THE FORMATION OF PHOSPHOLIPID BY THE HEPATECTOMIZED DOG AS MEASURED WITH RADIOACTIVE PHOSPHORUS

I. THE SITE OF FORMATION OF PLASMA PHOSPHOLIPIDS*

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Hevesy (1, 2) and Artom (3) have inferred from studies on the incorporation of labeled inorganic phosphate into phospholipid that plasma phospholipid is formed largely in the liver. This inference was based on a comparison of the specific activities of phospholipid phosphorus of plasma with those of other organs. Similar observations have been made in this laboratory (Table I). The phospholipid P\textsuperscript{32} per gm. of total phospholipid of several tissues of the dog was determined at 6, 18, 36, and 98 hours after the intraperitoneal injection of inorganic P\textsuperscript{32} (Table I). At the first interval the phospholipid P\textsuperscript{32} per gm. of phospholipid of the liver was higher than that of any other tissue. At the 18 hour interval the values for liver and plasma were about the same and higher than for small intestine, kidney, and muscle. At 36 hours the values for plasma and liver were still about the same, but higher than those for other tissues. At 98 hours the values for kidney and small intestine approximated those for liver and plasma.

Although the above observations do not contradict the view that the liver is the source of plasma phospholipids, interpretations based on comparisons of specific activities of phospholipid phosphorus are open to the criticism that they do not deal with a single substance but with a mixture of several different compounds. The following limitation should be noted even in the case of a single substance: the finding that the specific activity of an organ's lecithin P is lower than that of plasma serves to exclude that organ as the principal source of the plasma lecithin, but the observation that the specific activity of an organ's lecithin P is higher than that of plasma means only that the organ may be the source of the plasma lecithin. More definite criteria by which an organ can be determined as a source of plasma lecithin or cephalin, etc., were pointed out by Zilversmit et al. (4);

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the reasoning applied by them for the establishment of an immediate pre-
cursor holds equally well for the determination of an organ as source.

Evidence, other than the above, cited in support of the view that the
liver is the source of plasma phospholipid is of doubtful value. It is ques-
tionable whether the data of Nedswedsky and Alexandry support their
claim that the venous blood leaving the liver has a higher phospholipid
content than that of the portal vein (5). Leites' data show no consistent
differences in the phospholipid contents of the blood of portal vein, hepatic
vein, and of arterial blood (6). Zilversmit et al. have shown that in the
postabsorptive state 150 mg. of phospholipid are turned over per hour in
the plasma of an 8 kilo dog (7). Even if liver phospholipid were the sole
source of the plasma phospholipid being turned over at this rate, it is not
likely that measurable differences could be detected between the phospho-
lipid contents of the efferent and the afferent blood of the liver.

A more direct attack on the problem of the liver's rôle in the production
of plasma phospholipid is provided by the use of the liverless animal. In
the present investigation the capacity of the hepatectomized dog to synthe-
size phospholipid was tested with the aid of radioactive phosphorus.

Experimental Procedures

Normal Dogs (Table I)—The normal dogs were fed twice daily a mixture
of lean meat, sucrose, bone ash, Cowgill's salt mixture (8), and vitamin con-
centrates. The feeding of this diet was begun a week before the $\text{P}^{32}$ was
injected and was not interrupted during the course of the experiment.
$\text{P}^{32}$ was injected intraperitoneally as an isotonic solution of $\text{Na}_2\text{HPO}_4$ (14.6
mg. of $\text{Na}_2\text{HPO}_4$ per cc.) containing about 0.5 to 1.0 millicurie of radioac-
tive phosphorus. Each dog received from 1 to 30 cc. of this solution.
The dogs were sacrificed by an intracardiac injection of nembutal (30 mg.
per kilo). Blood for analysis was removed from the femoral artery just
before the administration of the nembutal, while tissues were rapidly ex-
cised immediately after its administration.

Hepatectomized and Control Dogs (Table II)—The livers of dogs were
excised by a modification of the one stage operation of Firor and Stinson
(9). In the dissection of the lower part of the vena cava as well as during
the insertion of the cannula, it was necessary to exteriorize the intestines.
They were protected, however, by application of warm saline packs. The
abdomen was not closed until the intestinal circulation was functioning
normally, as evidenced by the color of the intestines. In no case was the
caval or portal circulation occluded for more than 15 minutes, the usual
period being 5 to 10 minutes.

The hepatectomized dogs were kept in a warm environment. They re-
ceived intravenously at 30 minute intervals 5 gm. of glucose dissolved in
20 cc. of isotonic saline. Blood for analyses was removed by heart puncture.

