STUDIES ON GOITROGENIC AGENTS IN FOOD

III. GOITROGENIC ACTION OF SOME GLYCOSIDES ISOLATED FROM EDIBLE NUTS

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It has been shown in an earlier communication (Moudgal et al., '57) that the goitrogenic action of groundnut [1] is due to its glycoside (arachidoside) content. On the basis of increased excretion of phenols in the group of rats fed arachidoside, and of an increased iodine content in the phenolic fraction of the urine as compared with the control, it was suggested that this glycoside acted as an antithyroid compound by forming molecular compounds with elemental iodine in the gland. Confirmatory evidence for this hypothesis, obtained in studies using radioactive iodine (I^{131}) is presented in this communication. The study has been extended to cover other anthocyanin pigments isolated from the outer skin-covering of edible nuts similar in nature to groundnuts.

EXPERIMENTAL

Isolation of anthocyanin pigments. Red amorphous pigments were isolated from the outer red skin-covering of cashew nuts (*Anacardium occidentale*) and almonds (*Prunus amygdalus*) by the same method used earlier in the preparation of arachidoside from groundnuts (Moudgal et al., '57).

A red tannin pigment was also isolated from the common areca nut (*Areca catechu*) which is an article of human con-

[1] Peanut.

sumption in India. The commercially available nut is a processed variety containing only 15% of tannins whereas the original nut contains nearly 35% (The Wealth of India, '48). A 50% alcoholic extract of the processed areca nut was obtained by soaking the nuts overnight in alcoholic solution. The extract was filtered and the alcohol removed completely. The red amorphous powder thus obtained was purified by redissolving in a small amount of alcohol, followed by filtration and evaporation of the alcohol.

All 4 pigments are water-soluble. The pigment isolated from *Anacardium occidentale* has been named anacardioside, and this term has been used throughout in the present series of investigations.

Influence of arachidoside on the radioactive iodine (I^{131}) uptake and distribution in the thyroid glands of albino rats. Twelve albino rats weighing on an average 70 gm were divided into three groups of 4 each. Group I served as a control and was fed only the basal diet. Group II was the arachidoside-supplemented group, maintained on the basal diet plus a daily supplement of 25 mg of arachidoside per rat. Group III was similar to group II, except that a daily supplement of 10 μg of iodide as Kl per rat was given in addition to arachidoside. The composition of the basal diet and the method of feeding etc., have been reported earlier (Srinivasan et al., '57). At the end of a feeding period of 7 weeks, each rat was given an intraperitoneal injection of 100 μc of carrier-free radioactive iodine. Four hours after injection, the rats were sacrificed under ether anesthesia, and the thyroids dissected out, quickly weighed on a torsion balance and transferred to test tubes containing 6 ml of 3.5 N NaOH. The samples were fractionated into inorganic iodine, diiodotyrosine iodine and thyroxine iodine fractions according to the method of Morton and Chaikoff ('43). Suitable aliquots of the various fractions were transferred to steel planchets for the measurement of radioactivity using a Geiger counter attached to a "Panax" scaler. The results of this investigation are presented in table 1.

TABLE 1

Distribution of radioactivity in thyroid gland 4 hours after an injection of radioactive iodine (I^{131})

	GROUP	PERCENTAGE OF ADMINISTERED RADIOACTIVITY RECOVERED IN DIFFERENT FRACTIONS OF THYROID HYDROLYSATE			
		Inorganic fraction	Diiodotyrosine fraction	Thyroxine fraction	Total iodine
I	Control	1.06 (0.82–1.20)[1]	6.32 (5.52–7.80)	2.34 (1.98–2.70)	9.72
II	Arachidoside, 25 mg per rat per day	1.48 (1.18–1.63)	2.00 (1.48–2.66)	1.22 (0.95–1.50)	4.70
III	Arachidoside, 25 mg per rat per day plus KI, 10 μg per rat per day	1.02 (0.76–1.20)	3.25 (2.62–4.81)	1.43 (1.30–1.73)	5.70

[1] Values within parentheses represent range.

