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CARBONYL COMPOUNDS AND THE NON-ENZYMIC BROWNING OF LEMON JUICE

By K. MARY CLEGG and A. D. MORTON

During the non-enzymic browning of lemon juice the build-up of carbonyl compounds is believed to be associated with the breakdown of ascorbic acid. This has been studied by separation of their 2,4-dinitrophenylhydrazine derivatives on thin-layer chromatograms, and by spectroscopic examinations. The results have been compared with those from model systems. Twelve carbonyls were found in browned samples, half of these have been tentatively identified and the remainder have been classified as aldehydes or ketones. Work with model systems confirmed that the α,β-unsaturated carbonyls are potent browning agents and also that dicarbonyls of the glyoxal type make a contribution to browning in the early stages. The rôle of sugars in these systems has been considered.

Introduction

In a previous paper[1] the non-enzymic browning of lemon juice has been related to conditions such as pH, ascorbic acid and amino-acid contents. Sugar–amino-acid reactions, leading to a build-up of reactive carbonyl compounds, seemed unlikely to be the main contributors to the formation of melanoidin pigments because of the high acidity of the system. Under such conditions a more probable explanation was that the carbonyl compounds, which subsequently react with amino-groups and polymerise to give brown pigments, originated from the oxidation of ascorbic acid.[2, 3]

Studies on the mechanism of non-enzymic browning in natural products and model systems, whether by sugar–amino-acid condensation[4] or ascorbic acid decomposition,[5] have shown the development of furfural. This observation led to the idea that furfural and related compounds were the active carbonyls responsible for the formation of melanoidin pigments. More recent work of McWeeny & Burton[6] supports the hypothesis that furfural compounds are relatively inactive and tend to accumulate in the system while other more reactive carbonyls, which have formed as a result of oxidation and fragmentation of larger molecules, are mainly responsible for the development of browning.

An investigation of the build-up of all carbonyl compounds during the browning of lemon juice and simulated model systems has therefore been undertaken. An attempt has been made to classify some of these breakdown products with a view to following the reactions occurring during the non-enzymic browning of an acidic product with a high ascorbic acid content such as lemon juice (pH 2·5). Carbonyl compounds were extracted from the systems, both before and after reaction with various specific reagents for terminal groups, as their 2,4-dinitrophenylhydrazine derivatives. These complexes were then separated by thin-layer chromatography. The browning potential of different classes of carbonyls when added to these model systems has also been investigated.

Experimental

Materials

Commercial samples of pasteurised lemon juice, preserved with sulphur dioxide at a level of 350 p.p.m., of the following approximate composition per 100 ml. were used: citric acid 5·8 g., ascorbic acid (fortified) 100 mg., nitrogen 70 mg. and sugars 3 g. Samples at various stages of browning under aerobic conditions were obtained by incubating the juice, at 37° for different lengths of time, in filled 1-oz. screw-top bottles with two 1-mm. dia. holes in the caps. Freshly-squeezed lemon juice was also examined.

Preparation of 2,4-dinitrophenylhydrazine derivatives (DNPH's)

Preliminary extraction of the carbonyls with ethyl acetate was found to give no advantage over the simpler technique of precipitation by the direct addition of saturated 2,4-dinitrophenylhydrazine, in acidified carbonyl-free methanol, to the test solution. The washed and dried precipitate was usually dissolved in carbonyl-free dioxan for application to thin-layer chromatographic plates. No heat treatment was required by this method thus affording minimal loss of carbonyl compounds.

Thin-layer chromatographic technique (TLC)

In agreement with other workers[7, 8] the neutral adsorbent Kieselgel G in the particle size range 5–25 mμ was found to be successful in the chromatography of DNPH's. In the preliminary investigations the solvent system benzene/light petroleum (60–80°) 3 : 1 recommended by Dhont & de Rooy[9] was used but unresolved material remained at the origin indicating more polar solvents were required. A multiple development technique was then evolved in which the plates were first run in benzene/ethyl acetate 1 : 1, of intermediate polarity, for 6 cm., secondly in the non-polar solvent benzene/light petroleum 3 : 1 for 10 cm., and finally in the highly polar solvent ethyl alcohol/ethyl acetate 3 : 2 for 3 cm. This technique was found to give reasonably good separation of all the DNPH's obtained from a browned sample of lemon juice. Unfortunately it was impossible to separate the DNPH's of glucose and ascorbic acid which ran as one spot.