In two experiments (Dogs FH10 and FH14) 250 cc. of cream were fed 2 hours before hepatectomy. In all other experiments the dogs were in the postabsorptive state, the last meal having been ingested 18 hours before hepatectomy.

The control dogs were subjected to a sham operation; i.e., manipulation of the abdominal contents without removal of the liver. The animals were anesthetized with ethyl ether, their abdomens opened, and the visera sub-

### Table I

Recovery of Intraperitoneally Injected P³² As Phospholipid P³² of Plasma and Tissues of Normal Dogs

<table>
<thead>
<tr>
<th>Dog No.</th>
<th>Dog weight</th>
<th>Time after infusion</th>
<th>Organ weight</th>
<th>Phospholipid content at end of experiment, mg. per 100 gm. tissue</th>
<th>Recovery of phospholipid P³², as per cent of injected P³² per gm. phospholipid†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>hrs.</td>
<td>Liver</td>
<td>Kidney</td>
<td>Small intestine</td>
</tr>
<tr>
<td>15</td>
<td>17.1</td>
<td>6</td>
<td>326</td>
<td>44</td>
<td>216</td>
</tr>
<tr>
<td>16</td>
<td>9.6</td>
<td>6</td>
<td>308</td>
<td>41</td>
<td>226</td>
</tr>
<tr>
<td>14</td>
<td>15.0</td>
<td>18</td>
<td>284</td>
<td>57</td>
<td>198</td>
</tr>
<tr>
<td>7</td>
<td>10.5</td>
<td>18</td>
<td>213</td>
<td>37</td>
<td>278</td>
</tr>
<tr>
<td>9</td>
<td>9.8</td>
<td>18</td>
<td>217</td>
<td>42</td>
<td>200</td>
</tr>
<tr>
<td>10</td>
<td>18.7</td>
<td>18</td>
<td>315</td>
<td>49</td>
<td>265</td>
</tr>
<tr>
<td>5</td>
<td>9.5</td>
<td>36</td>
<td>292</td>
<td>52</td>
<td>276</td>
</tr>
<tr>
<td>6</td>
<td>10.2</td>
<td>36</td>
<td>246</td>
<td>50</td>
<td>266</td>
</tr>
<tr>
<td>17</td>
<td>7.0</td>
<td>98</td>
<td>228</td>
<td>27</td>
<td>201</td>
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<tr>
<td>19</td>
<td>7.0</td>
<td>98</td>
<td>243</td>
<td>32</td>
<td>136</td>
</tr>
<tr>
<td>20</td>
<td>6.0</td>
<td>98</td>
<td>225</td>
<td>46</td>
<td>190</td>
</tr>
</tbody>
</table>

* Labeled Na₂HPO₄ injected intraperitoneally.

† To obtain specific activities, i.e., phospholipid P³² phospholipid P³², multiply the values by 25.

jected to an amount of manipulation similar to that involved in hepatectomy (about 1 hour). The abdomen was then closed.

In experiments recorded in Table II the dogs received intravenously (by femoral vein) from 2 to 10 cc. of an isotonic solution of Na₂HPO₄ (14.6 mg. of Na₂HPO₄ per cc.) containing approximately 1 millicurie of P³². The P³² was injected 15 to 30 minutes after the closure of the abdomen. Blood for analyses was removed by heart puncture.

**Treatment of Tissues**—Blood was transferred to 15 cc. centrifuge tubes containing about 5 mg. of a heparin-urea mixture (1:19 parts). The
blood was then centrifuged for 10 minutes in a constant speed centrifuge at 3500 R.P.M. and the plasma removed.

Uniform samples of the liver were obtained by repeated grinding of the whole liver after all the gallbladder had been excised. The kidneys minus the pelves were also repeatedly ground before samples were taken. The gastrocnemius muscles were similarly treated after removal of visible fat.