Influence of anacardioside feeding on body weight, thyroid weight and thyroidal I^{131} uptake of albino rats. Fifteen albino rats weighing, on an average, 63 gm were divided into three groups of 5 each. Group I was maintained on the basal diet, whereas to group II a daily supplement of 20 mg of anacardioside per rat was given. Group III was given, in addition to anacardioside, a daily supplement of 15 μg of KI per rat. The composition of the basal diet and the feeding period were the same as those used in our earlier studies (Srinivasan et al., '57). At the end of the feeding period, 100 μc of carrier-free radioactive iodine was administered to each rat intraperitoneally, the rats transferred to individual metabolic cages and the urine collected for the subsequent 24 hours. The rats were then killed under ether anesthesia, the residuary urine in the bladder aspirated out as detailed earlier (Srinivasan et al., '57) and the thyroids dissected immediately and weighed on a torsion balance. The thyroid glands were then processed according to the method of Taurog and Chaikoff ('47), and the

radioactive iodine was fractionated into three portions — non-protein-bound iodine (NPBI), diiodotyrosine iodine and thyroxine iodine, according to the method of Taurog and Chaikoff ('46). The radioactivity in the various fractions was measured as in the earlier case using a Geiger counter attached to a "Panax" scaler. The results of these investigations are presented in tables 2 and 3. Thyroid glands from another set of rats grouped in the above manner were removed at the end of the experimental period for histological examination.

Influence of anthocyanin pigments and the areca catechin on the organic binding of I^{131} by thyroid tissue slices. The experimental techniques followed were essentially those of Franklin, Chaikoff and Lerner ('44). The reaction was carried out in 10-ml beakers in a Dubnoff metabolic shaking incubator. The composition of the reaction medium and the conditions of incubation are detailed in table 4. Each of these inhibitor compounds was tested in triplicate. In the control beakers, the inhibitor solution was replaced by an equal volume of Krebs, Ringer bicarbonate solution (KRB).

At the termination of the incubation period, the reaction media were decanted from the beakers and the slices immersed for 20 seconds in 3 ml of KRB solution. The solution was discarded and the operation repeated with a fresh 3-ml portion of KRB solution. The slices were then gently pressed between folds of a filter paper moistened with KRB and then ground well with 1.5 ml of 5% trichloroacetic acid and the ground mass transferred to centrifuge tubes. After centrifugation the residue was washed with 1 ml of 5% trichloroacetic acid, again centrifuged and the first and second supernatant solutions mixed and then made to a known volume. This represented the NPBI fraction.

The residue in the centrifuge tubes was hydrolysed for 8 hours over a steam bath with 6 ml of 2 N NaOH. The hydrolysates were then adjusted to pH 2.5 to 3.0 and repeatedly extracted with 10-ml portions of acidified n-butanol. The bu-

TABLE 2

Influence of anacardioside feeding on body weights and thyroid weights of albino rats

	GROUP	INITIAL WEIGHT	FINAL WEIGHT	GAIN IN WEIGHT	AVERAGE WEIGHT OF THYROID GLAND	RANGE OF THYROID WEIGHT
		gm	*gm*	*gm*	*mg*	*mg*
I	Control	64 (60–66)[1]	166 (157–171)	102	8.8	8.4– 9.2
II	Anacardioside, 20 mg per rat per day	63 (60–66)	143 (138–150)	80	16.4	13.0–21.0
III	Anacardioside, 20 mg per rat per day plus KI, 15 μg per rat per day	64 (61–67)	171 (160–182)	107	9.4	8.8–11.0

[1] Values within parentheses represent range.

TABLE 3

Distribution of radioactivity in thyroid gland 24 hours after an injection of radioactive iodine

	GROUP	PERCENTAGE OF ADMINISTERED ACTIVITY RECOVERED IN DIFFERENT FRACTIONS OF THYROID			PERCENTAGE OF ADMINISTERED RADIOACTIVE IODINE EXCRETED IN THE 24-HOUR URINE
		NPBI[1] fraction	Diiodotyrosine fraction	Thyroxine fraction	
I	Control	0.37	10.26	3.80	50 ± 2[2]
II	Anacardioside, 20 mg per rat per day	0.04	0.56	0.37	65 ± 1
III	Anacardioside, 20 mg per rat per day plus KI, 15 μg per rat per day	0.10	3.32	1.33	67 ± 3

[1] Non-protein-bound iodine.

[2] Standard deviation.

tanol extracts were pooled and made to a known volume. This represented the organic bound iodine fraction and included both thyroxine and diiodotyrosine fractions of Perlman et al. ('41). The radioactivity in the various fractions was measured as described earlier. The results are given in table 4.

"In vitro" formation of iododerivatives by the test compounds. It has been shown earlier (Moudgal et al., '57) that

TABLE 4

Influence of anthocyanin pigments and the areca catechin on the radioactive iodine (I^{131}) incorporating ability of surviving sheep thyroid slices

Three hundred milligrams of slices were incubated for three hours at 37°C in KRB [1] medium. Total volume was 3 ml. The inhibitors and I^{131} were added as KRB solutions; 25 μc of carrier-free I^{131} per beaker. No I^{127} was added.[2] Gas phase 95% O_2 and 5% CO_2.

