776. *Isolation of a New Plant-growth Hormone, 3-Indolylacetonitrile.*

By H. B. Henbest, E. R. H. Jones, and G. F. Smith.

3-Indolylacetonitrile, appreciably more active than the corresponding acid in *Avena* cell-elongation tests, is present in about 0·0002% in cabbage, and has been isolated (crystalline) by employing partition and chromatographic techniques. This is the first isolation of a growth hormone from the leaves and stems of actively growing higher plants. A preliminary account of this work has already been published (*Nature*, 1952, **169**, 485).

Naturally occurring plant-growth hormones, which have been isolated in a pure form, are auxin-a and auxin-b from urine, maize oil, and malt (Kögl, Erxleben, and Haagen-Smit, *Z. physiol. Chem.*, 1933, **214**, 241; 1934, **225**, 215), and 3-indolylacetic acid (heteroauxin) from urine (*idem*, *ibid.*, 1934, **228**, 90; Haagen-Smit, Leech, and Bergren, *Amer. J. Bot.*, 1942, **29**, 500), yeast (Kögl and Kostermans, *Z. physiol. Chem.*, 1934, **228**, 113), unripe maize kernels (Haagen-Smit, Dandliker, Wittwer, and Murneek, *Amer. J. Bot.*, 1946, **33**, 118), and dormant mature maize after alkaline hydrolysis (Berger and Avery, *ibid.*, 1944, **31**, 199). The great importance of auxins-a and -b makes a repetition of their isolation and a further investigation of their properties very desirable, expecially in view of the reports (Kögl, *Naturwiss.*, 1942, **30**, 395; Haagen-Smit *et al.*, *loc. cit.*, 1942) of failure of more recent attempts to repeat the isolation of these two compounds.

Researches on acetylenic compounds in these laboratories led to the realisation of a series of reactions making possible a synthesis of β-keto-δ-lactones, and therefore of auxin-b analogues. The *cyclo*pentenyl analogue of auxin-b lactone was synthesised (Brown, Henbest, and Jones, *J.*, 1950, 3634); it was also independently synthesised by a different route by Kögl and de Bruin (*Rec. Trav. chim.*, 1950, **69**, 729).

From a study of the chemical properties of the *cyclo*pentenyl analogue (in particular its stability in the lactone form), we concluded that the open-chain side-chain structure proposed by Kögl and his collaborators for auxin-b could not be correct. Since the synthesis of the proposed structure presents stereochemical problems of some magnitude, it was decided to reinvestigate the isolation of natural plant-growth hormones and, if auxin-b was rediscovered, to examine further the chemical evidence for its structure.

Our investigations have been made in collaboration with Dr. Joyce A. Bentley of the Botany Department of this University, who informed us that ethereal extracts of brussels sprouts were very rich in plant-growth hormone, as measured by the straight-growth *Avena* test (Bentley, *J. Exp. Bot.*, 1950, **1**, 201). It was soon found that nearly all the activity in this plant material resided in the ether-soluble *neutral* fraction, and that the corresponding acid fraction, which might have been expected to contain 3-indolylacetic acid and auxins-a and -b, exhibited almost negligible activity.*

* Unless stated otherwise, " activity " refers to the *Avena* straight-growth test.

It was therefore decided to concentrate upon the isolation of the hormone present in the neutral portion, and the following technique was adopted in order to reduce the risk of enzymic formation of artefacts during the first extraction stage. The plant material was frozen in solid carbon dioxide as soon as possible after harvesting, crushed, and extracted at 0—2° with ether or carbon tetrachloride. The hormone in such extracts was concentrated by partition between 90% aqueous methanol and light petroleum, all the activity passing into the aqueous-methanol layer. Further concentration was achieved by chromatography on alumina; dry ether eluted waxy materials first, followed by active fractions, and the green pigments remained strongly absorbed on the column. This concentrate was used in a number of experiments (Table) designed to give some information on the chemical nature of the hormone.

