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The Metabolism of Ortho-I^{131} Iodobenzoic Acid. I. Its Use As a Possible Liver Function Test[1,2]

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INTRODUCTION

The object of this preliminary study was to develop a test for liver function based on the conjugation of I^{131} labeled iodobenzoic acid to form I^{131} iodohippuric acid and other metabolites and to measure their urinary excretion.

Benzoic acid is detoxicated in man by forming hippuric acid and benzoyl glucuronide and analogously I^{131} labeled ortho-iodobenzoic acid forms the similar corresponding metabolites.

It was reported by Zieve and Hanson (1) that in man, benzoyl glucuronide appeared in the urine only after large doses of benzoate or when liver disease was present. Snapper and co-workers (2) had shown that in liver disease, decreased amounts of hippuric acid and increased amounts of benzoyl glucuronide were formed. In a group of 15 patients with liver disease, the recovered hippuric acid in 2 hours was equivalent to about ⅔ of the dose of 1.77 gms sodium benzoate given intravenously, this being 15 per cent less than the amount recovered in a similar period from normal adults (3).

Quick (4) based his test on the conjungation of sodium benzoate and reported that hepatic involvement resulted in a lower than normal excretion of hippuric acid.

The degree of conjugation with glycine and/or glucuronic acid varies with the particular isomer, *i.e.* ortho, meta or para (5) (6); ortho-halogen substituted acids being least conjugated with glycine, meta more and para most whereas the conjungation with glucuronic acid is in the reverse order, namely,

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the ortho-halogenated acid most, meta less and para least. Therefore, the ortho-iodobenzoic acid labeled with I^{131} was chosen for this preliminary study on the basis that if it were the most difficult to conjugate, then it might prove to be a more definitive test agent for liver function. Important in the excretion of the iodohippuric acid is the participation of the renal tubule in the transportation of these conjugates. In view of the small amount of sodium iodobenzoate used in our investigations, a slight impairment of kidney function should not prevent the total excretion of the metabolites (7).

MATERIALS AND METHODS

In the present study the I^{131} labeled ortho-iodobenzoic acid was used and this was prepared by exchange from NaI^{131} and unlabeled ortho-iodobenzoic acid (8). The specific activities obtained were variable but high and somewhat determined by the ratio of mM of acid used and the mc I^{131}. Typical preparations had specific activities up to 132 μc/mg. When freshly prepared and tested by descending chromatography using Whatman No. 1 paper and a solvent consisting of normal butanol:acetic acid:water 4:1:1 (9), showed an average Rf value of 0.93 for the I^{131} ortho-iodobenzoic acid, and an Rf of 0.16 to 0.18 for radioiodide. By quantitation of the radioiodide areas it was found that these constituted about 2 per cent of the total radioactivity but this was reduced drastically as described below.

For studies reported, a standard dose of 10 mg ortho-iodobenzoic acid as the sodium salt containing 25 μc I^{131} labeled acid, hereinafter referred to as OI*BA, was adopted. It was found most expeditious to prepare 2 solutions, one of the carrier, and the other, the OI*BA. The carrier solution was prepared by disolving 100 mgs. OIBA in sufficient N NaOH solution, adjusting to pH 7.2 to 7.4 with N HCl, adding sterile normal saline to 10 ml, filtering through a medium-porosity fritted glass filter into a pyrogen-free serum vial and sterilizing by heating in boiling water for 1 hour.

A sufficient quantity of the labeled acid to yield a solution of high specific activity, *i.e.* 150 to 200 μc/ml was made similarly but sterilized by filtration through a Millipore Filter Type HA, pore size 0.45μ and the Microfiber Glass Prefilter Discs in a Swinny Adapter XX30-012-00.[1] The sterilized solution was transferred to a sterile serum vial containing a "silver impregnated porcelain saddle"[2] used to remove radioiodide. This treatment was used to reduce and maintain the radioiodide content of the OI*BA solutions at a level of 1/100 of the original solution. The solutions of the OI*BA and the unlabeled OIBA were tested for sterility. The dose solution was prepared by mixing the requisite quantities of OI*BA and carrier OIBA solution in a sterile vial so as to provide 25 μc in 10 mg in a volume of about 1 ml.

