THE CONSTITUTION OF NATURAL COLOURING MATTERS, KUROMAMIN, SHISONIN, AND NASUNIN.

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"Kuromame", "Shiso", and "Nasu" are widely used vegetables in Japan, and as they are specially favoured for their bright colours, the chemical study of their colouring matters is of great importance as well as of much interest. However almost no literature has been found upon the subjects.

The authors were fortunately successful in isolating their main colouring matters in crystalline state, and after several investigations it was very curious to find that all three of these pigments are anthocyanin. Their short sketches have already been made[1], and now the following details will be recorded.

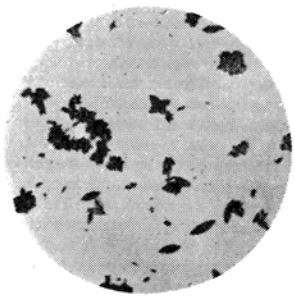
I. Kuromamin, The Colouring Matter of "Kuromame".

"Kuromame"[2] which belongs of *Glycine Soja Benth*, is given the special name because of its delicacy of taste and of the specially deep bluish colour of the seed-coat. Not only used as common food it is specially served as one of happy symbolical sweets in the typical ceremonial menu of New Year. It is also favoured as medicinal matter especially recommended to vocalists. As results of preliminary tests the black pigment was treated as anthocyanin; however, the pigment seemed at first to be a mixture of a water soluble colouring matter and another water insoluble bluish matter. Therefore the treatment was carried out accordingly. From the reddish purple aqueous solution obtained by leaving the beans immersed in cold water, a bluish-green precipitate was formed on addition of aqueous lead acetate. The lead salt was converted into chloride in the usual manner. Isolation of pure pigment from the chloride was exceedingly difficult. However, after several experiments the pigment was successfully obtained by purifying the lead salt with cold acetic acid. The chloride, obtained from the lead salt thus purified, still contained impurities, but the latter dissolved in CH_3OH easily, therefore the isolation of crystalline pigment was finally successful after removing the impurities by special treatment. The glucoside crystallized in green

(1) Kuroda and Wada, *Proc. Imp. Acad. Japan*, **9** (1933), 101; **9** (1933), 517; **11** (1935), 189; **11** (1935), 28; **9** (1933), 51; **11** (1935), 235.

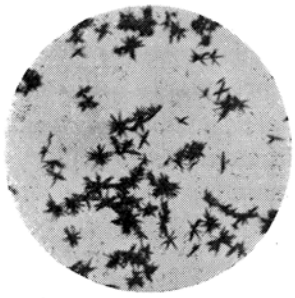
(2) "Kuro" means black and "Mame" bean in Japanese.

leaflets with metallic lustre. Its analytical results and decomposition products with boiling HCl proved to be monoglucoside of cyanidin chloride $C_{21}H_{21}O_{11}Cl+1^1/_2H_2O$.



Kuromamin chloride from water-soluble pigments from "Kuromame" from Hokkaido

Fig. 1.



Kuromamidin chloride from water-soluble pigments from "Kuromame" from Hokkaido

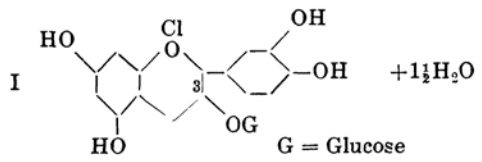
Fig. 2.

The dry seed-coat, after the removal of the above water-soluble colouring matter was treated with CH_3OH–HCl, and the pigment from the resulting red solution, was purified and isolated in crystalline state as chloride by using lead salt method as previously mentioned. The chloride was proved to be monoglucoside of cyanidin chloride $C_{21}H_{21}O_{11}Cl+1^1/_2H_2O$ by analysis and by its decomposition products as well as by the water-soluble pigment.

By these experiments, the nature of the colouring matter of the seed-coat was shown to be identical whether derived from the water-soluble portion or otherwise. This observation was verified by treating the seed-coat directly with CH_3OH–HCl instead of first treating it with water. The name kuromamin is given to the crystalline glucoside by the authors.

It was suggested that there may exist possible disagreement in the chemical structure of the colouring matter among different samples from unlike localities. Indeed there are amazingly numerous varieties of "Kuromame" on market, differing considerably in their appearances. Such a consideration led to our further studies to apply the above treatment to the beans from Manchuria (Mukden and Shing Tai Tzu). As the results, it was shown that the colouring matters of "Kuromame", regardless of the varieties and the localities, belong to the same kind of compound.

After several investigations, the above glucoside "kuromamin", in every respect, was found to be identical with chrysanthemin (I), namely cyanidin 3-monoglucoside.



I $+1\frac{1}{2}H_2O$ Kuromamin (= Chrysanthemin) chloride

G = Glucose

The procedures will be pointed out as follows :

(1) The analytical result of kuromamin chloride agreed with that of chrysanthemin chloride $C_{21}H_{21}O_{11}Cl+1^1/_2H_2O$ in literature[3] ; and m.p. 182° of kuromamin picrate also agreed with that of chrysanthemin picrate.

(2) By characteristic colour reactions, the position of glucose was determined to be "3", namely kuromamin chloride turning violet with Na_2CO_3 ; blue with NaOH or $FeCl_3$. In these respects they were exactly identical with chrysanthemin chloride[4], but distinctly different from cyanenin (synthetic, 5-monoglucoside of cyanidin) chloride[5] which is known to give bright bluish green colouration with Na_2CO_3 and to be oxidised rapidly with $FeCl_3$. Further confirmative colour reactions with buffered solution[6] were observed. Then, the results with kuromamin chloride and natural specimen[7] of chrysanthemin chloride were quite identical, and agreed with the report on synthetic[8] chrysanthemin chloride, and were distinctly distinguished from those of cyanenin chloride[9] and natural specimen of cyanin chloride (3,5-diglucoside of cyanidin), and mecocyanin chloride, (3-gentiobioside of cyanidin chloride[10], natural and synthetic).

(3) Distribution number was determined by Willstätter's method ; the results being 19 (1st) and 19.15 (2nd) in agreement with those of the literature (natural[11], 19 and 19.5 ; synthetic[12], 19 and 19.1).

(4) As results of observations of absorption spectrum, kuromamin chloride and chrysanthemin chloride were also proved to be quite identical and were distinguished from those of cyanin chloride (natural, from dahlia and "shiso")[13].

