Biochemical Pharmacology, 1961, Vol. 6, pp. 61–64. Pergamon Press Ltd., Printed in Great Britain.

# BIOCHEMICAL CONJUGATION PROCESSES

E. BOYLAND

Chester Beatty Research Institute, London, S.W.3.

**Abstract**—Biochemical processes of conjugation include the formation of glucuronides, sulphuric esters, sulphamates, phosphoric esters and mercapturic acid derivatives. A result of the conjugation is the production of compounds more ionized and more water soluble than parent compounds. The significance of these substances in the animal kingdom is pointed out. Mercapturic acid formation from naphthalene as well as the general significance of conjugation processes in detoxication are discussed.

KNOWLEDGE and understanding of the biochemical processes of conjugation have greatly increased during the past decade. These biochemical processes which include the formation of glucuronides, sulphuric esters, sulphamates, phosphoric esters and mercapturic acid derivatives generally lead to the production of compounds which are more water soluble and more readily ionized than the original substances. The recent discovery that 2-naphthylamine is excreted as bis (2-amino-1-naphthyl) phosphate suggests that many biochemical reactions of this type may still be discovered.

Brodie and his colleagues[1, 2] have shown how the ionized compounds are more readily excreted by the kidney. The ability to form these derivatives which are easily excreted is essential to animals living on land, and work from Brodie's laboratory shows that fish and aquatic amphibia do not have the ability to form glucuronides. The fact that mammalian foetal tissues, and new-born animals do not possess the ability to form the conjugates is in agreement with the idea that the development of these systems for the metabolism of unionized compounds is associated with, and probably essential for, terrestrial life.[3] The vertebrate animals which do not form glucuronides appear to lack the ability to oxidize uridine diphosphoglucose to uridine diphosphoglucuronic acid or "active" glucuronide.

The work of Smith[4] on the metabolism of foreign compounds by insects is of interest in this respect. This shows that these terrestrial animals convert phenols and other related compounds to glucosides instead of the glucuronides formed by mammals. This is an example of biochemical evolution in which the problem of maintaining the internal environment has been attempted in a different way.

The ionization theory which Brodie has developed extends earlier work such as that of Quick[5]. There are difficulties in the theory. The metabolic processes of conjugation produce ionized derivatives which cannot penetrate cell walls and so are not reabsorbed by the kidney. The reactions leading to products which cannot penetrate the cell membrane proceed inside the cell. In this case it must be difficult for these products to diffuse out of the cell so that they should accumulate within the cells.

The early work on glucuronides, which was mainly descriptive, showed that phenols, alcohols and carboxylic acids are excreted as glucosiduronic acids, but more recent investigations have revealed the occurrence of glucuronides of primary aromatic

amines[6] and in one case of a thiol (mercapto benzthiazol).[7] In view of these findings it might be of interest to see if aliphatic amines and secondary amines are excreted as N-glucuronides and if organic selenium derivatives are excreted in an analagous way to the mercapto benzthiazol.

Until a few years ago sulphate conjugation was only known to occur with phenols, but it is now known that aromatic amines are excreted as N-sulphates or sulphamates[6] and Roy[8] has shown that such compounds are formed *in vitro* when active sulphate is present. More recently Vestermark and Boström[9] have shown that simple alcohols and glycols are also excreted as ethereal sulphates.

The most remarkable of the new conjugates, however, is the bis (2-amino-1-naphthyl) phosphate found by Troll *et al.*[10] as a metabolite of 2-naphthylamine in dog urine. This derivative has two aromatic residues to each phosphate group and is unstable in solution. Dr. Manson has synthesized the derivative by reduction of the corresponding nitro compound and the pure product has now been isolated and has chromatographic properties identical with a substance sometimes present in the urine of dogs treated with 2-naphthylamine.

