decrease in the turbidity and iodine-staining power of glycogen-type polysaccharides, and its action is similar to that previously described for Z-enzyme on amylose β-dextrin in that it is increased by Ca^{2+} ions and decreased by EDTA.

SUMMARY

1. β-Amylase preparations from barley and soya beans, and β-glucosidase preparations from almond emulsin, show Z-enzyme activity, which is due to the presence of a trace of α-amylase. Extraction of the above-named plant materials with dilute calcium chloride solution has given preparations with α-amylolytic activity.

2. The above-named plant α-amylases attack glycogen and glycogen β-dextrin and are not therefore differentiated from normal α-amylases.

3. The α-amylase activity towards glycogen is increased by Ca^{2+} ions and decreased by complexones, thus resembling that of Z-enzyme on amylose.

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Comparative Studies of 'Bile Salts'

16. BILE SALTS OF MONOTREMES AND OBSERVATIONS ON GLYCINE CONJUGATION*

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The egg-laying mammals, order Monotremata, which are represented as living forms only by the platypus (*Ornithorhynchus anatinus*) and the spiny anteaters (*Tachyglossus* and *Zaglossus*), have been the objects of intense study (Simpson, 1945). In spite of this, their relationships to marsupial and placental mammals are poorly understood and it has been suggested that they may have had a separate reptilian origin (see Romer, 1945). We now report on the bile salts of the platypus and of *Tachyglossus* (*Echidna*) *aculeatus*, the Australian spiny anteater.

Bile acids conjugated with glycine have been found only in mammals, and we have come to think that they may be confined to Eutheria. We have therefore applied improved methods for the detection of glycine conjugates to the bile salts of the monotremes mentioned above and of six species of marsupials.

RESULTS

The bile of the monotremes apparently contained only C_{24} bile acids, and chiefly cholic ($3\alpha,7\alpha,12\alpha$-trihydroxycholanic) acid, with a small fraction

* Part 15: Anderson & Haslewood (1962).

running on paper chromatograms at the same rate as chenodeoxycholic (3α,7α-dihydroxycholanic) acid or deoxycholic (3α,12α-dihydroxycholanic) acid. Neither allocholic acid nor ketonic bile acids were detected.

Glycine conjugates, if present, comprised less than 0·05 % (w/w) of the bile salts of the monotremes examined and of the following marsupials: kangaroos (*Macropus rufus*, *M. canguru melanops*, *M. rufogrisea fructicus* and *Dorcopsis muelleri*); koala (*Phascolarctos cinereus*) and Tasmanian devil (*Sarcophilus harrisii*).

EXPERIMENTAL

General. Details were as given by Haslewood & Ogan (1959) and Haslewood (1961), except where otherwise stated.

Paper chromatography of bile salts. For taurine conjugates the system (T_2) was made by mixing 'amyl acetate' (Bridgwater, Briggs & Haslewood, 1962), light petroleum (b.p. 80–100°) and acetic acid (85:15:103, by vol.); water (47 parts, by vol.) was added: if two layers did not form, a little more water was added, until this occurred. The system was equilibrated by mixing; the upper ('amyl acetate'–light petroleum) was the moving phase, and the paper was prepared for use by washing with the stationary phase and heating until superficially 'dry' for a few minutes at about 90°. In this system platypus bile salts gave a spot corresponding to taurocholate and a faint spot running as taurodeoxycholate, and echidna bile salts only a spot running at the same rate as taurocholate.

For glycine conjugates, the system (G_4) was the dibutyl ether–'amyl acetate'–acetic acid–water mixture described by Bridgwater *et al.* (1962).