The dose of 10 mg represents less than 1/6000 of the LD-50 mouse dose as determined by the authors (8).

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[1]Millipore Filter Corp., Bedford, Mass.

[2]These "saddles" were generously supplied by Volk Radiochemical Co., Skokie, Ill.

TEST PROCEDURE

The following protocols were devised for the collection and fractionation of the urine. The patient protocol was as follows: (a) just prior to the injection of the OI*BA, the patient was given 2 glasses of water to insure adequate urine flow; (b) 10 mg OI*BA with a specific activity of 25 μc was injected into the antecubital vein; (c) all the urine voided during the first hour, the 2nd hour, the 3rd and 4th hours combined, and the 5th to 24th hours were collected as separate fractions. One ml aliquots of each collection were counted and the total excretion of activity during that period was calculated.

EXTRACTION PROCEDURE

Aliquots from each collection period were analyzed qualitatively and quantitatively by extracting a volume of 10 to 50 ml containing about 1 million counts. The urine was acidified with 3N HCl, 1 ml 10 per cent sodium tungstate solution added and extracted in a liquid/liquid extractor with 150 ml of ether for six hours (5), (10). The extracted urine was reserved for further isoamyl alcohol extraction.

The ether extract was extracted in a separatory funnel with 10 ml 1 per cent $NaHCO_3$ solution which removed the free and conjugated acids, and radioiodide quantitatively. The ether was then discarded.

The $NaHCO_3$ extract was acidified to pH 2 with N HCl and extracted with 25 ml of toluene which quantitatively separated the free or unconjugated acid. This was in turn extracted from the toluene with 3 ml 1 per cent Na HCO_3 solution, an aliquot counted, the $NaHCO_3$ extract evaporated to 0.5 to 1 ml, an aliquot of 5 to 25 μl chromatographed, radioautographed and quantitated by cutting out the spots and counting in a scintillation counter.

The previously acidified and toluene-extracted $NaHCO_3$ solution containing the conjugated acid, several other uncharacterized metabolites and free radioiodide was then neutralized and evaporated to a small definite volume, *e.g.* 2 to 3 ml. A small aliquot was counted and 5 to 25 μl chromatographed, radioautographed and quantitated.

The previously extracted urine which contained up to as much as 50 per cent of the original activity was then extracted successively with 4 or 5, 20 ml portions or isoamyl alcohol which removed the residual activity consisting of the radioiodide and some uncharacterized metabolites. The urine was then discarded.

The combined isoamyl alcohol extracts were then extracted with 5 ml of 1 per cent $NaHCO_3$ solution, concentrated to 1 to 2 ml, 10 to 25 μl chromatographed, radioautographed and quantitated.

The quantities of unconjugated OI*BA and each of the specific metabolites excreted during any time period was the sum of the quantities found in all of the fractions and was calculated as a percentage of the injected dose. A simplified summary of the extraction procedure is shown in Figure 1.

An attempt was made to measure the removal of OI*BA from the blood as a function of the conjugation and excretion of the test agent in a patient with

no known liver pathology. A dose of 25μc in 0.72 mg OI*BA was injected antecubitally and external monitoring performed over the side of the head and simultaneously monitoring the liver. At the same time radioisotope renography was attempted using dual probes over the kidneys. Readings were taken for 1 hour with all 3 probes. The results are reported below.

RESULTS AND CONCLUSIONS

The processes of conjugation and excretion were too slow to permit measurement of the removal from the blood by external monitoring. The head count decreased slightly for the first hour while the liver indicated a concomitant rise showing only a slight removal.

The radioisotope renograms were abnormal in form and although they were similar bilaterally, the right curve indicated higher activity ostensibly the result of liver accumulation.

Samples of each of the collections of urine were counted for the excretion of total radioactivity and fractionated according to the method described to characterize the conjugates and metabolites formed.

