In a recent paper (2) we postulated that during oxidative phosphorylation by isolated mitochondria the turnover rates of the adenine nucleotides within the mitochondria are much more rapid than their passage in and out of the mitochondria. This paper presents evidence to confirm that hypothesis by using radioactivity measurements as a more direct method of determining turnover and diffusion. The data also suggest that the mitochondrial enzyme, adenylate kinase (3-5), exists in two sites in the mitochondrion. Based on these results, a scheme is presented for certain aspects of the biochemical structure of the mitochondrion, and the possible rôle of such a structure in regulating oxidation is discussed.

EXPERIMENTAL

The rat liver mitochondria were prepared as described previously (6). In all experiments the mitochondria from 12 gm. wet weight of rat liver were used (12 equivalent gm. of Mt). In order to stop the enzymatic reactions before equilibrium was attained, the cold isotonic sucrose suspension of mitochondria was mixed with the cold oxidative medium (components given in Tables I to VI, total volume usually 26 ml.), and, immediately after mixing, the mitochondria were separated from the medium by centrifugation for 10 minutes at 7500 X g in the International refrigerated centrifuge kept at 0°. (For convenience this type of experiment is henceforth called centrifugation-incubation.) The mitochondria were washed once with cold isotonic sucrose and separated again as described above. The mitochondrial pellet was taken up in 8 ml. of cold water, 4 ml. of cold 1.5 N perchloric acid were added, and the suspension was left in the refri-
erator for 1 hour. After centrifuging the denatured protein, the precipitate was washed once with water, the washings were combined with the initial supernatant solution, and the whole was neutralized with 1.5 N KOH and left in the refrigerator overnight. After separation of the mitochondria from the oxidative medium, a one-tenth aliquot was taken from the medium, and an acid extract of the medium was prepared as described for the acid extract of the mitochondria. In both cases, the insoluble KClO₄ was centrifuged and the supernatant fluid was neutralized in preparation for ion exchange chromatography.

The Dowex 1 ion exchange columns were set up in glass tubing of 1.1 cm. diameter by 15 cm. long, sealed to the bottom of a 125 ml. Erlenmeyer flask. Their preparation and the placement of the neutralized extracts on the columns were as described previously (6). The successive 5.0 ml. eluents which were used for the consecutive elution of the various nucleotides are given in Fig. 1. The various nucleotides were located by placing samples of them on the columns, by a comparison of placement on similar columns described earlier (7), by their absorption spectra, and, in the case of the adenine and inosine phosphates, by the ratios of purine to stable phosphate to labile phosphate. Inorganic phosphate and glucose-6-phosphate were located by comparison with known samples and by determination of inorganic phosphate (Soyenko method (8)) and by the anthrone test (9). The presence of a flavin adenine dinucleotide was determined on the column by the coincidence of the peak of the yellow color (maximum at 445 μm) with that of the ultraviolet peak at 260 μm. In the columns described previously (6), in which elution was begun with formic acid, IP and G6P appeared just before the ADP peak. In order to effect a clear-cut separation of these compounds, 0.1 M ammonium formate was put through the column initially. This low concentration of ammonium formate had the effect of moving IP and G6P ahead on the chromatogram, the other compounds remaining stationary (Fig. 1; also Khym and Cohn (10)). When necessary, the IP and G6P could be separated from each other by using 0.04 M ammonium formate as the initial eluent, in which case the G6P was eluted sufficiently before the IP, and thus accurate specific activities could be obtained for both compounds. In the case of the acid extract from the medium, a simplified elution procedure was used, since the added adenine nucleotides were much greater in quantity than any other nucleotides which might be present. In this case, only the solu-

1 Abbreviations are used as follows: AMP, ADP, ATP = adenosine mono-, di-, and triphosphate; DPN and TPN = diphospho- and triphosphopyridine nucleotide; IP = inorganic orthophosphate; G6P = glucose-6-phosphate; CMP, GMP, and IMP = cytosine, guanosine, and inosine monophosphates; FAD = flavin adenine dinucleotide; ITP = inosine triphosphate; DNP = 2,4-dinitrophenol; UDX = derivative of uridine diphosphate; Mt = mitochondria.
tions for separating IP, AMP, ADP, and ATP (see Fig. 1) were put through the column. The micromoles contained in each tube were calculated by arbitrarily using 13,500 as the molecular extinction coefficient (at 260 με) of the adenine moiety, after first subtracting the known blank reading due to the non-specific absorption of the eluent.

Each fraction from the chromatogram was counted directly by means of a Geiger-Müller dip counter (obtained from Tracerlab, Inc., Boston) and a Berkeley scaler. Corrections were made for the radioactivity decay. The carrier-free radioactive IP32 was obtained from the Atomic Energy Commission, Oak Ridge, Tennessee.2 In one experiment all the fraction tubes from the column were counted, and there was no radioactivity other than when phosphate-containing compounds were found.