Experiment	Activity of: neutral hormone	3-indolylacetic acid
1. Distillation at 90°/10^{-5} mm.	Retained	—
2. Water at 100°/N_2/1 hr.	Retained	Retained
3. N-NaOH at 100°/N_2/1 hr.	20% retained in acid fraction	Retained
4. 10% KOH–MeOH at 20°/45 hr.	Retained	—
5. N-H_2SO_4 at 100°/air/1 hr.	Retained	Lost
6. N-H_2SO_4 at 100°/N_2/30 min.	Retained	50% retained
7. Satd. Aq. $NaHSO_3$	Retained in non-carbonyl fraction	—
8. Semicarbazide acetate at 20°/24 hr.	Retained	—
9. $LiAlH_4$–Et_2O	Lost	—
10. Zn–Cu couple in aq. HCl at 20°/4 hr.	Lost	Lost
11. 0·02N-HNO_2 (aq.) at 20°/1 hr.	Retained	Lost
12. 0·1% H_2O_2 (aq.) at 20°/24 hr.	Lost	Lost

It soon became evident that the hormone was an indole compound, for the ultra-violet light absorption of concentrates was similar to that of 3-indolylacetic acid. The stability of the hormone (cf. experiments 4, 5, 6), as well as its high biological activity, showed that it could not be 3-indolylacetaldehyde (Brown, Henbest, and Jones, *J.*, 1952, 3172).

Further chromatography of the neutral hormone concentrates from brussels sprouts yielded colourless viscous liquid fractions exhibiting activities much greater than that of 3-indolylacetic acid. On the assumption that the molecular weight was of the order of 200, analysis of the most active fraction showed that the molecule probably contained two nitrogen atoms.

At this stage it was found that cabbage contains an equally high concentration of hormone which did not appear to differ from that present in brussels sprouts. It was therefore used in subsequent work since it is a more convenient raw material for large-scale extractions. Further work (with Dr. S. Dunstan) has shown that extracts from other members of the Cruciferae family (radish, cauliflower, swede, and turnip) also yield neutral fractions of high activity. The hormone content of cabbage and closely related plants had been investigated previously (cf. Avery, Berger, and White, *Amer. J. Bot.*, 1945, **32**, 188; Linser, *Planta*, 1940, **31**, 49; 1951, **39**, 377). Linser demonstrated the presence of two hormones in broccoli, one of which was only weakly absorbed on alumina from ethanol and is probably identical with the neutral hormone encountered in the present investigation.

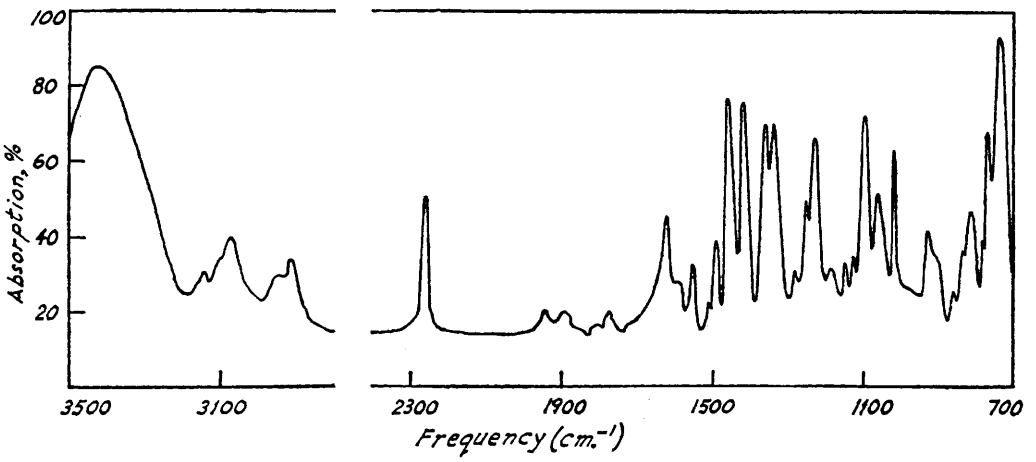
After several preliminary extractions, 500 kg. of cabbage were harvested, immediately frozen and powdered, and extracted with carbon tetrachloride at 0—2°. We are much indebted to Drs. W. A. Sexton and T. P. Metcalfe, of Imperial Chemical Industries Limited, for assistance in this large-scale extraction. The subsequent isolation is described in detail in the experimental section. A crystalline picrate was eventually obtained, from which the neutral hormone could be recovered quantitatively by chromatography on gypsum with ether as the eluting solvent. The nature of the substituent group present was suggested by the infra-red spectrum (see Figure) which showed a marked peak at 2255 $cm.^{-1}$ indicative of a nitrile grouping (Kiston and Griffith, *Analyt. Chem.*, 1952, **24**, 334) and, in addition, a number of peaks characteristic of 3-substituted indoles (Brown *et al.*, *loc. cit.*, 1952). Subsequent work revealed the neutral hormone to be 3-indolylacetonitrile. In view of the small quantities of pure natural material available, normal

degradative experiments were avoided. Once, however, infra-red and ultra-violet spectra and mixed melting-point determinations with the picrate had practically established the identity of the natural material with the synthetic nitrile, additional chemical evidence was obtained by reduction of the natural material to tryptamine with lithium aluminium hydride and conversion into 3-indolylacetic acid by alkaline hydrolysis. The synthetic nitrile was eventually obtained crystalline, m. p. 36—36·5°; hitherto it had only been obtained as an oil. Seeding with this product induced the " natural " nitrile to crystallise (m. p. 34·5—36°).

