PROCEEDINGS
OF THE
NATIONAL ACADEMY OF SCIENCES
Volume 30 October 15, 1944 Number 10

*VISION AND GUANINE PRODUCTION IN FISHES**

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Communicated August 29, 1944

Zoölogists have long known that the purine body guanine, alone or in some combination, is responsible for the silvery appearance of areas of skin in most fishes. It is also a familiar fact that crystals of this substance are contained in special cells, variously called guanophores, leucophores or iridocytes, which respond, as do other chromatophores, to stimuli received through the eyes. The paling of a fish upon a white background results in part from the withdrawal from view of dispersed melanin granules within the melanophores, but also in part from the centrifugal movement of guanine particles within the guanophores. Reverse relations, with respect to both substances, are manifest upon a black background.

That the same stimuli which call forth these rapid adjustments may result, when long continued, in actual large-scale changes in the amount of pigment present has been shown by a number of workers.[1] For melanin, fairly exact relations have now been established in certain fishes between the amount of this pigment and the albedo of the background to which they have been subjected.

For guanine, no exact relations of this sort have hitherto been determined, though several workers have recorded the occurrence of an increase in the number of guanophores or in the quantity of visible guanine in animals kept in white containers.[2] In general, however, guanophores have received relatively little attention from students of chromatophore activity. This is doubtless largely due to their inconspicuousness in comparison with melanophores, likewise to their ordinarily being rendered invisible in histological preparations. The only worker known to me who professes to have undertaken the measurement of guanine in connection with color changes in animals (Meyer, op. cit.) reports a purely negative result, concluding that any possible experimental differences were swamped by the individual variability among the animals studied. This writer fails to state the method which she employed in making these measurements.

After the somewhat extended assays which have been made in this laboratory of the black pigment melanin, in relation to the visual environment, a comparable series of quantitative determinations of the white pigment guanine seemed called for.[3]

A local marine fish, *Girella nigricans*, was employed for this purpose. Two hundred and forty sexually immature[4] specimens were apportioned among 20 painted glass bowls.[5] Of the latter, there were four each of five shades, ranging as follows in respect to albedo, relative to white as a standard.

White	100.00
Pale gray	37.74
Medium gray	17.80
Dark gray	6.90
Black	1.42

Each set of four nearly identical bowls was kept in a separate cabinet, lighted by a 100-watt lamp overhead. Running sea-water was supplied to the bowls.

Of the original number of fishes, 217 were alive at the end of four months, when they were killed with chloroform vapor, dried with paper towels and weighed, commonly in lots of six or seven. These were then dipped into boiling distilled water for three-fourths of a minute and the "skins," including the scales and fins, were removed and dried for several hours in an oven at 100°C. These dried "skins" were also weighed to the nearest tenth gram.

The fishes from the first bowl in each cabinet, which were fewer in number and much larger than the others, were sacrificed for preliminary tests of method. The occupants of the other three bowls of each set were divided into two lots of six or seven fishes each. There were thus six lots from each cabinet (thirty lots in all) which were available for guanine assay. Of these six lots only five have been used.

Thus the fishes under consideration are designated as follows: "1," "2.1," "2.2," "3.1," "3.2," "4.1," "4.2". Of these the "1" lots were used for preliminary tests, while the "4.2" lots have not yet been used at all.

The matter is further complicated by the fact that the solid material of the original "2.1" series was divided into two equal portions ("2.1*a*" and "2.1*b*") and each of these was treated as an independent sample. The results from these are not quite comparable with those from the subsequent series, in that the extraction with acid from the former was less complete than from the latter.

For the methods which I have employed in the extraction and measurement of guanine I am indebted to Dr. George H. Hitchings of the Wellcome Research Laboratories, Tuckahoe, New York, who is reporting upon his

own studies of some of the same material in another article in this issue of the PROCEEDINGS.[6] It is a pleasure to thank Dr. Hitchings for help and advice, kindly given at various stages of this undertaking, though I cannot hold him responsible for every minor step in my procedure. I must also acknowledge the important help of my colleague, Dr. Denis L. Fox, whom I have consulted frequently throughout these studies, as well as using some of the facilities of his laboratory. I must mention, too, the valuable technical assistance of Miss Ruth A. Larson during the earlier stages of these investigations.