Fig. 1 gives a typical chromatogram of the acid extract of mitochondria after oxidative phosphorylation had taken place in the presence of IP32. It can be seen that in ADP and ATP the radioactivity follows very closely the E260 absorption. In previous experiments with formic acid as the

2 The IP32 was allocated to Dr. Charles Heidelberger of this laboratory, to whom we are indebted for advice on the use of the counters.
initial eluent (see above), the AMP and DPN were entirely free of any IP,
and it was found that these two compounds were completely non-radio-
active. Also, the Ad-X could be more easily separated from ATP and it
contained no radioactivity. The specific activity of each compound was
determined by averaging those of from three to five of the peak tubes; the
specific activities of the individual tube contents rarely differing by more
than ±10 per cent of the average specific activity. In some cases, ADP
and ATP were rechromatographed on an ammonium formate column (7),
and the specific activity thus obtained agreed closely with that obtained
initially.

Hexokinase was prepared from bakers' yeast according to F. Loomis,3
and it contained no adenylate kinase or ATPase activities, but did contain
inorganic pyrophosphatase activity. AMP and ATP were obtained either
as the sodium or potassium salts from the Pabst Laboratories, Milwaukee.
In the experiments utilizing ADP$^{32}$, the radioactive ADP was made during
oxidative phosphorylation by mitochondria utilizing IP$^{32}$. The ADP$^{32}$
was obtained by ion exchange chromatography by using the initial washing
with ammonium formate to get rid of all contaminating IP$^{32}$ (see above).
The eluted ADP$^{32}$ solution was lyophilized and reprecipitated through
the barium salt.

Results

**Effect of Added Nucleotides on Quantities and Specific Activities of Intra-
and Extramitochondrial Nucleotides**—When mitochondria were incubated
at 25° for as little as 5 minutes with IP$^{32}$ and either AMP or ADP in the
oxidative medium, and then separated from the medium, it was found that
the specific activities of the intramitochondrial ADP and ATP were the
same as those in the medium. Thus in these experiments, in which 70 to
120 μmoles of IP were taken up, an equilibrium between the intra- and
extramitochondrial nucleotides was attained. In order to observe whether
there was any change in the concentrations of the intramitochondrial nu-
cleotides during oxidative phosphorylation, a centrifugation-incubation
experiment was set up (see “Experimental”) in which incubation took
place at 0° while the mitochondria were being separated from the medium
by centrifugation. It was found that under these conditions IP$^{32}$ was
taken up from the medium, ADP$^{32}$ and ATP$^{32}$ were formed, and a net
synthesis of ATP occurred. That oxidative phosphorylation can be shown
to occur at 0° is probably due to the large concentration of mitochondrial
enzyme used (cf. (12)). In these centrifugation-incubation experiments
the IP uptake was reduced to a value of 10 to 35 μmoles, and, under these

3 Unpublished method. This preparation corresponds to Fraction 3a of Berger
et al. (11).
conditions of lowered phosphorylation, differences were observed between the specific activities of the intra- and extramitochondrial nucleotides, depending upon whether AMP or ADP was added to the medium. All the following results refer to centrifugation-incubation experiments.

Table I shows that the specific activity of the ATP (per micromole of adenine) was always nearly twice that of the ADP. During the prepara-

**Table I**

Comparison of Specific Activities of Intra- and Extramitochondrial Nucleotides
When AMP or ADP Was Added Initially to Medium; Effect of KCN

In Experiment 1, each oxidative medium contained 200 µmoles of glutamate, approximately 60 µmoles of AMP or approximately 30 µmoles of AMP plus approximately 30 µmoles of ADP, 100 µmoles of IP, 63 µmoles of MgCl₂, 280 mg. of sucrose, and 0.2 ml. of IP³ containing approximately 250,000 c.p.m. The contents of the medium were the same in Experiment 2, except that 60 µmoles of ADP, or approximately 30 µmoles of AMP plus approximately 30 µmoles of ADP, or approximately 60 µmoles of AMP plus KCN at final concentration of 0.001 M were added in the appropriate flasks. Mixing of the mitochondria and media and the preparation of the acid extracts of mitochondria and media as described in the text. The specific activity in this and subsequent tables is given as P²² per micromole of purine as determined by absorption at 260 µm.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Additions</th>
<th>IP uptake, µmoles</th>
<th>IP* Intramitochondrial</th>
<th>ADP* Intramitochondrial</th>
<th>ATP* Intramitochondrial</th>
<th>ATP nucleotides, µmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AMP</td>
<td>2290 2350</td>
<td>1980 2120</td>
<td>3650 3840</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&quot; ADP</td>
<td>2280 2430</td>
<td>1570 750</td>
<td>2790 1770</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>ADP</td>
<td>13.4 3830</td>
<td>2560 520</td>
<td>4320 1080</td>
<td>1.50 1.07 0.99 0.93</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&quot; AMP</td>
<td>9.8 3970</td>
<td>2850 430</td>
<td>4520 1130</td>
<td>1.50 1.52 2.1 0.95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&quot; KCN</td>
<td>0.5 2420</td>
<td>1960 2450</td>
<td>1.95 0.79 0.56 0.59</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Specific activity.