It is difficult to estimate accurately the amount of nitrile present in the original plant material, but 915 mg. of very active product were isolated from 500 kg. of cabbage. This probably contained 400—700 mg. of the nitrile, the variation in the biological assays making a more exact estimate impossible.

There are not many reports of the isolation of nitriles from plants. Phenylacetonitrile, a close analogue of indolylacetonitrile, was isolated from several species in relatively high

3-*Indolylacetonitrile—infra-red absorption spectrum of melted film.*



yield (cf. Hofmann, *Ber.*, 1874, **7**, 518, 520, 1293). Subsequent work demonstrated the phenylacetonitrile to be an artefact produced (together with the *iso*thiocyanate) by the decomposition of glycosidic compounds (*e.g.*, glyconasturtin) during the steam-distillation (Gadamer, *Ber.*, 1899, **32**, 2335; Bottomley and White, *Roy. Austral. Chem. Inst. J. & Proc.*, 1950, **17**, 31). Another nitrile that has been isolated (from black mustard seed) is allyl cyanide, also structurally related to 3-indolylacetonitrile, and again it was an artefact produced by the decomposition of a precursor (sinigrin) (Will and Körner, *Annalen*, 1863, **125**, 277; Hérissey and Boivin, *Bull. Soc. Chim. biol.*, 1927, **9**, 947). We believe that the precautions taken in the present work to avoid enzymic or chemical changes during isolation rule out any possibility of indolylacetonitrile being similarly an artefact.

The isolation of ethyl 3-indolylacetate by ethanol-extraction of immature maize kernels in the relatively high yield of 50 mg. from 10 kg. of fresh plant material (Redemann, Wittwer, and Sell, *Arch. Biochem. Biophys.*, 1951, **32**, 80) is of great interest. The possibility of its being an artefact produced during the ethanol-extraction ought, however, to be investigated. Experiments carried out in this laboratory indicate that the nitrile does not react with ethanol *in vitro* either in neutral solution or in the presence of small concentrations of mineral acid over periods of up to a week. Conversion of the nitrile into the ethyl ester thus appears to be ruled out—however, enzymic catalysis of this conversion may take place in the presence of plant tissue.

The resistance of 3-indolylacetonitrile to hot dilute mineral acid in the presence or absence of oxygen (cf. expts. 5 and 6 in the Table) is noteworthy, in view of the general statements that are made about the instability of indole compounds under such conditions.

In this, the nitrile differs markedly from 3-indolylacetic acid, for the latter is unstable to mineral acid, especially when oxygen is also present. Dilute aqueous solutions of the nitrile have also been observed to be very stable, again in contrast with aqueous solutions of the acid, which slowly decompose.

A summary of the biological properties of 3-indolylacetonitrile has been given in our preliminary article (*Nature, loc. cit.*); more detailed treatments are given by Bentley and Housley (*J. Expt. Bot.*, 1952, **3**, 393) and by Bentley and Bickle (*ibid*, p. 406). By suitable paper chromatographic methods, 3-indolylacetonitrile is readily separated from 3-indolylacetic acid, and the use of such methods has suggested that the nitrile is present in small amounts in plants other than those of the Cruciferae family (Bennet-Clark, Tambiah, and Kefford, *Nature*, 1952, **169**, 452; Bennet-Clark and Kefford, *ibid.*, 1953, **171**, 645; Luckwill, *ibid.*, 1952, **167**, 375; von Denffer, Behrens, and Fischer, *Naturwiss.*, 1952, **39**, 550).

Experimental

All the work except where stated was carried out at *ca.* −5° in a cold room. Light petroleum (b. p. 60—80°) was purified by shaking it with concentrated sulphuric acid. Methanol was freed from formaldehyde by refluxing it over freshly precipitated silver oxide. A mixture of one volume of water and nine of methanol was used in the partitions. Ether was dried over sodium for not less than 4 weeks and distilled just before use. The absorbent was prepared by crushing pure mineral gypsum, passing it through a 90-mesh sieve, and heating it at 150° for 5 hr. All evaporations were carried out *under reduced pressure* (water-pump), the residues never being heated above 30° to avoid decomposition risks. M. p.s were determined on a Kofler block. In this section, IAA refers to 3-indolylacetic acid, and IAN to 3-indolylacetonitrile.