The following abbreviations are used hereinafter: OI*BA for ortho-I^{131} iodobenzoic acid; OI*HA for ortho-I^{131} iodohippuric acid and OI*BG for ortho-I^{131} iodobenzoyl glucuronide.

In Table I are shown the results obtained by extraction of the urines of 8 controls, 11 patients with clinical diagnoses of liver cirrhosis and 2 patients with diagnoses of hepatitis.

Qualitatively the same metabolites were found in all urines except that no OI*HA was detected in the urine of one of the cirrhotic patients. Another

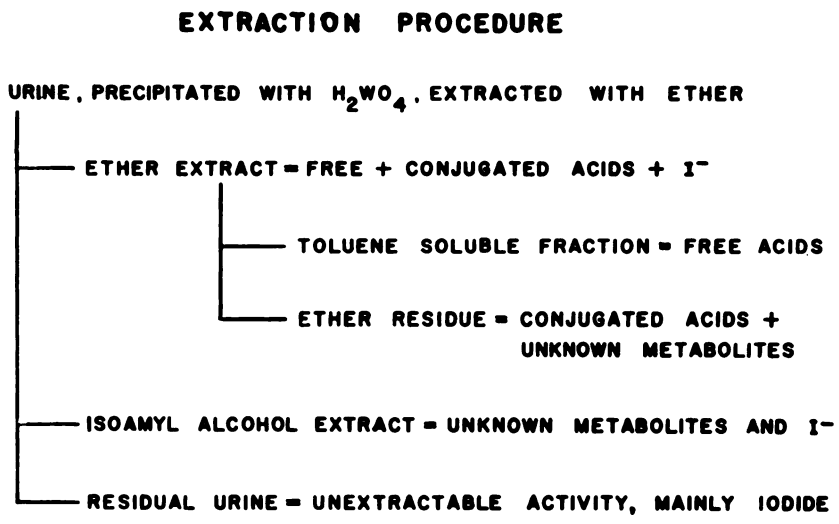

Fig. 1. The subjects in this preliminary study consisted of 8 patients with no liver pathology hereinafter referred to as "controls", 11 patients with clinical and laboratory findings compatible with Laennec's cirrhosis and 2 patients with acute toxic or infectious hepatitis.