Results

Development of carbonyl compounds in lemon juice

DNPH's were prepared from fresh juice immediately after squeezing the lemons, from untreated commercial lemon juice, and from commercial lemon juice which had been incubated for 2, 3, 6, 9, 12, 15, 19 and 26 days, and then separated by TLC (Fig. 1). Usually, a standard mixture of DNPH's of ascorbic acid, citral, furfural, hydroxymethylfurfural and diacetyl was run for comparison since R_F values alone cannot be relied upon with TLC.

The fresh lemon juice showed only three bands which, by comparison with an ascorbic acid standard, were thought to be glucose/ascorbic acid, dehydroascorbic acid and 2,3-diketogulonic acid (Fig. 2); ascorbic acid dissolved in de-ionised water is known to be unstable.[10] More than three bands were found in the commercial lemon juice, indicating that the first stages of non-enzymic browning had already begun. After 3 days' incubation, nine bands were detectable and after 26 days' incubation at least twelve carbonyl bands could be seen. An interesting observation was the development of a very pink band, compared with the orange colour of all the other bands, in the samples incubated for 2 and 3 days, which had disappeared by 6 days incubation. From the position of this spot on the chromatogram and its colour, it seemed likely to be α-ketogulonic acid, a breakdown product of ascorbic acid (Fig. 2) which is known to form a red DNPH; a standard sample of α-ketogulonic acid was not available for confirmation. Another feature was the late appearance of a band corresponding to hydroxymethylfurfural which was not seen until after 9 days' incubation, although a trace of furfural could be detected in the original commercial juice and increased in amount with incubation. A standard sample of methylfurfural was prepared from rhamnose[11] and converted to the DNPH which moved fractionally ahead of furfural, but it was not found in any of the lemon juice samples.

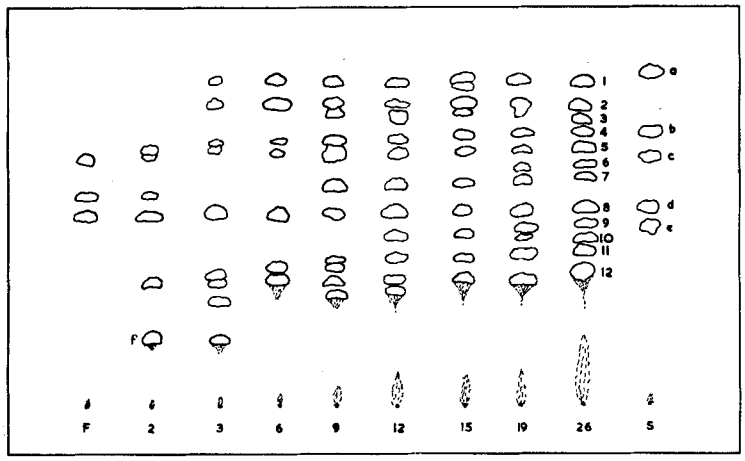

FIG. 1.—*Thin-layer chromatogram of DNPH's of fresh lemon juice (F), commercial lemon juice incubated 2–26 days, and a standard (S)*

a = citral; b = furfural; c = diacetyl; d = ascorbic acid; e = hydroxymethylfurfural; f = pink spot, α-ketogulonic acid?

Classification of carbonyl compounds found in browned lemon juice

The lemon juice which had been incubated for 26 days was taken as the standard material from which to classify tentatively the carbonyl compounds. The twelve bands on the chromatogram were numbered starting furthest from the origin.

(i) *Colour of DNPH's in alcoholic sodium hydroxide.*—Stadtman[12] noted a connexion between adsorption and the colour of DNPH's in alkaline alcoholic solution. The twelve bands were scraped individually off the plate and transferred to a small volume of ethyl alcohol which was made alkaline with 40% sodium hydroxide. The findings are summarised in Table I and show

