STUDIES ON RADIATION PASTEURISATION OF MEDIUM FATTY FISH

II. PRECURSORS OF YELLOW DISCOLOURATION IN IRRADIATED WHITE POMFRET (*STROMATEUS CINEREUS*)

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Triglycerides, phospholipids and sarcoplasmic proteins fractions of white pomfret produced considerable amounts of thiobarbituric acid reactive substances (TBRS) on irradiation. Incubation of malonaldehyde with pomfret skin under aseptic conditions developed yellow pigmentation of the skin tissues, similar in spectral characteristics to those produced on irradiation of the skin.

INTRODUCTION

A correlation between the yellow discolouration produced in the irradiated skin tissue of white pomfret *(Stromateus cinereus)* with the formation of 2-thiobarbituric acid (TBA) and 2,5 dinitrophenylhydrazine (DNPH) reactive carbonyl compounds has been shown in the first part of this series (Kamat and Kumta, 1972). Several other studies have shown that the food constituents like amino acids (Drake and Giffee, 1957; Ambe *et al* 1961), fatty acids (Saslaw *et al* 1963; Saslaw and Waravdekar, 1965), glycerol (Scherz, 1968) and carbohydrates (Scherz, 1970) produce 2-thiobarbituric acid reactive substances TBRS on irradiation. Kwon and Olcott (1969) have reported that malonaldehyde constitutes the principal TBRS in UV irradiated unsaturated fatty acids and squalene. Malonaldehyde has received much attention because of its potential reactivity with amino acids (Crawford *et al* 1966), proteins (Crawford *et al* 1967; Buttkus, 1967), and other food constituents (Kwon *et al* 1965). Formation of such carbonyl compounds in food products thus assumes importance because of their involvement in Maillard type of reactions leading to yellow to brown discolourations.

It may therefore be expected that the initiation of reactions causing browning could be a common feature of the irradiated fishery products. However, only a few of the irradiated fish species have been reported to develop brown discolouration during refrigerated storage (Matsuyama, 1966). Among the tropical fish varieties examined by us, only white pomfret developed a typical yellow discolouration. It was therefore of interest to examine i) the major constituents of white pomfret which yield high amounts of TBRS on irradiation, ii) the reactivity of malonadehyde, taken as a representative of TBRS, with the skin tissue of the fish in terms of yellow discolouration and iii) the spectral characteristics of the yellow pigment resulting from *in vitro* re-

action of malonaldehyde with skin tissue and that formed *in situ* in the irradiated white pomfret.

Experimental

Preparation of the fish samples:

Skin and muscle tissue samples of white pomfret were prepared as described in the first paper of the present series (Kamat and Kumta, 1972). The experiments relating to elucidation of the precursors of TBRS in skin and muscle tissues of the fish were repeated with three separate lots (each containing five specimen) of the fish using duplicate samples for each assay. The average values of the three lots have been repoted in the text.

Extraction of proteins:

Sarcoplasmic and fibrillar protein fractions were isolated from the fish muscle using the method of King (1966) as described in the previous paper (Kamat and Kumta, 1972). The water soluble and salt soluble protein fractions were also isolated from the skin tissue in a similar way. The protein fractions were dialysed for 10-12 hr to minimise the concentration of associated low molecular weight compounds and subsequently examined for the formation of 2-thiobarbituric acid reactive substances (TBRS) on irradiation. Protein nitrogen was estimated by nesselerisation (Umbriet *et al* 1957).

Preparation of non-protein fraction:

With a view to determining the contribution made towards TBRS formation by non-protein compounds such as amino acids, hexoses, pentoses etc, the non-protein fraction was prepared as follows:

To 50 ml protein solution were added 50 ml 8% trichloroacetic acid (TCA) to precipitate proteins. The precipitate so formed was then removed by filtering through Whatman No. 1 filter paper. The filtrate was then neutralized to pH 7 with 2N NaOH and made up to 100 ml. Nitrogen contents of this non-protein fraction was determined by nesselerisation (Umbriet *et al* 1957).

Isolation and fractionation of lipids:

Isolation of the extractable lipids from skin or muscle of white pomfret and their fractionation into triglycerides, free fatty acids and phospholipids were effected as described in the previous paper (Kamat and Kumta, 1972).