Monotreme bile salts

Platypus. (*a*) Two gall-bladders gave 0·24 g. and (*b*) one gall-bladder gave 0·29 g. of bile salts. Bile salts (0·23 g.) were hydrolysed at 137–141° in a metal bomb for 6 hr. in approx. 2N-KOH (2·5 ml.). A solid (20–30 mg.) which separated was apparently insoluble in $CHCl_3$, ethanol, acetone and benzene: it appeared to be polymeric and did not resemble bile alcohols examined by us. The alkaline liquors from this, with 2N-HCl and NaCl (excess), gave solid acids (118 mg. after drying), and the mother liquors, with $BaCl_2$, yielded $BaSO_4$ (15·3 mg.).

The acids (118 mg.) readily gave, with ethyl acetate, crystals (63 mg.) of m.p. 189–192°, not depressed by cholic acid. The ethyl acetate mother liquors were evaporated and the residue and the above-mentioned crystalline acid (50 mg.) were, separately, converted into the ethyl esters. The esters (50 mg.) from the crystalline acid consisted of ethyl cholate, m.p. 157–160°, giving only one spot on paper chromatograms, whose infrared spectrum in KBr did not suggest the presence of ethyl allocholate or any other contaminant. The ethyl esters (54 mg.) from the original ethyl acetate liquors were separated into 'ketones' and 'non-ketones' with Girard T reagent (Haslewood, 1954). The 'ketones' (about 5 mg.) did not appear to contain bile acids. The 'non-ketones' (47 mg.) in benzene–light petroleum (2:1, v/v) were separated on a column of neutralized Al_2O_3 (1 g.). Elution was as follows (fraction no., ml. of solvent, mg. eluted): I, 13 benzene–light petroleum (2:1, v/v), 22·5 (oil); II, 55 benzene, 4·0 (gum + crystals); III, 10 benzene–$CHCl_3$ (1:1, v/v), 2·8 (gum); IV, 65 $CHCl_3$, 17·0 (solid); V, 30 ethanol, about 1 (brown gum). Total eluted, 46 mg. Paper chromatography in systems A and B_5 of Bush (1952) gave the following results: fractions I and II contained no bile acid esters, fraction III gave a spot corresponding to ethyl deoxycholate, fraction IV consisted largely of ethyl cholate, but gave a faint spot corresponding to ethyl deoxycholate and (possibly) a substance running slightly faster than ethyl cholate, and fraction V contained ethyl cholate. Dr I. D. P. Wootton reported that the infrared spectrum of fraction III showed that it was a mixture probably containing ethyl deoxycholate and possibly also ethyl chenodeoxycholate. In summary: 118 mg. of crude acids contained more than 80 mg. of cholic acid, at least 30 mg. of material free from bile acids and about 3 mg. rich in deoxycholic acid or chenodeoxycholic acid or both.

Echidna. One gall-bladder gave 930 mg. of bile salts. These (645 mg.) were hydrolysed in 3N-NaOH (5 ml.) in a metal bomb at 120° for 12 hr. After cooling and diluting with water, the liquid (which contained no precipitate) was extracted with benzene (twice), then acidified (HCl) and extracted twice with ethyl acetate. Evaporation of the washed extract left crude acids, which were converted into ethyl esters (287 mg.). These were separated into 'ketones' (about 50 mg.) and 'non-ketones' (200 mg.) with Girard T reagent as above. The highly coloured 'ketones' contained no identifiable bile acid esters. The 'non-ketones' were dissolved in benzene and separated on Al_2O_3 (6 g.). Fractions (20 ml.) of eluate were collected. Elution of combined fractions was as follows (fraction no., ml. of solvent, mg. eluted): I, 60 benzene, 30 (oil); II, 80 (total) of ether–benzene and ether, 3; III, 60 of 10% (v/v) acetone–ether, 9; IV, 60 of 25% (v/v) acetone–ether, 7; V, 60 of 50% (v/v) acetone–ether, 15; VI, 60 acetone, 10; VII, 60 of 10% (v/v) methanol–acetone, 98; VIII, 20 of 25% (v/v) methanol–acetone, 7. Total eluted, 179 mg. Paper chromatography with system A of Bush (1952) showed that fractions I–III contained no bile acid esters, fractions IV, V and VI contained esters running at the same rate as ethyl deoxycholate and ethyl cholate and fraction VII consisted almost entirely of ethyl cholate. The fractions showing spots corresponding to both ethyl deoxycholate and ethyl cholate were separated in system A on paper, as described by Haslewood (1954). The 'ethyl deoxycholate' fraction (total about 9 mg.) was a mixture that could not be identified. Fraction VII, from 50% (v/v) methanol–water, gave ethyl cholate, m.p. 162–163°, whose infrared spectrum in KBr showed no indication of ethyl allocholate or other contaminant. Thus of 179 mg. eluted, about 110 mg. was ethyl cholate and 42 mg. contained no bile acid esters: the remaining mixture, which was not separated, did not appear to contain esters of bile acids other than those of cholic acid, deoxycholic acid or chenodeoxycholic acid.