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(3) Willstätter and Bolton, *Ann.*, **421** (1916), 146. S. Murakami, Robertson, and R. Robinson, *J. Chem. Soc.*, **1931**, 2670.

(4) S. Murakami, R. Robinson, and A. Robertson, *ibid.*, **1931**, 2668.

(5) Leon and Robinson, *ibid.*, **1932**, 2222.

(6) R. Robinson and A. Robertson, *Biochem. J.*, **23** (1929), 35.

(7) From flower of deep red *chrysanthemum indicum*.

(8) Murakami, Robertson, and Robinson, *J. Chem. Soc.*, **1931**, 2671.

(9) A. Leon and Robinson, *ibid.*, **1932**, 2222.

(10) Willstätter and Weil, *Ann.*, **412** (1916), 231. Grove, Inubuse, and Robinsen, *J. Chem. Soc.*, **1934**, 1611.

(11) Willstätter and Bolton, *Ann.*, **412** (1916), 146.

(12) Murakami, Robinson, and Robertson, *J. Chem. Soc.*, **1931**, 2670.

(13) Kuroda and Wada, *Proc. Imp. Acad. Japan*, **11** (1935), 28.

(5) The aglucone of kuromamin was again verified exactly to be cyanidin, on comparing its properties (solubilities, colour reactions, and appearances, etc.) with those of our specimen of synthetic cyanidin chloride[14] (for which the writers thank Miss Yashiro for her kind help in a part of this synthesis) and of the aglucone from "Shiso" pigment and was also identified by the absorption spectrum[13].

(6) Upon the decomposition with acid, kuromamin yielded one mol each of cyanidin and glucose. The quantity of glucose was determined exactly by Pavy's method; the result was found to be in agreement (Found: 35. Calc.: 35%).

(7) Furthermore by Karrer's method[15] the position of glucose was also proved to be "3", namely by action of perhydrol.

(8) Kuromamin chloride was compared with the report on idaein chloride, 3-monogalactoside of cyanidin chloride[16] $C_{21}H_{21}O_{11}Cl+2^1/_2H_2O$; then they were actually distinguished from each other in their solubilities, quantities of water of crystallisation, and in their osazones (glucosazone from kuromamin, m.p. 203°; galactosazone, m.p. 186°).

Experimental.

Isolation of Kuromamin Chloride from Water-soluble Portion. When the beans (36 *l.*) from Hokkaido were immersed in cold water for several hours, a reddish purple aqueous solution was obtained. From the aqueous solution, bluish green precipitate was formed on addition of saturated aqueous lead acetate which was then filtered, dried on a tile quickly, powdered (yield, 0.5% of the beans) and triturated with cold glacial acetic acid. From the purple acetic acid solution thus obtained, a pure indigo blue lead salt of the pigment was precipitated upon the addition of several times its volume of ether (yield, 17 g.). When the above lead salt was treated by 2% CH_3OH–HCl, a red solution was obtained, and the chloride of the colouring substance was precipitated by addition of ether. And the chloride was triturated with a small quantity of absolute CH_3OH to remove easily soluble impurities. It crystallized out from dry CH_3OH–HCl in leaflets with a metallic lustre (Fig. 1) (yield, 0.4 g.). On analysis, the result agreed with a monoglucoside of cyanidin chloride (Found: C, 49.38; H, 4.73. Calc. for $C_{21}H_{21}O_{11}Cl+1^1/_2H_2O$: C, 49.30; H, 4.69%).

Hydrolysis of Kuromamin Chloride (from Water-soluble Portion) with Acid. The above kuromamin chloride (air dry, 0.1 g.) was dissolved in hot 0.1% aqueous HCl (6 c.c.), conc. HCl (6 c.c.) added, and the mixture was boiled for three minutes. On cooling, an aglucone (Fig. 2) crystallized out in reddish brown needles. Yield, 0.0642 g. (air dry, 64.2%. calc. 65.3%). It showed all the qualitative reactions of cyanidin chloride. The aglucone was recrystallized from 2% CH_3OH–HCl for analysis (Found: C, 52.79; H, 4.14. Calc. for

(14) A. Robertson and R. Robinson, *J. Chem. Soc.*, **1928**, 1526.
(15) Karrer and Meuron, *Helv. Chim. Acta*, **15** (1932), 1212.
(16) Willstätter and Mallison, *Ann.*, **408** (1915), 15.

cyanidin chloride $C_{15}H_{11}O_6Cl+H_2O$: C, 52.89; H, 3.82%). Micro-Zeisel estimation proved the absence of methoxyl groups in the aglucone.

Sugar. (1) *Estimation.* The above filtrate removed from the aglucone was neutralized with alkali carefully, evaporated on the water bath to dryness, then dissolved again in water, and filtered to remove completely any insoluble matter, and was dried on the water bath; the dry matter dissolved in H_2O (pale yellow solution), was made up to 100 c.c. of about 10% NH_3 with conc. NH_3 and the sugar estimated with Pavy's solution.

(2) *Osazone.* The resulting acid solution obtained upon decomposition of kuromamin chloride with 20% aqueous HCl was neutralized with alkali carefully and dried. The dry matter was dissolved in water and filtered. When the filtrate was heated with C_6H_5NH-NH_2·HCl and sodium acetate, glucosazone was formed: washed with CH_3OH, and ether, m.p. 205°, both alone and after admixture with the authentic specimen.

Isolation of Kuromamin Chloride from Water-insoluble Portion. The dry seed-coat, after the removal of the water-soluble pigment, was treated with 0.5% CH_3OH–HCl and to the resulting red solution, methyl alcohol solution of lead acetate was added drop by drop, and the bluish purple lead salt of the pigment was collected after removing the initial white precipitate of lead chloride. The purification of both the above lead salt and chloride of pigment was carried out as in the case of the water-soluble pigment. The chloride (0.5 g.) thus obtained was recrystallized from dry CH_3OH–HCl twice. The kuromamin chloride separated in leaflets with green metallic lustre. (Found: C, 49.24; H, 4.43. Calc. for $C_{21}H_{21}O_{11}Cl+1^1/_2H_2O$: C, 49-30; H, 4.69%.)