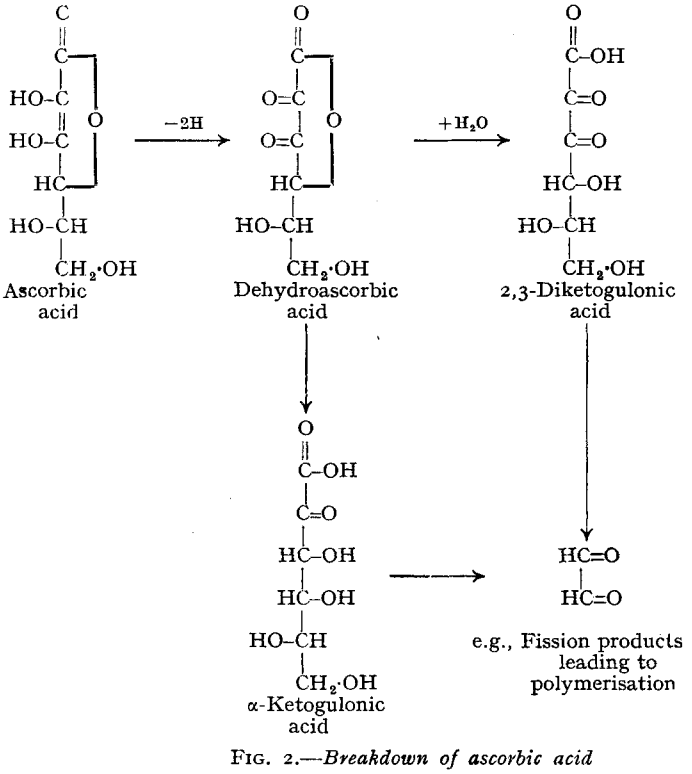

FIG. 2.—*Breakdown of ascorbic acid*

Table I

Classification of DNPH's of browned lemon juice separated by TLC

Band no. (see Fig. 1)	Colour of DNPH in alkaline alcoholic solution*	Formation of bisulphite complex†	Reaction with Tollens reagent‡	Comparison with standard DNPH's	Tentative carbonyl classification
1	pink	yes	positive	—	aldehyde
2	lilac	no	none	methylglyoxal	—
3	,,	no	none	glyoxal	—
4	pink	yes	positive	furfural	—
5	deep pink	no	none	—	ketone
6	lilac	yes	positive	—	aldehyde
7	blue	yes	,,	—	aldehyde-dicarbonyl ?
8	,,	no	none	glucose/ascorbic acid	—
9	violet	yes	positive	hydroxymethylfurfural	—
10	blue	yes	none	acetylacetone	—
11	,,	yes	,,	—	methylketone-dicarbonyl ?
12	,,	yes	positive	—	aldehyde-dicarbonyl ?

* Blue colour indicates carbonyls capable of forming bis-hydrazones

† No complex formation shows that these carbonyls are ketones other than methyl ketones; glucose is an exception as it also does not complex with bisulphite

‡ No reaction indicates that these carbonyls are ketones

that the colour was blue for the bands near the origin and pink for those less strongly adsorbed; the exceptions were bands 2 and 3 which were bluish. It is known that compounds such as glucose, glyoxals and dicarbonyls which are capable of forming bis-hydrazones give blue colour changes in alcoholic sodium hydroxide.

(ii) *Specific reagents.*—The browned lemon juice was treated with the following reagents which react characteristically towards certain types of carbonyls. By this means it should be possible to classify the DNPH bands; for example, after preliminary reaction of the lemon juice with a reagent which specifically combines with the aldehyde group, any DNPH bands which are subsequently developed must be of ketonic origin. However, it was realised that the presence of unsaturated ketones, for example, might confuse some of the observations.

(*a*) *Saturated sodium bisulphite solution.* This reagent combines with all aldehydes and methyl ketones; glucose is an exception as it fails to form a bisulphite complex. Bands 2, 3, 5 and 8 for DNPH's were obtained which indicated that these carbonyls are probably ketones, other than methyl ketones.

(*b*) *Tollens reagent* (ammoniacal silver nitrate). This reagent is specific for aldehydes. Bands, 2, 3, 5, 8, 10 and 11 for DNPH's were found from the ketones in the lemon juice which had not reacted with the silver nitrate. These results for reagents (*a*) and (*b*) indicate that bands 10 and 11 may be methyl ketones.

(*c*) *Fehling's solution.* This reagent is also specific for aldehydes but proved unsuccessful because heat is necessary for the reaction and there was loss of volatile carbonyls.