...
extramitochondrial ADP by the added ADP. However, the ATP which was synthesized was also lower in specific activity in the medium than in the mitochondria. This lowered specific activity is considered to be due to a dilution of the radioactive ATP formed during oxidative phosphorylation by non-radioactive ATP formed by some other process, these two sources of ATP not contributing their ATP to an immediately common pool. The results obtained by Cohn (14), using IP with labeled O¹⁸ and adding unlabeled ATP, are suggestive of the same conclusions. Since the two radioactive phosphates of ATP were approximately equal in specific activity, it would appear that the ATP was being diluted as a whole.

To be noted from Table I is that 0.001 M KCN reduced the specific activity of the intramitochondrial ATP more than that of the intramitochondrial ADP, probably because the residual oxidative phosphorylation was enough to turn over all of the labile phosphate in ADP and only a part of the phosphates in ATP. The results with KCN would indicate that most of the radioactivity in the ATP is not due to exchange reactions between IP and ATP, as described by Boyer et al. (15). It can also be seen that KCN also greatly reduced the mitochondrial concentrations of ADP, ATP, and Ad-X, while it increased that of AMP, indicating that these mitochondrial bound nucleotides are probably generated by oxidative phosphorylation (6).

Table II presents a comparison of the specific activities obtained when AMP or ADP or AMP plus ATP was added initially. The results with AMP and ADP are similar to those of Table I. When AMP plus ATP was added, not only was the specific activity of the extramitochondrial ATP less than that of the intramitochondrial ATP (through dilution by the added ATP), but the specific activity of the extramitochondrial ADP was also less than that of the intramitochondrial ADP. This result is probably due to the fact that there is another source of ADP (non-radioactive) which dilutes the radioactive ADP formed through oxidative phosphorylation plus adenylate kinase action and which is in a relatively slow equilibrium with it. The only known mitochondrial reaction which could produce non-radioactive ADP when AMP plus ATP is added, and non-radioactive ATP when ADP is added, is the mitochondrial adenylate kinase reaction (3-5). This suggests that when ADP is added it probably reacts immediately with a surface adenylate kinase to form AMP and non-radioactive ATP. The AMP goes into the mitochondria, to be acted upon by the interior adenylate kinase and through oxidative phosphorylation to form radioactive ADP and ATP. Under the conditions of the experiment, the rates of these reactions are higher than the rates of passage.

4 Actually, for the purpose of these experiments, it would not matter how the radioactivity got into the ATP, as long as it was an enzymatic reaction.
of the radioactive nucleotides out of the mitochondria, thus accounting for the differences in specific activities between the intra- and extramitochondrial nucleotides. The same explanation can be given when AMP plus ATP is added initially.

It can be seen from Tables I and II (and found in all cases) that when ADP was added the specific activity of the extramitochondrial ATP was always greater than twice that of the extramitochondrial ADP. This result may be further proof of the theory outlined above, for, while the radioactive ADP is being diluted only by non-radioactive ADP, the radioactive ATP is diluted by ATP produced by the surface adenylate kinase, this ATP being generated from both non-radioactive ADP and from radioactive ADP. Thus the radioactivity in the extramitochondrial ATP phosphates would always be greater than the radioactivity of the ADP phosphates.

**Effect of Added ADP\(^{32}\) on Specific Activities of Intra- and Extramitochondrial Nucleotides**—If the foregoing hypothesis is correct, it must be that when ADP is added the surface adenylate kinase reaction converts the ADP to AMP very rapidly, and it is AMP which goes into the mitochondrial interior to be utilized in oxidative phosphorylation. This can be demonstrated by using ADP\(^{32}\) (Table III). It can be seen from Table III that the specific activity of the intramitochondrial ADP and ATP is much less than that of the extramitochondrial nucleotides, while the specific activity of the intramitochondrial IP is much higher than that of the extramitochondrial IP. Thus it can be postulated that the added ADP\(^{32}\) gives

### Table II

**Comparison of Quantities and Specific Activities of Intra- and Extramitochondrial Nucleotides When AMP or ADP or AMP Plus ATP Is Added to Medium**

Experimental conditions as for Table I. When used, approximately 60 μmoles of AMP or approximately 60 μmoles of ADP or approximately 40 μmoles of AMP plus approximately 40 μmoles of ATP were added. The theoretical IP uptake (see Table IV) was 33.4 μmoles with AMP, 25.5 μmoles with ADP, and 17.1 μmoles with AMP plus ATP. S.a. = specific activity.