Isolation of 3-Indolylacetonitrile.—After much preliminary work with similar batches of brussels sprouts and cabbage, the following procedure was used for the main extraction.

Cabbages (var. 1st Early Market) were picked (end of June 1951), the outer green leaves discarded, and the centres (approx. 500 kg.) immediately packed in drums with crushed solid carbon dioxide contained in small paper bags (in order to obviate contamination with the small amounts of mineral oil in the carbon dioxide). After 3 hr. the frozen material was crushed in a large, pre-cooled, stone, roller mill. Partial thawing occurred towards the end of the crushing process but the temperature of the material was still well below 0° when it was introduced into a large vat containing pre-cooled carbon tetrachloride (pure commercial grade, *ca.* 750 l.). The mixture was pummelled and stirred at between −2° and 2° (10 hr.). The filtered carbon tetrachloride extract (*ca.* 650 l.; activity equiv. to 9—12·5 g. of IAA) was evaporated to 25 l. (≡ 5 g. of IAA), and then to 1·5 l. (≡ 3·5 g. of IAA : it must be noted, however, that at a later stage in the isolation, the total activity was equivalent to 4·8—6·0 g. of IAA).

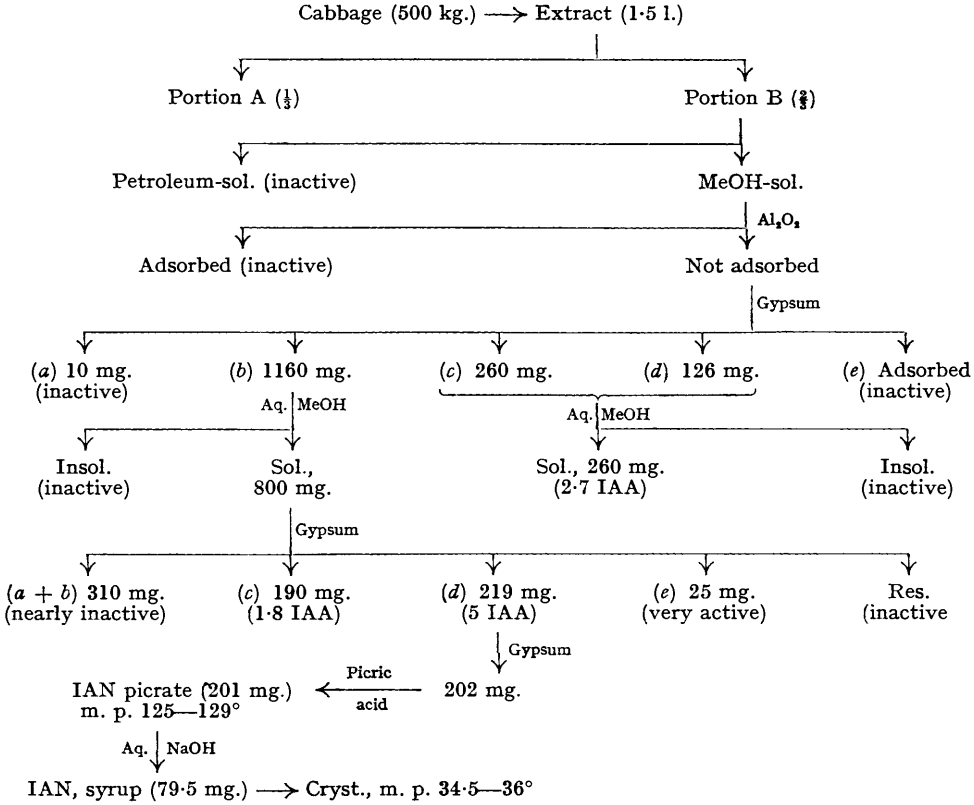
The dark viscous concentrate was worked up in two portions : A, approx. one-third (IAN picrate isolated), and B (two-thirds) from which pure IAN was obtained.