(*d*) *Thiobarbituric acid.* This reagent has been used to identify aromatic and α,β-unsaturated aldehydes from other carbonyls;[13] for example, substances of the furfural type give precipitates. However, side reactions with glyoxals can occur[14] and no conclusive evidence was obtained with thiobarbituric acid.

(*e*) *Isonicotinyl hydrazide.* This reagent combines with Δ^4-3- and Δ^{1-4}-3-ketones.[15] The results for this trial were disappointing, but bands 2 and 3 were missing after this treatment, indicating that they may be ketones of this nature.

(iii) *Comparison with standard DNPH's.*—A large number of pure recrystallised DNPH's were prepared from commercial carbonyl compounds, subjected to TLC, and compared with lemon juice incubated for 26 days.

Carvone, which has been isolated from grapefruit concentrates,[8] was not present in lemon juice, nor was α-ketoglutaric acid which has been postulated as an oxidation product of reductic acid.[16]

A series of homologous methyl ketones, acetone, β-ionone, diacetyl and acetylacetone showed that acetylacetone moved in a similar manner to band 10 in lemon juice. The reactions

with bisulphite and Tollens reagent had indicated that band 10 probably was a methyl ketone, also a dicarbonyl had been expected from the blue colour in alcoholic sodium hydroxide.

A series of straight-chain aldehydes from acetaldehyde to heptaldehyde, crotonaldehyde, citral, methylglyoxal, glyoxal, furfural and hydroxymethylfurfural indicated that the last four were present in browned lemon juice corresponding to bands 2, 3, 4 and 9, respectively. From the previous tests the aldehyde nature of bands 4 and 9 was expected; bands 2 and 3 were thought to be ketones, but their α,β-dicarbonyl structure could have interfered with the usual chemical reactions. Citral was found to be the major component of lemon essence but its concentration in the juice was too low to be detected by TLC and it did not constitute one of the twelve bands.

Glucose and ascorbic acid gave DNPH's which moved together equivalent to band 8. As ascorbic acid is known to disappear after 3 days under these standardised conditions, band 8 in the juice which had been incubated for 26 days must be due to glucose. This is in agreement with the bisulphite finding but not with the DNPH derivative for glucose which was observed after treatment with Tollens reagent but would be expected to be missing. Ascorbic acid yielded two subsidiary DNPH bands which were taken to be dehydroascorbic acid and 2,3-diketogulonic acid, and corresponded to the three bands found in freshly squeezed lemon juice. However, these bands have also disappeared after prolonged incubation and are not included in the 12 bands under observation. Similarly, the transient pink band found after a short incubation, and believed to be α-ketogulonic acid, cannot be numbered in the 12 carbonyls of 26-day-incubated juice.

The results of this attempted classification are summarised in Table I which shows that six of the 12 carbonyls separated from browned lemon juice have been tentatively identified. The remaining six have been classified as aldehydes or ketones.

Browning of model systems

The evidence, so far, indicated that during the non-enzymic browning of lemon juice both aldehydes and ketones were found. The ultra-violet spectrophotometric analysis of the separated DNPH bands[17–19] showed that the absorption maximum in the near visible region gradually broadened and shifted from 370 mμ to 420 mμ, with concurrent decrease in relative absorption, from band 1 to band 12. These characteristics indicated increasing complexity of the DNPH'S due to conjugated unsaturation of the parent carbonyl.[19, 20] The browning potential of different classes of carbonyl compounds therefore was investigated using model systems.

In the first instance the browning of ascorbic acid alone, and in the presence of other components of the model system (Table II), was determined before investigating the effect of added carbonyls. All solutions were adjusted to pH 2·5 and withdrawn from the incubator at 37° after varying lengths of time. The degree of browning in the visible range was measured at 400 mμ[1] and the comparative build-up of conjugated unsaturated carbonyl compounds was assessed by absorption in the ultra-violet range at 285 mμ.[19]

Visually, ascorbic acid alone gave little browning even after 30 days' incubation; addition of citric acid and citric acid + amino-acids gave increased browning. The complete model system D gave considerable browning, but with a plateau between 15–22 days' incubation followed by a further increase in pigmentation with longer incubation. Browning of the lemon juice control was linear throughout and therefore appeared greater than model system D.

