137. THE CHEMICAL NATURE OF ROYAL JELLY¹

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Most chemical investigations of substances important in nutrition have dealt with foods used by mammals. The work to be reported is an attempt to determine the chemical nature of the components of royal jelly, the peculiar nutriment which is fed to queen bees by their subjects throughout life. Royal jelly is a thick, milky material which is generally believed to be a secretion of the pharyngeal glands of the nurse bees (young workers). For a proper appreciation of the nutritional significance of royal jelly the following facts about the natural history of bees should be recalled.

A colony of bees consists of one queen, several hundred drones or males and from 25,000 to 100,000 worker bees, sexually undeveloped females. This large number of workers are all the daughters of one queen. As the bees die off constantly the queen may, during the whole season, become the mother of a quarter of a million bees. At the height of the season, queens have been known to lay more than 2000 eggs (over their own weight in eggs) in one day. Such fecundity must be associated with a very active metabolism and the nature of the food which makes this possible is of interest. But of greater nutritional significance is the fact that the anatomical and physiological differentiation of the female larvae is dependent upon the nature of their diet. Two types of eggs are laid by the queen: (1) unfertilized eggs which give rise only to drones (male bees), and (2) fertile eggs which give rise to females, either workers (sexually immature females) or queens. By grafting young larvae, it has been proven that a nutritional difference determines whether a fertile egg is to become a worker or a highly specialized egg-laying organism, the queen. All female larvae are fed on royal jelly for the first 2-3 days after hatching and during this period their anatomical development is similar. Only the queen continues to receive this special diet. Any larva from a fertile egg, if given royal jelly throughout its larval period, develops sexually so that it becomes a perfect or true female bee, or what is called a queen; otherwise, the larva develops into a sexually immature worker. The queen is structurally much the same as the workers but with these important differences: the pollen-gathering apparatus remains undeveloped, the mouth parts and sting are modified, while the spermatheca and ovaries are highly developed.

Previous chemical work on the nature of royal jelly is very limited. The first three papers dealt only with proximate analyses, since the workers were mainly concerned with nutritional aspects of the problem. The early analyses carried out by Von Planta [1888] and more recently confirmed by Aeppler [1922] and

¹ In view of the fact that one of the authors (G. F. T.) is changing his post, it has been considered advisable to publish this preliminary report, although several of the fractions have not yet been investigated very thoroughly.

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Elser [1929] indicate a similarity in the food of the worker and the queen larvae during the first 2-3 days of larval development. The food of the larvae destined to become workers is changed on the third day to one much richer in carbohydrate material (honey and pollen) while the queen continues to be raised on royal jelly. Several months ago Melampy & Jones [1939] published the results of a careful study of the proximate chemical composition and vitamin content of royal jelly collected in Louisiana, U.S.A. The average values indicated 66% moisture, 0.82% ash, 12.34% protein, 5.46% total lipid, 12.49% total reducing substance (by the dinitrosalicylic acid method of Sumner) and 2.84% undetermined. Vitamin A could not be detected, vitamin B₁ was present (1-1.5 international units per g.), but little, if any vitamin C was found in the sample studied.

With the discovery of vitamin E, it was thought that possibly royal jelly owed its astonishing sex-differentiating property to the presence of large quantities of this vitamin. Hill & Burdett [1932] performed experiments to test this interesting hypothesis and claimed that if pregnant rats on vitamin E-deficient diets were fed 50 mg. of royal jelly per day it would prevent resorption of the embryos. In 1936, Melampy & Mason subjected the above work to critical examination and found several possible sources of error in both the technique and conclusions of Hill & Burdett. Melampy & Mason repeated the experiments under better controlled conditions and found that even as much as 1 g. of royal jelly fed daily throughout the period of gestation does not supply sufficient vitamin E to permit the completion of a normal pregnancy in E-deficient female rats. This finding has been thrice confirmed [Schoorl, 1936; Evans, *et al.* 1937; Haydak & Palmer, 1938]. It would appear, therefore, that some nutritional factor other than vitamin E is responsible for the production and maintenance of queen honey bees.