Irradiation:

N-heptane solutions of the lipid fractions and dialysed protein solutions in phosphate buffer(0.01M,pH 7.5)were used for irradiation. The concentration of protein fractions was 0.5 mg/ml while that of lipid fractions was 1 mg/ml. Protein, non-protein and lipid fractions were irradiated at different dose levels ranging from 0.1 to 1.0 Mrad in Gamma Cell 220 (3991 curies, Co^{60} source) at the dose rate of 0.16 Mrad/hr. During irradiation, temperature was maintained at 0-2°C.

Estimation of 2-thiobarbituric acid reactive substances (TBRS):

2-thiobarbituric acid reactive substances (TBRS) formed by irradiation of lipid, protein and non-protein fractions were estimated as in earlier paper (Kamat and Kumta, 1972).

Preparation of malonaldehyde (MA):

0.1 M solution of malonaldehyde (MA) was prepared by acid hydrolysis of 1, 1, 3, 3 - tetraethoxy propane (TEP) as described by Buttkus (1967).

Reaction of malonalhyde with the skin tissue:

This was carried out under aseptic conditions. The solutions of malonaldehyde and phosphate buffer (0.01 M, pH 7) were sterilized by passing through bacterial filters. 2 g fine pieces of the skin tissue were allowed to incubate aseptically with 10μ moles malonaldehyde for 30 hr at 30°C and pH 7. The reaction was followed up

by estimating at intervals the amount of malonaldehyde reacted with the skin tissue.

RESULTS

Radiation-induced TBRS formation by lipid, protein and non-protein fractions isolated from white pomfret:

Since a number of compounds such as amino acids, fatty acids, glycerol, carbohydrates etc are known to produce a variety of carbonyl compounds as a result of irradiation, it was of interest to understand the behaviour of different chemical constituents of white pomfret towards irradiation. Protein and lipid fractions, which form the major constituents of fish, were therefore examined for the effects of irradiation. Table I incorporates the data on distribution

TABLE I DISTRIBUTION OF CHEMICAL COSTITUENTS IN SKIN AND MUSCLE TISSUES OF WHITE POMFRET AND THEIR CONTRIBUTION TO RADIATION-INDUCED TBRS FORMATION

Distribution of chemical constituents			TBRS values,[1] μM MA				
			Radiation dose: Mrad				
Individual constituent	Tissue system	Quantity: %	Unirradiated	0.1	0.25	0.5	1.0
Triglyceride fraction	Skin	11.00	88.0	336.0	608.0	1507.0	2112.0
	Muscle	2.35	13.2	51.2	83.7	230.0	367.0
Phospholipid fraction	Skin	2.50	14.2	88.0	108.0	138.5	133.0
	Muscle	0.95	4.0	9.6	10.0	12.4	13.2
Free fatty acid fraction	Skin	0.40	2.2	16.5	32.1	53.6	64.2
	Muscle	0.08	0.5	3.0	4.5	7.5	8.0
Salt soluble protein fraction	Skin	1.20	0.5	3.5	8.3	8.3	4.0
	Muscle	12.15	5.6	25.0	57.0	60.0	49.0
Water soluble protein fraction	Skin	0.60	0.3	2.3	2.9	5.0	4.0
	Muscle	3.65	3.1	49.3	153.0	197.0	185.0
Non-protein nitrogen (NPN) fraction	Skin	0.28	0.8	2.0	5.1	5.1	4.3
	Muscle	0.24	1.9	7.1	10.6	10.5	14.2

[1] The values show the contribution made by individual chemical constituents of skin and muscle, in proportion to the quantities present in the tissues, to TBRS formation.

Skin and muscle tissues of white pomfret were separately analysed for lipids, proteins, and non-protein compounds as per the methods described in the text.