The procedure here adopted was as follows:

1. The oven-dried skin was extracted with $N/1$ H_2SO_4, in the proportion of 10 cc. of the dilute acid per gram of the original wet weight of the fishes. Extraction here comprised grinding of the dampened material in a mortar, followed by four cycles[7] of prolonged shaking and centrifuging, in alternation, using a new fraction of the acid for each cycle.
2. Measured portions of this acid extract were heated for one-half hour in 100-cc. centrifuge tubes in a water bath.
3. These were made slightly alkaline with NaOH, using phenolphthalein as an indicator.
4. The contained purines were precipitated with copper sulfate, followed by sodium bisulfite, at boiling temperature.
5. The precipitate was brought down by centrifuge.
6. Precipitate washed twice with boiling water, followed each time by centrifuging.
7. Precipitate redissolved in boiling dilute HCl.
8. Copper precipitated by H_2S, passed through the heated solution.
9. Last precipitate removed from the guanine-HCl solution by filtration through a sintered glass filter.
10. Phenol reagent[8] (Folin-Denis-Ciocalteu) added to the filtrate, followed by Na_2CO_3.
11. Colorimetric determinations made of the resulting blue solution by means of a Bausch and Lomb Visual Spectrophotometer.

The degree of specificity to be expected from this method is discussed by Hitchings and Falco (pp. 294–297 of this issue). These workers have likewise added independent evidence, by the application of an altogether different method, that we are actually dealing here with guanine. This is important, since the procedure which I have employed is not sufficiently specific to distinguish between guanine and certain closely related compounds. It is gratifying to note that the values obtained by Hitchings and Falco, by the use of these two methods agree very closely; and it is further gratifying to note the reasonably close agreement between their figures and mine for the four samples which were assayed independently in the two laboratories

by the copper precipitation method (Black 3.1, 3.2; White 3.1, 3.2). My figures should be compared with those in the middle column of Hitchings and Falco (0.285, etc.).

In making the spectrophotometer determinations, absorption values (logarithmic) were recorded by me at 550, 600 and 650 mμ. Colorimetric

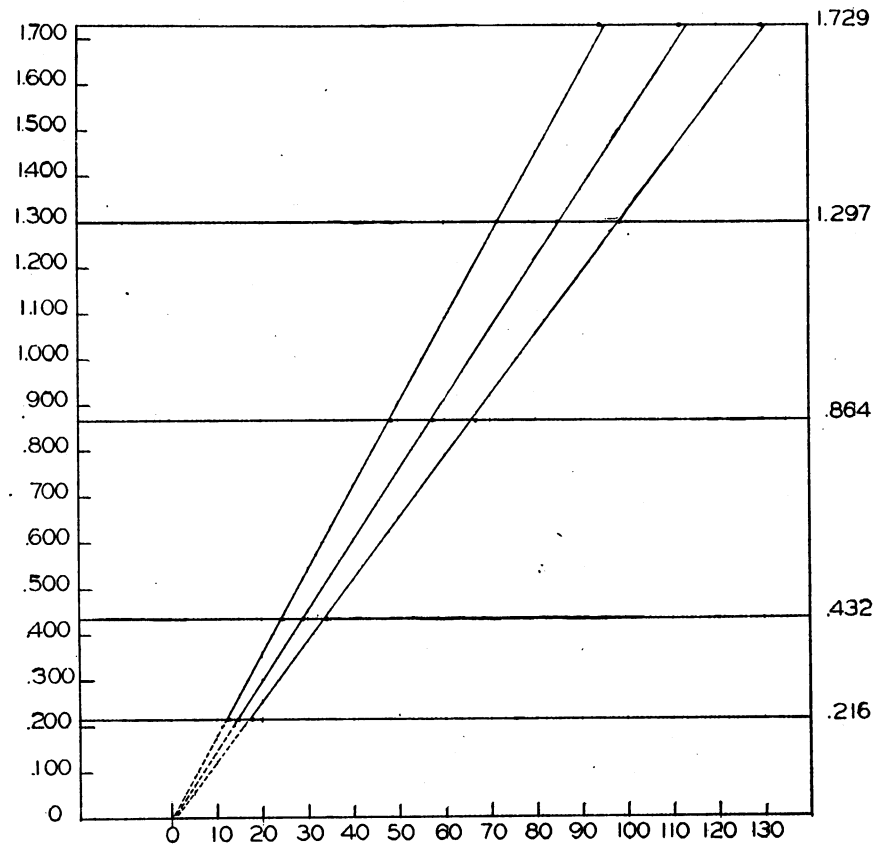

FIGURE 1

Readings with dilutions of standard guanine-HCl solutions. Abscissae = spectrophotometer readings (logarithms of absorption at 550, 600 and 650 mμ). Ordinates = mg. guanine per 100 cc. of solvent.