Hydrolysis with Acid. When the acid decomposition of the glucoside (from water-insoluble portion) (0.1 g.) was carried out as previously mentioned, cyanidin chloride (0.065 g.) separated in brown-red needles. Yield, 65%; calc. 65.3%. For analysis, the aglucone was recrystallized from 2% CH_3OH–HCl (Found: C, 52.70; H, 3.62. Calc. for $C_{15}H_{11}O_6Cl+H_2O$: C, 52.86; H, 3.82%. Estimation of sugar: Found: 34.05. Calc.: 35.22%).

Direct Isolation of Kuromamin Chloride from "Kuromame" from Hokkaido. The seed-coats were separated from the beans by the help of an electric fan, grinding in a hand mill at room temperature. They were then immersed in 2% CH_3OH–HCl for three hours, and into the resulting red solution, methyl alcohol solution of lead acetate was added drop by drop in order to precipitate the lead salt of pigment. On carrying out the subsequent treatment as already mentioned, the glucoside crystallized out; 1.5 g. of the crystalline chloride was derived from 8 *l.* of beans or 370 g. of seed-coat, showing a notable increase in the yield, hence the advantage of the present method (over the one employed formerly when beans were first immersed in water). (Found: C, 49.21; H, 4.79. Calc. for $C_{21}H_{21}O_{11}Cl+1^1/_2H_2O$: C, 49.30; H, 4.60%.) The conversion of the glucoside led to a quantitative yield of crystalline aglucone (Found: C, 52.72; H, 3.62. Calc. for cyanidin chloride $C_{15}H_{11}O_6Cl+H_2O$: C, 52.92; H, 3.82%).

Isolation of Kuromamin Chloride from "Kuromame" from Mukden. The seed-coat (550 g.) of beans from Moukden was treated with 2% CH_3OH–HCl directly and purified by the foregoing method. The glucoside obtained in crystals with a green metallic lustre was decomposed into one molecule each of cyanidin chloride and glucose, and analytical results of both the glucoside (Found: C, 49.02; H, 4.59. Calc.; C, 49.30; H, 4.69%) and the aglucone (Found: C, 52.51; H, 3.46. Calc.: C, 52.94; H, 3.82%)

agreed with cyanidin chloride-3-monoglucoside. 0.5 g. of the crystalline glucoside was derived from 550 g. of seed-coat.

Isolation of Kuromamin Chloride from "Kuromame" from Shing Tai Tzu. Seed-coat of beans from Shing Tai Tzu was treated under the same condition as that from Mukden with the same results: Analytical results of both the glucoside (Found: C, 48.98; H, 4.55%) and the aglucone (Found: C, 52.87; H, 3.97%) agreed with 3-monoglucoside of cyanidin chloride. 0.5 g. of kuromamin chloride was derived from 580 g. of seed-coat.

Kuromamin Picrate. Kuromamin chloride was dissolved in 0.05% aqueous HCl, and saturated aqueous picric acid was added. In every respect the picrate that separated as a deep brownish-red prisms with bronze glance in mass (decomposition point, 190–192°), was found to be identical with chrysanthemin (cyanidin-3-monoglucoside) picrate.

The Alkali Fusion of Kuromamin Chloride. Kuromamin chloride (1 g.) was fused with KOH (3 g.) and H_2O (small quantity) at 203°. After cooling, the product was dissolved in water, acidified with conc. HCl, extracted with ether, and was evaporated. From the ethereal residue, a lead salt was precipitated by the addition of lead acetate. The lead salt filtered was decomposed with dilute H_2SO_4 and extracted with ether; the ethereal solution was treated with animal charcoal, and evaporated. When the residue was purified with hot water, protocatechuic acid crystallized out in white needles; m.p. 193°, both alone and after admixture with an authentic specimen (the quantity was not enough for analysis). The filtrate from the lead precipitate was treated with dilute H_2SO_4; after removing $PbSO_4$, the filtrate was extracted with ether. From the ethereal solution, phloroglucinol was obtained in white long needles; m.p. 210°, both alone and after admixture with an authentic specimen (Found: C, 44.45; H, 6.26. Calc. for $C_6H_6O_3+2H_2O$: C, 44.44; H, 6.17%).

The Oxidation of Kuromamin Chloride with H_2O_2. To the chloride (1 g.) dissolved in 1% aqueous Na_2CO_3 (80 c.c.) 3% aqueous H_2O_2 (80 c.c.) was added and the mixture was left at room temperature for three hours, then acidified with H_2SO_4 and extracted with ether. From the ethereal extract protocatechnic acid was isolated in white needles; m.p. 193°, both alone and after admixture with the authentic specimen. (Found: C, 48.97; H, 4.75. Calc. for $C_7H_6O_4+H_2O$: C, 48.83; H, 4.65%.)

The Position of Sugar. Kuromamin chloride (0.3 g.) was dissolved in hot H_2O (3.5 c.c.) and after cooling, into the solution, 30% H_2O_2 (2.5 c.c.) was added and the mixture left at room temperature. When yellow coloured solution thus obtained, was neutralized with 2N NaOH very carefully, the excess of H_2O_2 was decomposed with Pt-black, and the whole was dried by distillation in vacuum. From the aqueous solution of the residue, glucosazone (m.p. 205°, both alone and after admixture with the authentic specimen) was obtained by heating with $C_6H_5NH\cdot NH_2\cdot HCl$ and sodium acetate (yield, 0.05 g.). In this result, the position of glucose was proved to be "3". The filtrate from the above osazone was treated as in the case of shisonin (see following pages), however, no osazone from position "5" has been formed.

Preparation of Chrysanthemin Chloride from Chrysanthemum. The fresh dark red flowers were treated with 2% CH_3OH–HCl and from the resulting red solution, a lead salt was precipitated by the addition of lead acetate. The lead salt was also converted into chloride as usual; the raw chloride dissolved in 0.05% aqueous HCl was converted into picrate as previously mentioned. And the picrate was reconverted into chloride by aid

of 2% CH_3OH–HCl, and was purified as in the case of kuromamin chloride, then chrysanthemin chloride crystallized out in leaflets with green metallic lustre and was found to be quite identical with kuromamin in every respect.

Experiments with Buffered Solution. Colour reactions in a range of buffered solution of graded *p*H were examined. The method of the Robertson and Robinson[14] was applied and the numbers refer to solutions of *p*H from 3.2 (1) to 11.0 (14) and then to more alkaline solution of unknown *p*H. The chloride of kuromamin and natural chrysanthemin gave quite identical results as follows: (1) Salmon-red; (2) salmon-red, fading; (3) bluish-red, fading; (4) bluish salmon-red to pink fading; (5) pink; (6) brown-violet; (9) (10) (11) brown-violet-red (keeping considerably); (13) (14) brown-violet; (15) violet; (16) bluish-violet.