Portion A. This was divided into two portions for partition. Each half was dissolved in light petroleum (1 l.) and the dark solution, which contained much suspended material, was extracted with 90% methanol (1 l.). Of the three layers which formed, the lower clear dark methanol layer was run off; the next, an emulsion of light petroleum and methanol containing the insoluble material, was filtered, the filtrate separating into two layers of which the lower was added to the main methanol extract. The combined methanol solutions were washed with light petroleum (250 c.c.) and evaporated. The (wet) syrup was thoroughly extracted with ether, and the extracts were dried (Na_2SO_4). The two portions (I and II) were chromatographed separately on alumina (P. Spence, grade H) : I (15 × 6·5 cm.) (*a*) (ether 750 c.c.), 75 mg.; (*b*) (900 c.c. of ether + 2½% of methanol) 120 mg.; (*c*) (350 c.c. of ether + 2½% of methanol), 590 mg.; (*d*) (180 c.c. of ether + 2½% of methanol), 62 mg.; (*e*) (methanol 500 c.c.), 210 mg. : II (13 × 6·5 cm.) (*a*) (ether 400 c.c.), 50 mg.; (*b*) (500 c.c. of ether + 2% of methanol), 205 mg.; (*c*) (130 c.c. of ether + 2% of methanol), 695 mg.; (*d*) (270 c.c. of ether + 2% of methanol), 130 mg. The majority of the activity was to be found in (the 2) fractions (*c*), which were therefore combined and chromatographed on activated gypsum (12 × 5 cm.), with ether as eluting solvent : (i) (140 c.c.) 29 mg. (inactive); (ii) (40 c.c.) 222 mg. (≡ 1675 mg. of IAA); (iii) (75 c.c.) 139 mg. (≡ 630 mg. of IAA); (iv) (40 c.c.) 46 mg. (≡ 160 mg. of IAA); (v) (190 c.c.) 106 mg. (≡ 190 mg. of IAA). Fraction (iii) was dissolved in methanol (10 c.c.) and gradually treated with water (10 c.c.). The precipitated material (inactive) was filtered off and the filtrate was

freed from methanol by warming under reduced pressure and then extracted with ether. This yielded a syrup (112 mg.) which in methanol was treated with picric acid. The dark solution deposited orange prisms (33 mg.), m. p. 120—129·5°, on cooling. The mother-liquor was evaporated and the residue crystallised from benzene (3 c.c.), to give sheaves of orange needles (59 mg.), m. p. 122—129°. The benzene mother-liquor was evaporated and the residue introduced on to gypsum (9 × 2·7 cm.) from ether. The picric acid was adsorbed strongly and the eluate yielded a pale brown syrup (57 mg.), which in benzene (1 c.c.) was treated with picric acid (65 mg.), to yield a picrate (75 mg.), m. p. 122—129°. The mother-liquor on being treated with light petroleum (2 c.c.) yielded a dark brown crystalline podwer (17 mg.), m. p. 121—128°, sintering at 117°. (The total weight of impure picrate was 184 mg., which corresponds to 75 mg. of IAN.)

Fraction (ii) in methanol was treated slowly with water (10 c.c.). The gum (inactive) was separated and the filtrate evaporated to a yellow syrup (124 mg.; ≡ 580 mg. of IAA). This was dissolved in benzene (3·5 c.c.), and picric acid (145 mg.) added: the very dark solution yielded impure picrate (117 mg.), m. p. 116—129°; a second crop (23 mg.) obtained by addition of light petroleum (2 c.c.) had m. p. 114—125° (total weight of impure picrate: 140 mg. corresponding to 57 mg. of IAN). Crystallisation from benzene gave the pure picrate, m. p. 125—129° (Found: C, 49·6; H, 3·0; N, 17·55. Calc. for $C_{10}H_8N_2,C_6H_3O_7N_3$: C, 49·8; H, 2·9; N, 18·2%). Ultra-violet spectrum (in ethanol) of material recovered from picrate: Max. 2190, 2710, 2790, and 2890 Å; ε = 33,700, 6200, 6250, and 5100 respectively. Majima and Kotake (*Ber.*, 1925, **58**, 2042) give m. p. 127—128° for the picrate.

Summary of extraction.