Examination of glycine conjugates

Bile salts of the platypus (30 mg.), echidna (50 mg.) and marsupials [*Macropus rufus* (51 mg.), *M. canguru melanops* (51 mg.), *M. rufogrisea fructicus* (2·5 g.), *Dorcopsis muelleri* (51 mg.), *Phascolarctos cinereus* (60 mg.) and *Sarcophilus harrisii* (50 mg.)] were examined as described below.

A preliminary examination by paper chromatography in system G_4 (above) did not detect glycine conjugates. Glycine conjugates of cholic acid or deoxycholic acid could be readily separated from the corresponding taurine conjugates by extraction from 0·01 N-HCl with ethyl acetate, and bile salts (weights are given in parentheses after the names) of the above-named mammals were purified by a countercurrent method as described below, the echidna being selected as an example.

Crude echidna bile salts (50 mg.) were dissolved in 0·01 N-HCl (3 ml.), and the solution was equilibrated in a separating funnel (no. 1) with ethyl acetate (3 ml.). The lower (aqueous) phase was transferred to a second funnel (no. 2) containing ethyl acetate (3 ml.) and, after equilibration, the process was repeated for a total of nine funnels, with restoration of 0·01 N-HCl (3 ml.) to the first funnel for the first three transfers, and thereafter water (3 ml.). In this separation, fats remained in funnel no. 1, and inorganic salts and taurine conjugates accumulated in the aqueous layers. The ethyl acetate layers finally left in funnels 2, 3 and 4 were combined and evaporated and the residue (4·7 mg.) was loaded on to Whatman paper 3MM (previously washed with methanol and dried) and run with glycocholate and glycodeoxycholate as 'markers' in system G_4. The marker strips were cut off and sprayed and corresponding areas cut out from the paper loaded with the above-mentioned residue, as described by Haslewood (1954). These areas were eluted with methanol and one-half of the residues left on evaporation of the eluates run again on paper in system G_4. After development, no spots corresponding to glycocholic acid, glycodeoxycholic acid or any other available glycine conjugate could be detected.

In trials with glycocholic acid and glycodeoxycholic acid added to taurine conjugates, it was found that (*a*) 70–80% of the glycine conjugates were found in funnels no. 2, 3 and 4 and (*b*) that the whole process would detect less than 0·05% (w/w) of glycine conjugates added to bile salts not containing these.

DISCUSSION

Monotreme bile. The bile of about 80 species of mammals has been found to contain only C_{24} bile acids. With a few exceptions (Suidae, Murinae, Pinnepedia), these are cholic acid and chenodeoxycholic acid and their derivatives, such as deoxycholic acid, ursodeoxycholic ($3\alpha,7\beta$-dihydroxycholanic) acid and lithocholic (3α-hydroxycholanic) acid, probably formed by microbial action during the enterohepatic circulation: the status of 3α-hydroxy-7-oxocholanic acid in this respect is uncertain (Haslewood, 1962).