Quite recently Heyl [1939] has described observations which suggest that a gonadotropic hormone occurs in royal jelly. Dilute NaOH extracts or aqueous pyridine extracts of royal jelly, when injected subcutaneously for 5 days into 21-day-old female rats, caused an increase in weight and in follicular activity of the ovaries. This observation may be related to the finding in this laboratory, that the ether-soluble fraction of royal jelly, when added to the diet of fruit-flies (*Drosophila*), caused a marked increase in the rate of reaching sexual maturity and also increased the number of eggs laid per day (by about 60%). These experiments will be reported in detail elsewhere.

Experimental

The collection of sufficient royal jelly for investigation is a matter of great difficulty since normally one finds only a very few queen cells per hive and the amount of material per cell is very small. The royal jelly was collected from the bottom of queen cells containing larvae 2-3 days old. The queen cell was cut from the frame, the larva removed and the jelly squeezed out.

The small individual samples of royal jelly were pooled and kept in a refrigerator at about 0° until all the available material had been collected. This amounted to about 100 g., over 20 g. of which was used in feeding experiments to be described elsewhere. Royal jelly from different parts of Ontario was kept separate. Each sample was weighed and dried over CaCl₂. Towards the end of the drying period the material was powdered, screened and returned to the desiccator. It was finally dried over P₂O₅ *in vacuo*, at room temperature, until it reached constant weight. This process took approximately 6 weeks. The various samples of royal jelly, as received, were found to vary in moisture content from 45 to 65%.

Preliminary fractionation

The dried material was extracted exhaustively with ether in a Soxhlet extractor and the residue, suspended in water, was dialysed. Four main fractions were thus obtained as shown in Table 1.

Examination of the fractions

Fraction 1. The ether-soluble material was a creamy coloured semi-crystalline solid of waxy consistency with a faint flowery to spicy odour. It contained 0.07% phosphorus and 0.3% nitrogen. The fraction was redissolved in ether and shaken with *N* NaOH to remove acids and phenols. After several such treatments about 90% of the ether-soluble material went into the alkaline aqueous layer. The complete fractionation is shown in Table 2.

After the treatment with 1*N* NaOH, the ether layer ("lipid" fraction) was dried with anhydrous Na₂SO₄, evaporated to dryness and weighed. The residue was redissolved in the smallest possible amount of ether and poured into several vol. of acetone. A small precipitate (about two-thirds of the "lipid" fraction or 6% of fraction 1) was formed. This acetone precipitate should contain phosphatides and waxes. After being dried and weighed, it was taken up in ether and poured into alcohol. The resulting precipitate, which contained neither N nor P, melted sharply at 69° and on reprecipitation the M.P. remained unchanged. It accounted for about 5% of fraction 1 and was apparently beeswax. Purified beeswax from different sources melts at temperatures between 65 and 70° and is precipitated by alcohol or acetone from ether solution in exactly the same manner. The minute amount of alcohol-soluble, acetone-insoluble material (about 0.4% of fraction 1) is apparently phospholipin but it has not been examined.

The ether-acetone solution was evaporated to dryness and weighed. This "fat" fraction accounts for about 3-4% of fraction 1. One portion of the small residue was dissolved in chloroform and tested for cholesterol. The Liebermann-Burchard reaction was strongly positive. Another portion was used for a micro-determination of the saponification number; the values found were 116 and 118. These low figures may indicate sterol esters or glycerides mixed with free sterols.

Table 2. *Fractionation of ether-soluble material extracted from dried royal jelly*

Owing to the minute amount of material available, this fraction was not further characterized.

The alkaline extract obtained from the original ether solution of fraction 1 was treated with CO₂ to set free the phenols. A small amorphous precipitate with the characteristic flowery to spicy odour (reminiscent of cloves) was obtained. The aqueous solution was extracted with ether to remove completely any phenols. Phenols accounted for 4 and 10%, respectively, of fraction 1 in two different samples of royal jelly which were worked up separately to this stage. The crude phenol melted at 65°; when recrystallized from dilute alcohol and then from aqueous acetone it melted at 69°. An attempt to identify it as the aryloxyacetic acid derivative gave no crystalline product.