In the ultra-violet range, readings for ascorbic acid alone increased up to 10 days' incubation and then showed a slight falling off in carbonyl content. Model systems B and C followed a similar pattern but the maximum was not reached until 17 days after and the presence of amino-acids had a depressing effect. Model system D behaved like ascorbic acid alone but the falling off ceased by 17 days when another increase in carbonyls began, perhaps due to the presence of glucose. With the lemon juice control a carbonyl content approximately three times that ever reached during 30 days' incubation of any of the model systems was obtained after 3 days; between the 3rd and 10th days there was a slight increase after which a linear increase took place for 8 days when the readings remained constant for the rest of the incubation period. These findings with model systems suggested that amino-acids were implicated in ' mopping up '

Table II

Composition of model systems

Model system	Ascorbic acid, %	Citric acid,%	Amino-acids,* %	Glucose, %
A	0·1	—	—	—
B	0·1	5·0	—	—
C	0·1	5·0	0·44	—
D	0·1	5·0	0·44	3·0

* Hydrolysed casein

carbonylic fragments; also, since ascorbic acid/citric acid systems can brown, some of the pigments must arise from polymerisation of the primary fragments.

Development of carbonyl compounds in model systems

DNPH's of carbonyl compounds which had developed during 13 days' incubation of model systems B, C and D were investigated by TLC. In model system B, containing only ascorbic acid and citric acid, roughly the same bands for 12 DNPH's could be seen as were found in browned lemon juice. In model system C which contained additional amino-acids, the only bands which were detectable were the two or three moving near the solvent front which indicated that the other free carbonyl groups had been blocked by the amino-acids at this stage of browning. The presence of glucose in model system D almost restored the picture to that of ascorbic acid/citric acid only, except that the faster-moving bands which remained in model system C were relatively faint.

Browning of model system + carbonyl compounds

Model system D of definable composition, rather than lemon juice, was taken as the basal medium for the investigation of the browning capacity of various carbonyl compounds, even though this system had failed to produce the same intensity of browning, and had developed less than a third of the carbonyl material found when lemon juice was incubated under identical conditions. The following carbonyls, obtained commercially and without further purification, were chosen as representative of different classes and were incubated at 1 millimolar concentrations in model system D: acetaldehyde (aliphatic aldehyde), glyoxal (bis-aldehyde), furfural, crotonaldehyde (α,β-unsaturated aldehyde), methyl isobutyl ketone, diacetyl (diketone), hydroxymethylfurfural, methyl vinyl ketone (α,β-unsaturated ketone).

The results over a 21-day incubation period showed that acetaldehyde appeared to inhibit the browning of model system D, and methyl isobutyl ketone had no effect. Carbonyls of the type which would arise from fragmentation of larger molecules, such as glyoxal and diacetyl, accelerated browning in the early stages and then levelled off. The furfurals were potent additives giving a marked linear increase in browning over the incubation period; browning of the model system + furfural was approximately equal to that of lemon juice and slightly greater with hydroxymethylfurfural as the additive. The straight-chain α,β-unsaturated carbonyls, crotonaldehyde and methyl vinyl ketone, gave the most marked increases in browning, especially the latter; by the 15th and 10th days, respectively, the absorption at 400 mμ was double that of the model system alone after 25 days' incubation. The rate of browning of model system D + methyl vinyl ketone was even greater than the lemon juice control. No latent period was observed in the presence of the additives except for the plateau reached with the 'fragmentary' carbonyls.

Discussion

Compared with freshly squeezed lemon juice the commercial sample was in the first stages of non-enzymic browning. The one or two extra DNPH bands in the commercial juices were near the origin of the TLC plates and the blue colour in alcoholic sodium hydroxide of these more strongly adsorbed compounds would indicate dicarbonyls. Also, bands 2 and 3, which were tentatively identified as the dicarbonyls methylglyoxal and glyoxal and gave a bluish/pink colour in alcoholic sodium hydroxide, were clearly visible after 2 days' incubation. This early

appearance of dicarbonyls, and especially those of a 'fragmentary' nature, indicates their important association with the initiation of non-enzymic browning. Supporting evidence comes from the browning capacity of carbonyls added to a model system in which glyoxal gave the greatest initial boost to the development of brown pigments, even though the potential was not maintained compared with other carbonyls.