The monotremes contained the most general type of 'modern' bile salts, i.e. taurocholate, with possibly taurochenodeoxycholate and taurodeoxycholate. These bile salts are also to be found in birds, snakes and most of the teleostean fishes examined and are probably to be regarded as the usual end point towards which bile-salt evolution has proceeded.

There was no convincing indication in monotreme bile of bile alcohols or of C_{27} bile acids (e.g. $3\alpha,7\alpha,12\alpha$-trihydroxycoprostanic, tetrahydroxysterocholanic, varanic) characteristic of reptiles (Crocodilia, Chelonia and the lizard *Varanus*) which have apparently changed little during a very long period. The bile did not, apparently, contain allocholic acid, which has, tentatively, been regarded as having been discarded during the evolutionary pathways leading to mammals (Anderson & Haslewood, 1962).

Thus the processes which have caused 'modernization' of bile salts in other mammals have also had this effect in monotremes, which Romer (1945) described as 'very primitive as well as highly specialized'. Such processes have not operated, at least to the same extent, in other vertebrate groups (e.g. Selachii, Amphibia), which include modern forms with those having primitive characters, and this may suggest that all mammals have evolved from reptiles in which the bile salts had already reached the 'modern' (C_{24}) type, or at least that 'modernization' of bile salts occurred very early in mammalian evolution. We have not found substances in monotreme bile which could, in the present state of understanding, throw any other light on the origin of these mammals. It is not known whether the process cholesterol $\rightarrow C_{24}$ bile acids has the same chemical sequence in all mammals: it has, in fact, been partially elucidated only in rats (Bergström, Danielsson & Samuelsson, 1960) and we believe that it may be different in fishes (Bridgwater *et al.* 1962). It would be interesting to know whether the process is indeed the same in eutherians, marsupials and monotremes, and studies of this kind might help to elucidate the history of mammalian evolution.

Glycine conjugation. Our failure to detect this in monotremes and marsupials agrees with previous work in which modern methods have been used; for example, Nakayama & Johnston (1957) did not find glycine in the bile of the opossum, *Didelphys marsupialis virginiana*. Earlier authors had suggested its occurrence in kangaroo bile (see Haslewood, 1962) and we cannot claim to have proved its absence, even from the species studied by us. In Eutheria, the occurrence of glycine conjugation is irregular, and glycine conjugates are apparently absent from some species (Haslewood & Ogan, 1959). These conjugates are only gradually established in the human infant (Bergström *et al.* 1960). Such facts suggest the possibility that glycine conjugation arose, as a single evolutionary event, in ancestral mammals and, if this is the case, the distribution of glycine conjugates may prove to be a valuable guide for studies of mammalian geographical 'radiation'.

Glyco bile salts are found in large proportions in certain vegetarian and omnivorous eutherians but, in view of their absence from other vegetarian

species (including fishes, chelonians and marsupials) it is difficult to suggest selection pressures that may have led to their retention and (in some species) dominance. Smyth (1962) has drawn attention to the effects of certain bile salts on the intestinal parasite *Echinococcus granulosus*: he finds that taurine and glycine conjugates have quite different influences on the survival of these parasites *in vitro*. It is therefore tempting to suggest that reactions to infestation may have played a part in the establishment of glycine conjugation, once enzymes capable of causing this had arisen during evolution.

SUMMARY

1. Bile salts of the platypus (*Ornithorhyncus anatinus*) and the echidna (*Tachyglossus aculeatus*) have been shown to contain taurocholate and taurine conjugates of mixtures of substances chromatographically similar to deoxycholic acid and chenodeoxycholic acid. Bile alcohols or C_{27} bile acids were not found. Monotreme bile salts are therefore of a 'modern' general type, and biological implications of this are briefly considered.

2. With improved methods, glycine conjugates were not detected in the monotremes mentioned above and in six species of marsupials. Glycine conjugates may possibly be confined to eutherian mammals and, if their emergence during evolution was a single event, their distribution may be of value in studying geographical 'radiation' of mammals.

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