THE SITE OF THE SYNTHESIS OF HIPPURIC ACID AND PHENYLACETURIC ACID IN THE DOG*

BY ARMAND J. QUICK

WITH THE TECHNICAL ASSISTANCE OF MARY A. COOPER

(From the Department of Surgical Research, Cornell University Medical College, New York City)

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At present the view is commonly accepted that hippuric acid is produced chiefly by the kidney in most animals and exclusively by that organ in the dog. This conclusion is based largely on the well known work of Bunge and Schmiedeberg (1) who were able to demonstrate that hippuric acid was formed when blood containing benzoic acid and glycine was perfused through dog kidney. Furthermore, they failed to find hippuric acid after injecting sodium benzoate and glycine into dogs in which the renal blood vessels were tied, but could find it when the ureters were ligated and the vascular supply of the kidneys left intact. Although these findings appear to constitute almost conclusive proof that in the dog, the synthesis of hippuric acid is effected only by the kidney, Kingsbury and Bell (2) challenged these conclusions. They reported that after injecting sodium benzoate and glycine into a nephrectomized dog, they were able to find hippuric acid in the blood and other tissues. Unfortunately, they did not differentiate between combined benzoic acid and hippuric acid, which is a serious error inasmuch as in the dog the conjugation with glucuronic acid is the more prominent. In only one experiment did they record the actual isolation of hippuric acid without, however, giving any information as to the amount. Snapper, Grünbaum, and Neuberg (3) criticized their conclusions and proceeded to reinvestigate the problem. They corroborated the work of Bunge and

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Site of Hippuric Acid Synthesis

Schmiedeberg, Kochs (4), and Bashford and Cramer (5), finding that dog kidney on perfusion with sodium benzoate and glycine yielded hippuric acid. They further found that the same held true for the kidney of the human, of the pig, and to a limited extent of the sheep. They failed to find even a trace of hippuric acid in the tissues of nephrectomized dogs after injecting large doses of sodium benzoate and glycine. They concluded that, contrary to Kingsbury and Bell, they were able to establish the second conclusion of Bunge and Schmiedeberg: in the dog, hippuric acid is formed only in the kidney and in no other organ.

In species other than the dog, there is good evidence that the synthesis of hippuric acid is not confined solely to the kidney. Kühne and Hallwachs (6), in 1857, voiced the opinion that the synthesis took place within the vessels of the liver and in the presence of bile constituents. The reliability of their experimental work is subject, however, to grave doubt, and even in 1866 their conclusions were questioned by Meissner and Shepard (7) who considered the kidney as the more likely site for the synthesis. Their experimental results are, however, somewhat conflicting. They found, following the feeding of sodium benzoate, hippuric acid in the blood of a rabbit in which the renal blood vessels were ligated, but they also found it in the blood of rabbits and cats in which the portal vein had been tied. These observations are somewhat comparable to those made by Bunge and Schmiedeberg (1) on the frog. In this animal neither the removal of the kidney nor of the liver apparently stopped the synthesis of hippuric acid. Salomon (8) isolated hippuric acid from the blood of nephrectomized rabbits. In 1911, Friedmann and Tachau (9) found hippuric acid after perfusing a rabbit liver with blood containing sodium benzoate. Significantly, the addition of glycine did not appear to increase the production of hippuric acid.

Snapper and Grünbaum (10) made the interesting observation that phenylpropionic acid and phenylvaleric acid when perfused through dog kidney yielded hippuric acid, while phenylacetic acid and phenylbutyric acid gave phenylaceturic acid.

While the bulk of the evidence is in favor of the hypothesis that the synthesis of hippuric acid in the dog occurs only in the kidney, it was felt that the problem is of such profound theoretical importance that further study seems justified.
EXPERIMENTAL

Analytical Methods

Free Benzoic Acid in Blood—Defibrinated blood was treated by the original Folin-Wu method for the removal of blood proteins. The filtrate, after concentration by distillation under reduced pressure to approximately 40 cc., was made acid to Congo red and extracted with three 25 cc. portions of toluene. The combined fractions of toluene were washed with a slightly acidified saturated solution of sodium chloride, filtered through a dry filter, and titrated with 0.1 N sodium alcoholicate, phenolphthalein being used as indicator.

Hippuric Acid in Blood—The acidified filtrate after extraction with toluene was divided into 10 or 15 cc. portions and extracted with ether in a continuous extractor. On boiling off the ether, hippuric acid when present could easily be detected by its characteristically high refractive and glistening needle crystals. The hydrolysis of hippuric acid was effected with 20 per cent hydrochloric acid. The determination of glycine by the formol titration was the same as in previous studies (11). The reliability of the method is attested by the fact that normal blood shows no hippuric acid and that on mixing 100 mg. of hippuric acid with 100 cc. of blood a recovery of over 80 per cent can be obtained.