<table>
<thead>
<tr>
<th>Additions</th>
<th>IP</th>
<th>ADP</th>
<th>ATP</th>
<th>AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S.a.</td>
<td>μmoles</td>
<td>S.a.</td>
<td>μmoles</td>
</tr>
<tr>
<td>AMP</td>
<td>Intramitochondrial 7140 5710</td>
<td>12,200</td>
<td>8.2</td>
<td>35.8</td>
</tr>
<tr>
<td></td>
<td>Extramitochondrial 8900 6350 17.0</td>
<td>16,020</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td>Intramitochondrial 5810 4280</td>
<td>9,190</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Extramitochondrial 7900 2550 15.9</td>
<td>5,800</td>
<td>34.7</td>
<td>9.2</td>
</tr>
<tr>
<td>AMP + ATP</td>
<td>Intramitochondrial 5350 4030</td>
<td>8,020</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Extramitochondrial 7680 1650 25.1</td>
<td>4,320</td>
<td>37.1</td>
<td>20.1</td>
</tr>
</tbody>
</table>
rise to ATP$^{32}$ and non-radioactive AMP via the surface adenylyl kinase; some of the ATP$^{32}$ enters the mitochondria, mixes with the mitochondrial ATP, and is broken down to IP$^{32}$ and ADP$^{32}$. These become involved in mitochondrial oxidative phosphorylation, and an equilibrium is quickly established between mitochondrial ADP, ATP, and IP. The breakdown of ATP to ADP and IP is inferred to have occurred at the same general site of oxidative phosphorylation. Thus it can also be suggested that the rate of passage of ADP and ATP into (as well as out of) the mitochondria is much less than the rate of interconversion of these nucleotides within the mitochondria. Also, since in this experiment there was a net oxidative synthesis of ATP, it must be that AMP was the form of the adenine nucleotide which entered the mitochondria to be utilized in this ATP synthesis.

### Table III

**Comparison of Specific Activities of Intra- and Extramitochondrial Nucleotides When ADP$^{32}$ Is Added to Medium**

Experimental conditions as for Table I, except that approximately 30 μmoles of ADP$^{32}$, containing approximately 500,000 c.p.m. of P$^{32}$ and 100 μmoles of non-radioactive IP, were added. The calculated IP uptake was 10.0 μmoles.

<table>
<thead>
<tr>
<th>Determined</th>
<th>Intramitochondrial, s.a.</th>
<th>Extramitochondrial</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S.a.</td>
<td>μmoles</td>
</tr>
<tr>
<td>IP</td>
<td>1880</td>
<td>350</td>
</tr>
<tr>
<td>AMP</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ADP</td>
<td>2070</td>
<td>6,400</td>
</tr>
<tr>
<td>ATP</td>
<td>4130</td>
<td>12,680</td>
</tr>
</tbody>
</table>

**Comparison of Calculated with Actual Dilutions of Nucleotide Radioactivity**—Another way of explaining the results, when ADP is added together with IP$^{32}$ in an oxidative system, is to say that the amount of dilution of the radioactivity in the ATP of the medium is a function of the amount of ATP which was synthesized by oxidative phosphorylation within the mitochondria compared to that produced by adenylyl kinase action at some other mitochondrial site. The procedure for the calculation of the theoretical results and a comparison of the calculated and actual results are given in Table IV. The agreement is very good, even when AMP plus ATP is added and the radioactivity of the ADP of the medium is diluted.

**Validity of Centrifugation-Incubation Method**—The interpretations mentioned in the preceding paragraphs are based on the assumption that the method of incubation (incubating while separating the mitochondria from the medium at 0°) does not introduce an artifact into the results. From a theoretical standpoint, the measurements on the intramitochondrial nu-
cnoletides can be regarded as yielding data that are the resultant of processes occurring (a) while the mitochondria are being spun down with opportunity to equilibrate with the medium (see below), and (b) while they are tightly packed in the pellet and lacking such opportunity (see below). During period (b) the differences in passage of compounds in and out of the mitochondria will be suppressed, but during period (a) such differences are expressible. Since the data represent the sum of differences during (a) and (b), differences between compounds should be detectable. Using

Table IV

Comparison of Observed with Calculated Dilution of Radioactivity of Extramitochondrial Nucleotides

Experiments performed as for Table I with added ADP only, except when noted below. The theoretical dilution was obtained as follows. When ADP was added, the amount of ATP formed in the medium was the resultant of two reactions: adenylate kinase (ADP → AMP + ATP), and oxidative phosphorylation (ADP → ATP). Therefore, by subtracting the amount of AMP in the medium at the end of the experiment from the amount of ATP in the medium, the ATP obtained via oxidative phosphorylation could be approximated.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 ×</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S.a. extramitochondrial ATP</td>
<td>25</td>
<td>73</td>
<td>58</td>
<td>53</td>
<td>50</td>
<td>63</td>
<td>50</td>
<td>59</td>
<td>26</td>
<td>26</td>
<td>41*</td>
</tr>
<tr>
<td>S.a. intramitochondrial ATP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 × ATP by oxidative phosphorylation</td>
<td>31</td>
<td>78</td>
<td>57</td>
<td>63</td>
<td>42</td>
<td>73</td>
<td>58</td>
<td>59</td>
<td>30</td>
<td>26</td>
<td>40</td>
</tr>
</tbody>
</table>

| Total ATP     |    |    |    |    |    |    |    |    |    |    |    |

* This experiment was with added AMP plus ATP; hence that figure = 100 × (s.a. of extramitochondrial ADP)/(s.a. of intramitochondrial ADP).