analysis of this sort obviously requires the previous plotting of graphs, based upon various dilutions of a standard guanine solution (Fig. 1). The values given in the tables, and in figures 2 and 3, are the average values obtained for these three wave-lengths. Five readings at each wave-length were comprised in each determination. It must be added that two to five subsamples of the acid extract were employed in the case of every lot of

fishes, and that the means here presented are based upon all the values thus obtained, commencing with "2.1*a*," except for a few cases in which the treatment had been varied for special purposes.

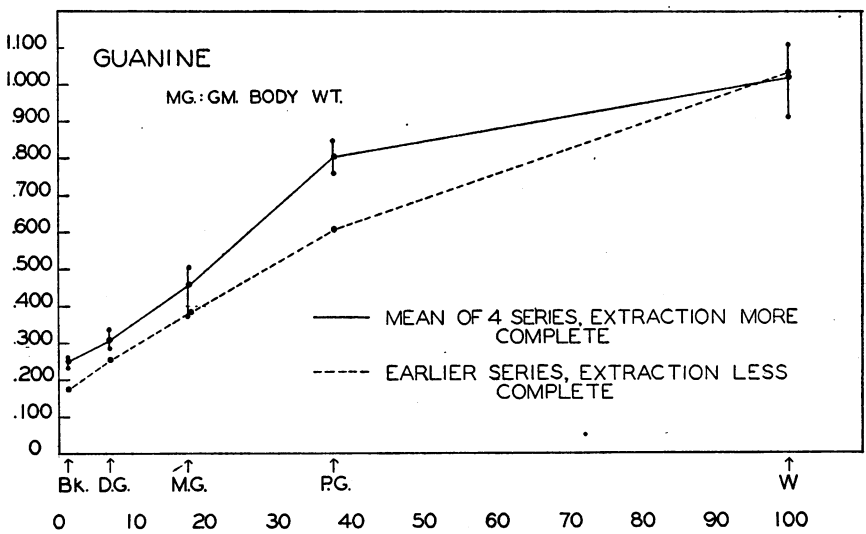

FIGURE 2

Guanine values for *Girella* series. Abscissae = albedos of the five shades of background. Ordinates = mg. guanine per g. of wet weight of fishes. Continuous line based on means of series "2.2," "3.1," "3.2," "4.1." Range of each series indicated by vertical bars. Broken line based upon means of "2.1*a*" and "2.1*b*."

TABLE 1

GUANINE, FIRST EXTRACTION

	BLACK		DARK GRAY		MEDIUM GRAY		PALE GRAY		WHITE	
	MG./G TOTAL WT.	MG./G DRY SKIN	MG./G TOTAL WT.	MG./G DRY SKIN	MG./G TOTAL WT.	MG./G DRY SKIN	MG./G TOTAL WT.	MG./G DRY SKIN	MG./G TOTAL WT.	MG./G DRY SKIN
2.1*a*	0.175		0.243		0.366		0.604		1.036	
2.1*b*	0.179		0.264		0.397		0.618		1.021	
Means	**0.177**		**0.253**		**0.381**		**0.611**		**1.028**	
2.2	0.231	3.61	0.286	4.27	0.371	5.80	0.756	10.65	0.972	13.89
3.1	0.260	3.71	0.293	4.58	0.483	6.90	0.766	11.27	1.084	15.27
3.2	0.239	3.62	0.300	4.48	0.504	6.00	0.851	11.35	0.913	13.63
4.1	0.256	3.77	0.333	5.05	0.467	6.97	0.851	12.33	1.110	16.32
Means	**0.246**	**3.68**	**0.303**	**4.59**	**0.456**	**6.42**	**0.806**	**11.40**	**1.020**	**14.78**

While occasional considerable differences were encountered between subsamples which should have yielded identical results, the mean deviation of such values from their average was 2.5 per cent (extremes: 0 and 6.6 per cent). Such differences are mainly small in comparison with the differences among the various experimental lots which had been subjected to different backgrounds.