Distribution Number. Amyl alcohol for this experiment was purified as follows: commercial amyl alcohol was washed with 0.5% aqueous HCl several times, then washed with water to remove a trace of acid and distilled (b.p. over 110°). Kuromamin chloride (air dry, 0.01 g.) was dissolved in 0.5% aqueous HCl (50 c.c.) and was shaken with the above amyl alcohol. The operation was repeated twice. After standing the amyl alcoholic layer was kept for use. The preparation of standard solution: kuromamin chloride (0.0019 g., air dry) was dissolved in hot 2% CH_3OH–HCl (2 c.c.), to the solution, amyl alcohol was added and the whole made up to 50 c.c. and was used as standard. The result of 1st operation; 0.0019 g. in amyl alcohol layer, distribution number 19; 2nd: 0.00155 g. in amyl alcohol layer, distribution number 19.15.

The Colour Reactions with Buffered Solutions. The three specimens, kuromamidin, shisonidin (17) and synthetic cyanidin chlorides gave the identical results. (1) Red, pink in 30 seconds; (2) bluish red, colourless in 30 seconds; (3) reddish violet, colourless in 30 seconds; (4) blue reddish violet, colourless in 30 seconds; (5) permanganate, colourless in 1 hour; (6) bluer than (5) permanganate in 30 seconds; (9) violet red, deep permanganate in 30 seconds.; (10) similar but a little bluer; (11) violet blue; (13) dark blue violet dichroic; (14) similar to (13); (15) dark blue violet; (16) blue, dark yellowish gray in 1 hour.

Absorption Spectra of Kuromamin, Chrysanthemin, Kuromamidin, and Synthetic Cyanidin Chloride. Photographs were taken for each substance dissolved in CH_3OH (N/10000), with iron arc as the light source. They were reproduced in another paper.[17]

The authors desire to thank the Imperial Academy for a grant, and Viscount Masatoshi Okochi, the President of the Institute, for his kindness for obtaining samples of the beans from Manchuria.

II. Shisonin, The Colouring Matter of "Shiso".

The pigment of "Shiso" leaves (*Perilla Ocimoides L. var Crispa Benth*)[18] is extensively used for colouring "Umeboshi" which is a kind of plum pickle favoured throughout Japan. The chemical study on this pigment was made

(17) Kuroda and Wada, *Proc. Imp. Acad. Japan*, **11** (1935), 189.

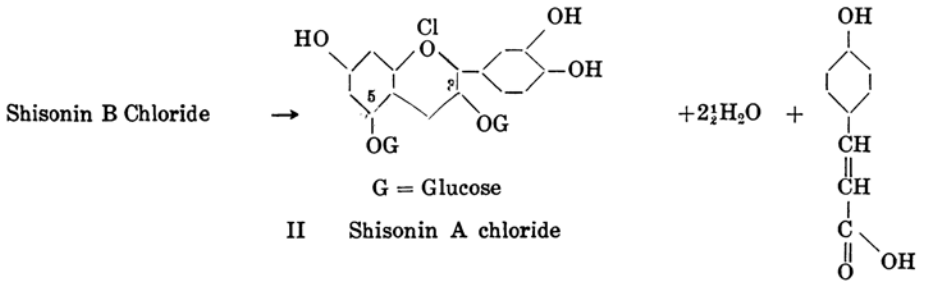
(18) Japanese special name "Chirimen Shiso".

by Dr. Kaoru Kondo,[19] a crystalline pigment "perillanin" was isolated and considered to be delphinidin-mono-glucoside combined with protocatechuic acid.

The present authors have undertaken the study of the anthocyan pigment obtained from the leaves cultivated in Tokyo, and some interesting new observations were made.

From "Shiso" leaves, a pigment which was named shisonin by the authors, was isolated as chloride. After several investigations shisonin was shown to be a mixture of glucosides, shisonin A and another new glucoside shisonin B. Shisonin A was isolated in a crystalline state as its chloride from the shisonin chloride by use of a suitable solvent. Shisonin B chloride was confirmed to be a compound of shisonin A chloride and *p*-coumaric acid, because the above two components were isolated, when shisonin B chloride was treated with cold alkali in H_2 atmosphere.

Shisonin A chloride was identified to be cyanin chloride, 3,5-diglucoside of cyanidin (II) in every respect.



G = Glucose

II Shisonin A chloride

For the direct comparisons, cyanin chloride was prepared from dahlia.[20] Appearances of crystals obtained in both cases under similar conditions were quite identical rhombic plates (Fig. 3) or fine needles (Fig. 4), m.p. (d.p.) being 195° as cyanin chloride. Moreover all the colour reactions, solubilities (except that, it was almost insoluble in cold water, or in dilute aqueous HCl, differing in this respect from mecocyanin[21]), and the analytical results ($C_{27}H_{31}O_{16}Cl+2^1/_2H_2O$)[22] were in agreement.

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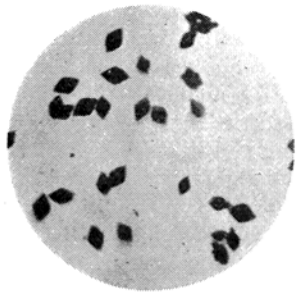
(19) *J. Pharm. Soc. Japan*, **51** (1931), 254.

(20) Kōzō Hayashi, *J. Bot. Japan*, **47** (1933), 396.

(21) Mecocyanin chloride, 3-bioside of cyanidin chloride $C_{27}H_{31}O_{16}Cl\cdot 3H_2O$: Willstätter and Weil, *Ann.*, **412** (1916), 231. A. Leon and R. Robinson *J. Chem. Soc.*, **1931**, 2737.

(22) Willstätter and Nolan, *Ann.*, **408** (1915), 1. Willstätter and Mallison, *Ann.*, **408** (1915), 147.



Shisonin A chloride from "Shiso"

Fig. 3.

Shisonin A chloride from "Shiso"

Fig. 4.

Shisonidin chloride from "Shiso"

Fig. 5.