TEST COMPOUND	CONCENTRATION	I^{131} OF RINGER'S SOLUTION RECOVERED IN SLICES AS		
		NPBI [3]	Organic iodine	Total iodine
	%	%	%	%
None	—	55.0	15.0	70
Arachidoside	0.0008	39.8	12.2	52
Anacardioside	0.0008	41.3	6.4	48
Almond anthocyanin	0.0008	41.0	5.4	46
Areca catechin	0.0008	38.5	7.3	46

[1] Krebs, Ringer bicarbonate.

[2] The only I^{127} contained in the reaction medium was that due to impurities in the reagent grade chemicals used in the preparation of the media.

[3] Non-protein-bound iodine.

arachidoside forms a stable iododerivative containing 19.8% iodine on treatment with iodine-potassium iodide solution at 37°C in phosphate buffer pH 5.8. Using a similar procedure, iododerivatives of the other three compounds were prepared. The iodine contents of these derivatives are shown in table 5.

Influence of the iododerivatives of the test compounds on the organic binding of I^{131} by thyroid tissue slices. The experimental technique adopted was the same as described earlier for uniodinated pigments. The concentration of iododerivatives in

the reaction medium, however, was of the order of 0.0016% (50 μg) in a final volume of 3 ml. The results of this study are presented in table 5.

Effect of test compounds on the monoiodotyrosine (MIT) synthesizing ability of sheep thyroid homogenates. The

TABLE 5

Influence of iodinated glycosides on the organic binding of radioactive iodine by surviving sheep thyroid tissue slices

The composition of the incubation medium and the conditions and duration of incubation are the same as detailed earlier in table 4.

IODINATED DERIVATIVE OF	IODINE CONTENT	CONCENTRATION OF IODINATED DERIVATIVE IN THE INCUBATION MEDIUM	I^{131} OF RINGER'S SOLUTION RECOVERED IN THE ORGANIC FRACTION
	%	%	%
None	—	—	22.5
Arachidoside	19.8	0.0016	19.3
Anacardioside	18.6	0.0016	18.0
Almond anthocyanin	27.0	0.0016	21.4
Areca catechin	25.8	0.0016	22.2

TABLE 6

Influence of anthocyanin pigments and areca catechin on the monoiodotyrosine (MIT) synthesis in cell-free preparations of sheep thyroid tissue

The experiment was carried out in test tubes containing the following reaction medium: 2 ml of 20% isotonic KCl homogenate, 0.5 ml of 0.1 M phosphate buffer pH 7.4, 0.2 ml of 0.04 M tyrosine, 0.2 ml of 0.04 M $CuCl_2$ solution, 0.05 ml of I^{131} solution containing 10 μc of I^{131} and 0.4 ml of inhibitor solution or distilled water, final volume 3.55 ml. Incubation for three hours at 37°C.

INHIBITOR	CONCENTRATION OF INHIBITOR	MIT SYNTHESIS [1]
	μg	
Arachidoside	25	+
	50	—
Anacardioside	25	++
	50	+
Almond anthocyanin	25	+
	50	+
Areca catechin	25	++
	50	—

[1] ++ No inhibition; + partial inhibition; — total or nearly total inhibition of MIT synthesis.

method followed for the preparation of the homogenate, incubation and the post-incubation treatment was essentially the same as that for the tyrosine iodinase system adopted by Fawcett and Kirkwood ('53). The composition of the incubation medium and the conditions of incubation are indicated in table 6. The radioautograph was developed according to the method of Fawcett and Kirkwood ('53). The results are presented in table 6.

RESULTS AND DISCUSSION

From the results presented in table 1, it can be seen that feeding arachidoside to albino rats brings about a considerable reduction in the I^{131} content of diiodotyrosine and thyroxine fractions of the thyroid gland. These results thus provide confirmatory evidence for the hypothesis that arachidoside is goitrogenic by virtue of its capacity to interfere with the organic binding of iodine. The slight compensatory increase in the radioactivity of the inorganic iodine fraction (table 1) however, is not equivalent to the observed decrease of radioactivity in the organic fraction. The total iodine of the thyroid gland of rats fed arachidoside is nearly 50% less than that present in the thyroid of control rats. This suggests that arachidoside, besides interfering with the organic binding of iodine, may directly inhibit the iodine uptake by the thyroid. This inhibitory effect on the iodine concentrating capacity of the thyroid gland could not be observed in the earlier study (Srinivasan et al., '57), presumably owing to the mildness of the goitrogenic effect of defatted groundnut cake. Small amounts of KI added as supplements to the arachidoside diet afford a partial protection against the goitrogenic action. This is in keeping with the results reported earlier with defatted groundnut cake (Srinivasan et al., '57).

