A COMPARISON OF METHODS FOR QUANTITATIVE ESTIMATION OF HYPOXANTHINE, XANTHINE, AND URIC ACID IN INSECT MATERIAL[1]

J. L. NATION AND K. K. THOMAS
Zoology Department, University of Florida, Gainesville

The purine uric acid is the usual nitrogenous metabolite of protein catabolism in insects. Hypoxanthine and xanthine are purines which are normally converted into uric acid and therefore do not appear in insect excreta; however, since the discovery of all three of these purines in excreta of the sheep ked, *Melophagus ovinus* Meig., (Nelson 1958) various workers have found them in excreta of three additional species. There is no quantitative information available at present on excretion of hypoxanthine and xanthine in any species.

Excretory hypoxanthine and xanthine may be detrimental to insects by interfering with the normal water reabsorption process occurring in the rectum. The possibility of some adverse effect has been suggested by Mitchell *et. al.* (1959), who showed that there is about fifty per cent failure to successfully emerge from the pupal stage at 25°C by a *Drosophila melanogaster* Meig. mutant, *rosy*,[2] which excretes hypoxanthine instead of uric acid. Both hypoxanthine and xanthine are approximately 25 times as soluble as uric acid (Dawson *et. al.* 1959), which means that more water must be available for excretion, or that more energy must be expended in reabsorbing needed water against an increased osmotic gradient when these two purines make up a significant amount of the nitrogen excreted.

The larvae of the wax moth, *Galleria mellonella* (L.), excrete hypoxanthine and xanthine, although uric acid is the major nitrogenous metabolite (Nation and Patton 1961, Nation 1963). Quantitative information concerning these three purines in *Galleria* would clearly be of value in assessing the possible effects on water conservation, and also might be expected to yield fundamental information concerning the physiology of nitrogen metabolism.

There are several methods for purine analysis available, but a careful study of their suitability for use on crude preparations of insect tissue is needed. A method should be specific and accurate at the microgram level, for volumes of haemolymph or excretory material available from many insects are exceedingly small.

The quantitative methods reported upon in this paper are well known methods which have been used successfully to estimate purines in a variety of biological material. The purpose of the present study has been to determine which, if any, of these methods are suitable to estimate purines in insect tissues and excreta.

The colony of *Galleria* was started from field collections of larvae near Gainesville, Florida, and maintained in the laboratory on Haydak's diet (1936). Hypoxanthine, xanthine, and uric acid were purchased from Nutritional Biochemicals Corporation. Folin phenol reagent was purchased from Curtin and Company.

---

[1] This research was supported in part by NSF grant GB-1088.

Extracts of excretory material were made by grinding the large pellets from *Galleria* larvae in the last or next to last instar with 3 ml portions of 0.01 M lithium carbonate in a mortar. The solution of the purines was removed from an insoluble residue by centrifugation. Repeating the extraction and centrifugation procedure on the residue three additional times extracted all detectable purines in up to 100 mg of excretory material. The supernatants from each extraction were combined and diluted with distilled water as dictated by the sensitivity of the method being used.

The differential spectrophotometric method following separation of purines by chromatography and electrophoresis was tested for hypoxanthine estimation (Vischer and Chargaff 1948). Xanthine was determined with the Folin phenol reagent on crude, unpurified extracts using the procedure of Litwack, *et. al.* (1953) and also following a purification step according to Williams (1950). Uric acid was estimated colorimetrically with the arsenophosphotungstic acid reagent prepared according to Benedict (1922). Kalckar's method (1947) was used to estimate all three of the purines concerned in this study in crude, unpurified extracts of excreta. Xanthine oxidase for this method was prepared from fresh milk by the method of Horecker and Heppel (1949). Purification of the xanthine oxidase was stopped after the second ammonium sulfate fractionation. Thus prepared the enzyme had an activity of 1000 units/ml enzyme solution in the assay of Plesner and Kalckar (1956). The enzyme was stored at −18°C until needed. Commercial uricase powder (Nutritional Biochemicals Corporation) was suspended in distilled water to produce an activity of 100 units/ml and stored at −18°C (Plesner and Kalckar 1956).

A Beckman DU spectrophotometer with an ultraviolet light source was used in the methods requiring measurements in the ultraviolet region of the spectrum, and a B & L Spectronic 20 instrument served for the colorimetric determinations.

TABLE 1.—COMPARISON OF QUANTITATIVE METHODS FOR DETERMINATION OF PURINES IN CRUDE EXTRACTS OF *Galleria* LARVAL EXCRETA.

Compound	mg/g Excreta	Method
Hypoxanthine	8.46 ± 2.65*	Vischer and Chargaff (1948)
	2.91 ± 0.52	Kalckar (1947)
Xanthine**	17.57	Litwack et al. (1953)
	8.68	Williams (1950)
	6.38	Kalckar (1947)
Uric Acid†	51.50	Benedict Reagent (1922)
	36.81	Kalckar (1947)

* Standard deviation of mean; at least six determinations made, but the same excretory samples were not used in each method.