Upon the decomposition with acid, from shisonin A chloride (m.p. 195°), one molecule of cyanidin chloride and two molecules of glucose were yielded quantitatively as anticipated. The estimation of the glucose was carried out by Pavy's method, in which the result agreed. And the positions of the glucose were proved to be "3" and "5" by Karrer's method.

The aglucone (brown crystals, Fig 5) was exactly proved to be cyanidin chloride ($C_{15}H_{11}O_6Cl+H_2O$), by its properties, analysis, and by the following decomposition products. When fused with alkali, it gave phloroglucinol and protocatechuic acid. The protocatechuic acid was obtained, however, in a better yield when the aglucone was oxidized with H_2O_2 in a sodium carbonate solution.

More confirmative colour reactions with buffered solution[14] were observed. The results with shisonin and cyanin chloride (from dahlia) were quite identical, and agreed with the report on synthetic cyanin chloride,[23] and were distinctly distinguished from those of chrysanthemin chloride, obtained from natural pigments, and also of cyanenin (synthetic, cyanidin 5-monoglucoside) chloride[9] and mecocyanin chloride[8] (natural).

As the results of estimation of distribution number and of observation of absorption spectrum, shisonin A was also proved to be quite identical with cyanin chloride.

In view of the above experimental results, the anthocyanin of "Shiso" is considered to be a mixture of shisonin A and shisonin B, the latter being a compound of *p*-coumaric acid and shisonin A; further study on the arrangement of *p*-coumaric acid in the interesting second glucoside shisonin B is progressing.

(23) R. Robinson and A. R. Todd, *J. Chem. Soc.*, **1932**, 2496.

Experimental.

Preparation of Sh sonin Chloride. A dry powder, which was cautiously prepared from the leaves (obtained from the vegetable market in Tokyo) was soaked in cold petroleum ether to remove the yellow pigment. On separating the yellow solution, it was then extracted with 2% CH_3OH–HCl. On addition of ether to the above resulting red solution a heavy red flocculent crude chloride of the colouring matter was precipitated and then, by dissolving this in H_2O and adding lead acetate the pigment was purified as lead salt. The lead salt was reconverted into chloride by aid of CH_3OH–HCl and ether by the similar method as previously mentioned for kuromamin chloride and purified.

The Isolation of Shisonin A Chloride. Amorphous shisonin chloride (2 g.) dissolved in water (50 c.c.) was treated with 4 N aqueous NaOH (50 c.c.) in H_2 and left for two hours at room temperature; then acidified with conc. HCl (calculated quantity) and extracted with ether. The residue (0.1 g.) of ethereal solution was washed with a small quantity of cold water, then was recrystallized from hot water by aid of animal charcoal twice. *p*-Coumaric acid, m.p. 207°, both alone and after admixture with the authentic specimen. (Found: C, 65.64; H, 5.06. Calc. for $C_9H_8O_3$: C, 65.64; H, 5.06%.)

When the above aqueous red solution, obtained free from *p*-coumaric acid, was left at room temperature, a dark brown coloured crystalline substance with metallic lustre gradually separated (the separation finished in 2 or 3 days); the dry matter (0.5 g.) thus obtained was washed with cold CH_3OH, was dissolved in hot 0.01% aqueous HCl (small quantity), to which an equal volume of 3% C_2H_5OH–HCl was added and left. Shisonin A chloride (decomposition point 195°) crystallized out in small rhombic plates (Fig. 3) with metallic lustre. It was recrystallized three times for analysis. (Found: C, 47.03; H. 5.54. Calc. for $C_{27}H_{31}O_{16}Cl+2^1/_2H_2O$: C, 46.86; H, 5.21%).

The Decomposition of Shisonin A Chloride with Acid. Shisonin A chloride (0.1024 g., air dry) was boiled with 20% aqueous HCl (5 c.c.) on the sand bath for three minutes, then cooled with ice; an aglucone crystallized out in reddish brown needles. Yield, 0.05 g. (air dry, 48.81%, calc. 49.2%). It showed all the qualitative reactions of cyanidin chloride. For analysis, a specimen was recrystallized from 2% CH_3OH–HCl. (Found: C, 52.72; H, 4.06. Calc. for cyanidin chloride $C_{15}H_{11}O_6Cl+H_2O$: C, 52.86; H, 3.82%). Micro-Zeisel estimation proved the absence of methoxyl groups in the aglucone.

Sugar. (1) *Estimation.* Crystalline shisonin A chloride (0.0985 g.) was boiled with 2)% aqueous HCl for 3 minutes. After cooling, the filtrate removed from the aglucone was treated as in the case of kuromamin and sugar was estimated by Pavy's method (Found: sugar, 50.44. Calc. for $C_{27}H_{31}O_{16}Cl+2^1/_2H_2O$: 52.10%).

(2) *Osazone.* Glucosazone from the resulting acid solution obtaind by acid hydrolysis of shisonin A chloride was formed in a similar method to the case kuromamin chloride.

(3) *The position of sugar.* Shisonin A chloride (0.3 g.) was treated with 30% H_2O_2 as in the case of kuromamin and glucosazone from position "3" was obtained, m.p. 205°, 0.04 g. The filtrate from the above osazone was heated with 15% aqueous HCl on the water bath for $1^1/_2$ hours, then filtered. The filtrate was neutralized with alkali and dried in vacuum. From the residue, glucosazone from position "5" was isolated by aid of $C_6H_5NH{\cdot}NH_2{\cdot}HCl$ and sodium acetate as usual. After purifying, yield 0.06 g.; m.p. 203°, both alone and after admixture with the authentic specimen.

Isolation of Shisonin A Chloride by the Use of Solvent. When amorphous shisonin chloride (5 g.) was warmed with absolute CH_3OH on the water bath and left for a few days, a pigment free from *p*-coumaric acid (microscopic crystals upon treatment with CH_3OH) was separated (dry matter 1.5 g.); this was sucked. This crystallized out in small rhombic plates (decomposition point 195°) with golden metallic lustre, when the dry matter was dissolved in hot 0.5% aqueous HCl and an equal volume of 3% C_2H_5OH–HCl was added. Recrystallized twice for analysis (Found: C, 46.74; H, 5.42. Calc. for $C_{27}H_{31}O_{16}Cl+2^1/_2H_2O$: C, 46.86; H, 5.21%.) From the compound, an aglucone was produced. (Found: C, 52.84; H, 4.06. Calc. for $C_{15}H_{11}O_6Cl+H_2O$: C, 52.86; H, 3.82%.)