TABLE I

EXCRETION OF METABOLITES AFTER INJECTION OF 10 MGS SODIUM ORTHO-RADIOIODOBENZOATE

TIME	AT END OF 1ST HR.			AT END OF 2 HRS.			AT END OF 4 HRS.			AT END OF 24 HRS.		
Patients	Co[x]	C[x]	H[x]	Co	C	H	Co	C	H	Co	C	H
Total radioactivity excreted as % injected dose	6.8(7)[a]	12.2(8)	17.2(2)	12.5(8)	18.8(11)	32.4(2)	21.7(8)	33.7(11)	52.7(2)	71.9(8)	82.1(11)	91.4(2)
range %	1.0–14.0	1.5–24.2	11.8–22.5	0.7–30.0	6.6–34.3	23.5–41.3	2.5–54.0	13.2–57.0	42.5–62.9	38.5–92.9	54.1–98.4	85.0–97.7
OI*BA‡												
mgs. excreted	0.30(6)	0.37(6)	0.34(2)	0.48(7)	0.47(9)	0.60(2)	0.84(8)	0.82(10)	1.22(2)	2.21(8)	1.69(11)	1.64(2)
range	0.00–0.76	0.01–0.91	0.14–0.53	0.04–1.64	0.01–1.08	0.53–0.66	0.02–2.57	0.01–1.95	1.09–1.35	0.47–4.97	0.01–4.86	1.12–2.15
rate[r]–mg/hr.	0.30	0.37	0.34	0.22	0.22	0.26	0.21	0.21	0.32	0.07	0.05	0.02
OI*HA‡												
mgs. excreted	0.07(6)	0.12(6)	0.24(2)	0.10(7)	0.17(9)	0.31(2)	0.18(8)	0.25(10)	0.72(2)	0.36(8)	0.49(11)	0.72(2)
range	0.00–0.15	0.00–0.27	0.16–0.31	0.00–0.34	0.00–0.31	0.25–0.37	0.01–0.59	0.00–0.61	0.45–0.98	0.01–1.22	0.00–1.08	0.45–0.98
rate[r]–mg/hr.	0.07	0.12	0.24	0.04	0.09	0.08	0.05	0.05	0.21	0.01	0.01	0.00
OI*BG‡												
mgs. excreted	0.24(6)	0.80(6)	0.67(2)	0.50(7)	1.35(8)	1.58(2)	0.58(8)	1.76(10)	2.38(2)	2.14(8)	2.99(11)	3.32(2)
range	0.00–0.62	0.38–1.53	0.44–0.89	0.00–1.53	0.00–2.66	1.35–1.80	0.00–1.88	0.11–4.20	2.06–2.70	0.00–5.22	0.18–5.57	2.70–3.94
rate[r]–mg/hr.	0.24	0.80	0.67	0.29	0.75	0.91	0.07	0.34	0.41	0.08	0.07	0.05
Rf 0.33–0.40												
mgs. excreted	0.01(6)	0.04(6)	0.04(2)	0.03(7)	0.07(8)	0.04(2)	0.03(8)	0.11(10)	0.04(2)	0.04(8)	0.30(11)	0.28(2)
range	0.00–0.05	0.00–0.08	0.00–0.07	0.00–0.14	0.00–0.13	0.00–0.07	0.00–0.14	0.00–0.39	0.00–0.07	0.00–0.14	0.00–1.76	0.00–0.55
rate[r]–mg/hr.	0.01	0.04	0.04	0.02	0.04	0.0005	0.0005	0.03	0.00	0.0005	0.01	0.01
Radioiodide as % inj. dose‡										0.3(8)	0.8(11)	1.7(2)
range										0.00–1.10	0.06–2.28	1.26–2.06
rate–% dose/hr.										0.01	0.03	0.07

Co[x] = controls, C[x] = cirrhotics, H[x] = hepatitics

a = averages of the numbers in parentheses

r = rates of formation and excretion during 1st, 2nd, 3rd and 4th, and 5th to 24th hrs.

‡ = these values represent the substances extractable from the urines

metabolite with an Rf of 0.71 to 0.73 was found in the urines of control and cirrhotic patients but not in the urine of hepatitic patients.

Although only two hepatitic patients were studied, the results are included for comparative purposes.

Referring to Table I, it is seen that the 2 hepatitics excreted more total radioactivity in all time periods than the cirrhotics and controls and that the range was definitely higher despite some overlap. This is partially due to somewhat greater excretion of I^{131} iodobenzoyl glucuronide, partially due to OI*HA, and to a lesser degree to 2 unknown metabolites having Rf values of 0.57 to 0.59 and 0.33 to 0.40 which occurred in relatively small amounts.

Referring to the excretion of unchanged OI*BA, it is seen that the cirrhotics and hepatitics excrete the OI*BA at a slightly higher rate and in slightly greater total amount than the controls do, in the first 4 hours. This might be expected since the damaged liver would be unable to conjugate the OI*BA. However, between 5 and 24 hours the rate of excretion of the controls is 1.5 times that of the cirrhotics and 3 times that of the hepatitics and the total excretion is about ⅓ higher than the others. This is discussed later.