We have been interested in mercapturic acid formation from hydrocarbons such as naphthalene. When *p*-bromophenyl mercapturic acid was isolated by Baumann and Preusse,[11] these authors were aware that the material was excreted in the urine in some form which was changed on acidification, because there was a large irreversible change in optical activity on acidification of the urine. Although the precursor of *p*-bromophenyl mercapturic acid has not been isolated, the precursor of naphthyl mercapturic acid has been isolated as the ammonium salt in my laboratory.[12] The precursor is decomposed by cold acid or by cold alkali, but is stable enough to be crystallized as the ammonium salt. It is 1 : 2-dihydro-2-hydroxy-1-naphthyl-S-acetylcysteine which readily eliminates water to give the 1-naphthyl mercapturic acid, first isolated by Bourne and Young[13].

Studies of the intermediate reactions in the biosynthesis of the naphthalene mercapturic acid precursor by rat tissue homogenates[14] have shown that the first stage appears to be the formation of 1 : 2-dihydro-2-hydroxy-1-naphthyl-S-glutathione by liver tissue. This is then broken down to the corresponding cysteine derivative by kidney enzymes. The dihydrohydroxynaphthyl cysteine is then acetylated in the liver to give the mercapturic acid precursor. These steps must occur rapidly in the animal as we have detected the naphthyl mercapturic acid precursor in the urine of rats within 20 min of administration of naphthalene.

The naphthalene derivative which reacts with glutathione could be the hypothetical 1 : 2-epoxide which we have been unable to synthesize chemically. We have, however, administered 1 : 2-dihydro-3 : 4-epoxynaphthalene to rats and this is excreted as the corresponding mercapturic acid precursor. In this case the corresponding tetrahydro-hydroxynaphthylcysteine (i.e. not acetylated) is also excreted and has been isolated.

These experiments support the hypothesis that the microsomes convert naphthalene and other hydrocarbons into epoxides or similar derivatives which can then react either with water to yield dihydrodiols or with glutathione to yield eventually mercapturic acid precursors.

Dr. Manson has evidence that 2-naphthylamine is excreted both as the 1-mercapturic acid and as a precursor of a 5- or 6-mercapturic acid. These derivatives are, however, difficult to isolate or to synthesize.

The conjugation reactions with sulphate or with glucuronide may be used as transporting mechanism. If a hormone inactivated by conjugation is carried by the blood stream to an organ or tissue rich in β-glucuronidase the active hormone could be released locally and produce its particular biological effect. Although such a mechanism is possible there is no proof that it actually occurs. To attribute a purpose to biochemical processes is difficult in this as in other cases.

Although conjugation processes usually lead to detoxication there is a possibility that the conjugation of the *o*-aminophenols formed by metabolism of carcinogenic aromatic amines changes the site of action of the parent amine. Men exposed to 2-naphthylamine and dogs treated with 2-naphthylamine develop cancer of the bladder, and cancer of the bladder only. We think that the parent amine is not carcinogenic but that one of the first formed metabolites, 2-amino-1-naphthol, is carcinogenic locally. When 2-naphthylamine is adsorbed it is metabolized mainly in the liver to 2-amino-1-naphthol. In the liver it is immediately conjugated with glucuronide, sulphate and presumably phosphate. These conjugates are then excreted and concentrated in the kidney. In the urine the glucuronide or the phosphate can be hydrolysed enzymatically to give the free carcinogenic 2-amino-1-naphthol. This mechanism would explain the localized action of aromatic amines such as 2-naphthylamine on the bladder. The corresponding sulphate is not hydrolysed by urinary sulphatase but it might be hydrolysed by some other mechanism.

Levvy[15] showed that the hydrolysis of glucuronides by β-glucuronidase is inhibited by 1 → 4 saccharolactone much more strongly than by 3 → 6 saccharolactone. Investigation of this inhibition at different pH values has shown that the inhibition is much less at pH values above the optimum.[16] The pH–enzyme activity curve and the pH–inhibition curve are parallel, which agrees with the idea that the substrate and inhibitor combine with the same centre on the enzyme. The 1 → 4 saccharolactone has only a fraction of the inhibitory effect at pH 7 compared with its effect at the optimu pH for the enzyme pH 5.

