrial integrity (Connelly and Hallstrom, 1966b). Thus, if ADP could be produced through the action of this enzyme on AMP and ATP in the presence of oligomycin, which does not influence kinase activity, one should observe an increase in TO similar to that seen in the system shown in Figure 4. The results of such an experiment are shown in Figure 7. Only those reaction mixtures containing oligomycin and substrate have TO's substantially increased over that of the phosphate control. A further increase is noted in that curve which represents the system containing oligomycin, substrate, and the AMP–ATP combination which would, through the kinase reaction, result in ADP. This experiment eliminates the possible explanation that ADP functions through its conversion to ATP *via* the adenylate kinase reaction since the effectiveness of ATP, however produced, is blocked by oligomycin. The results of the experiments described in this report are collected in

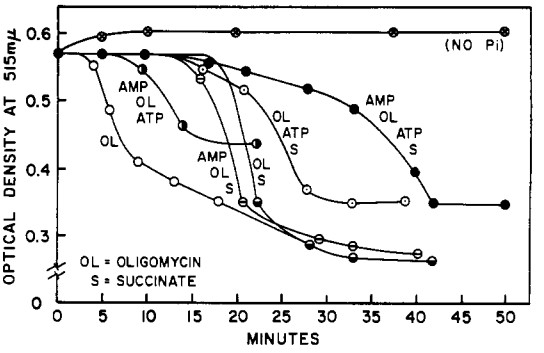

FIGURE 7: The effect of AMP on TO in the presence of oligomycin, ATP, and succinate. The media are the same as in Figure 2 with ATP = 5×10^{-4} M, AMP = 5×10^{-4} M, succinate = 1.67×10^{-4} M, and oligomycin = 1.67 μg/ml.

Figure 8 for the purpose of visualizing the possible interrelationships among a number of reactions which are concerned with respiration, ADP, and TO. Reaction A together with D represents the schematic oxidative phosphorylation where I denotes the functional high-energy intermediates. Reaction B symbolizes the interdependent use of respiratory-generated intermediates and ADP in the cyclic generation of ADP-I, used to denote the conditions favorable toward increasing TO. Since magnesium is also known to function as a protective agent, it is included in this reaction. Reaction E indicates the adenylate kinase activity. If the maintenance of mito-

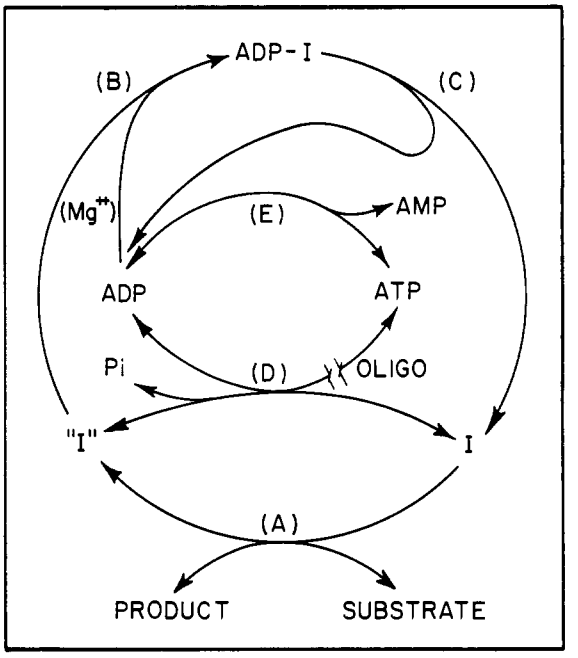

FIGURE 8: The interrelationships of various factors affecting the time of onset of mitochondrial swelling. See text for description of reactions A–E.

chondria is dependent upon the level of ADP-I it is necessary that the reaction rates of A and B must be rapid in comparison to C. According to this representation ATP should be able to act to provide ADP and I toward the increase in TO, whereas ADP alone would be dependent upon the generation of I. Furthermore, in the presence of oligomycin, all of the I produced and enough ADP to saturate this I would be available for ADP-I production since the use of I for ATP production would be blocked. Finally, in accordance with Figure 7, TO will be increased by substrate, oligomycin, and ATP only if AMP is present.

Discussion

Although a clear description of the chemistry involved in the maintenance of mitochondrial integrity is not yet available, the results of experiments described herein strongly support the idea that the uptake of water by mitochondria is opposed by an active chemical force involving ADP and factors related to the metabolism of certain compounds including succinate. Furthermore, the participation of the terminal portion of the oxidative phosphorylation mechanism in this process has been eliminated. There now remains, however, the need to describe the means by which ADP functions and likewise the chemistry involved in the respiratory substrate contribution.