Referring to the excretion of OI*HA, it is seen that the hepatitics excrete this at 4 times the rate of the controls and 2 times that of the cirrhotics during the first 4 hours and that the total quantities are 4 times that of the controls and 3 times that of the cirrhotics. In the 5 to 24 hour period, the rate of the hepatitics drops to zero while that for the others continues at a low value and the total

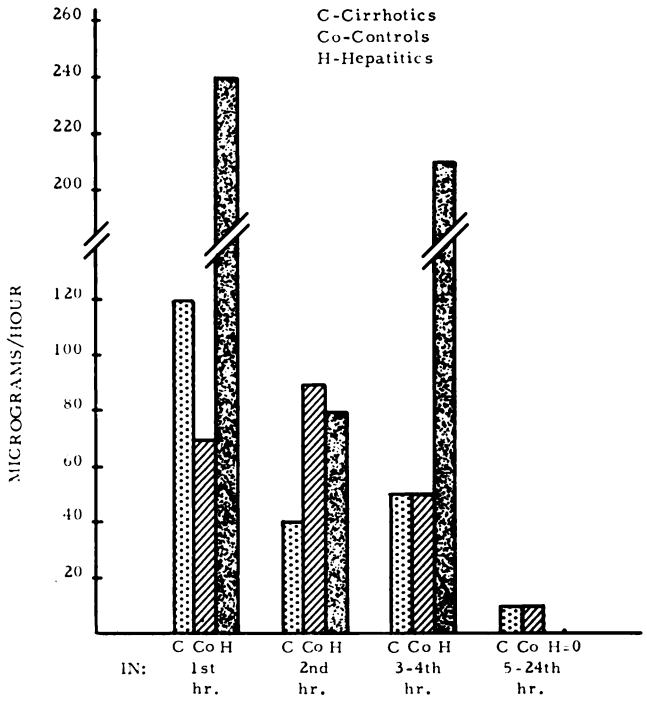

Fig. 2. Av. Rates of Excretion of o-Radioiodohippuric Acid

quantity in 24 hours is 2 times that of the controls and 1.5 times that of the cirrhotics. In other words, the hepatitics produced all of their OI*HA during the first 4 hours. The average rates of excretion of OI*HA are shown in Figure 2.

Referring to the production of the metabolite I^{131} iodobenzoyl glucuronide, OI*BG, the cirrhotics and hepatitics produced this at a rate 3 to 4 times that of the controls during the first 4 hours and the total quantities are similar multiples whereas in the 5th to 24th hours, the rate for the controls is slightly greater than that of the cirrhotics and about twice as high as the average for the hepatitics. The total quantity, however, produced by the controls is about ⅔ that of the cirrhotics and hepatitics. In the controls, cirrhotics and hepatitics, the total quantities of OI*BG formed in 24 hours was 5 to 6 times that of the OI*HA, suggesting that the major pathway for the detoxication was via the formation of the glucuronide. The average rates of excretion of OI*BG are shown in Figure 3.

In addition to these major metabolites, there were several others identified only by chromatography and radioautography whose significance and composition are unknown except that they contain I^{131}.

One group of metabolites had Rf values of 0.33 to 0.40 which were similar to the Rf values of sulfur-containing amino acids in combination with OI*BA which we prepared. These compounds occurred in quantities which were ca. ⅕ to 1/10 that of OI*HA and even smaller fractions of the quantities of the OI*BG. The total excretion in 24 hours in the controls was ⅛ that of the cirrhotics and 1/7 that of the hepatitics.