N-heptane solutions of isolated lipids, dialysed protein solutions in phosphate buffer (pH7.5) and non-protein nitrogen (NPN) compounds fractions were irradiated at different doses and subsequently assessed for TBRS production.

of lipids, proteins and non-protein compounds in skin and muscle of white pomfret and contribution made by these constituents towards the formation of TBRS on irradiation. It can be seen that the triglyceride fraction isolated from skin and muscle, phospholipid fraction from the skin and the sarcoplasmic protein fraction of the muscle tissue contributed appreciably towards the production of TBRS as compared to the other constituents of the fish. Table 2 describes the data on TBRS yields per gram of the irradiated lipid, protein and non-protein fractions. It can be seen that for the same amount of the tissue sample subjected to irradiation, the yields of TBRS in increasing order were free fatty acids > triglycerides > phospholipids > protein or

TABLE II

RADIATION-INDUCED TBRS FORMATION BY THE CHEMICAL CONSTITUENTS OF WHITE POMFRET

Chemical constituents	Tissue System	TBRS value, μM MA/g* Radiation dose: Mrad				
		Unirradiated	0.1	0.25	0.5	1.0
Triglyceride fraction	Skin	8.9	30.6	54.5	137.0	192.0
	Muscle	5.6	21.8	35.6	98.0	156.0
Phospholipid fraction	Skin	5.7	35.3	43.5	55.4	53.4
	Muscle	4.2	10.1	10.7	13.0	13.9
Free fatty acid fraction	Skin	5.6	41.2	80.2	134.0	160.5
	Muscle	6.9	38.3	56.3	94.0	112.5
Water soluble protein fraction	Skin	0.5	3.9	4.9	8.3	6.8
	Muscle	0 9	13.5	41.8	54.0	46.1
Salt soluble protein fraction	Skin	0.4	2.9	6.9	6.9	3.8
	Muscle	0.5	2.1	4.8	5.0	4.1
Non-protein nitrogen (NPN) fraction	Skin	2.7	7.3	15.2	18.0	15.2
	Muscle	8.1	29.8	44.1	47.8	68.0

* The values represent the contribution made by the individual chemical constituents, on weight basis to TBRS production on irradiation.

N-heptane solutions of isolated lipid fractions, dialysed protein solutions in phosphate buffer (pH 7.5), and non-protein-nitrogen (NPN) fraction (aqueous solution) were irradiated at different doses and subsequently assessed for TBRS formation.

NPN fraction for skin and free fatty acids > sarcoplasmic protein fraction > triglycerides > phospholipids, salt soluble protein fraction or NPN fraction for muscle respectively.

Fig. I incorporates data on the amount of malonaldehyde treated with skin tissue of the white pomfret and yellowing produced by the reaction. A slight but distinct yellow colouration could be detected in the skin tissue within 3 hr of incubation period. Intensity of this yellow colouration further increased consistently as a function of time up to 30 hr. Concomitantly, there occurred a fall in the concentration of malonadehyde in the reaction medium.

At 8, 24 and 30 hr of incubation period when the yellow colouration was more, the extractant employed (acetone-HCl-methanol) did not extract the pigment completely. Nevertheless, the colour yields were appreciably high. Complete extraction of the yellow pigment could be effected only with 6 N NaOH, indicating the formation of insoluble protein-pigment complex. Thus although the results shown in Fig. 1 did not give the extent of yellow pigment formed, hey suggested the possible involvement of malonaldehyde-like compounds in causing yellow discolouration of the skin of white pomfret.

