An artefact in the chromatographic analysis of 2,4-dinitrophenylhydrazones of keto acids

The present study arose from a chance observation during our work on the production of keto acids by incubated brain homogenates from normal and ethanol intoxicated rats (Fig. 1). Because an unknown substance *x*, which separated from the 2,4-dinitrophenylhydrazone of α-ketoglutaric acid, seemed to be related to the effects of ethanol, we decided to collect it in amounts sufficient for identification. The complete separation of substance *x* was achieved when the lid of the chromatographic vessel was left slightly ajar toward the end of the run for the evaporation of the solvent. The unknown substance was not detected in the preparations from other sources (urine, blood, liver and muscle). Even in different experiments with brain the

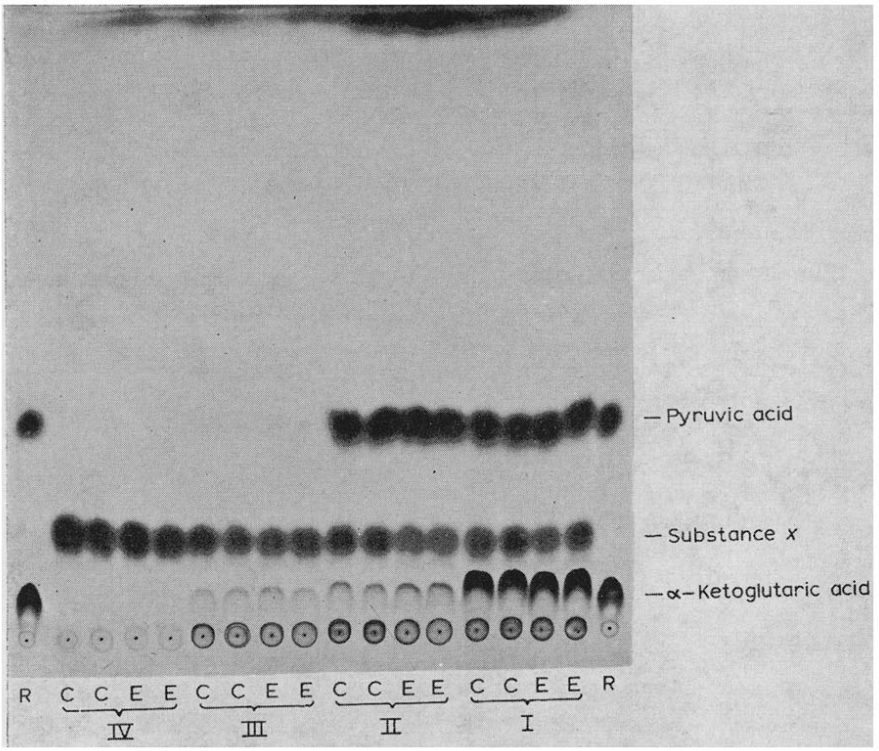

Fig. 1. Thin-layer chromatogram of 2,4-dinitrophenylhydrazones of acid carbonyl compounds of brain homogenates, incubated under various conditions (photographed in U.V. light). E = samples from ethanol intoxicated rats; C = samples from control rats; R = references; I = homogenates incubated for 60 min at + 37° in the presence of glucose and glutamic acid; II = in the presence of glucose; III = without glucose or glutamic acid; IV = not incubated at all. Otherwise the incubation medium was that of DiPietro and Weinhouse[1] with slight modifications. The preparation and isolation of the acid hydrazones were based on the work by Seligson and Shapiro[3]. The plates were coated with "Silica gel G according to Stahl" (E. Merck AG, Darmstadt, No. 7731). The powder (30 g) was suspended in 60 ml of propionic acid–water mixture (1:2, v/v), spread with the applicator to a 0.2 mm nominal thickness, and heated at + 110° for 30 min. The solvent system consisted of petroleum ether–ethyl formate–propionic acid (65 ml:35 ml:0.1 moles)[2]. The run lasted at room temperature for 3–4 h. When the front had reached the top of the plate, the lid was left slightly ajar.

amount of substance x was variable, sometimes almost negligible, and it was unstable at room temperature.

The spot containing the unknown substance x was scraped off from several chromatograms, and eluted with water, ethanol or dioxane–phosphate buffer[2] for rechromatography.

The unknown substance was rather pale yellow, and it was best observed in ultraviolet light. It had two absorption maxima: the first at 278 mμ, with no shift in N HCl, 10 % Na_2CO_3 (w/v) and 5 % NaOH (w/v), the second absorption maximum was at 340 mμ in HCl, at 410 mμ in Na_2CO_3 and at 400 mμ in NaOH. In contrast to the 2,4-dinitrophenylhydrazones of the keto acids, the absorption was not increased in NaOH by comparison with the colour in Na_2CO_3.