The mean guanine values of the various lots of fishes herein discussed are presented in table 1. These values have been computed in two ways. In one column is presented the amount of guanine in the "*skin*" (as defined above) per gram of *total original wet weight* of the fishes. This may not seem to be a very logical value to employ, since the very considerable amount of guanine present in some other tissues of the body was not ordinarily known. In the other column we have the amount of guanine in the skin, in proportion to *the dry weight of the skin*. This last would doubtless be the preferable value to employ, except for the circumstance that the "skin," as here dealt with, was inevitably accompanied by a small but rather variable amount of adhering muscle. This would somewhat affect its weight, probably without correspondingly affecting its absolute guanine content. Thus the values in the first of these columns seem to furnish a fairer index of the changes in the concentration of this substance. Actually, the figures based upon the dry weight of the skin yield a graph very similar to those shown in figure 2.

No calculation of probabilities seems necessary in support of the relations shown by these graphs. Considering the four fully comparable series, "2.2," "3.1," "3.2," and "4.1," we find that the guanine content of fishes from the white bowls is 4.15 times that of those from the black ones, when ratios to total wet weight are considered; 4.02 when ratios to dry skin weight are considered.

In view of the readily visible guanine deposits in the peritoneum of these fishes, it is not surprising that considerable of this substance may be extracted from their "bodies" after removal of the "skins." Only two lots were tested for this purpose, but in these the total amount contained in the "bodies" was absolutely greater than that in the "skins" (1.06 and 1.19:1). Since the latter constituted only about 23 per cent[9] of the dry weight of the fishes, the concentration of guanine was obviously much greater in the skins.

It was early found that even after four "cycles" of extraction, as above described, the fish material continued to yield some substance which gave distinct traces of the blue reaction with phenol reagent and sodium carbonate. The material from the "4.1" series was subjected to these additional extractions, these being carried through four cycles, as had been done originally. The results are shown in table 2.

The mean absolute values of these "second extractions" thus do not differ significantly for the different lots. Indeed, their magnitude appears to bear no relation to the source of the chromogen, a circumstance which is in striking contrast to the figures for the "first extractions," which range from 0.256 to 1.110, as we pass from black to white. This fact suggests that we are dealing with a different chromogen in the earlier and later extractions, and that in these last the amount is not influenced by optical conditions.[10]

I may add that a third extraction (i.e., series of extractions) yielded 0.009 and 0.008, respectively, for "black" and "white" lots (the other lots were not tested). These last figures are of about the same magnitude as was obtained when the H_2SO_4 alone, of the same dilution, was employed, without any known contamination by organic matter. It would seem that all of these guanine measurements are subject to a small constant error, which perhaps could be determined more precisely and applied to the results presented in the tables.

Several earlier writers have stressed the reciprocating relations between guanophores and melanophores, resulting from changes in the background, but without attempting to measure either of the pigments concerned. Thus Kuntz (1917) remarks that "complete adaptation to a white background involves a rearrangement and probably an increase in the number of the guanophores and a material reduction in the number of melanophores in the layer just beneath the epidermis" (op. cit.).

TABLE 2

GUANINE, SECOND EXTRACTIONS

	FIRST EXTRACTIONS	SECOND EXTRACTIONS 1	2	MEAN	% OF FIRST EXTRACTION
Black	0.256	0.021	0.017	0.019	7.4
Dark gray	0.333	0.024	0.020	0.022	6.6
Medium gray	0.467	0.024	0.024	0.024	5.1
Pale gray	0.851	0.022	0.019	0.021	2.5
White	1.110	0.025	0.018	0.021	1.9

Murisier (1920–1921) notes that melanin and guanine seem to be substances which may substitute for one another, the amount of one which is formed being inverse to that of the other. Like melanin, it is formed under the influence of retinal stimuli. Murisier suggests that both guanophores and melanophores form an accessory excretory system, but one which retains its products instead of discharging them.

Millot (1923) sets forth these same ideas at greater length: "The three pigments (melanin, guanine, lipochrome) have almost the same physiological value and belong to the category of waste materials" (trans.). Although guanine is an excretory product, its abundance in the tissues is altogether independent, he asserts, of the food consumed or of the activity or integrity of the renal apparatus.

"Experiment reveals in a number of cases the existence of an equilibrium between the melanin and the guanine such that any diminution of the quantity of one pigment brings about a compensatory augmentation of the other."