Table II
Recovery of Intravenously Injected $^{32}P$ As Phospholipid $^{32}P$ of Plasma and Tissues of Hepatectomized Dog

<table>
<thead>
<tr>
<th>Dog</th>
<th>Weight</th>
<th>Type of operation</th>
<th>Time killed after per injection</th>
<th>Phospholipid content at end of experiment, mg. per 100 gm. tissue</th>
<th>Recovery of phospholipid $^{32}P$, as per cent of injected $^{32}P$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>kg.</td>
<td></td>
<td>hrs.</td>
<td>Plasma</td>
<td>Kidney</td>
</tr>
<tr>
<td>FH1</td>
<td>18.0</td>
<td>Hepatectomy</td>
<td>2</td>
<td>270</td>
<td>2380</td>
</tr>
<tr>
<td>FH2</td>
<td>17.7</td>
<td>&quot;</td>
<td>3</td>
<td>163</td>
<td>2030</td>
</tr>
<tr>
<td>FH10</td>
<td>20.2</td>
<td>&quot;</td>
<td>4</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>FH14</td>
<td>20.0</td>
<td>&quot;</td>
<td>4.5</td>
<td>210</td>
<td></td>
</tr>
<tr>
<td>FH3</td>
<td>22.0</td>
<td>&quot;</td>
<td>4.5</td>
<td>344</td>
<td>1820</td>
</tr>
<tr>
<td>FH4</td>
<td>22.2</td>
<td>&quot;</td>
<td>6</td>
<td>153</td>
<td>1470</td>
</tr>
<tr>
<td>FH5</td>
<td>19.8</td>
<td>&quot;</td>
<td>6</td>
<td>204</td>
<td>1870</td>
</tr>
<tr>
<td>FH6</td>
<td>20.0</td>
<td>&quot;</td>
<td>6</td>
<td>315</td>
<td>1860</td>
</tr>
<tr>
<td>FC1</td>
<td>19.5</td>
<td>Sham operation $^\dagger$</td>
<td>6</td>
<td>232</td>
<td>2530</td>
</tr>
<tr>
<td>FC2</td>
<td>19.5</td>
<td>&quot;</td>
<td>6</td>
<td>282</td>
<td>2040</td>
</tr>
</tbody>
</table>

* Both kidneys in these dogs weighed 82 gm. (FH2), 116 gm. (FH3), 96 gm. (FH4), 82 gm. (FH5), 101 gm. (FH6), 82 gm. (FC1), 76 gm. (FC2).
† The small intestine in these dogs weighed 327 gm. (FH2), 430 gm. (FH3), 352 gm. (FH4), 360 gm. (FH5), 309 gm. (FH6), 427 gm. (FC1), 350 gm. (FC2).
‡ Fed 250 cc. of cream 2 hours before the start of the operation. All other dogs recorded in this table were in the postabsorptive state; viz., they received their last meal 18 hours before the start of the operation.
§ For a description of the sham operation see the text.

and connective tissue. The small intestine was freed of mesenteric fat and carefully washed before grinding. From 10 to 20 gm. of these tissues were usually taken for analysis.

Determination of Radiophospholipid of Plasma—5 cc. samples of plasma were added to 150 cc. of Bloor's solution (3 parts of 95 per cent ethyl alcohol to 1 part of ethyl ether) and extracted for 2 hours at 56°. The extract was decanted and the residue reextracted for 1 hour with 120 cc. (24 vol-
umes) of Bloor's solution. The mixture was then filtered. The residues and filter paper were further extracted in a Soxhlet apparatus for 8 hours with ethyl ether. The alcohol-ether and ether extracts were then combined in a Kjeldahl flask, and 1.0 cc. of a saturated Na₂HPO₄ solution as well as 50 mg. of finely ground Na₂HPO₄ was added. This was done in order to "dilute" any inorganic P³² which may have been carried into the extracts. The combined extracts were then concentrated under a vacuum at 56° to a volume of about 1 cc., the last 20 to 30 cc. being removed under an atmosphere of CO₂. 20 cc. of ethyl ether were now added to the Kjeldahl flask, and the mixture thoroughly agitated and transferred to a glass-stoppered Erlenmeyer flask with a small side arm. This type of flask has been described elsewhere (10). The supernatant ether layer was poured off, the lower phase being caught in the side arm. The latter was then re-extracted twice and these extracts combined in an Erlenmeyer flask that also had a side arm.

In order to remove any remaining inorganic P³² the volume of the combined extracts was reduced to about 15 cc. on a hot water bath (about 50°), and an excess of Na₂HPO₄ was again added, both as the saturated solution and as the powdered salt. The flask was then stoppered and the mixture violently agitated for 15 minutes; the ether phase was separated from the water phase by means of the side arm and the latter extracted twice with ethyl ether. The water phase was then tested for radioactivity. If the amount of radioactivity contained in the water phase was not reduced to negligible quantities, a second "washing" with Na₂HPO₄ was carried out. In most cases the washing procedure served only as a precautionary measure, since the previous steps were sufficient to effect a complete separation of the phospholipid P³² from non-phospholipid P³².