The alkaline aqueous extract (freed of phenols) was acidified with H₂SO₄ and extracted with ether. The ether solution was dried over anhydrous sodium sulphate and evaporated. An amorphous residue with low indistinct melting point resulted. It was taken up in hot alcohol, and water was added to produce turbidity. The solution was warmed to clear it; on cooling slowly crystals separated. They were not well formed, were of a soft, waxy consistency and melted over a wide range (45-56°). When recrystallized from aqueous acetone, long prisms and poorly formed plates melting at 45-56° were obtained. Titration in dilute alcohol with 0.1 N NaOH gave a sharp end-point corresponding to an equivalent weight of 178. Fractional crystallization from aqueous acetone and from aqueous alcohol failed to resolve this acid fraction into materials of sharper melting point or of different equivalent weight. It appears to soften about 46 to 48° and to melt at 58°, but not sharply. The acid is probably not yet pure; further attempts to fractionate the material are being made. In the meantime, the following data may be recorded. A 10% solution in absolute alcohol is optically inactive. The free acid is insoluble in cold water and only slightly soluble in hot water; it is soluble in alcohol, ether, acetone, chloroform and benzene, but it is insoluble in light petroleum. The Na and Ba salts are soluble in water, the Pb and Cu salts are insoluble. It does not contain N, S or P.

A micro-analysis (Schoeller) gave C=64.23, 64.12% and H=9.48, 9.63% with a trace (0.19%) of ash residue. These figures agree fairly well with the

formula $C_{10}H_{18}O_3$; calculated C=64.45%, H=9.68%, mol. wt.=186. The acid (or mixture) does not absorb Br in chloroform solution and is therefore not unsaturated. It does not lose weight when kept slightly above its melting point for several hours *in vacuo* over P_2O_5 and is apparently not an anhydride-acid or lactone-acid mixture, since titrations at 0 and 100° give the same values. Further work on this acid will be done as soon as more material can be obtained.

Fraction 2, the water-soluble, dialysable material, was evaporated *in vacuo*. The resulting syrupy solution was transferred to a wide dish and put in a vacuum desiccator over P_2O_5 . The drying agent was renewed frequently for several weeks and the sticky residue was kept broken up to hasten drying. When brought to constant weight the toffee-like residue accounted for about 55% of the dried royal jelly. It contained 1.2% N, 0.37% inorganic P and 0.82% total P. Upon ignition, 227 mg. left a residue of 7.8 mg., corresponding to 3.4% ash. It gave negative tests for chloride and sulphate. The test for Ca was positive.

A portion of fraction 2 was dissolved in water to give a 20% solution, from which 5 and 1% solutions were prepared. The 1% solution was acid in reaction (pH 3.6). Sufficient of the 5% solution to contain exactly 100 mg. of the residue required 5.2 ml. 0.01 N NaOH to neutralize it to phenolphthalein. Another preparation of this fraction obtained a year later required 6.4 ml. 0.01 N Ba(OH)₂ at 0° and 7.3 ml. at 50°. Such neutralized solutions reduced Fehling's solution strongly; even in the cold there was a slight reduction in 7-8 min. The behaviour suggested the presence of uronic acids as well as sugars. Tollen's naphthoresorcinol test, with benzene extraction [Neuberg & Saneyoshi, 1911] was negative when applied directly, but since glucose is known to interfere [Brule, *et al.* 1925] the solution (containing 100 mg. fraction 2) was treated first with 0.3 ml. 20% lead acetate and then with 2 ml. basic lead acetate to precipitate any uronic acids, thus separating them from sugars. The precipitates were separately washed twice with water, treated with 1:1 HCl and 0.5 ml. 1% alcoholic naphthoresorcinol solution on a boiling water bath and the cooled mixtures were extracted with benzene. The neutral lead precipitate gave rise to a green-red fluorescent coloration and a dark turbidity in the aqueous layer; the benzene extract was a pale reddish-brown by transmitted light, faintly bluish-purple by reflected light. While this was not a typical positive test for uronic acids, the behaviour seemed to be suggestive. Fructose, if present, would cause a reddish-brown colour in the aqueous solution and a yellow-brown coloration of the benzene extract, but interference is not due to fructose, since in known solutions it was readily separated by the lead procedure. The basic lead precipitate gave an even less satisfactory test. Even when 5 mg. glucurone were added to 100 mg. of the fraction, the test (after precipitation with lead) was weaker and more reddish-brown in colour than the blue-violet coloration obtained from 5 mg. glucurone in simple solution treated similarly. If a uronic acid is present, interfering substances obscure the test. Attempts to improve the colour by fractional precipitations with lead were unsuccessful. Ascorbic acid was found to behave somewhat similarly to the unknown in royal jelly; with naphthoresorcinol it gave a pale reddish-brown colour in the benzene extract. However, the aqueous solution of fraction 2 failed to reduce $AgNO_3$ in acid solution, although ascorbic acid in simple solution or added to fraction 2 did so even at room temperature. Identification of the material responsible for the reaction observed awaits further investigation.

