Biochemical Studies on Carbohydrates.

CII. On the Jelly of Toad and Frog Eggs.

First Report.

By

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Having worked on frog oviducts, Schulz and Dittthorn¹⁾ said that the jelly-forming protein is made up of galactosamine and an amino acid-grouping. Their belief that the amino sugar in this protein is galactosamine rested on a false basis that mucic acid had been obtained after oxidation of the oviduct and spawn with nitric acid. Mucic acid is obtainable from galactose but not from galactosamine. Nevertheless, the hexosamine hydrochloride, which they separated, exhibited different solubility than glucosamine hydrochloride and is not accepted as identical with the latter hexosamine, although Schulz replaced the claim later on with Becker²⁾ (1935) for the opinion that the above protein involves glucosamine and galactose in an equimolecular proportion. He always did not try to isolate the protein, but derived the recent conclusion from analysis of the dry powder of the oviduct which they also took as a whole for the aim of identifying the sugars.

Whether the mucinogen to be secreted for covering the eggs is the only carbohydrate-containing protein in the oviduct is extremely doubtful, because according to our experiences animal organs have in general a glycidamin or glycidamins constructing the proper tissue. In the oviduct therefore one may assume that there are present at least two glycidamins, one the mucinogen and the other the glycidamin playing an architectonic rôle and that glucosamine of Schulz and Becker and "galactosamine" of Schulz and Dittthorn originated in different glycidamins. So when Schulz and Becker obtained glucosamine hydrochloride from the oviduct hydrolysate as crystalline deposits, "galactosamine" hydrochloride, which was much more soluble, is possible to have remained in the mother liquid. Similar suspicion might be given as to the nitric acid-oxidation derivatives of these amino sugars of which the authors separated norisaccharic acid but

not 2,5-anhydrotalomucic acid. In the earlier experiment, Schulz could not separate glucosamine which readily crystallizes, but the amount of the jelly-forming glycidamin in the gland has close relationship with rut, and in the season when it is produced abundantly, "galactosamine," if this is its constituent, will predominate over glucosamine in yield so that the former is missed.

Before the investigations referred to, Giacosa³⁾ and Wolfenden⁴⁾ had studied the protein from the envelopes of deposited frog eggs and found it become strongly reducing to copper salts on acid hydrolysis. In addition, Wolfenden obtained non-fermenting sugar-like crystals with sweet taste but did not follow up its nature, only describing, "it"—the crystalline mass—"does not appear to be a carbohydrate, since it yields ammonia."

We have recently discovered A group-specificity of the glycidamin from the outer coverings of toad eggs, notwithstanding that the glycidamin of frog egg coverings was devoid of this potency. The finding led us to take up the research of these proteins again, particularly in connection with the carbohydrate in them, because the anti-isohemagglutinative activity of glycidamins is assumed to consist mostly in this moiety (cf. Masamune⁵⁾).

EXPERIMENTAL.

Preparation of Mucin and Mucinogen from Toad- and Frog-Egg Jelly.

The glycidamin from the outer coverings of toad (Bufo vulgaris formosus Boulenger) eggs.

Eggs deposited in ponds were collected and stood in a vessel with constant change of water. On appearance from the cords of polypous processes with simultaneous movement of the eggs thereinto, the coverings were dissected and the jelly separated from the eggs as well as from their outer thin layer stained. The jelly was then strained through loose cotton gauze, wrapped in a cotton bag and washed for several days in a stream of tap water.

1. To 1 l. of the jelly was added 0.1 n. HCl to pH 4.0 (42 cc. of the hydrochloric acid solution were used). The jelly began to contract in 30 minutes. Centrifuged after 10 hours. The centrifugate (420 cc.) was washed twice with double the volume of water acidulated similarly, then with alcohol many times and finally with ether, and dried in vacuo—mucinogen hydrochloride. Crop, 2.3 gm.

2. A second 1 l. portion of the jelly was caused to shrink by standing with 100 cc. of 30% acetic acid (pH of the mixture, 2.0) for 24 hours and centrifuged. The volume was reduced to one third. It was agitated twice in two volumes of 3% acetic acid and then once in alcohol, extending each

for several hours. It was further washed with many changes of alcohol, and dried, after replacing alcohol with ether, in vacuo—mucinogen acetate. 1.2 gm. yield was obtained.

3. Mucin. A third 400 cc. portion of the jelly was suspended in an equal volume of water and the saturated $\text{Ca}(\text{OH})_2$ added up to pH 8.5 (15 cc. of the $\text{Ca}(\text{OH})_2$ solution were required) and stirred up. In one hour pH was lowered to 8.0. By further addition of 5 cc. of the $\text{Ca}(\text{OH})_2$ solution and 2 hours' agitation, the jelly dissolved into a nearly homogeneous solution, which was percolated into an equal volume of 10% acetic acid. The precipitated flakes were washed with many portions of 3% acetic acid, until calcium was not detected anymore, and subsequently with alcohol and ether and dried. The room temperature throughout the course was below 20°C . Crop, 0.35 gm.