** Portions of the same sample of excreta used in each method.

† Mean of nine samples determined by both methods.

We were aware that many substances in a crude extract might interfere in the direct spectrophotometric and colorimetric methods being used, and, as Table 1 shows, different methods used on the same sample

produced widely divergent values. By means of paper chromatography and paper electrophoresis we were able to separate from the crude extract at least four substances, some of which, when eluted from the paper and added to standard solutions of the purines, interfered in one or another of the direct methods. No completely successful scheme for separation of the purines from all interfering substances in the crude extract was perfected. Careful experimentation with these same substances failed to show any interference in the method of Kalckar (1947). All the methods except those of Kalckar and Vischer and Chargaff suffer from the further drawback that they are not applicable to all three purines which we wished to determine.

Table 2 shows that Kalckar's method is sensitive enough to determine purines at levels to be expected in insect tissues and excreta, and the accuracy even at the lower limit of the method, which is about 0.3 μg/ml (Kalckar 1947), is within an acceptable range.

TABLE 2.—EXPERIMENTS ON PER CENT RECOVERY AFTER ADDITION OF PURINES TO AN EXTRACT OF *Galleria* LARVAL EXCRETA. PURINES DETERMINED BY THE METHOD OF KALCKAR (1947). PER CENT RECOVERY CALCULATED AS (FOUND)/(PRESENT + ADDED).

Exp. no.	Purine	Present in extract μg/1.2 ml*	Added μg	Total found μg	Recovery %
1	Hypoxanthine	2.20	1.50	3.50	94.6
	Xanthine	5.79	2.50	8.44	101.8
2	Hypoxanthine	1.85	1.50	3.55	105.9
	Xanthine	6.37	2.50	7.80	87.9
3	Uric Acid	2.73	5.00	6.96	89.9

* Final volume of reaction mixture was 3.0 ml.

We have used Kalckar's method to determine purines in *Galleria* haemolymph, uric acid in haemolymph of *Periplaneta americana* L., and purines in excreta of several other insects without experiencing any difficulties or inadequacies of the method.

SUMMARY

We have made comparative studies on several potentially suitable methods for quantitative estimation of purines in insect tissues and excreta. The results show substances in excreta of the wax moth, *Galleria,* which interfere in some of the methods; however, no interference is indicated when the method of Kalckar (1947) is used. Further advantages of Kalckar's method are speed, accuracy, and sensitivity compatible with quantities of purines to be expected in insect tissues and excreta.

LITERATURE CITED

Benedict, S. R. 1922. The determination of uric acid in blood. J. Biol. Chem. 51: 187-207.

Dawson, R. M. C., D. C. Elliott, W. H. Elliott, and *K. M. Jones.* 1959. Data for biochemical research. Oxford Univ. Press, Oxford, England. 299 p.

Haydak, M. H. 1936. Is wax a necessary constituent of the diet of wax moth larvae? Ann. Ent. Soc. Amer. 29: 581-588.

Horecker, B. L., and *L. A. Heppel.* 1949. The reduction of cytochrome c by xanthine oxidase. J. Biol. Chem. 178: 683-690.

Kalckar, H. M. 1947. Differential spectrophotometry of purine compounds by means of specific enzymes-I. Determination of hydroxypurine compounds. J. Biol. Chem. 167: 429-443.

Litwack, G., J. W. Bothwell, J. N. Williams, Jr. and *C. A. Elvehjem.* 1953. A colorimetric assay for xanthine oxidase in rat liver homogenates. J. Biol. Chem. 200: 303-310.

Mitchell, H. K., E. Glassman, and *E. Hadorn.* 1959. Hypoxanthine in *rosy*^{2} and *maroon-like* mutants of *Drosophila melanogaster.* Science 129: 268-269.

Nation, J. L. 1963. Identification of xanthine in excreta of the greater wax moth, *Galleria mellonella* (L.). J. Insect Physiol. 9: 195-200.

Nation, J. L., and *R. L. Patton.* 1961. A study of nitrogen excretion in insects. J. Insect Physiol. 6: 299-308.

Nelson, W. A. 1958. Purine excretion by the sheep ked, *Melophagus ovinus* (L.). Nature, London. 182: 115.

Plesner, P., and *H. M. Kalckar.* 1956. Enzymatic microdeterminations of uric acid, hypoxanthine, xanthine, adenine, and xanthopterine by ultraviolet spectrophotometry. Methods in Biochemical Analysis 3: 97-110.

Vischer, E., and *E. Chargaff.* 1948. The separation and quantitative estimation of purines and pyrimidines in minute amounts. J. Biol. Chem. 176: 703-714.

Williams, J. N., Jr. 1950. A micromethod for the determination of xanthine and guanine in urine. J. Biol. Chem. 184: 627-632.