Fraction 2 gave positive Seliwanoff and Ihl-Pechmann tests for ketoses. On one sample a Willstätter-Schudel titration indicated that 40% of fraction 2 was aldose; a sample obtained later contained less aldose, about 20% of the

fraction. Total reducing sugars were determined on the first sample by copper reduction according to the A.O.A.C. methods before and after fermentation with washed yeast (*S. cerevisiae*), indicating 49 % and less than 1 % respectively.

A differential fermentation was carried out on the second sample of fraction 2 through the kindness of Dr T. F. Nicholson. This sample contained 53 % of total reducing sugars, calculated as glucose, determined by a modified Shaffer-Somogyi copper reagent [Harding & Downs, 1933]. By the use of a series of micro-organisms with specific fermentative or "removal" ability [Harding & Nicholson, 1933; Nicholson, 1936] this particular sample of fraction 2 was found to contain 52 % of its reducing material as fructose, 42 % as glucose and 6 % as non-fermentable matter. No other sugar was recognized by this procedure. There was no significant increase in reduction after hydrolysis with cold 2 *N* H_2SO_4 for 5 hr. or hot 0.5 *N* H_2SO_4 for 30 min.

An attempt to isolate the acid material was made as follows: Several grams of the original residue (fraction 2) were taken up in about 20 ml. water. A small insoluble portion which was filtered off was found to be soluble in alcohol; it is still unidentified. The water solution was treated with an excess of BaCO_3 to form Ba salts and evaporated on a water bath to a thick syrup. This was rubbed up four times with 95 % alcohol to remove sugars. The alcohol-insoluble Ba salts were dissolved in water and the excess BaCO_3 filtered off. The Ba salts in solution (volume reduced to 4 ml.) were precipitated with 6 vol. of alcohol. The precipitated Ba salt was taken up in water and precipitation was repeated twice more with 6 vol. of alcohol. In each case, about one-fourth of the bulk of the precipitate failed to redissolve in water. The salt was dissolved in water, Ba removed exactly with dilute H_2SO_4 and the solution was evaporated to a small volume, *in vacuo*, but would not crystallize on long standing. It was finally brought to constant weight over P_2O_5 *in vacuo*. An attempt to estimate the approximate equivalent weight of this material gave a value of 346. If this figure be accepted, the original titration indicates that the acid accounts for about 20 % of fraction 2. We have not as yet been able to isolate a pure compound in crystalline form or any crystalline derivative of this acid.

The alcoholic supernatant, freed of Ba and concentrated to remove alcohol, was made *N* with respect to H_2SO_4 . It gave a precipitate with phosphotungstic acid, but not all of the nitrogen in the solution was precipitated. The nitrogenous compounds in fraction 2 have not yet been definitely identified.

Fraction 3, the water-soluble protein, was dried over P_2O_5 *in vacuo* to constant weight and found to contain 0.5 % ash, 14.9 % N, 0.3 % P and 0.89 % S. Qualitative tests for tryptophan, tyrosine, cystine and arginine were found to be positive; the test for histidine was faintly positive. The protein (1.07 g.) was hydrolysed with 8 *N* HCl for 12 hr., at which time the amino-N was found to be at a maximum (80 % of the total nitrogen). After removing as much HCl as possible by repeated evaporation *in vacuo*, the mixture was diluted with H_2O and the black insoluble melanin filtered out. This was found to make up 1.7 % of the original weight and to contain 0.5 % of the total nitrogen.

The amino-acid solution was brought to a definite volume. One portion was used to determine a conventional N-distribution and smaller aliquots were removed for special tests.