the same reasoning, Bartley and Davies (16), in their successful attempt to show concentration gradients between mitochondria and medium, employed centrifugation methods in which a large gravitational force was reached in a very short time and kept for a very short time in order to minimize process (b). In their process (a), these authors incubated at 20°, while in our process (a) we could, owing to the very large amounts of mitochondria used, incubate at 0° during the centrifugation.

A second type of possible artifact might arise if there were two or more kinds of mitochondria (17–19) in our preparation, the lighter ones containing relatively more adenylate kinase activity than the heavier ones. In this case an apparent separation of adenylate kinase activity from oxidative phosphorylation might occur. However, a variety of experiments
indicated that during the incubation-centrifugation of our mitochondrial preparation both adenylate kinase and oxidative phosphorylation activities came down together, and, moreover, the final supernatant medium contained no determinable adenylate kinase activity.

Intramitochondrial Dilution of Exogenous Inorganic Phosphate—In all experiments except one it was found that the specific activity of the intramitochondrial IP was less (sometimes by as much as 40 per cent) than that of the medium IP, confirming Crane and Lipmann (20) and Bartley and Davies (16). However, when AMP was added initially (20), the specific activities of the intra- and extramitochondrial ADP were approximately equal, and the specific activity of the ADP was the same as that of the intramitochondrial IP, in conformity with results with brain mitochondria (21), but not the same as that of the extramitochondrial IP, as was found previously (20). It would appear that there is a dilution of the added IP32 by non-radioactive mitochondrial phosphate, and it is this pool of phosphate which is tapped preferentially during oxidative phosphorylation. It has been found that in the 12 equivalent gm. of Mt used there was 1 to 2 μmoles of inorganic orthophosphate present initially.6 Considering that in some experiments more than 17 μmoles of ATP were synthesized via oxidative phosphorylation and at the same time there is a dilution of some 50 per cent in the specific activity of the intramitochondrial IP, this small amount of mitochondrial inorganic orthophosphate could not account for the large continuing dilution observed.6 We also do not think that ATP is a source of this phosphate, as elsewhere postulated (16), since, when AMP is the initial nucleotide, the specific activities of extra- and intramitochondrial ATP are equal, no non-radioactive ATP is formed, and there still exists the intramitochondrial dilution of the IP. The only large sources of mitochondrial phosphate able to account for the dilution are the phospholipide and phosphoprotein fractions (22, 23), but the rôle of these fractions has not been studied in detail.7

6 After oxidative phosphorylation had taken place, the inorganic orthophosphate content of the mitochondria rose to 4 to 8 μmoles per 12 equivalent gm. of Mt. Thus the mitochondria can apparently increase their IP concentration (16), in contrast to the adenine nucleotides (6).

6 Even in experiments run at 25°, when approximately 70 μmoles of ATP were synthesized oxidatively, there was still a dilution of the radioactivity of the intramitochondrial IP of approximately 20 per cent.

7 It has been found that the phosphates of both phosphoproteins (24) and phospholipides (25) are very rapidly turned over in respiring mitochondria. We have found (unpublished observations) that, upon incubation of mitochondria in isotonic sucrose at 30°, all of the initial inorganic orthophosphate was lost from the mitochondria and no acid-insoluble phosphate fraction was lowered; when incubated with 0.01 M phosphate buffer (pH 7.2), approximately 20 per cent of the phospholipide phosphate and phosphoprotein phosphate was lost from the mitochondria (cf. (6));
Action of 2,4-Dinitrophenol—The specific activities obtained when various concentrations of DNP were added to the oxidative medium containing ADP are presented in Table V. As the concentration of DNP was increased, with increasing uncoupling of phosphorylation, the specific activities of both intra- and extramitochondrial ADP and ATP decreased (cf. (12, 21, 27, 28)). This effect of DNP also took place if AMP was substituted for ADP in the oxidative medium. The results would agree with the formulation of Hunter (29), of Lee and Eiler (30), and of Lardy and Wellman (28) in that little of the radioactive IP would be presumed to appear in ATP (under conditions of complete inhibition), but that an intermediate between IP and ATP (Y-PO₄ of Hunter (29)) would be broken down by DNP (see also (31–36)). The decrease in the specific activity of the intramitochondrial IP produced by DNP (also found by Abood (21)), small as it is, might reflect the discharge phenomenon of Teply (32), in that non-radioactive phosphate from some other source (“gel phosphate”)

when incubated with DNP, the IP was lost completely, and approximately 40 per cent of the phospholipide phosphate and phosphoprotein phosphate was lost. Phospholipide and phosphoprotein phosphates were determined by the method of Schneider (26).
of Teply (32)) would dilute the added radioactive IP. This effect would also be comparable to the “replacement” of added IP postulated by Loomis and Lipmann (33) and to the “sparing” of added IP postulated by Judah (34).