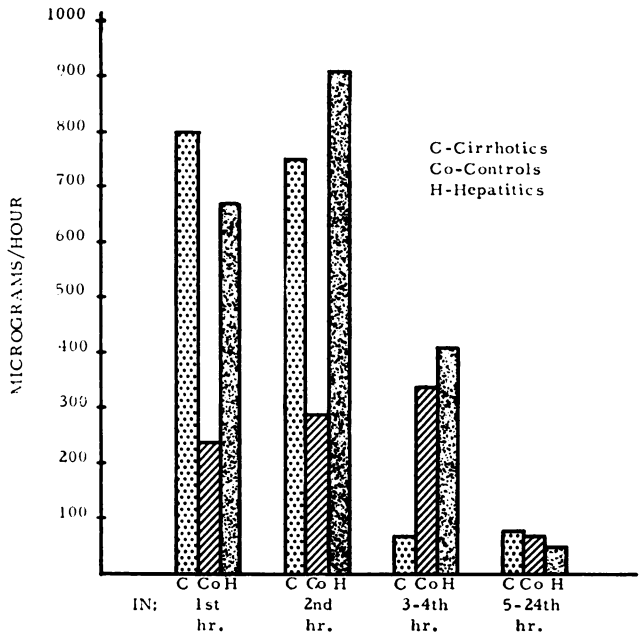

Fig. 3. Av. Rates of Excretion of o-Radioiodobenzoyl Glucuronide

One metabolite, Rf 0.57-0.59 occurred in almost all urines, and was greatest in the hepatitic urines. The quantities excreted were several times as great as OI*HA and almost as large as the OI*BG. Its composition and significance are unknown.

Other compounds with Rf values of ca. 0.28, 0.46 occurred in small quantities but were not found in any of the hepatitic urines. A metabolite with an Rf of 0.71 to 0.73 occurred in larger quantities in the urines of the controls than in cirrhotics at all times.

The composition and significance of these metabolites are unknown at present.

Radioiodide was released or formed in only small percentages of the dose as shown in Table I, and was found to be about 3 times greater in the cirrhotics and 6 times greater in the hepatitics than in the controls, as if the OI*BA or its metabolites were deiodinated to a greater degree in the pathological livers.

In one control, the effect of an added standard dose of 1.77 grams of sodium benzoate, used in the Quick test (7), on the excretion of total urinary activity was investigated. The first test dose consisted of 2.6 mgs OI*BA with an activity of 26 μc and the second dose of 18.6 mgs OI*BA with an activity of 25 μc plus the sodium benzoate. Whereas the percentages of the dose excreted as total radioactivity in the 1st and 4th hours were similar in both tests, 76 per cent of the dose as total radioactivity was eliminated in the 5th to 24th hours with the dose of sodium benzoate compared to 54 per cent excretion of the OI*BA alone representing a 40 per cent increase.

DISCUSSION

Using ortho-I^{131} iodobenzoic acid and separating the metabolites by our procedure, we have been able to isolate measurable quantities of ortho-I^{131} iodohippuric acid from the urines. Quick (5) had previously reported that "there was little or no conjugation of glycine with ortho-substituted benzoic acids." It is significant to note that the pathological livers produced more of the glycine conjugate, OI*HA, than the liver of the controls, which is contrary to expectations. However the major metabolite was not OI*HA but the glucuronide OI*BG which was found in several times the quantities of OI*HA at all time periods in the controls and patients with the highest OI*BG/OI*HA ratios in the cirrhotics.

This is in general agreement with previous reports in the literature. Borgström (11) reported that liver disease caused an increase in benzoyl glucuronide excretion after benzoic acid administration and that this synthesis was disturbed later than the hippuric acid synthesis in disease. Snapper and Saltzman (2) found no glucuronide in the urines of normals after 5 gm doses of benzoic acid but found considerable amounts in the urine of patients with impaired liver function.

Despite the use of only 10 mgs of OI*BA used in our study compared to the 1.77 gms of benzoic acid used in the standard Quick test (7), significant amounts of the unchanged OI*BA were isolated from the urines and more in the 24 hour period in the urine of normals than in the cirrhotics and hepatitics.

This is contrary to expectations, namely that the conjugative function of normal liver should be greater and therefore less unchanged acid should be excreted.

Regarding the metabolites with Rf values of 0.33 to 0.40, presently presumed to be combinations of OI*BA and the sulfur-containing amino acids cysteine, methionine and glutathione, these have not been reported previously and their significance in normals and liver disease is unknown. They may indicate the existence and actions of enzyme systems of importance. The occurrence of these compounds in very much larger quantities in the hepatitic and cirrhotic urines may be significant.

In the case of the control who received the sodium benzoate in addition to the OI*BA, one interpretation of the findings may be that the liver was forced to utilize its reserve function (7) and therefore conjugated and excreted more of the injected OI*BA under these conditions. The standard Quick test (7) which measures the conjugation of benzoic acid was done also on the first hour urine and showed this patient to be on the low side of the normal range, corroborating the low normal value of excreted radioactivity.