While this reciprocal relation between guanine and melanin has been affirmed by several previous writers, it has never, so far as I know, been sup-

ported by quantitative data. Although Millot discusses a method for isolating guanine, he gives no figures to support his claims that the amount of this substance may be influenced by visual stimuli.

In figure 3 of the present paper I have placed in comparison the curve for guanine production in *Girella nigricans* with that for melanin production in this same species, which was obtained under almost identical experimental conditions.[11] The approximate character of the values upon which both of these curves are based has been freely admitted.

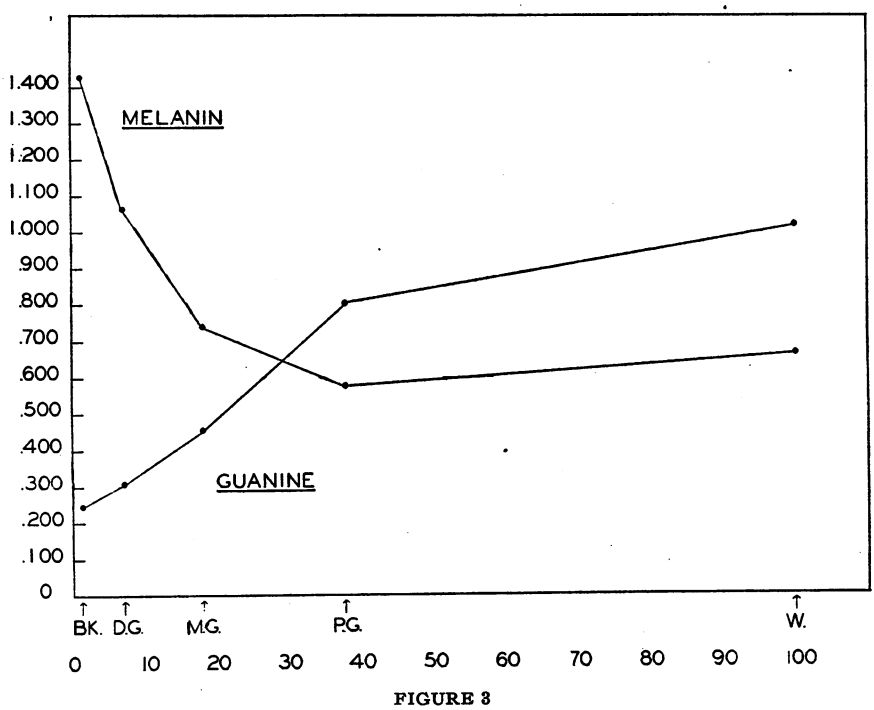

FIGURE 3

Comparison of curves for guanine and melanin in *Girella*. Guanine curve = continuous line of figure 2. Melanin curve is taken from Sumner, 1943.

While the melanin values, from black to pale gray, vary inversely as the logarithm of the albedo, no such arrangement holds for the guanine values. However, the two curves agree in displaying a marked change in their trend beyond pale gray, that for melanin ceasing to fall (indeed, rising slightly) at this point, that for guanine undergoing a marked reduction in the rate of increase. It is, of course, unfortunate that an intermediate albedo between "pale gray" and white was not intercalated in this series, but the relative length of this interval was not realized by eye at the time of mixing the paints.

The outstanding fact revealed by these experiments is that the course of protein metabolism, and the production of two important metabolites, melanin and guanine, are strongly influenced by the visual field of the fish, and influenced in such a way as to make for the animal's concealment.

These fundamental organic processes are thus caused to change their course in the interest of superficial appearance, and this with no apparent effect upon the growth of the fishes. It is true that the strict accuracy of this last statement should be tested more carefully than was done in the present experiments, in which the original mode of selection of the animals, at the time they were distributed to the various bowls, appears to have led to minor size differences which are irrelevant to the question at hand.

That this mechanism for concealment may be of life-saving value has been shown by considerable experimental evidence.[12]

If these two pigments (three, if we follow Millot) actually function as excretory products, it is surely surprising that the necessary excretory balance should be maintained through reciprocal adjustments in the production of substances so widely unrelated chemically. Plainly, there are physiological (shall we not say psychobiological?) problems here which call for extensive future investigation.

* Contributions from the Scripps Institution of Oceanography, New Series, no. 235.