Because the so-called 1 → 4 saccharolactone is such an effective competitive inhibitor of β-glucuronidase one would expect the saccharolactone and glucuronide molecules to have similar structures. The glucuronides have, however, pyranose structures, while the so-called 1 → 4 saccharolactone would have a furanose ring. Dr. Lumley Jones has found that the carbonyl stretching band of the so-called ammonium 1 → 4 saccharolactone is at 1744 $cm^{-1}$ which would be in agreement with a pyranose structure. The potassium salt of 3 → 6 saccharolactone has a band at 1780 $cm^{-1}$, in agreement with a furanose ring structure. On the other hand the lactone at present designated as the 1 → 4 form reacts very slowly with one molecule of periodate, in agreement with the furanose structure.

One difficulty in the study of conjugation processes is the variation in the reaction. For example many workers have observed the production of free naphthalene on acidification of the urine of rats dosed with naphthalene. Ten years ago "naphthalene urine" always contained the naphthalene precursor. Our rats and rabbits treated with naphthalene now do not excrete the naphthalene precursor. In the past few years we have administered 2-naphthylamine to many animals. In the first experiments in which $^{14}C$ labelled 2-naphthylamine was injected into dogs, 2-amino-l-napthyl sulphate was the only metabolite detected in the urine. In later experiments under the same conditions 2-amino-1-naphthyl glucosiduronic acid, 2-amino-1-naphthyl sulphate,

the phosphoric ester and other products were excreted. These examples of apparent variation indicate that there are many factors concerned in the metabolism of foreign compounds which are not yet understood.

In the study of conjugation reactions, there are probably new types of reactions still to be discovered, although the main part of the descriptive phase is probably complete. Much remains to be investigated in the conjugation of products of normal metabolism as compared with drugs and other foreign compounds. We know a great deal about the mechanism of the reactions and we appear to understand their function or purpose better than we did. It still remains, however, an interesting field for research.

## REFERENCES

1. B. B. BRODIE, *J. Pharm., Lond.* **8**, 1 (1956).
2. B. B. BRODIE and C. A. HOGBEN, *J. Pharm., Lond.* **9**, 345 (1957).
3. F. W. JONDORF, R. P. MAIKEL and B. B. BRODIE, *Fed. Proc.* **18**, 407, 418 (1959).
4. J. N. SMITH, *Biol. Rev.* **30**, 455 (1955).
5. A. J. QUICK, *J. Biol. Chem.* **97**, 403 (1932).
6. E. BOYLAND, D. MANSON and S. F. B. ORR, *Biochem. J.* **65**, 417 (1957).
7. J. W. KLAPP, *J. Biol. Chem.* **223**, 207 (1956).
8. A. B. ROY, *Biochim. Biophys. Acta* **30**, 193 (1958).
9. A. WESTERMARK and H. BOSTRÖM, *Acta Chem. Scand.* **13**, 827 (1959).
10. W. TROLL, S. BELLMAN and N. NELSON, *Proc. Soc. Exp. Biol., N.Y.* **100**, 121 (1959).
11. E. BAUMANN and C. PREUSSE, *Ber. dtsch. Chem. Ges.* **12**, 806 (1879).
12. E. BOYLAND and P. SIMS, *Biochem. J.* **68**, 440 (1958).
13. N. I. C. BOURNE and L. YOUNG, *Biochem. J.* **28**, 803 (1934).
14. J. BOOTH, E. BOYLAND and P. SIMS, *Biochem. J.* **74**, 117.
15. A. A. LEVVY, *Biochem. J.* **52**, 464 (1952).
16. E. BOYLAND, J. N. DAVIES and K. WILLIAMS, *Biochem. J.* **72**, 28 P (1959).