Portion B. The partition was carried out as described for portion A. The ethereal solutions were combined and passed on to alumina (P. Spence, grade H: 10 × 5 cm.). Elution with 2½% of methanol in ether (1 l.) gave material (2—2·5 g.) which was dissolved in ether and chromatographed on gypsum (ether elution). Four fractions were collected: (*a*) (170 c.c.) 10 mg. (inactive); (*b*) (110 c.c.) 1·16 g. (≡ 450 mg. of IAA); (*c*) (150 c.c.) 260 mg. (≡ 1460 mg. of IAA);

(*d*) (180 c.c.) 126 mg. (≡ 180 mg. of IAA). Fractions (*c*) and (*d*) were combined and dissolved in methanol (12 c.c.), and water (8 c.c.) was gradually added. The partly crystalline precipitate (inactive) was filtered off and the filtrate evaporated to a syrup (260 mg.; ≡ 700 mg. of IAA). Fraction (*b*) was dissolved in methanol (75 c.c.) and treated with water (50 c.c.); the mixture was left for 30 min. at −5°, and then filtered. The resinous precipitate (243 mg.) had low activity (12·5 mg. of IAA); the filtrate was evaporated and a pale reddish-brown oil was obtained (803 mg., ≡ 2000 mg. of IAA). This material was rechromatographed on gypsum (21 × 2·7 cm.) with ether as eluting solvent. Five fractions were collected: (i) (50 c.c.) 26 mg.; (ii) (12 c.c.) 283 mg. (i + ii ≡ 19 mg. of IAA); (iii) (22 c.c.) 190 mg. (≡ 340 mg. of IAA); (iv) (250 c.c.) 219 mg. (≡ 1000 mg. of IAA); (v) (200 c.c.) 25 mg. (≡ 290 mg. of IAA). Fraction (iv), which had darkened on being kept even at −5°, was passed through gypsum (10 g.) with ether. The yellow syrup thus obtained (201 mg.) was dissolved in ethanol (2·5 c.c.) and treated with picric acid (250 mg.). The whole was warmed until a clear solution was obtained and the crystals which separated on cooling had m. p. 124—130° (307 mg., corresponding to 125 mg. of IAN). It was recrystallised from ethanol (2·5 c.c.), to yield a product (249 mg.), m. p. 125—129°. This m. p. was unchanged by a further crystallisation from ethanol (2 c.c.) which yielded beautiful orange prisms (201 mg.). This material was decomposed as rapidly as possible between aqueous sodium hydroxide and ether, and the ethereal layer after having been twice washed with water, was dried and passed through gypsum (2 × 1·5 cm.) by means of ether. A red ring formed at the top of the column and the eluate yielded a colourless syrup (79·5 mg.), which was distilled in a short-path apparatus at 10^{-5} mm. and a bath-temperature of 90° on to a surface cooled by acetone–solid carbon dioxide (Found: C, 76·4; H, 5·15. Calc. for $C_{10}H_8N_2$: C, 76·9; H, 5·15%).

The remainder of the distilled natural product was dissolved in ether (1 c.c.) at −5°, light petroleum was added to incipient turbidity, and the solution seeded with synthetic IAN. Light petroleum was added at intervals until altogether 5 c.c. had been added. The colourless product (25 mg.) which crystallised melted between 35° and 80°, most being liquid at 36°. The mother-liquor yielded colourless crystals (13·5 mg.), m. p. 29—35·5°, mostly from 33°. The infra-red spectrum of the latter material (after short-path distillation at 10^{-5} mm.) was identical with that of synthetic 3-indolylacetonitrile. The compound was recovered from the infra-red examination, and crystallised from ether–light petroleum, to afford crystals, m. p. 34·5—36°, undepressed on admixture with synthetic nitrile.

Synthetic 3-Indolylacetonitrile.—Gramine (33·5 g.) in methanol (500 c.c.) was treated with potassium cyanide (25 g.) dissolved in water (50 c.c.). Methyl iodide (65 g.) was then added slowly, at <35°. The solution was kept at 20° overnight, most of the methanol was removed by evaporation under reduced pressure, and the nitrile was extracted with ether. The ethereal layer was washed with water, dilute sulphuric acid (some viscous red material separated), and sodium hydrogen carbonate solution, and dried (Na_2SO_4). The nitrile was distilled, to give 24 g., b. p. 157°/0·05 mm. It solidified at 0°, and crystallisation from ether–light petroleum gave prisms, m. p. 36—36·5° (Found: C, 76·6; H, 5·1%). Ultra-violet absorption (in 95% EtOH): Max., 2190, 2730, 2790, and 2880 Å; ε = 35,300, 6000, 6100, and 4800 respectively: Min., 2410, 2750, and 2860 Å; ε = 1700, 5900, and 4600 respectively. Infra-red spectrum (liquid film, cf. Figure): strong peaks at 3430 (NH), 2255 (CN), 1620, 1461, 1425, 1360, 1340, 1230, 1095, 1010, 770, and 740 $cm.^{-1}$.

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