✓ *The glycidamin from the envelopes of frog (*Rana japonica*
Guenther) eggs.*

Fresh clean eggs (the coats of the eggs deposited among water grasses are not stained and hence are favourable for the purpose) were washed under running water and subjected without delay to mechanical separation of the jelly which was achieved easily by suction through a narrow glass tube.

The mucinogen hydrochloride and acetate were prepared similarly as those from the coverings of toad eggs. The average yields were 2.2 and 2.3 gm. respectively per l. of the jelly.

✓ Tests and Analyses of the Glycidamins and Identification of the Hexose.

In qualitative aspects the glycidamins resembled mutually:—

Molisch test gave intensive violet colour. By the *p*-dimethylaminobenzaldehyde reagent of Ehrlich was produced a red dye from them immediately as well as after treatment with hot alkali (Osaki and Turumi⁶⁾). Widsoe-Tollens, Oshima-Tollens and Rosenthaler tests (for methylpentose) were given (tested on the distillate with hydrochloric acid). Neuberg and Saneyoshi (for hexuronic acid) and aniline acetate and Bial's orcinol (for pentose) tests resulted in negative. Phosphorus and hydrolysable sulfur were barely detected.

Quantitative analyses gave figures embodied in Table I. The colour intensities developed by the indole, orinol and diphenylamine methods coincided fairly well with one another as expressed as galactose. The methylpentose content thus appeared minute. Galactose was separated as methylphenylhydrazone after hydrolysis and fractioning of toad mucinogen (1 gm.) in the manner described by Turumi and Sasaki⁷⁾. When recrystallized twice from 95% alcohol, it melted constant at 190°C , and the

TABLE I.
Quantitative Analyses of Mucinogens and Mucin from Toad Egg Jelly and Mucinogen from Frog Egg Jelly.
 (In=indole method. O=orcinol method. D=diphenylamine I method.)

Preparation	Analysis	Nitrogen	Hexosamine	Galactose				Ash
				In	O	D	Average	
Toad	Mucinogen hydrochloride Prep. I	8.63	20.2 1.0	30.75	29.5	30.08	30.08 1.49	
	" Prep. II	8.68	19.96 1.0	30.91	30.58	29.78	30.42 1.51	4.60
	Mucinogen acetate Prep. I	8.32	20.92 1.0	31.45	31.25	31.04	31.28 1.49	4.40
	" Prep. II	8.38	20.75 1.0	31.21	30.92	31.04	31.05 1.49	4.31
Frog	Mucin Prep. I	8.34	20.48 1.0	31.05	30.70	30.25	30.66 1.50	3.94
	Mucinogen hydrochloride Prep. I	8.86	14.39 1.0	28.83	27.72	27.50	28.02 1.94	4.36
	Mucinogen acetate Prep. I	8.85	14.76 1.0	28.11	27.55	27.27	27.64 1.88	4.31

mixed melting point test showed no depression. Measured as a 1% solution in pyridine, it did not rotate D-light at all⁸⁾ (temp., 3°C). N (micro Dumas) 9.86% (calc., 9.85%). From the mucinogen fraction precipitated by mercuric acetate at neutral reaction, the hexosamine could not be separated.

The hexosamine and galactose were present in the molecular ratio of 2:3 in the toad mucinogen and mucin and in that of 1:2 in the frog mucinogen. The carbohydrate content diverged to a certain extent between the hydrochloride and acetate of toad mucinogen, and the corresponding content in the mucin from the same source lay intermediately. But the discrepancies are not considered essential. The both salts of the frog mucinogen showed no difference from each other in the present respect.

✓ Group Specificness.

The substances were taken up (partially dissolved and partially suspended) in physiological saline solution and faintly alkalinized according to Sinokawa⁹⁾ to submit to the assay by our usual procedure. The mucin and mucinogen from the jelly of the toad eggs inhibited isoagglutination of A erythrocytes specifically at a dilution of 1:10⁵, whereas the mucinogen from the frog-egg-coverings exhibited no anti-agglutinative activity even at only 1:100 dilution. The used blood sera both possessed agglutinin titre 160.

✓ SUMMARY.

1. Glycidamins were prepared from the jelly of toad (*Bufo vulgaris formosus* Boulenger) and frog (*Rana japonica* Guenther) eggs.

2. The toad mucinogen and mucin involved hexosamine and galactose in the molecular ratio of 2:3 and the frog mucinogen in the corresponding ratio of 1:2. Methylpentose was detected but was small.

3. Galactose of the toad mucinogen hydrochloride was identified as the methylphenylhydrazone.

4. The preparations from the toad jelly were found A group-active, but those from the frog jelly not.

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