Alkali Fusion of Shisonin A Chloride. Shisonin chloride (0.7 g.) was fused with KOH (2.1 g.); from the product, phloroglucinol (m.p. 207°) and protocatechuic acid (m.p. 196°, both alone and after admixture with the authentic specimen) were obtained.

The Oxidation of Shisonin A Chloride with H_2O_2. The oxidation with 3% H_2O_2 was similarly carried out as in the case of kuromamin; protocatechuic acid was isolated in good yield, m.p. 196°. (Found: C, 48.90; H, 4.55. Calc. for $C_7H_6O_4+H_2O$: C, 48.83; H, 4.65%.)

Cyanin Chloride from Dahlia. The dark coloured flowers of dahlia ("Chishima", "Ubatama", "Black diamond" about 100 g.) was immersed in 2% CH_3OH–HCl (160 c.c.) in cold for three hours. When the resulting red solution was concentrated at 35° under reduced pressure and left, the pigment crystallized out in needles. It was filtered, dried, and to the solution of the dry matter dissolved in 0.01% hot aqueous HCl (1 part), 3% C_2H_5OH–HCl (1 part) was added. Cyanin chloride crystallized in small rhombic plates with golden lustre (decomposit on point 195°). To remove a trace of monoglucoside, the above chloride was dissolved in 0.5% aqueous HCl saturated previously with *n*-butyl alcohol and was shaken with butyl alcohol, saturated with 0.5% aqueous HCl twice, and then with isoamyl alcohol. Now, the aqueous layer was warmed and 3% C_2H_5OH–HCl was added. Pure cyanin chloride was isolated from the solution.

Another Comparison of Shisonin A and Cyanin Chloride. All the apparent characters of the specimens were identical and they furnished solutions (aqueous and alcoholic) having the same colour and behaviour under all conditions. All the ordinary reactions, the colours with Na_2CO_3 (blue) and aqueous $FeCl_3$ (violet) were observed identical.

The colour reactions in a range of buffered solutions were investigated and the same results were obtained as follows: (1) Rose, fading; (2) (3) bluish rose, colourless in 30 seconds; (4) cherry red, fading; (5) permanganate fading less rapidly; (9) more intense and more stable; (9) (10) (11) reddish-violet; (13) violet, fading; (14) blue violet, fading; (15) (16) blue, fading rapidly.

Comparison of Distribution. Shisonin A chloride (10 mg.) was dissolved in 0.5% aqueous HCl (50 c.c.) previously saturated with *n*-butyl alcohol. A similar solution of cyanin chloride (from dahlia) was prepared and both solutions were shaken twice with *n*-butyl alcohol (50 c.c. each time) previously saturated with 0.5% aqueous HCl. Each butyl alcohol layer was matched colorimetrically and was found to be identical.

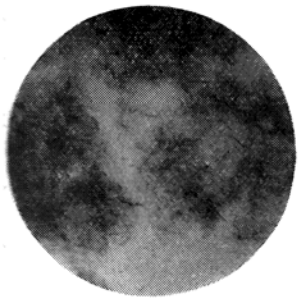
Absorption Spectra of Shisonin A, Cyanin (from Dahlia) and Shisonidin Chloride. Photographs were taken for each substance dissolved in CH_3OH (N/10000), with iron arc as a light source. They were reproduced in another paper.[24]

(24) Kuroda and Wada, *Proc. Imp. Acad. Japan*, **11** (1935), 189.

III. Nasunin, Colouring Matter of "Nasu".[25]

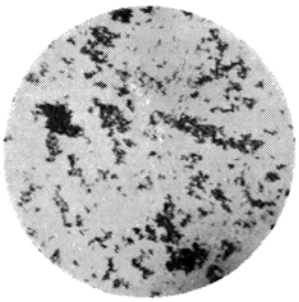
The vegetable fruit of egg-plant (*Solanum Melongena L. var erculentum Ness*) is not only a popular and useful vegetable food material from summer to autumn Japan, but the colour of its epidermis is noted for the beautiful dark purple which is taken as a standard, in dyeing from the olden time, being called as "Nasu blue." As cares are taken even in cooking to preserve this colour unaffected, the authors have been attracted to the study of the pigment. As the colour was extremely delicate and changeable it called for absolutely tedious methods with a special precautions to isolate the pigment. After preliminary tests, as the bright colouring matter of "Nasu" was shown to belong to an anthocyanin, the treatments were carried out by using the lead salt method accordingly.

The isolation of "Nasu" pigment in a crystalline state, was extraordinarily difficult; however, after several experiments, a new crystalline glucoside which was named nasunin by the authors was successfully obtained as picrate (Fig. 6), and finally as chloride.



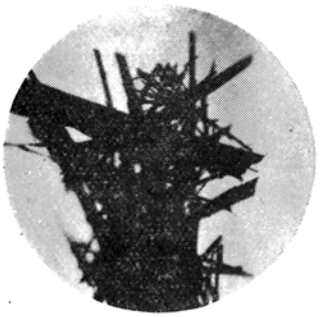
Nasunin Picrate from "Nasu"

Fig. 6.



Nasunin A chloride from "Nasu"

Fig. 7.



Nasunidin chloride from "Nasu"

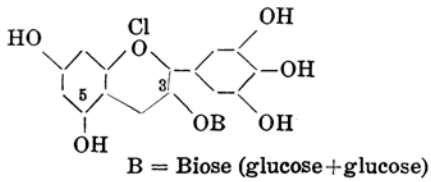
Fig. 8.

Then, as the results of careful investigations, nasunin was confirmed to be a compound of *p*-coumaric acid (1 mol) and a new glucoside which was named nasunin A by the authors was isolated as chloride, and was shown to be 3-bio-di-glucoside of delphinidin. The procedures are as follows: (1) Analytical results of nasunin chloride and picrate agreed with $C_{36}H_{37}O_{19}Cl+10H_2O$, $C_{42}H_{9}O_{26}N_3+4H_2O$, respectively. (2) The one component *p*-coumaric acid in nasunin was isolated quantitatively, when nasunin chloride was carefully treated with cold dilute aqueous NaOH in H_2. From the same glucoside, the above acidic component was also obtained by action of fused alkali. (3) On

(25) "Nasu" is the Japanese name of egg-plant.