The ether extract was then made up to volume and a suitable aliquot taken for precipitation of its phospholipids. The phospholipid P³² was measured in the manner previously described (11).

Determination of Total Phospholipid—A 5 cc. sample of plasma was treated as described above and brought to the stage where the extracts were concentrated in vacuo to a volume of about 1 cc. No phosphate was added before the extract was concentrated. The concentrate was then extracted with petroleum ether. Phospholipids were determined by the oxidative procedures recorded previously (12).

The procedures used for the determination of phospholipid of kidney, small intestine, and muscle have been described elsewhere (12).

Results

Recovery of Phospholipid P³² in Plasma, Kidney, and Small Intestine of Hepatectomized Dog (Table II)—Eight hepatectomized dogs were sacrificed
at various intervals after the intravenous injection of labeled Na$_2$HPO$_4$. The plasma, small intestine, and kidneys were then analyzed for phospholipid P$^{32}$ and total phospholipid.

*Plasma*—Dogs FC1 and FC2 served as controls for the hepatectomized animals. After their abdominal cavities were opened under ether anesthesia, they were subjected to visceral manipulation after the manner described above. The P$^{32}$ was introduced intravenously into these two dogs soon after their abdominal walls were sutured and the phospholipid P$^{32}$ of their plasma measured 6 hours later.

The recoveries of phospholipid P$^{32}$ are expressed as percentages of the injected P$^{32}$ per cc. of plasma and as percentages of the injected P$^{32}$ per gm. of plasma phospholipid. 6 hours after Dog FC1 was injected with P$^{32}$, the recovery of phospholipid P$^{32}$ was $170 \times 10^{-6}$ per cent per cc. of plasma or $72 \times 10^{-3}$ per cent per gm. of plasma phospholipid. Higher values were found in Dog FC2; namely, $318 \times 10^{-6}$ per cent per cc. of plasma and $110 \times 10^{-3}$ per cent per gm. of plasma phospholipid.

The values found in the hepatectomized dogs are in marked contrast to those of the controls. As late as 6 hours after the injection, the recovery of phospholipid P$^{32}$ was only $3$ to $7 \times 10^{-6}$ per cent per cc. of plasma or $2$ to $3 \times 10^{-3}$ per cent per gm. of plasma phospholipid (Dogs FH4, FH5, and FH6). The highest recovery of phospholipid P$^{32}$ in the entire series of eight hepatectomized dogs was found in Dog FH14; it amounted to $19 \times 10^{-6}$ per cent per cc. of plasma or $9 \times 10^{-3}$ per cent per gm. of plasma phospholipid. Even this maximum recovery is but a small fraction of the recoveries observed in the control animals.

*Kidney*—The recoveries of radiophospholipid in the kidney were about the same in the hepatectomized and control dogs. Thus in the control dogs the phospholipid P$^{32}$ recovered was 0.26 to 0.43 per cent per gm. of kidney phospholipid; in the hepatectomized dogs the values ranged from 0.18 to 0.40 per cent per gm. of phospholipid.

Approximately 0.5 per cent of the total P$^{32}$ injected into the dog had been incorporated into phospholipid in both kidneys of the hepatectomized dog. Since each dog received about $4 \times 10^8$ counts per minute of P$^{32}$, this means that about 2 million counts per minute were recovered from both kidneys as phospholipid P$^{32}$. In the entire plasma of the hepatectomized animal, only about 20,000 counts per minute were recovered as phospholipid P$^{32}$ or $5 \times 10^{-8}$ per cent of the injected P$^{32}$. The recoveries of phospholipid P$^{32}$ in both kidneys were about 100 times as great as in the entire plasma. These observations lead to the conclusion that kidney phospholipid is not readily available to the plasma.

*Small Intestine*—The recoveries of radiophospholipid in the small intestine did not differ widely in the hepatectomized and control dogs. That
there exists a barrier with respect to phospholipid between plasma and tissues such as kidney and small intestine is shown by the results obtained here. The phospholipid $\text{P}^{32}$ recovered in both kidneys plus small intestine amounted to approximately 1 per cent of the total $\text{P}^{32}$ injected or about 4 million counts per minute; yet (as noted above) very little phospholipid $\text{P}^{32}$ was recovered from the entire plasma of the hepatectomized dog.