In accordance with the classification provided by Stuart and Williams (1966) and by Klingenberg (1965) three general types of effects of ADP on mitochondrial metabolism must be considered. These include the indirect effects wherein ADP influences metabolism (1) by means of varying the redox status of pyridine nucleotides or other respiratory chain components, or (2) by allowing the production of ATP which in turn affects enzymatic activities; and (3) by direct effect of ADP which might be considered coenzymic or allosteric in nature. The action of oligomycin would differentiate clearly between the indirect and direct effects since both of the indirect effects require operation of the oxidative phosphorylation mechanism. As a consequence of this reasoning it appears that in the presence of oligomycin and substrate, the effect of ADP on TO is direct and very possibly allosteric in nature. Observations from a number of laboratories are consistent with this capability of ADP (Chen and Plaut, 1963; Klingenberg *et al.*, 1965; Keech and Utter, 1963; Stuart and Williams, 1966; Wellner and Meister, 1966). The direct effect of ADP upon mitochondrial structure or upon the mechanisms regulating structural variation has generally been interpreted on the basis of the function of ADP as a phosphate acceptor (Hunter *et al.*, 1959; Corwin and Lipsett, 1959; Emmelot, 1960). However, in the presence of oligomycin, it is unlikely that "the actual synthesis of ATP (would) appear as an important requirement for maintenance of both function and structure" (Gamble, 1962). Chappell and Greville (1961) and Azzi and Azzone (1965) suggest that substrate-level phosphorylation accounts for the protective action of ADP in the presence of oligomycin although this explanation would not appear to suffice when succinate is substrate. Furthermore, it has been observed (Hallstrom and Connelly, unpublished data) that malate provides results similar to those obtained with succinate.

The unique ability of minimal amounts of ADP to affect TO could very likely be a gross manifestation of the activity of reactions such as the adenyl-exchange enzyme (Pfaff *et al.*, 1965) which exhibit a preference for ADP over AMP and ATP. It is pertinent that the binding of adenine nucleotides occurs very rapidly and that once bound the adenine compounds are not easily removed (Brierley and O'Brien, 1965; Pressman, 1958). Secondly, Pfaff *et al.* (1965) and Carafoli *et al.* (1965) have noted the preferential accumulation of ADP by oligomycin-treated mitochondria during limited uptake of calcium. Whether this latter phenomenon is a reflection of the role of ADP in structural control is speculative.

Recent observations by Hackenbrock (1966) show that the addition of ADP to mitochondria is accompanied by morphological changes in the inner membrane and matrix of those particles. Although these changes appear to be related to the various respiratory states it is conceivable that ADP, in addition to its role as a phosphate acceptor, is also functioning in a nonphosphorylative capacity. Thus, there remains the possibility that the observation made by Hackenbrock and the effect of ADP reported here might in fact be separate reflections of a single phenomenon, occurring independently of ATP synthesis.

Beyond the likely possibility that respiratory substrate acts to influence TO by affecting the status of respiratory chain or phosphorylation intermediates, no comprehensive explanation of its action is available, nor does the present work simplify this situation. Nevertheless, it is apparent that under the conditions employed here, substrate functions not to decrease TO (*i.e.*, to swell) but to increase TO proportionate to the substrate concentration. This is in agreement with earlier observations (Kaufman and Kaplan, 1960; Connelly and Lardy, 1964a,b; Emmelot *et al.*, 1960). Packer (1962) suggests that the reversal of electron transport and factors affecting this process might contribute to the control of TO. More in agreement with observations presented here, Emmelot (1960) concluded that the "process which prevented the entry of water into the mitochondria was dependent upon added oxidizable substrate and adenine nucleotides (as distinct from the situation in which a high concentration of ATP added singly may prevent swelling). "It appears, nevertheless, that substrate, in the presence of ADP, under conditions which prevent oxidative phosphorylation, is transformed by a process related to structural control of mitochondria. The nature of the action of ADP suggests that this molecule influences this process so as to markedly increase the efficiency of substrate utilization for increased TO. As a correlate to this proposition, one would expect the general action of swelling agents to oppose this process either by affecting the binding of ADP or by interfering with the substrate utilization or with the disposition of the reaction products. Studies designed to investigate these possibilities

are currently under way in this laboratory.