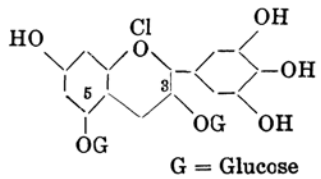
partial hydrolysis of nasunin chloride with cold 20% aqueous HCl, nasunin A as the another component in nasunin was isolated in crystalline state as chloride (Fig. 7). The analytical result ($C_{27}H_{31}O_{17}Cl+3H_2O$) was in agreement with diglucoside of delphinidin chloride. Decomposition of the glucoside with boiling HCl gave 1 mol of delphinidin chloride (Fig. 8) and 2 mols of glucose quantitatively.

Now one of the positions of glucose in nasunin was proved to be "3" by applying Karrer's[15] perhydrol method to nasunin chloride. Accordingly, the two formulae as di-glucoside are possible as indicated below:



B = Biose (glucose+glucose)

III Nasunin A chloride



G = Glucose

IV Awobanin chloride

IV was obtained from "Awobana".[26] Although nasunin A in question corresponds well to the sample of IV from "Awobana" in almost every respect (absorption spectrum, appearance, and the results of analyses), there are some disagreements as follows: (1) Notwithstanding the fact that the glucoside of "Awobana" is confirmed to be 3,5-diglucoside by Karrer's perhydrol method, in the case of egg-plant, there are some experimental evidences against the glucose linkage "5". (2) Comparing the two substances in reactions in buffered solutions at 15 grades of *p*H[14], "Awobana" gives slightly more pink colouration than the other. (3) Solubilities of the two, in CH_3OH, H_2O, and HCl are also somewhat different, "Awobana" being less soluble. The decomposition point was a little different from that of "Awobana".

With such experimental facts in consideration, of the two proposed formulæ, III seems more probable.

Now, as to the manner of combination of one mol of *p*-coumaric acid, it may be linked as an ester to the biose at "3". A favourable example of such a view is violanin[27] (VI). Considering this example with the previously mentioned nasunin A which seemed to be 3-bioside of delphinidin, for the formula of nasunin chloride, V may be much more probable. Further studies will be made to confirm the present consideration.

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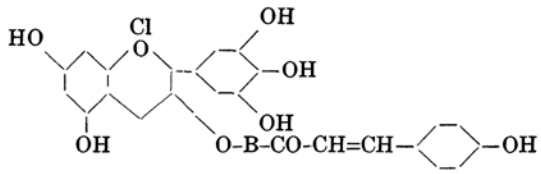
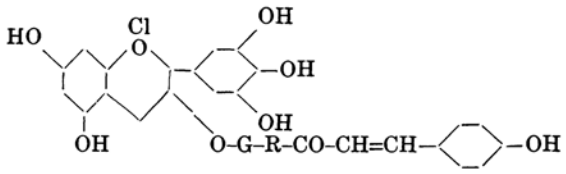
(26) See the foregoing paper.

(27) Karrer and Meuron, *Helv. Chim. Acta*, **16** (1933), 292.



V Nasunin chloride

B = Biose (glucose+glucose)



VI Violanin chloride

G = Glucose R = Rhamnose

Experimental.

Isolation of "Nasu" Pigment. The skins which were quickly peeled off from the fresh fruits were immediately immersed in 3% CH_3OH-HCl. Then in a few hours, the solution which turned bright red was taken out, and to this, a methyl alcohol solution of lead acetate was added drop by drop; the resulting white precipitate, lead chloride, was previously removed, then the indigo blue salt of the pigment was sucked, washed well with cold water, dried on a tile quickly, and powdered (150 fruits yielded 15 g. of the lead compound). The lead salt thus obtained was converted into a chloride solution by treating again with 2% CH_3OH-HCl. From the solution, the chloride of the colouring matter was precipitated upon the addition of several times its volume of ether, as the reddish purple and crystalline powder (150 fruits yielded about 1.5 g. of the chloride).

From this material dissolved in methyl alcohol, the colouring matter was precipitated by the addition of ether, this process being repeated for the purification.

Nasunin Picrate. To the solution of amorphous nasunin chloride (5 g.) in hot 1% HCl, powdered picric acid (3 g.) was added, warmed on the water bath for a few minutes, and left at room temperature for few days, then a reddish picrate gradually crystallized out (5 g.). The picrate thus obtained was dried, powdered, triturated with ether carefully to remove picric acid, and then was recrystallized twice from hot aqueous solution of picric acid saturated in cold (yield 2.5 g.). The picrate separated in beautiful red slender needles (copper-red mass in dry state, Fig. 6); for analysis, the specimen was purified several times (Found: C, 46.85; H, 4.82. Calc. for $C_{42}H_{39}O_{26}N_3+4H_2O$: C, 46.96; H, 4.37%). It agreed with picrate of delphinidin diglucoside combined with 1 mol of *p*-coumaric acid.

Nasunin Chloride. To the solution of the above pure picrate (2 g.) in 2% CH_3OH-HCl, ether was added, then reddish purple coloured nasunin chloride was isolated. The quantity of picric acid obtained from the above mother liquor (mixture of CH_3OH and ether) was determined (Found: picric acid, 0.38 g. or 38%. Calc. for $C_{42}H_{39}O_{26}N_3+4H_2O$: 0.38 g. or 38%).

The chloride was purified several times from CH_3OH–HCl or dilute aqueous HCl; the appearance of the chloride was similar to the picrate. (Found: C, 44.03; H, 5.7; Cl, 3.33. Calc. for $C_{36}H_{37}O_{19}Cl+10H_2O$: C, 43.73; H, 5.7; Cl, 3.31%.)

The Decomposition of Nasunin Chloride with Cold Alkali. Nasunin chloride (0.1 g.) dissolved in water (2.5 c.c.) was treated with cold 16% aqueous NaOH (5 c.c.) and kept for $2^1/_2$ hours at room temperature in H_2. The product was acidified, extracted with ether. From the ethereal solution, *p*-coumaric acid (white crystal, m.p. 207°) was isolated quantitatively (Found: 0.015 g. or 15%. Calc. for $C_{36}H_{37}O_{19}Cl+10H_2O$: 16.6%. Found: C, 65.61; H, 4.64. Calc. for $C_9H_8O_3$: C, 65.85; H, 4.87%).