**Phospholipid Contents of Plasma and Tissues of Hepatectomized Dog**—The phospholipid contents of plasma, kidney, and small intestine of the hepatectomized and control dogs are recorded in Table II. Values for normal dogs are shown in Table I. Significant amounts of phospholipids were present in the tissues of the dog 6 hours after excision of its liver.

**DISCUSSION**

The observation that radiophospholipid was recovered in the kidney and small intestine of the hepatectomized dog leaves no doubt that the liver is not the only site of phospholipid formation in the animal body. The recoveries of phospholipid $\text{P}^{32}$ per gm. of kidney phospholipid or per gm. of small intestine phospholipid in the liverless dog did not differ significantly from those found in the intact dog. Yet only negligible amounts of phospholipid $\text{P}^{32}$ were recovered in the plasma of the hepatectomized dog as late as 6 hours after excision of the liver. These results lead to the conclusion that plasma phospholipids are derived mainly from the liver.

The finding that in the hepatectomized dog the recovery of phospholipid $\text{P}^{32}$ per gm. of kidney phospholipid and per gm. of phospholipid in the small intestine is much higher than per gm. of plasma phospholipid is indeed striking. 6 hours after the injection of the labeled phosphate, the values for phospholipid $\text{P}^{32}$ per gm. of tissue phospholipid were about 100 times greater in the kidney than in the plasma. At this time the total radiophospholipid present in both kidneys and small intestine would, if delivered completely to the blood stream, raise the phospholipid $\text{P}^{32}$ per gm. of plasma phospholipid to a value of about 0.3 per cent of the injected $\text{P}^{32}$. It seems reasonable to conclude that the transfer to the plasma of phospholipids formed in the kidney and small intestine, if this occurs, is not a rapid process. But the results do not exclude a slow transfer. Evidence indicating that certain structures are but slowly permeable to phospholipid has been presented (13, 14). No such barrier for the transfer of phospholipids exists, apparently, between liver and plasma. McCarrell, Thayer, and Drinker (15) point out that the liver cells are bathed in blood plasma and in this respect are perhaps in a unique position in the mammalian body. It is this peculiar arrangement between the liver cells and plasma that probably accounts for the early appearance of radiophospholipid in the plasma of the intact animal and the almost complete absence of radiophospholipid.
in that of the hepatectomized dog. The differences in the barriers that exist between liver and plasma on one hand and between plasma and kidney or small intestine on the other hand are well shown in the experiment of Zilversmit et al. (7), in which plasma containing radiophospholipid was introduced into the bloodstream of the normal dog. The uptake of radiophospholipid by the liver greatly exceeded that by kidney and small intestine.\(^1\)

Although it is now clearly established from studies \textit{in vitro} that such tissues as liver, kidney, and brain can form both ester bonds of phosphate in the phospholipid molecule (10, 16, 17), the recovery of radiophospholipid in a given tissue of the hepatectomized or intact dog provides no information on the exact reaction carried out by the given tissue. The radiophospholipid or newly formed phospholipid in the kidney and small intestine of the hepatectomized dog is probably not derived from plasma, for the specific activities of the phospholipid found in the plasma of this preparation were much lower than those in the kidney and small intestine. Since in the hepatectomized dog practically no radiophospholipids were delivered to these tissues by the plasma, the recovery of phospholipid P\(^{32}\) in the kidney and small intestine does demonstrate that at least the last step in the formation of the phospholipid molecule can occur in each of these tissues \textit{in vivo}. This last step need not involve the formation of the ester bonds of phosphate. For example, it is conceivable that the conversion of inorganic P\(^{32}\) to glycerophosphorylcholine P\(^{32}\) takes place in one tissue and that this compound is then carried to another tissue where by the addition of fatty acids it is converted to phospholipid. No evidence in support of such a mechanism, however, exists at the present time.

\textbf{SUMMARY}

The rôle of the liver in the formation of plasma phospholipids was investigated. Inorganic P\(^{32}\) was injected into normal and hepatectomized dogs and its recovery as phospholipid P\(^{32}\) compared in these animals.

1. Plasma phospholipids are formed mainly in the liver. Excision of the liver reduced the recovery of phospholipid P\(^{32}\) of the plasma to very small quantities.

2. The recovery of phospholipid P\(^{32}\) in kidney and small intestine was not reduced by excision of the liver. Apparently phospholipids synthesized by these two tissues are not readily available to the plasma.

\(^1\)At the end of 5 hours, the liver contained approximately 1500 counts per minute per gm.; kidney and small intestine contained 600 and 300 counts per minute per gm., respectively.
BIBLIOGRAPHY

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