References

Azzi, A., and Azzone, G. F. (1965), *Biochim. Biophys. Acta 105*, 253.

Brierley, G., and O'Brien, R. L. (1965), *J. Biol. Chem. 240*, 4532.

Carafoli, E., Rossi, C. S., and Lehninger, A. L. (1965), *J. Biol. Chem. 240*, 2254.

Chappell, J. B., and Greville, G. D. (1961), *Nature 190*, 502.

Chen, R. F., and Plaut, G. W. E. (1963), *Biochemistry 2*, 1023.

Connelly, J. L., and Hallstrom, C. H. (1966a), *Biochemistry 5*, 570.

Connelly, J. L., and Hallstrom, C. H. (1966b), *Biochemistry 5*, 2255.

Connelly, J. L., and Hallstrom, C. H. (1966c), *Federation Proc. 25*, 736.

Connelly, J. L., and Lardy, H. A. (1964a), *J. Biol. Chem. 239*, 3065.

Connelly, J. L., and Lardy, H. A. (1964b), *Biochemistry 3*, 1969.

Corwin, L. M., and Lipsett, M. N. (1959), *J. Biol. Chem. 234*, 2453.

Emmelot, P. (1960), *Nature 188*, 1197.

Emmelot, P., Bos, C. J., Brombacher, P. J., and Reyers, I. H. M. (1960), *Nature 186*, 556.

Gamble, J. L. (1962), *Am. J. Physiol. 203*, 886.

Hackenbrock, C. R. (1966), *J. Cell Biol. 30*, 269.

Hunter, F. E., Jr., Levy, J. F., Fink, J., Schultz, B., Guerra, F., and Hurwitz, A. (1959), *J. Biol. Chem. 234*, 2176.

Kaufman, B. T., and Kaplan, N. O. (1960), *Biochim. Biophys. Acta 39*, 322.

Keech, D. B., and Utter, M. F. (1963), *J. Biol. Chem. 238*, 2609.

Klingenberg, M. (1965), *in* Control of Energy Metabolism, Chance, B., Estabrook, R. W., and Williamson, J. R., Ed., New York, N, Y., Academic, p 149.

Klingenberg, M., Goebell, H., and Wenske, G. (1965), *Biochem. Z. 341*, 199.

Lehninger, A. L. (1964), The Mitochondrion, New York, N. Y., Benjamin.

Packer, L. (1962), *J. Biol. Chem. 237*, 1327.

Pfaff, E., Klingenberg, M., and Heldt, H. W. (1965), *Biochim. Biophys. Acta 104*, 312.

Pressman, B. C. (1958), *J. Biol. Chem. 232*, 967.

Stuart, S. C., and Williams, G. R. (1966), *Biochemistry 5*, 3912.

Wellner, V. P., and Meister, A. (1966), *Biochemistry 5*, 872.

Isolation of a Phospholipid Renin Inhibitor from Kidney*

Subha Sen, Robert R. Smeby, and F. Merlin Bumpus

ABSTRACT: The reactivity of renin added to plasma from nephrectomized dogs increases independent of increase in renin substrate concentration. Addition of plasma proteins from normal dogs to plasma from a nephrectomized dog reduces the reactivity of renin. This suggests the presence of a renin inhibitor in normal dog's plasma of renal origin. The inhibitor was isolated from dog's kidney and shown to be a phospholipid similar to bovine phosphatidylserine but differs in fatty acid content and the structure of the amino acid. The phospholipid completely inhibits the reaction of dog renin with dog renin substrate *in vitro* and single, daily intramuscular injections of the compound reduces the blood pressure of chronic renal hypertensive rats.

Renin, an enzyme released from kidney, reacts with an α-2-globulin in plasma (renin substrate) to release a decapeptide, angiotensin I. Converting enzyme, also present in plasma, rapidly splits histidylleucine from the C-terminal end of angiotensin I to yield an octapeptide, angiotensin II. This latter, the only substance of this system with direct biological activity, is the most potent, natural, pressor substance known. Both peptides are rapidly degraded by peptidases named angiotensinases. The renin–angiotensin system, recently reviewed by Peart (1965) and Page and Bumpus (1961) and summarized by the reactions in Figure 1, may play an important role in the regulation of normal blood pressure, salt metabolism, and some types of experimental and clinical hypertension.

The recent intensified interest in measurement of plasma renin concentration (Page *et al.*, 1965) has demonstrated the need to determine if substances

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