In agreement with Burton *et al.*[21a] the α,β-unsaturated carbonyls were found to be the most potent browning agents and maintained a linear increase over the 21-day incubation period. These workers have also suggested that furfural and hydroxymethylfurfural are relatively inert in the browning of sugar–amino-acid model systems but may play a more important part in the browning of fruit juices.[21b] The present investigation has shown that in ascorbic acid/citric acid/amino-acids/glucose model systems these cyclic carbonyls do in fact have a high browning capacity, but in the natural product although furfural was detected in the early stages hydroxymethylfurfural was late in forming.

The fluctuations in the carbonyl content during browning were indicated by the readings at 285 mμ of model systems. In solutions of ascorbic acid alone there was no linear increase; instead, a maximum carbonyl development was recorded after 10 days' incubation, after which this level must have been reduced by polymerisation. In the presence of citric acid and citric acid + amino-acids, the maxima were delayed another 7 days. Although the amino-acids depressed the carbonyl level as measured by absorption at 285 mμ, absorption was increased in the visible range at 400 mμ. This indicated that carbonyl/amino interaction, in addition to polymerisation, had taken place and eliminated some of the free carbonyl groups.

Carbonyl development in model systems containing glucose was similar, but at a reduced level, to that of lemon juice except that in the latter the initial maximum was earlier, probably due to the lemon juice having undergone the first stages of browning before the incubation experiments began. The presence of glucose as a contributor to the build-up of carbonyls appeared not to take effect until after 18 days' incubation. Under acid conditions hydroxymethylfurfural can be formed from sugars.[22, 23] Possibly in non-enzymic browning mainly due to ascorbic acid breakdown, glucose makes a separate delayed contribution to α,β-unsaturated carbonyl development. Burton *et al.*[21a] have found that straight-chain saturated carbonyls retard browning in glucose/glycine systems at pH 6·5; a similar result has now been shown with acetaldehyde in a model system at pH 2·5.

Considering that the test carbonyls were added to a model system at a low concentration and that the α,β-unsaturated carbonyls produced such striking increases in browning, it is not surprising that the 'complete' model system failed to reproduce the browning capacity of lemon juice. The natural product need only contain a trace of carbonyl to give it this lead. Citral could be a factor contributing to the difference between lemon juice and the model system, although its concentration was so low as to be undetectable by TLC; however, addition of 0·05% citral to lemon juice was not found to augment the rate of browning. Other trace substances must account for the discrepancy; the rôle of trace elements has not been included in this study.

After 12 days' incubation under aerobic conditions at 37°, 12 DNPH's could be separated by TLC and the pattern remained constant with further incubation up to 26 days. Stadtman[12] also isolated twelve carbonyls from browned apricot concentrates, which is of interest since apricots are less acidic (pH 3·5) and browning of this product is generally attributed to sugar–amino-acid reactions. Only furfural and hydroxymethylfurfural were identified and it may be coincidental that the number of carbonyls was the same as found in the present investigation, rather than proof of similar chemical reactions in apricots. In this study an attempt has been made to identify more of the carbonyls as their DNPH's which were precipitated from lemon juice (Table I). The blocking of specific carbonyl groups with various reagents has proved to be a useful technique. The inconclusive results with isonicotinyl hydrazide were not unexpected since it has been primarily used with more complex ketosteroid compounds;[15] the two carbonyls which reacted with this reagent could have been an artefact as they were subsequently thought to be methyl glyoxal and glyoxal. The general classification of the 12 DNPH's with sodium bisulphite and Tollens reagent was shown to be generally correct for some of the carbonyls identified by comparison with standard DNPH's. All of the DNPH's

which have been identified and gave a bluish colour in alcoholic sodium hydroxide are carbonyls which form bis-hydrazones, and it is likely that unidentified bands 7, 11 and 12 are also dicarbonyls. Fractionation of the carbonyls in browned lemon juice and incubated model systems into volatile and non-volatile groups could possibly assist their identification.

The evidence from this investigation shows that carbonyls are the reactive compounds in the non-enzymic browning of an acidic product such as lemon juice. Hodge[24] and Burton & McWeeny[23] also stress the rôle of carbonyls in sugar–amine systems under non-acidic conditions. The melanoidin end-products of the two different types of system may therefore be similar.

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