The 2,4-dinitrophenylhydrazones of keto acids yield on reduction the corresponding amino acids[4,5]. Therefore several attempts were made to reduce the unknown substance x to identifiable products. Treatment with tin and hydrogen chloride gas[6] rapidly yielded a ninhydrin-positive product but only ammonia could be identified. Hydrogenation in the presence of Raney nickel or of Adams' catalyst produced ninhydrin-positive substances from the 2,4-dinitrophenylhydrazone of α-ketoglutarate (glutamic acid with Raney nickel, and glutamic and γ-aminobutyric acid with Adam's catalyst) and from the 2,4-dinitrophenylhydrazone of pyruvate (alanine), but

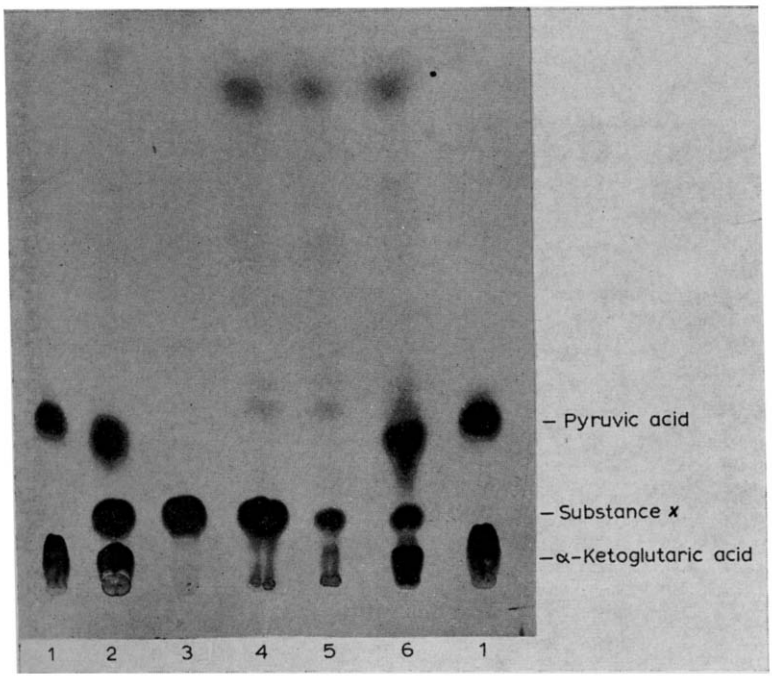

Fig. 2. Thin-layer chromatogram of mixed samples including the substance x. Conditions were the same as explained in the legend of Fig. 1. 1 = reference containing 2,4-dinitrophenylhydrazones of pyruvic acid and α-ketoglutaric acid; 2 = reference mixed with substance x derived from 2,4-dinitrophenylhydrazine itself; 3 = substance x derived from 2,4-dinitrophenylhydrazine; 4 = substance x derived from 2,4-dinitrophenylhydrazine mixed with substance x from brain homogenate; 5 = substance x from brain homogenate; 6 = substance x from brain homogenate mixed with reference.

none from the unknown substance in spite of several attempts under different conditions. 2,4-Dinitrophenylhydrazine itself gave ninhydrin-positive substances (presumably ammonia and aniline) on reduction with tin and hydrochloric acid.

When 2,4-dinitrophenylhydrazine was treated similarly to the 2,4-dinitrophenylhydrazones of keto acids, *i.e.*, by repeated extractions with ethyl acetate, sodium carbonate solution and (after acidification) again with ethyl acetate, the final product yielded the unknown substance *x* on chromatography (Fig. 2, sample 3). The spectrum (in the range 320–700 mμ) of the substance *x*, which had been derived from pure 2,4-dinitrophenylhydrazine, resembled that of the original substance *x*. When 2,4-dinitrophenylhydrazine was dissolved in *N* HCl, 10% Na_2CO_3, 5% NaOH, dioxane–phosphate buffer or ethyl acetate, the absorption maxima of substance *x* were not observed, even after storage of the solutions.

We concluded that the substance *x* was an artefact arising from the 2,4-dinitrophenylhydrazine during the manipulations and therefore further work for the identification was abandoned.

It remains to be explained, why in the first experiments less of the unknown substance *x* was formed on incubation of the brain homogenates from the ethanol intoxicated rats. The presence of substances in the homogenates which affect the formation of this artefact from the hydrazine during the repeated extractions must be assumed.

The question also arises, whether this artefact has to be accounted for in the routine chromatographic analyses of 2,4-dinitrophenylhydrazones of keto acids. If the artefact appears as a clearly separated spot, it might be confused with the 2,4-dinitrophenylhydrazones of other acid carbonyl compounds. However, as a rule it does not separate from the 2,4-dinitrophenylhydrazone of α-ketoglutaric acid. The colour yield of the unknown substance in NaOH solution is comparatively low, but in an unfavourable case it may be noticeable. For accurate analyses of α-ketoglutaric acid as its 2,4-dinitrophenylhydrazone, the possibility of this artefact should be kept in mind.

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