Site of Action of Added Hexokinase—In a previous paper (2), it was postulated that added yeast hexokinase acted in or on the mitochondria

Table VI

Effect of Exogenous Hexokinase on Specific Activities and Quantities of Intra- and Extramitochondrial Nucleotides and of Glucose-6-phosphate

Experimental conditions as for Table I, except that approximately 60 μmoles of ADP and 0, 20, and 40 μmoles of glucose were added to the appropriate flasks with the hexokinase solution. The G6P was isolated as described in “Experimental,” and its specific activity is given as P22 c.p.m. per μmole of G6P determined by the anthrone test. The total ATP synthesized by oxidative phosphorylation could be calculated (assuming no breakdown of the G6P) to be 17.5 μmoles with zero hexokinase, 12.1 μmoles with 2 ml. of hexokinase, and 12.0 μmoles with 4 ml. of hexokinase.

<table>
<thead>
<tr>
<th>Hexokinase added</th>
<th>S. a.</th>
<th>μmoles</th>
<th>S. a.</th>
<th>μmoles</th>
<th>S. a.</th>
<th>μmoles</th>
<th>S. a.</th>
<th>μmoles</th>
<th>Ad-X</th>
<th>S. a.</th>
<th>μmoles</th>
<th>S. a.</th>
<th>μmoles</th>
<th>G6P</th>
<th>μmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>ml.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 Intramitochondrial</td>
<td>3200</td>
<td>5.3*</td>
<td>0</td>
<td>1.59</td>
<td>3480</td>
<td>0.94</td>
<td>6430</td>
<td>0.83</td>
<td>0</td>
<td>0.83</td>
<td>Trace</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extramitochondrial</td>
<td>6640</td>
<td>48.7*</td>
<td>0</td>
<td>10.4</td>
<td>1460</td>
<td>18.2</td>
<td>3420</td>
<td>27.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Intramitochondrial</td>
<td>3620</td>
<td>7.1*</td>
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* Estimated through the radioactivities of all the tubes in the IP peak.

in its stimulation of respiration. Since we have found conditions (Tables I, II, V) in which the intra- and extramitochondrial ATP can be differentiated, by the differences in specific activity, it was possible to delineate more closely the site of action of hexokinase. Table VI shows the result of an experiment in which G6P was collected as described in “Experi-

8 It could be that DNP acts to prevent the initial “activation” of added IP by preventing its possible pooling with other sources of mitochondrial phosphate (see above), but the figures in Table V would seem to rule this out, since the radioactive dilution of intramitochondrial IP is still noted in the presence of DNP.
mental" and its specific activity compared with that of the intra- and extramitochondrial ADP and ATP. Since there is only a small breakdown of the G6P\(^9\) once it is formed, its final measured specific activity is an integration of its initial specific activity of 0, from initially unlabeled ATP, to that of its maximal specific activity, from the radioactive ATP at the end of the experiment. Therefore, since the specific activity of the G6P fell between those of the high energy phosphates of the intra- and extramitochondrial ADP and ATP, it must mean that a preponderance of the phosphate of G6P must have come from the more highly radioactive intramitochondrial ATP. It can be seen from Table VI that, unlike the action of DNP (Table V), the presence of the hexokinase system produces only small changes in the intramitochondrial nucleotides, including ATP. This indicates that the added hexokinase does not act on the intramitochondrial ATP (6, 40), but probably acts at the surface of the mitochondria, mostly on the ATP which has been formed within the mitochondria and is coming out into the medium (see Fig. 2). Ernster and Lindberg (41) have also postulated that the increased activity of hexokinase when mitochondria were added to the mitochondria could be split, and it could be calculated that in the centrifugation-incubation conditions employed approximately 2.5 \(\mu\)moles of G6P would have been broken down.

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**Fig. 2.** Representation of biochemical structure of rat liver mitochondrion in terms of oxidative phosphorylation mechanisms.