Hippuric Acid in Liver, Muscles, and Other Tissues—The tissue immediately after removal was passed through a meat grinder and extracted with boiling water. The aqueous extract was concentrated by boiling and then treated with 3 volumes of alcohol. The resulting precipitate was removed by centrifuging, and the clear alcoholic solution evaporated on a water bath to a thick syrup which was dissolved in a small amount of hot water, and made acid to Congo red. The precipitate which formed was removed by centrifuging, and the clear supernatant solution extracted with ether in a continuous extractor. The determination of hippuric acid was the same as before. On mixing 100 mg. of hippuric acid with one-half of a ground liver, a recovery of about 50 per cent was obtained. In no experiment was either hippuric acid or phenylaceturic acid found; indicating that even if present the amount must be exceedingly small.
No synthesis of hippuric acid could be demonstrated in a nephrectomized dog. This is in conformity with the observations of Bunge and Schmiedeberg and others, and furnishes additional evidence for the conclusion that in the dog the synthesis of hippuric acid occurs only in the kidney. The validity of this conclusion is especially well illustrated by a comparison of the results obtained on two dogs (Dogs 3 and 6) one with both kidneys removed, the other with ureters tied. After each had been given sodium benzoate and excess glycine intravenously, no hippuric acid could be found in the nephrectomized dog, but 81 mg. were found in the blood of the dog with the kidneys intact, but the ureters tied.

It will be noted that the concentration of hippuric acid in the blood is not only strikingly small, but also that it does not progressively increase even when the ureters are tied. This suggests that an equilibrium exists between the rate of synthesis of hippuric acid and its hydrolysis. It was previously shown that the conjugation of benzoic acid can best be explained by applying the law of mass action (12). The two basic equations are:

\[
\text{Benzoic acid} + \text{glycine} \rightleftharpoons \text{benzoylglycine (hippuric acid)} + \text{water}
\]
\[
\text{Benzoic acid} + \text{glycuronic acid} \rightleftharpoons \text{glycuronic acid monobenzoate} + \text{water}
\]

Since the ligation of the ureters stops the excretion of hippuric acid and glycuronic acid monobenzoate, these two products will tend to accumulate until the respective speeds of their hydrolysis will equal the speeds of synthesis when a condition of equilibrium will be established. Thus, it can readily be seen that hippuric acid cannot keep on accumulating in the blood but will reach a fixed maximum concentration which is necessarily small, since hippuric acid is present not only in the blood but is distributed through the other tissues of the body.

When the kidneys are removed, the mechanism for the synthesis of hippuric acid is destroyed, but the organism still possesses the power to hydrolyze hippuric acid, for apparently the hydrolytic enzyme is not localized in any one organ. Theoretically one can predict that on injecting hippuric acid, hydrolysis will begin immediately and go to completion since the mechanism for the reverse or synthetic process is absent. Ultimately only free benzoic acid and glycuronic acid monobenzoate should be found. This is
borne out by actual experiments. In one nephrectomized dog 2
gm. of hippuric acid given intravenously disappeared completely
in 24 hours, and both free benzoic acid and glycuronic acid mono-
benzoate could be detected in the blood stream (Dog 7).

On feeding sodium benzoate to a nephrectomized rabbit, hip-
puric acid was readily isolated from the blood, liver, and muscles.
This is in agreement with the observations of Salomon, and consti-
tutes conclusive proof that the synthesis of hippuric acid in the
rabbit is not, as in the dog, localized in the kidney. Friedmann
and Tachau have demonstrated by means of perfusion experiments
that the synthesis can take place in the liver, but it is not known
whether other organs can also produce hippuric acid.

Phenylaceturic acid was definitely isolated from the blood of a
nephrectomized dog following the injection of phenylacetic acid
(Dog 9). This proves that the synthesis of phenylaceturic acid
unlike that of hippuric acid does not take place solely in the kidney.
It is even probable that the kidney is not even the main site of the
synthesis in the normal animal, for on feeding phenylacetic acid
and excess glycine, phenylaceturic acid can be found in the blood
(Dog 10), indicating that the synthesis has been effected before
the phenylacetic acid reaches the kidney. Hippuric acid, in agree-
ment with the observations of Meissner and Shepard, is consist-
ently absent from the blood (Dog 11) unless it is dammed back
from the kidney, which occurs when the ureters are ligated.