When more of this fraction became available, a slightly larger portion (3.8 g.) of the protein was hydrolysed in the same manner. After removal of excess HCl and of melanin, the solution was made alkaline with baryta and concentrated *in vacuo* (below 40°) to a small volume. The solution of the Ba salts of the amino-acids was treated with 4 vol. of alcohol to precipitate the dicarboxylic acid

Table 3. *Nitrogen distribution of water-soluble protein*

(15.08 % N, on moisture and ash-free basis)

Melanin-N	<1 % of total N
Ammonia-N	13
Dicarboxylic acid-N	10
Basic N	21
Non-amino-N	20
Mono-amino-N	35

fraction. This fraction was taken up in the smallest possible volume of water and reprecipitated once with 3 vol. of alcohol and finally with 2 vol. of alcohol. The dicarboxylic acid fraction thus obtained contained nearly 20 % of the total N; however, both tyrosine and arginine were still present in small amount and some glycine, although not definitely identified, was probably also present. No glutamic acid could be isolated either before or after removal of aspartic acid. The latter was isolated as the copper salt in a yield corresponding to 9.9 % of aspartic acid in the original protein. The combined filtrates, after removal of alcohol, were freed of Ba with H_2SO_4 , of Cl with Ag_2SO_4 and of Ag with H_2S .

The filtrate from this procedure was made *N* with respect to H_2SO_4 and treated with phosphotungstic acid to precipitate the basic amino-acids. The filtrate from this was freed of all inorganic ions and concentrated *in vacuo*, upon which 17 mg. tyrosine crystallized out; the remaining amino-acids were converted into copper salts.

The phosphotungstate precipitate was dissolved in aqueous acetone and freed of inorganic ions with baryta. Arginine was precipitated as flavianate (m.p. 249°), corresponding to 10 % of the original protein. Neither histidine nor lysine could be obtained as crystalline derivatives, so no quantitative data concerning them are yet available, but they cannot be present in more than traces since the arginine isolated accounts for practically all of the basic N.

The copper salts have not yet been adequately examined.

Fraction 4, the water-insoluble protein, dissolves only in alkalis, e.g. 0.5 % Na_2CO_3 . It contains 13.4 % N and 0.16 % P. It contains S, but no Pb-blackening S. Tests for tyrosine, tryptophan and arginine are positive. It has yet to be investigated.

SUMMARY

Royal jelly has been separated into four main fractions: (1) ether-soluble, (2) water-soluble and dialysable, (3) water-soluble, non-dialysable and (4) water-insoluble.

Fraction 1 appears to consist principally of an organic acid (or mixture of acids), m.p. 58° , equivalent weight, 178. Small amounts of phenolic material and smaller amounts of beeswax, sterol, phospholipin and a saponifiable substance also occur in this fraction.

Fraction 2, the largest fraction, is about 50 % sugars; glucose and fructose account for most of the reducing material. An unidentified acid, inorganic salts and nitrogen compounds are also present.

Fraction 3 is protein in nature. Its N distribution has been determined; aspartic acid, arginine and tyrosine were isolated; colour tests for tryptophan, cystine and histidine are positive.

Fraction 4 is a protein, soluble only in alkalis. It gives positive colour tests for tyrosine, tryptophan and arginine.

There is some evidence that fraction 1 contains the physiologically active material responsible for sexual development of the queen bee.

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REFERENCES

- Aeppler (1922). Gleanings in Bee Culture, **50**, 151.
Brule, Garban & Amer (1925). *Chem. Zbl.* **11**, 1375.
Elser (1929). *Markische Bienen Ztg.* **19**, 232.
Evans, Emerson & Eckert (1937). *J. econ. Ent.* **30**, 642.
Harding & Downs (1933). *J. biol. Chem.* **101**, 487.
—— & Nicholson (1933). *Biochem. J.* **27**, 1082.
Haydak & Palmer (1938). *J. econ. Ent.* **31**, 576.
Heyl (1939). *Science*, **89**, 540.
Hill & Burdett (1932). *Nature, Lond.*, **130**, 540.
Melampy & Jones (1939). *Proc. Soc. exp. Biol., N.Y.*, **41**, 382.
—— & Mason (1936). *Proc. Soc. exp. Biol., N.Y.*, **35**, 459.
Neuberg & Saneyoshi (1911). *Biochem. Z.* **36**, 56.
Nicholson (1936). *Biochem. J.* **30**, 1804.
Von Planta (1888). *Hoppe-Seyl. Z.* **12**, 327.
Schoorl (1936). *Z. Vitaminforsch.* **5** (4), 246.