The influence of anacardioside feeding on the body and thyroid weights of albino rats can be assessed from the data in table 2. Anacardioside, unlike arachidoside (Moudgal et al., '57), does not possess any growth-promoting property. On

the other hand, it can be seen that anacardioside supplementation inhibits the growth of rats. The thyroid weights of rats fed anacardioside are considerably increased. Supplementation of the diet with small amounts of KI reverses the inhibitory effect of anacardioside on growth and further helps to maintain the thyroid weight within the normal range.

The distribution of radioactivity in the various fractions of the thyroid, and the pattern of urinary excretion of radioactive iodine by rats fed anacardioside are presented in table 3. That anacardioside interferes with the normal uptake of I^{131} by thyroid is evident from the low radioactivity present in the NPBI fraction. It is also seen from the I^{131} content of the diiodotyrosine and thyroxine fractions, that anacardioside inhibits the organic binding of iodine as well. The increased urinary excretion of radioactivity in rats fed anacardioside is similar to the effect observed earlier with defatted groundnut cake (Srinivasan et al., '57).

Anacardioside supplementation thus results in an increase in thyroid weight of rats, a decrease in uptake as well as organic binding of iodine by the thyroid gland and an increase in the urinary excretion of radioactive iodine. These observations clearly point to this glycoside being a potent goitrogen.

The histological examination of thyroids of rats fed anacardioside showed increased colloid spaces filled with faintly staining colloid and thus provides further evidence for the goitrogenic action of this glycoside. The picture was that of a typical colloid goitre without any accompanying hyperplasia. Similar examination of thyroid slices of rats fed anacardioside and potassium iodide indicated a partial reversal of the goitre.

The results obtained with the above two anthocyanin pigments suggested a strong possibility that this goitrogenic action might be a property shared by other anthocyanin pigments and related compounds. Hence the *in vitro* effect of three such glycosides, as well as of a catechin, on the uptake and organic binding of I^{131} in thyroid tissue slices was stud-

ied. From table 4, it is clear that all of the 4 test compounds significantly inhibit the organic binding of iodine in surviving thyroid tissue slices. Unlike the thiocarbamides (Pitt-Rivers, '50), these compounds bring about a significant decrease in the iodine concentration of the non-protein fraction also.

Fawcett and Kirkwood ('53) from their *in vitro* experiments conclude that all aromatic compounds capable of forming substituted derivatives with iodine and possessing electron-donating groups are potential thyroid inhibitors. Anthocyanins and their closely related flavonol derivatives, like catechins, contain polyphenolic components in their moieties and one of the products of total breakdown of these compounds is phloroglucinol. The reactivity of these polyphenolic groups towards iodine is dependent upon the total number of the hydroxyl groups present in the molecule and the orientation of these hydroxyl groups with respect to each other. It can be seen from table 5, that all of the 4 compounds are capable of forming stable iododerivatives containing large amounts of iodine. This suggests a probable mechanism whereby these glycosides interfere with normal thyroid function. Thus they may act as competitors with tyrosine for elemental iodine in the thyroid gland. To test this possibility, the influence of the iododerivatives of these compounds on the organic binding of radioactive iodine in surviving thyroid tissue slices was studied. It is evident from table 5 that inclusion of these iododerivatives even at twice the concentration of the corresponding noniodinated analogues does not bring about any significant change in the organic binding of iodine.

Phloroglucinol, which is known to be the final breakdown product of many of these polyphenolic compounds, has been shown to be a powerful goitrogen (Arnot and Doniach, '52). The formation of iodophloroglucinol in *in vitro* systems has been observed by Fawcett and Kirkwood ('53). The fact that the test polyphenolic compounds form iododerivatives directly suggests that the formation of phloroglucinol from these com-

pounds in an *in vitro* system is not a prerequisite for these substances inhibiting normal thyroid function.