Since a nephrectomized dog can produce phenylaceturic acid
but not hippuric acid, it must be concluded that these two com-
ounds require different enzymes for their synthesis. For the
sake of convenience, the enzyme which is responsible for the
conjugation of glycine with benzoic acid is named hippuricese,
while the one which effects the union of glycine with phenylacetic
acid is designated phenaceturicese. No logical explanation for their
peculiar distribution can be given. In the dog hippuricese is
present only in the kidney, while in the rabbit it is present in the
liver and possibly in the kidney and other organs. Phenace-
turicese, on the other hand, appears to be present both in the liver
and kidney of the dog. In the human the problem is more compli-
cated, for phenylacetic acid is conjugated with glutamine (13),
while some of the substituted phenylacetic acids are combined
with glycine (14).

The enzyme commonly called histozone, which hydrolyzes hip-
puric acid, is found in various organs of the dog. It seems quite
definite that this enzyme is entirely distinct from the synthetic
enzyme, hippuricese. It is also doubtful whether true histozyme
can hydrolyze phenylaceturic acid.

One cannot regard the presence of the conjugating enzymes in
the kidney as merely accidental; nor can one set aside as incidental
the fact that the higher phenyl-substituted aliphatic acids can
undergo β-oxidation within this organ as was shown by Snapper
and Grünbaum. These are probably important facts that must
be properly evaluated in attempting to arrive at a comprehensive
understanding of the function of the kidney.

SUMMARY

No hippuric acid was detected in the blood or liver of nephrec-
tomized dogs that had been given sodium benzoate and glycine,
but was found when the ureters were ligated.

On feeding sodium benzoate to nephrectomized rabbits, hippuric
acid was found in the blood, liver, and muscles.

After injecting phenylacetic acid into a nephrectomized dog,
phenylaceturic acid was isolated from the blood and definitely
identified.

It was found that on injecting hippuric acid into nephrectomized
dogs the compound gradually disappeared from the blood stream
and free benzoic acid and glycuronic acid monobenzoate appeared.
The data obtained on several dogs and a rabbit are given in the
accompanying protocols.

CONCLUSIONS

The synthesis of hippuric acid is effected by an enzyme, hip-
puricese, which in the dog is apparently present only in the kidney,
whereas the production of phenylaceturic acid is brought about by
a different enzyme, designated phenaceturicese, which occurs not
only in the kidney but also in some other organs. In the rabbit
hippuricese is not localized in the kidney, since the nephrectomized
animal can still synthesize hippuric acid.

The enzyme, histozyme, which hydrolyzes hippuric acid is
distinct from hippuricese, since the nephrectomized dog can still
hydrolyze but cannot synthesize hippuric acid.
BIBLIOGRAPHY


PROTOCOLS

All operations were performed under ether anesthesia. Benzoic acid, phenylacetic acid, and hippuric acid were always given as the sodium salt.

Fate of Benzoic Acid in Nephrectomized Dogs

Dog 1, weight 22 kilos. June 3, 10.00 a.m., 15 gm. of gelatin given by stomach tube; 11.00 a.m., bilateral nephrectomy; 11.30 a.m., 3 gm. of benzoic acid given intravenously; 7.00 p.m., 1 gm. of benzoic acid and 1 gm. of glycine fed. June 4, 9.30 a.m., 0.5 gm. of benzoic acid and 3 gm. of gelatin fed; 11.00 a.m., animal exsanguinated. 1000 cc. of blood obtained. Benzoic acid combined as hippuric acid found in total blood, 0.016 gm.

All attempts to isolate hippuric acid failed. The recorded analysis although expressed as hippuric acid probably represents impurities such as phenylaceturic acid and p-hydroxyphenylaceturic acid.

Dog 2, weight 13 kilos. October 1, 11.00 a.m., bilateral nephrectomy. October 2, 10.00 a.m., 2 gm. of benzoic acid, 15 gm. of gelatin, and 150 cc. of milk fed; 12.00 m., 0.5 gm. of benzoic acid, 2.5 gm. of gelatin, and 75 cc. of milk fed; 2.00 p.m., animal exsanguinated. 550 cc. of blood obtained. Benzoic acid combined as hippuric acid found in total blood, 0.008 gm.

Dog 3, weight 8.6 kilos. October 8, 11.00 a.m., bilateral nephrectomy; 11.30 a.m., 2 gm. of benzoic acid and 5 gm. of glycine given intravenously; 2.30 p.m., animal exsanguinated. 250 cc. of blood obtained. Benzoic acid combined as hippuric acid found in total blood, 0.003 gm.

Dog 4, weight 12 kilos. October 19, 10.00 a.m., bilateral nephrectomy. October 20, 10.00 a.m., 2 gm. of benzoic acid, 15 gm. of gelatin, and 150 cc. of milk fed; 12.00 m., 0.5 gm. of benzoic acid, 2.5 gm. of gelatin, and 75 cc. of milk fed; 2.00 p.m., animal exsanguinated. 550 cc. of blood obtained. Benzoic acid combined as hippuric acid found in total blood, 0.008 gm.
of milk fed; 12.30 p.m., 0.5 gm. of benzoic acid and 5 gm. of gelatin fed; 4.30 p.m., animal exsanguinated. 400 cc. of blood obtained. Benzoic acid combined as hippuric acid found in total blood, 0.008 gm.