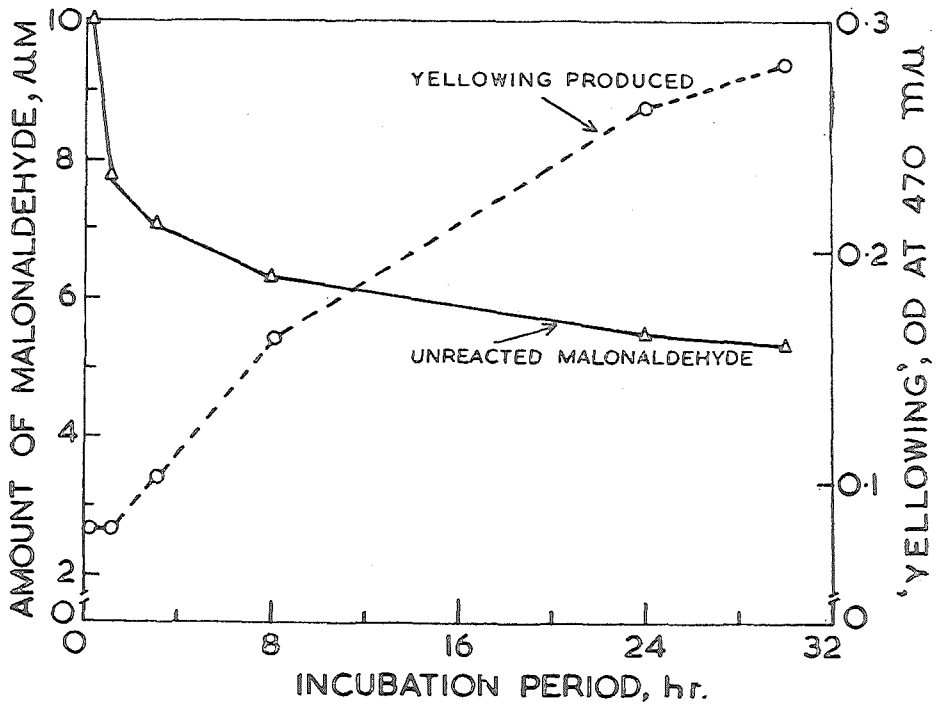

Fig. 1: Reactivity of malonaldehyde with white pomfret skin.

White pomfret skin was incubated with malonaldehyde for 30 hr at 30°C and pH 7. At different intervals during incubation the skin samples were assessed for 'yellowing' by extracting the pigment with acetone- HCl- methanol and measuring its absorption at 470 mμ

The spectral characteristics of the yellow colour resulting from *in vitro* reaction of malonaldehyde with pomfret skin and of that formed *in situ* in the irradiated white pomfret are shown in Fig. 2. All the three types of the yellow colouration showed two distinct absorption maxima at 360 and 470 mμ respectively. This suggested that the three pigments may be of similar nature.

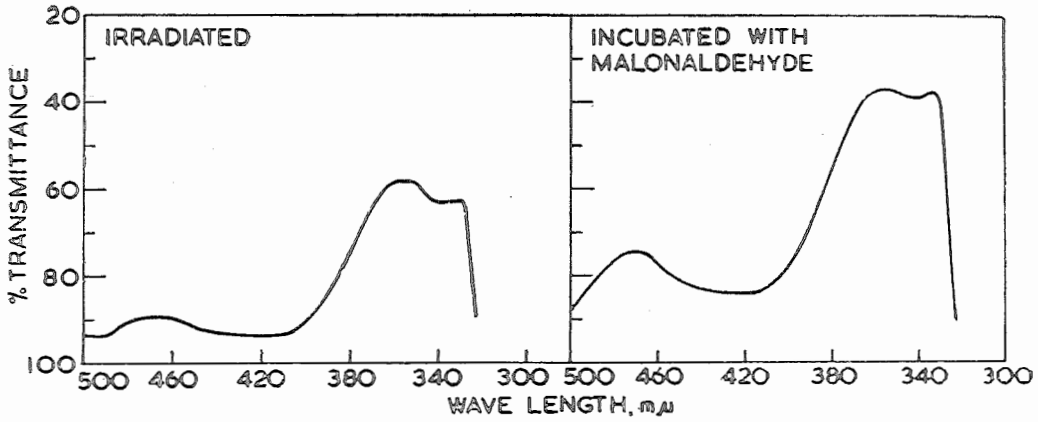

Fig 2: Mechanisms of 'yellowing' in aerobically irradiated white pomfret.

Discussion

Non-enzymatic reactions involving carbonyl and amino compounds (Danehy and Pigman, 1951; Reynolds, 1965), which may be already present initially or may be formed as a result of processing and/or storage, lead to development of yellow to brown discolouration in fishery products. Thus, degradation of nucleotides, glycogen (Tarr, 1966), proteins (Amlacher, 1961) and other nitrogenous compounds like TMAO (Castell *et al* 1968) in fish may give rise to several compounds having free amino or carbonyl group, which in turn may initiate reactions leading to the discolouration.