Fawcett and Kirkwood ('54) have reported the preparation of the enzyme tyrosine iodinase which catalyses the single-step iodination of tyrosine to monoiodotyrosine. Since the action of the enzyme is independent of the formation of elemental iodine from inorganic iodide (cupric ion is responsible for this oxidative conversion), the enzyme system offers itself as a convenient tool for studying the possible action of suspected goitrogenic agents at the stage of the synthesis of MIT. Hence, in the present case, the action of the above test compounds on the MIT synthesis by cell-free preparations of thyroid tissue was studied. The results are presented in table 6. Since the same volume of the incubation mixture (30 µl) was delivered on to the chromatogram paper in each case, the intensity of the MIT spot on the radioautogram gives an idea of the extent of inhibition brought about by a particular test compound. It can thus be seen that generally the above test compounds are inhibitory at the 50 µg level but have no action at the 25 µg level. Anacardioside even at the 50 µg level does not cause total inhibition of MIT synthesis. This observation, together with the fact that anacardioside feeding to rats results in a marked reduction of radioactivity, even in the NPBI fraction (table 3) suggests more than one mode of action for this compound, namely, that it may act both by inhibiting the uptake of inorganic iodine and by blocking the organic binding of iodine. It is also of interest to note that in experiments with iodinated derivatives of these anthocyanin pigments, the percentage of I^{131} recovered from the KRB medium is the least in the case of iodinated anacardioside (table 5). The fact that areca catechin totally inhibits MIT synthesis in the present case and loses its entire activity on iodination (table 5) shows that this compound mainly interferes with the organic binding of iodine. Arachidoside and the almond anthocyanin may have a mode of action very similar to anacardioside.

Thus the present investigation shows that all of the 4 compounds are goitrogenic by virtue of the fact that they compete with tyrosine for elemental iodine, forming stable iododerivatives at the gland site. Hence their goitrogenic activity can be partially overcome by supplementing the diet with small amounts of KI.

SUMMARY

The inclusion of arachidoside and anacardioside, the pigments isolated from the outer skin-covering of groundnuts and cashewnuts respectively, in the diet of rats, at a level of 20 mg per rat per day for 7 weeks results in goitre. Incorporation of potassium iodide in the diet counteracts partially the goitrogenic action of the anthocyanins. Results of studies on the distribution of radioactive iodine in the thyroid glands of the rats suggest that the pigments inhibit the organic binding of radioactive iodine.

An anthocyanin pigment and a catechin have been isolated from almonds and areca nuts respectively. *In vitro* experiments employing the tissue slice as well as homogenate technique reveal that all of the above 4 test compounds interfere with the organic binding of radioactive iodine. The test compounds form stable iododerivatives which, however, do not possess any significant inhibitory effect on the organic binding of radioactive iodine. The implications of the results are discussed.

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LITERATURE CITED

ARNOT, D. G., AND I. DONIACH 1952 The effect of compounds allied to resorcinol upon the uptake of radioactive iodine (I^{131}) by the thyroid of the rat. Biochem. J., *50:* 473.

FAWCETT, D. M., AND S. KIRKWOOD 1953 The mechanism of antithyroid action of iodide ion and of the "aromatic" thyroid inhibitors. J. Biol. Chem., *204:* 787.

———— 1954 Tyrosine iodinase. Ibid., *209:* 249.

FRANKLIN, A. L., I. L. CHAIKOFF AND S. R. LERNER 1944 The influence of goitrogenic substances on the conversion *in vitro* of inorganic iodine to thyroxine and diiodotyrosine by thyroid tissue with radioactive iodine as indicator. Ibid., *153:* 151.

MORTON, M. E., AND I. L. CHAIKOFF 1943 The formation *in vitro* of thyroxine and diiodotyrosine by thyroid tissue with radioactive iodine as indicator. Ibid., *147:* 1.

MOUDGAL, N. R., V. SRINIVASAN AND P. S. SARMA 1957 Studies on goitrogenic agents in food. II. Goitrogenic action of arachidoside. J. Nutrition, *61:* 97.

PERLMAN, I., M. E. MORTON AND I. L. CHAIKOFF 1941 Radioactive iodine as an indicator of the metabolism of iodine. II. The rates of formation of thyroxine and diiodotyrosine by the intact normal thyroid. J. Biol. Chem., *139:* 449.

PITT-RIVERS, R. 1950 Mode of action of antithyroid compounds. Physiol. Revs., *30:* 194.

SRINIVASAN, V., N. R. MOUDGAL AND P. S. SARMA 1957 Studies on goitrogenic agents in food. I. Goitrogenic action of groundnut. J. Nutrition, *61:* 87.

TAUROG, A., AND I. L. CHAIKOFF 1946 The determination of thyroxine in the thyroid gland of the rat. J. Biol. Chem., *163:* 323.

———— 1947 The metabolic interrelations of thyroxine and diiodotyrosine in the thyroid gland as shown by a study of their specific activity-time relations in rats injected with radioactive iodine. Ibid., *169:* 49.

WEALTH OF INDIA 1948 A dictionary of Indian raw materials and Industrial products. Published by Publications Division, Council of Scientific and Industrial Research, New Delhi (India), *1:* 110.