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\(^9\) Even though the impure yeast hexokinase preparation had inorganic pyrophosphatase activity, there is far too little inorganic pyrophosphate in the flasks, or likely to be formed, to account for the observed inorganic phosphate output. Also, even though it has been found that the glucose-6-phosphatase of liver is associated with the microsome fraction (37), the presence of some activity in the mitochondria (38, cf. (39)) could be significant in view of the larger amounts of mitochondria used. Thus, it was found that G6P added to the mitochondria could be split, and it could be calculated that in the centrifugation-incubation conditions employed approximately 2.5 \(\mu\)moles of G6P would have been broken down.
are added to it might be due either to an activation of the enzyme by mitochondria or else that hexokinase acts on the mitochondrial surface, while Slater and Holton (42) found that free ATP formed from the endogenous ADP of heart muscle sarcosomes is capable of reacting with soluble proteins like hexokinase.

To test the possibility that yeast hexokinase added to a mitochondrial suspension might in part come down with the mitochondria upon centrifugation, experiments were performed in which hexokinase was mixed with mitochondria, and, after immediate centrifugation, the activity of the added hexokinase was compared with the activity in the mitochondria and medium fractions. Only 90 per cent of the added hexokinase was recoverable in the medium, the other 10 per cent coming down with the mitochondria. Similar results showing absorption of added acid phosphatase by mitochondria, microsomes, and nuclei have been found by Berthet et al. (38). However, this 10 per cent of the added yeast hexokinase activity associated with the centrifuged mitochondria had from 3 to 9 times (according to the amount of hexokinase added) the activity of the added hexokinase (cf. (41)). It was found that the increased activity of this mitochondrially bound hexokinase was associated with a marked production of IP₃, which was proportional to the amount of the hexokinase originally added to the mitochondrial suspension. Since under these conditions it has been found that the mitochondria have very little ATPase activity, this IP₃ output may well have come from the breakdown of G6P. The reason for the increased activity of the hexokinase bound to the mitochondria is not known, but it might be due either to the increase in local concentrations of the reactants or to a relief of possible enzyme inhibition by the products (reports on yeast hexokinase inhibition by G6P are contradictory (43, 44)). It was also noted that insulin added to the mitochondrial suspension along with yeast hexokinase had no effect on the observed binding of the enzyme by the mitochondria.

Effect of Fluoride on Specific Activities of Intra- and Extramitochondrial Nucleotides—Since fluoride has been found to inhibit mitochondrial adenylate kinase (3–5), the action of F on the specific activity of the various nucleotides was tested. In the presence of IP₃ and with either AMP or ADP as the initial nucleotide, no effect of 0.01 M F was noted on either the amount of oxidative phosphorylation observed or on the specific activity of the intra- and extramitochondrial nucleotides. It is not clear therefore that F acts in inhibiting oxidative phosphorylation under certain conditions (3, 6) solely by its observed inhibition of adenylate kinase. The observed

10 In contrast to the results of Crane and Sols (43), we have found very little liver hexokinase activity associated with the mitochondrial fraction.

11 In extension of experiments mentioned earlier (5), we have found that the inhibition of adenylate kinase by 0.01 M F with much smaller amounts of mitochondria
lack of phosphorylation with AMP when the adenylate kinase of rat liver mitochondria was inhibited by F (3) or when rat heart muscle suspensions with low adenylate kinase activity were used (42), and the lag period of phosphorylation observed when AMP is added to a system with active adenylate kinase (4, 42), could be due to a necessity of activating AMP for entry into the site of mitochondrial phosphorylation. This might mean that, when ADP is added, the adenylate kinase at the mitochondrial surface (see Fig. 2) produces not AMP, but an AMP derivative, and the necessity for adding equimolar amounts of F to bind the derivative could explain the observed variations in the inhibitory effects of F.

DISCUSSION

Deductions regarding Structure of Rat Liver Mitochondria—From the results of Paper I (6) and of this paper, an attempt has been made to devise a representation of the biochemical structure of the mitochondria in terms of their oxidative phosphorylation mechanisms (Fig. 2). This diagram is based on the assumption that the two enzyme activities (adenylate kinase and oxidative phosphorylation) which come down together in the centrifuge are indeed present in the same mitochondrion. The intramitochondrial nucleotides are represented as being bound (as \([ATP]\)) to some site within the mitochondrion (6). The mitochondria are represented as having an inner and an outer zone which could be interpreted to conform with the electron micrographs by Palade (45). The two sites of adenylate kinase postulated are shown on the diagram as being in the mitochondrial membrane or outer zone and also within the mitochondria at the site of the oxidative phosphorylation mechanism. It should again be mentioned that DNP is presumed to act between the hypothetical phosphate intermediate and \([ATP]\) at the site of oxidative phosphorylation (Table V), that any ATPase activity of the mitochondria presumably takes place not at the surface of the mitochondria but within the mitochondria (Table III), as distinct from that of the ATPase of the yeast cell (46), and that added hexokinase acts mostly on or in the surface of the mitochondria on the ATP which was produced within the mitochondria and which is moving out into the medium (Table VI).