The test using OI*BA may be adjunctive to other tests for liver pathology. Since OI*BA is more difficult to conjugate than benzoic acid it may test the liver function as well as the larger quantities of benzoic acid. Due to the small quantity used, it is unlikely that the excretion of metabolites would be affected by even a moderate impairment of kidney function, although this has not been specifically demonstrated. The OI*BA test is not affected by ingested benzoic acid in the diet and can be applied to patients on low sodium diets.

SUMMARY

The characterization and quantitation of the excretion of the metabolites of ortho-I^{131} iodobenzoic acid may possibly serve as a basis for the development of a new diagnostic test for liver function.

Methods for the preparation and determination of purity of the test agent, the performance of the test and the quantitation are described.

The conjugation and excretion were too slow to permit measurement of the removal from the blood by external monitoring over the side of the head. Similarly it was not possible to perform the customary renographic study in situ over the kidneys.

Results of the test in 8 controls, 11 patients with clinical diagnoses of liver cirrhosis and 2 patients with diagnoses of hepatitis indicate qualitative and quantitative differences in the excretion of total radioactivity, unchanged OI*BA, OI*HA, OI*BG and other metabolites.

Methods of analysis were devised which indicated several radioactive metabolites not reported heretofore. These may indicate enzyme systems which have some significance in pathological states.

Our studies indicate that cirrhotics and hepatitics produce more OI*HA and OI*BG than the controls do in all time periods tested.

These studies indicate that the major pathway for the detoxication of OI*BA is the formation of iodobenzoyl glucuronide and not iodohippuric acid.

REFERENCES

1. ZIEVE, L. AND HANSON, M.: Studies of Liver Function Tests. IV. Effect of Repeated Injections of Sodium Benzoate on the Formation of Hippuric Acid in Patients with Liver Disease. *J. Lab. Clin. Med.* **42**:872, 1953.
2. SNAPPER, I. AND SALTZMAN, A.: Quantitative Aspects of Benzoyl Glucuronate Formation in Normal Individuals and in Patients with Liver Disorders. *Am. J. Med.* **2**:327, 1947.
3. SNAPPER, I., ROBINSON, B. D. AND ROSENTHAL, D. J.: An Evaluation of a Fractional Intravenous Sodium Benzoate Test for Liver Function. *J. Mt. Sinai Hosp.* **18**:203, 1951.
4. QUICK, A. J.: The Synthesis of Hippuric Acid: A New Test of Liver Function. *Am. J. Med. Sci.* **185**:630, 1933.
5. QUICK, A. J.: The Relationship Between Chemical Structure and Physiological Response. I. The Conjugation of Substituted Benzoic Acids. *J. Biol. Chem.* **96**:83, 1932.
6. WILLIAMS, R. T.: Detoxication Mechanisms. Second Ed., New York, N. Y. John Wiley & Sons, Inc., 1959.
7. QUICK, A. J.: The Hippuric Acid Test as a Means for Determining Hepatic Function. *Acta Med. Scand.* **104**:216, 1940.
8. TUBIS, M., ENDOW, J. S. and RAWALAY, S. S.: The Preparation and Properties of I^{131}-Labeled Iodobenzoic Acids. *Int. J. Appl. Rad. Isotopes. In press.*
9. GAFFNEY, G. W., SCHREIER, K., DI FERRANTE, N. and ALTMAN, K.: The Quantitative Determination of Hippuric Acid. *J. Biol. Chem.* **206**:695, 1954.
10. BRAY, H. G., CLOWES, R. C., THORPE, W. V. and WOOD, P. B.: The Fate of Certain Organic Acids and Amides in the Rabbit. *Biochem. J.* **50**:583, 1952.
11. BORGSTRÖM, B.: Detoxication of Benzoic Acid by Glycuronic Acid under Normal Conditions and in Liver Disease. *Acta Med. Scand.* **133**:7, 1949.