Formation of TBRS:

Lipids (Chipault, 1962), proteins and amino acids (Drake and Giffee, 1957; Ambe *et al* 1961), carbohydrates (Scherz, 1970) and glycerol (Scherz, 1968) have been known to form carbonyl compounds on irradiation. Saslaw *et al* (1963) and Saslaw and Waravdekar (1965) noted the formation of TBRS in unsaturated fatty acids irradiated under aerobic conditions. Among the chemical constituents of pomfret analysed for TBRS formation, triglycerides (from skin and muscle), phospholipids (from skin) and sarcoplasmic protein fraction (from muscle) were found to produce considerable amounts of TBRS. Contribution made by the other constituents was comparatively less. Sarcoplasmic proteins appear to undergo scission or cleavage to yield amounts of TBRS higher than those formed by fibrillar protein fraction which appears to undergo aggregation by irradiation. Such type of differences in the type of damage to the sarcoplasmic and fibrillar protein fractions have also been reported by Kumta and Gore (1970).

Chipault and Mizuno (1960) distinguished between radiation degradation of methyl esters of saturated and unsaturated fatty acids; the latter contributing to high levels of peroxides formed. No peroxides were found when fatty acids esters were irradiated under vacuum. Similarly, it was pointed out that the radiation-induced degradation of glycerol results in the formation of carbonyl compounds (Scherz, 1968). The high amounts of TBRS found in the irradiated triglyceride fraction, thus appear to arise from oxidative degradation of glycerol and unsaturated fatty acid moieties.

Reactivity of malonadehyde with skin tissue of white pomfret:

Incubation of malonaldehyde with the pomfret skin resulted in the development of yellow pigmentation on the tissue. Malonaldehyde has been recognised for its high potential reactivity with the proteins (Buttkus, 1967; Crawford *et al* 1967; Kwon and Brown, 1965; Kwon and Olcott, 1966), amino acids (Crawford *et al* 1966) and other food constituents (Kwon *et al* 1965; Kwon and Norgard, 1966). It was also

observed by us earlier, (unpublished data) that the reaction of malonaldehyde with amino acids and amines led to the development of characteristic yellow colouration having absorption maximum at 390 mμ. Kwon *et al* (1965) reported that the reactivity of malonaldehyde with water soluble protein fraction of the chicken muscle resulted in the formation of insoluble yellow coloured precipitate.

Formation of insoluble yellow coloured aggregates were also observed by interaction of fibrillar proteins of white pomfret with malonaldehyde (unpublished data). This may explain low extraction of yellow pigment found on the skin after 30 hours incubation with malonaldehyde. The yellowing produced by the incubation of malonaldehyde with the pomfret skin thus appears to be due to the interaction of malonaldehyde with the available sites in the fish tissue. It may be concluded therefore that the yellow patches encountered on the skin surface of irradiated white pomfret fillets may be a manifestation of the two major reactions: i) formation of TBRS by radiation and ii) reactivity of TBRS with the amino nitrogenous compounds in the fish as described in Fig. 3.

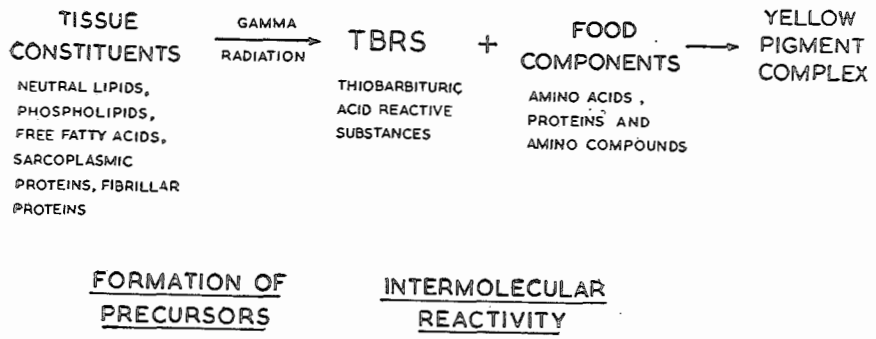

Fig. 3.

SUMMARY

Among the major chemical constituents of white pomfret examined, the triglycerides isolated from skin and muscle tissues, phospholipids from skin and sarcoplasmic proeins of the muscle contributed appreciably towards 2-thiobarbituric acid reactive substances (TBRS) on irradiation. Contribution by the other constituents of the fish was comparatively less. *In vitro* reaction under aseptic condition of the skin tissue with pure malonaldehyde produced yellow colouration of the former. Comparison of the spectral characteristics of yellow colour formed by this *in vitro* reaction with those of the colours formed *in situ* in irradiated skin samples, suggested that the three pigments were similar in nature.

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