The data clearly show that during mitochondrial oxidative phosphorylation the turnover of phosphate in the intramitochondrial adenine nucleo-

(25 equivalent mg. of Mt) varies from 10 to 100 per cent in different experiments. Furthermore, in those oxidative experiments in which F lowered the P:O ratio when AMP was the initial nucleotide (3), there was no inhibition of the simultaneously measured adenylate kinase reaction, in either direction. In those experiments in which F did inhibit adenylate kinase, the inhibition was shown to be non-competitive in nature. In this connection the preincubation effect of F should also be noted (6).
tides under the conditions of the experiments mentioned is more rapid than the diffusion of the nucleotides in and out of the mitochondria. This conclusion is in agreement with that reached in an earlier paper (2) under different experimental conditions. Since it has been found ((6), see above) that the concentration of each of the adenine nucleotides within the mitochondria remained constant during active oxidative phosphorylation, it is possible that the rate of oxidative phosphorylation is regulated not only by the intramitochondrial (2), or extramitochondrial (2, 35, 40, 47, 48), turnover of the adenine nucleotides, but also by the rate of entry of phosphate acceptor, in the form of some adenine nucleotide, into the mitochondria. This regulation of the rate of entry might well be the function of the surface adenylate kinase (Fig. 2). The equilibrium constant of the adenylate kinase reaction can be calculated to be close to 1 (5), and, therefore, this enzyme could function effectively in the capacity suggested, since any small changes in concentrations of any of the reactants would shift the reaction very quickly. Reactions which tend to use ATP and produce ADP would, as a result of adenylate kinase action, make AMP or its product available for entry into the oxidative phosphorylation site. On the other hand, if ATP piled up, the adenylate kinase reaction would be shifted towards the formation of ADP; since this would reduce the amount of AMP present, it would reduce nucleotide entry and phosphorylation. This rate of entry of AMP into the mitochondria could well be the immediate means of the regulation of mitochondrial oxidation. Somewhat the same view has been expressed by Lindberg and Ernster (49), in that they postulated that AMP was indispensable for the maintenance of the generation of phosphate bond energy.

There is considerable evidence (see the review by Schneider (19)) that mammalian mitochondria contain a semipermeable, perhaps double (44, 45), membrane. It is conceivable that the enzyme adenylate kinase is a protein comprising part of this membrane. If we make the assumption that the membrane has little permeability to the adenine nucleotides, then this vantage point for adenylate kinase would confer a more specific and intimate means of regulating the oxidative rate of mitochondria.

Bartley and Davies (16) reported that mitochondria can accumulate Na⁺, K⁺, and various substrates, while these authors (16) and we have found that the mitochondria can increase their concentration of IP. However, in the presence of AMP or ADP, non-oxidizing mitochondria can bring in, but cannot accumulate, these nucleotides, and oxidizing mitochondria cannot retain the ATP which they have synthesized from these nucleotides. In other words, the mitochondria act like a secretory body for the production of ATP. They can take the ATP from the intramitochondrial site of formation and use it at other sites within the mitochondria...
for synthetic work (2) and for the transport of solutes across their membranes (50), or they can pass the ATP out to the rest of the cell for synthetic use there. It may be noted that recent reports by Harman and coworkers (51, 52) question the occurrence of mitochondrial membranes in the morphological sense. If their views are substantiated and extended to the mitochondrial material studied here, it will be desirable to consider what type of mitochondrial structure could yield the behavior found in the biochemical studies. The idea of inner zones and outer zones in the mitochondrial substance, which has been arrived at by purely biochemical studies, may prove to be an acceptable picture to both schools of morphologists. This view of mitochondrial structure appears to receive support from the recent studies of Glimstedt et al. who refer to matrix substance (53) and a granulated inner body (54) in rat liver mitochondria, in addition to an outer membrane.

**SUMMARY**

1. Mitochondria which had been carrying out oxidative phosphorylation in the presence of radioactive inorganic phosphate were separated from the incubation medium, and the specific activities of the intra- and extramitochondrial adenine nucleotides were determined.

2. It has been suggested from the results of these and similar experiments that there may be two sites of mitochondrial adenylate kinase activity, one at the surface of the mitochondria and the other at the site of oxidative phosphorylation within the mitochondria.

3. The results would indicate that during oxidative phosphorylation the turnover of the phosphates of mitochondrial ADP and ATP was greater than the passage of these compounds in and out of the mitochondria.

4. The data indicated that added inorganic phosphate mixes in the mitochondria not only with inorganic phosphate but with some other source of phosphate, and this mixture constitutes a pool of phosphate which is drawn upon during oxidative phosphorylation.

5. The results indicate that dinitrophenol acts on some phosphate intermediate between inorganic phosphate and adenosine triphosphate.

6. Added yeast hexokinase has been shown to act in part at the surface of the mitochondria upon the adenosine triphosphate which has been formed within the mitochondria and which is being released into the medium.

7. A scheme of the biochemical structure of the mitochondria as regards oxidative phosphorylation is presented, and the rôle of adenylate kinase is discussed in relation to the regulation of mitochondrial oxidation.

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