Alkali Fusion of Nasunin Chloride. Nasunin chloride (1 g.) was fused with KOH (2 g.) (compare, fusion of nasunidin). When the ethereal extract of the product was triturated with minimum quantity of water, *p*-coumaric acid was obtained from the water-insoluble part, purified from hot water; the acid was obtained in white needles, m.p. 208° (Found: C, 65.54; H, 5.15. Calc. for $C_9H_8O_3$: C, 65.85; H, 4.88%). Other phenolic products obtained from delphinidin were also found.

Decomposition of Nasunin Chloride with Acid. When nasunin chloride (0.1275 g.) was boiled with 20% aqueous HCl for three minutes, and was cooled, delphinidin chloride separated out in an amorphous state as in the acid hydrolysis of nasunin A. The filtrate obtained free from the above aglucone was extracted with ether to remove *p*-coumaric acid, and the sugar content in the acid solution was determined by Pavy's method (Found: sugar, 0.05083 g. or 39.47. Calc. for $C_{36}H_{37}O_{19}Cl+10H_2O$: 36.44%).

Nasunin A Chloride. The solution of nasunin chloride (1 g.) dissolved in cold 20% aqueous HCl was left for a few days at room temperature. A bluish purple precipitate gradually separated (0.3 g.), which was washed with 0.5% HCl, and dried; this was dissolved in a minimum quantity of 0.5% hot aqueous HCl and an equal volume of 3% CH_3OH–HCl was added. On leaving for some time, nasunin A chloride crystallized out in golden lustrous fine needles (Fig. 7). It was recrystallized twice (decomposition point 197°) for analysis (Found: C, 44.90; H, 5.30; Cl, 4.45. Calc for $C_{27}H_{31}O_{17}Cl+3H_2O$: C, 45.25; H, 5.17; Cl, 4.6%).

Hydrolysis of Nasunin A Chloride with Acid. To the purified nasunin A chloride (0.1 g.) dissolved in hot dilute aqueous HCl an equal volume of conc. HCl was added and the mixture boiled for two minutes. On cooling in contact with ice, an aglucone separated out as an apparently amorphous, nearly black precipitate. It was filtered, washed well with 20% HCl, and dried. The amorphous product was dissolved in hot dry CH_3OH, touched with dry HCl, then an aglucone crystallized out in plates with green metallic lustre (Fig. 8) and was dried in the air. It showed all the qualitative reactions of delphinidin (Found: C, 46.09; H, 4.57. Calc. for delphinidin chloride $C_{15}H_{11}O_7+3H_2O$: C, 45.91; H, 4.57%).

Micro-Zeisel estimation proved the absence of methoxyl groups in the aglucone. A delphinidin chloride hydrate of this composition has not previously been isolated, but it was already observed that the quantity of water of crystallization in such a substance depends on the conditions of crystallization.

Sugar. (1) *Estimation.* The above filtrate removed from the aglucone was neutralized with alkali, dried on the water bath, then the sugar was determined by Pavy's method as previously mentioned (Found: sugar, 0.0502 g. or 50.2%. Calc. for $C_{27}H_{31}O_{17}Cl+3H_2O$: 50.28%).

(2) *Osazone.* Glucosazone from the resulting acid solution obtained upon decomposition of nasunin A chloride was formed in the similar method as in the case of kuromamin chloride.

(3) *The position of sugar.* Nasunin A chloride (0.3 g.) was treated with 30% H_2O_2 as in the case of kuromamin, and glucosazone from position "3" was obtained in a considerable quantity, m.p. 205°. The filtrate from the above osazone was treated as in the case of shisonin, however, osazone from the position "5" was not found distinctly.

Alkali Fusion of Nasunidin (Aglucone of Nasunin). Nasunidin chloride (0.8 g.) was fused with NaOH (2.5 g.) and a small quantity of water at 203°. After cooling, the product was acidified with HCl, extracted with ether, and was evaporated. From the ethereal residue dissolved in water, a yellowish brown lead salt was precipitated by addition of lead acetate. The lead salt was filtered (filtrate A), washed with water, decomposed with dilute H_2SO_4, and extracted with ether. The ethereal residue dissolved in small quantity of CH_3OH was methylated with $(CH_3)_2SO_4$ and 50% aqueous KOH in H_2; the reaction product was heated on the water bath to decompose the ester and to evaporate CH_3OH, then acidified, extracted with ether, evaporated, and the residue was again extracted with hot benzene. The benzene-soluble part was crystallized from hot water, then gallic acid trimethyl ether (m.p. 165°, both alone and after admixture with the authentic specimen) was isolated. From filtrate A treated with dilute H_2SO_4, phloroglucinol was isolated (m.p. 208°, both alone and after admixture with the authentic specimen). No *p*-coumaric acid was found in this decomposition.

Direct Comparison of Nasunin A and Awobanin A Chloride. Both specimens were recrystallized in the same way with a different result as follows: The compound was dissolved in a minimum quantity of 0.5% hot aqueous HCl and an equal volume of 3% C_2H_5OH–HCl was added. After cooling, nasunin A chloride crystallized out in fine needles with golden lustre, while the other separated in small hexagonal plates with golden lustre.

The colour reactions of the above two specimens in buffered solutions were compared. The colours given by the two pigments were almost identical except that nasunin A became blue at a lower *p*H than awobanin A. (1) Rose-pink, fading rapidly; (3) (5) bluer, fading rapidly; (4) bluish-pink, fading to colourless rapidly; (9) (10) blue-violet; (11) violet; (13) violet-blue; (15) deep blue.

Both specimens were very sparingly soluble (or insoluble) in cold water, alcohol, and dilute aqueous HCl but readily in hot dilute aqueous HCl. All the ordinary reactions, the colours with Na_2CO_3 (a bright pure blue) and alcoholic $FeCl_3$ (violet-blue) were observed identical.

In buffered solution the colour reactions of both specimens, namely the aglucones from nasunin and awobanin chlorides, gave quite identical results agreeing with those of delphinidin chloride in the literature. (1) red, pink in 80 seconds; (2) violet red. colourless in 30 seconds; (3) permanganate, colourless in 30 seconds; (4) similar to (3); (5) violet; (6) deep violet red; (9) blue violet; (10) similar to (9); (11) blue violet; (13) blue violet dichroic, pale yellow in $1^1/_2$ hours; (14) indigo blue, pale yellowish gray in $1^1/_2$ hours; (15) (16) similar to (14).

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