In none of these four dogs was it possible to find or isolate crystals of hippuric acid by extracting the concentrated blood filtrate with ether.

**Fate of Benzoic Acid in Dogs with Ureters Ligated**

Dog 5, weight 8.5 kilos. June 22, 10.00 a.m., 15 gm. of gelatin given by stomach tube; 11.00 a.m., both ureters ligated; 11.30 a.m., 3 gm. of benzoic acid given intravenously; 7.30 p.m., 1 gm. of benzoic acid given orally. June 23, 10.00 a.m., animal exsanguinated. 425 cc. of blood obtained. Benzoic acid combined as hippuric acid found in total blood, 0.029 gm.

Dog 6, weight 9 kilos. October 5, 11.30 a.m., both ureters ligated; 12.30 p.m., 2 gm. of benzoic acid and 3 gm. of glycine given intravenously; 2.00 p.m., 70 cc. of blood withdrawn. Benzoic acid combined as hippuric acid found in total calculated volume of blood (470 cc.), 0.059 gm. October 6, 10.00 a.m., animal exsanguinated. 400 cc. of blood obtained. Benzoic acid combined as hippuric acid found in total blood, 0.034 gm.

Crystals of hippuric acid were found in the ether extract of the blood filtrate of both specimens.

**Fate of Injected Hippuric Acid in Nephrectomized Dogs**

Dog 7, weight 7 kilos. June 25, 11.00 a.m., bilateral nephrectomy; 11.30 a.m., 2 gm. of hippuric acid given intravenously. June 26, 11.00 a.m., animal exsanguinated. 175 cc. of blood obtained. Benzoic acid combined as hippuric acid found in total blood, 0.003 gm.

Free benzoic acid was present and the naphthoresorcinol test for glycuronic acid was strongly positive.

Dog 8, weight 12 kilos. October 13, 10.00 a.m., bilateral nephrectomy; 11.00 a.m., 3 gm. of hippuric acid given intravenously; 12.00 m., 25 cc. of blood withdrawn. Benzoic acid combined as hippuric acid found in total calculated volume of blood (325 cc.), 0.033 gm. Crystals of hippuric acid appeared in the ether extract. October 14, 10.30 a.m., animal exsanguinated. 300 cc. of blood obtained. Benzoic acid combined as hippuric acid found in total blood, 0.018 gm. No crystals of hippuric acid were found in the ether extract.

Free benzoic acid was found in the blood and liver.

**Fate of Phenylacetic Acid in a Nephrectomized Dog**

Dog 9, weight 10 kilos. June 26, 10.00 a.m., 15 gm. of gelatin given by stomach tube; 11.00 a.m., bilateral nephrectomy; 12.00 m., 3 gm. of phenylacetic acid given intravenously. June 27, 10.00 a.m., animal exsanguinated. 570 cc. of blood obtained. Phenylacetic acid combined as phenylaceturic acid found in total blood, 0.218 gm.

From the filtrate of 350 cc. of blood, 0.065 gm. of pure phenylaceturic acid was isolated. M.p. 141° uncorrected.
Presence of Phenylaceturic Acid and Hippuric Acid in Blood

Dog 10, weight 9 kilos. February 24, 2.30 p.m., 6 gm. of phenylacetic acid and 15 gm. of gelatin given by stomach tube; 6.30 p.m., animal exsanguinated. 450 cc. of blood obtained. Phenylacetic acid combined as phenylaceturic acid found in total blood, 0.062 gm.

Dog 11, weight 9 kilos. January 24, 10.00 a.m., 5 gm. of benzoic acid and 10 gm. of gelatin given by stomach tube; 2.00 p.m., animal exsanguinated. 400 cc. of blood obtained. Benzoic acid combined as hippuric acid found in total blood, 0.0 gm.

Fate of Benzoic Acid in Nephrectomized Rabbit

Rabbit 1, weight 2.25 kilos. January 16, 10.30 a.m., bilateral nephrectomy; 10.45 a.m., 0.25 gm. of benzoic acid and 0.5 gm. of glycine injected intravenously; 11.00 a.m., 1.5 gm. of benzoic acid and 15 gm. of gelatin given by stomach tube; 8.00 p.m., animal exsanguinated. Benzoic acid combined as hippuric acid found in 50 cc. of blood was 0.057 gm.; in 45 gm. of liver, 0.016 gm.; and in 500 gm. of muscle, 0.063 gm.

The characteristic crystals of hippuric acid appeared in the ether extract of each of the reported analyses.
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