[1] A summary of observations in this field was published by Sumner, *Biol. Rev.*, **15**, 351–375 (1940). Some later results have been reported by Dawes, *Jour. Exper. Biol.* **18**, 26–49 (1941); by Sumner and Doudoroff, *Biol. Bull.*, **84**, 187–194 (1943); and by Sumner, *Biol. Bull.*, **84**, 195–205 (1943).

[2] Kuntz, *Bull. U. S. Bur. Fisheries*, **35**, 1–29 (1915–1916); Murisier, *Revue Suisse de Zoologie*, **28**, 45–97, 149–195, 243–299 (1920–1921); Millot, *Bull. Biol. de la France et de la Belge*, **57**, 261–363 (1923); Hewer, *Phil. Trans. Roy. Soc. London*, **B215**, 177–200 (1927); Meyer, *Zool. Jahrb. (Abt. f. allgem. Zool.)*, **49**, 231–270 (1931); Sumner and Wells, *Jour. Exper. Zool.*, **64**, 377–403 (1933); Odiorne, *Proc. Nat. Acad. Sci.*, **19**, 750–754 (1933).

[3] I think that guanine may here be legitimately rated as a *pigment*, inasmuch as it actually functions as a pigment, i.e., influences the *color* (in the broad sense) of the animal.

[4] This point is important to note, since it makes certain that no sexual differences in pigmentation can have complicated the picture.

[5] This is the same set of bowls that was employed in the writer's melanin experiments with the same species (*Biol. Bull.*, **84**, 197 (1943)). They were of clear, colorless glass, painted on the outside, and sand-blasted within to eliminate gleams of reflected light.

[6] Hitchings and Falco, *Proc. Nat. Acad. Sci.*, **30**, 294–297 (1944). See also Hitchings, *Jour. Biol. Chem.*, **139**, 843–854 (1941); Hitchings and Fiske, **140**, 491–499 (1941).

[7] Only one cycle in the case of "2.1*a*" and "2.1*b*."

[8] Folin, *Laboratory Manual of Biochemistry*, D. Appleton-Century, 1934; Folin and Ciocalteu, *Jour. Biol. Chem.*, **73**, 629 (1927).

[9] Based upon only one lot of 3 fishes.

[10] Of course, a certain amount of this second chromogen may be supposed to occur in the earlier extractions.

[11] The latter from Sumner, *Biol. Bull.*, **84**, 195–205 (1943).

[12] Sumner, Amer. Nat., **69**, 245–266 (1935); *Proc. Nat. Acad. Sci.*, **20**, 559–564 (1934); **21**, 345–353 (1935).

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*THE IDENTIFICATION OF GUANINE IN EXTRACTS OF GIRELLA NIGRICANS. THE SPECIFICITY OF GUANASE**

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Communicated August 29, 1944

Sumner in the preceding article (pp. 285–294) has shown that the apparent concentration of guanine in the skin of *Girella nigricans* varies with the albedo of the background against which the fish are kept.

Guanine was identified as a constituent of fish scales about eighty years ago[1,2] and commercial "pearl essence" from this source is believed to consist essentially of free guanine.[3] The existence of guanine in fish scales may therefore be accepted, though with some reservations, since the chemical identification was carried out before isoguanine (2-hydroxy-6-aminopurine) which has properties nearly identical to those of guanine,[4] became available. Within recent years a new series of pigments closely allied to the purines has been studied in detail by Wieland and associates.[5,6] These include isoguanine ("guanopterin")[7] and a number of derivatives of the pteridine (azinepurine) heterocycle. These pigments were isolated first from butterfly wings, but at least one member now is known to exist in fish skin.[8] Therefore, though the identification of guanine as a major constituent of fish skin and scales may be accepted, the absence of similar substances is by no means certain.

The procedure adopted by Dr. Sumner for the determination of guanine, involves a preliminary separation of the material by a precipitation with copper and bisulfite, and finally a colorimetric estimation with phenol reagent. The copper-bisulfite precipitation limits the number of substances which may be present to those of the purine group and substances closely allied chemically[9] but the reaction with the phenol reagent is relatively unspecific.[10] Isoguanine, for example, and some of the pteridines might be expected to be determined as guanine by these procedures. Therefore a quantitative chemical or biochemical procedure was sought which would increase the specificity of the method. Ideally such a procedure, applied to small amounts of material, would distinguish between guanine and all other compounds known to behave like guanine with respect to copper precipitation and the phenol reagent. This would enable one to determine