The maximum and shape of the curve of thiazoline carboxylic acid in phosphate buffer near the neutrality are very similar to those found for the metabolic product of thiazolidine carboxylic acid by liver preparations[2]. This finding strengthens the hypothesis that a thiazoline derivative is the first product of the biological oxidation of thiazolidine carboxylic acid.

D. CAVALLINI, B. MONDOVÌ, and C. DE MARCO

Institute of Biological Chemistry of the University of Rome and Centro di Enzimologia del C.N.R., Rome (Italy), June 29, 1957.

Riassunto

La formilcisteina in HCl 12 *N* dà un assorbimento nell'ultravioletto indicante la formazione di un anello tiazolinico. La reazione è più rapida per la formilcisteina che per il glutatione e dipende dalla concentrazione dell'acido. Trattando la formicilsteina con HCl 12 *N*, è stato preparato un composto avente le proprietà dell'acido tiazolin carbossilico, che in soluzione di fosfati a pH 7,4 dà una curva spettrofotometrica sovrapponibile a quella ottenuta in un precedente lavoro da un metabolita dell'acido tiazolidin carbossilico. Questo risultato avvalora l'ipotesi che il fegato di ratto è capace di ossidare l'anello tiazolidinico a tiazolinico.

Biochemical Studies on the Degradation Products of Diazinone

During chemical and biochemical studies on some organophosphorus insecticides[1], it was found that diazinone (O,O-diethyl O,2-*iso*propyl-4-methyl-pyrimidyl thionophosphate) is degradated to various derivatives by chemical and other means. It was also observed that plants sprayed with diazinone contained biochemically active residues even after comparatively long periods of time. The results of these experiments are reported in the following:

Methods and material[2]. Plants (*Impatiens balsami*) were sprayed with a 0·02% aqueous diazinone solution, approximately 100 ml per m[2]. Samples (3·5 g of fresh leaves) were taken after various periods of time, and homogenized in water (10 ml). The homogenates were analysed for chemical activity by measuring the cholinesterase-inhibiting effect on an acetylcholine-cholinesterase system with the Warburg technique[3]. The enzyme used was a purified preparation from human blood serum. Before the addition of substrate (acetylcholine chloride), the enzyme was incubated for 50 min with the extracts. Samples taken from unsprayed plants and treated in the same way were used as controls.

Paper chromatograms of various diazinone preparations were carried out with filter paper (Munktell OB) impregnated with silicone or vaseline from a 5% hexane or benzene solution, and air dried. The solvent used was the upper phase from a mixture containing water-ethanol-chloroform (6:10:10)[4]. After air drying, the paper was developed by spraying first with a N-bromo-succinimide solution (0·09 g in 50 ml chloroform and afterwards diluted 1:5 with the solvent) and then with a fluorescein solution (0·33 g in 100 of 0·1 *N* NaOH and afterwards diluted 3:100 with ethanol)[5]. The colour development could only be favorably performed when the paper was treated with vaseline.

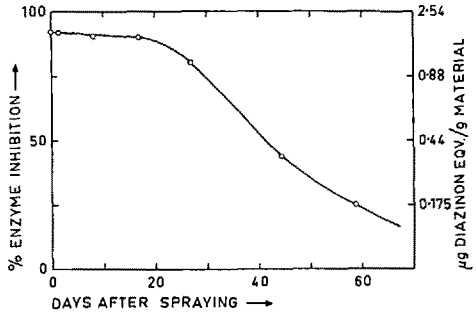

Fig. 1.—Biochemical effect of residues in plants sprayed with diazinone.

Chromatograms not sprayed with the colour reagents were used to study the cholinesterase inhibition by separated constituents. The chromatogram was cut into sections of the same size, usually 20 sections from the starting point to the solvent front, and extracts of each section were made in a bicarbonate buffer solution (the same solution as used in enzyme activity determination[3]). Cholinesterase inhibition by these extracts was assayed using the technique mentioned above. The location of active constituents thus obtained (Fig. 3) was evaluated as per cent enzyme inhibition by each section of the chromatogram, and compared with a chromatogram developed with the colour reagents.

Analysis of residues from diazinone-sprayed plants. The first sample of the plant sprayed with diazinone was taken 1 h after spraying, followed by sampling during one to two months (duplicate series of experiments carried out). The results obtained from one of these experiments are recorded in Figure 1. They indicate that 50% of the active components was present 25 days after spraying. After 9 weeks, the activity of the residues was approximately 20% of the original biochemical activity, corresponding to approximately 0·15 μg of diazinone equivalents per gram plant material. The original amount of diazinone (based on the amount used in spraying) was approximately 10 μg/g material.

It is most probable that diazinone is easily isomerised or converted to analogues (e.g., by oxidation) in a way similar to that demonstrated for parathion[4]. Some of these isomers and analogues might be more active biochemically than diazinone, in the same way as was found for parathion. In order to find support for this view, the following experiments were performed.

Enzyme inhibition by diazinone preparations treated in various ways. The cholinesterase inhibiting activities of pure (99·9%) diazinone and of a one year old preparation containing 95% pure diazinone (chemical analysis) differed greatly, as demonstrated in Figure 2. Even after keeping for four months at room temperature, a preparation behaved much the same as the one year old prepara-

[1] K.-B. AUGUSTINSSON, Acta agr. Scand. *7*, 165 (1957).

[2] We are grateful to Dr. SIV RENVALL of the Swedish Plant Protection Institute, Stockholm, for carrying out the spraying of the plants and taking samples for analysis. The diazinone preparations used were kindly supplied by Messrs. J. R. Geigy, Basel.

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tion. The enzyme inhibition was increased by heating a pure preparation for several hours at 140°C, and this increase in activity is assumed to be due to the formation

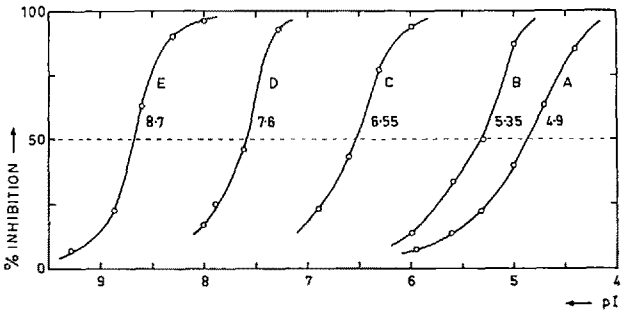

Fig. 2. — *In vitro* inhibition of cholinesterase activity (purified enzyme from human blood serum) by diazinone preparations treated in various ways. (*A*) Pure diazinone; (*B*) Pure diazinone heated for 65 h at 140°C; (*C*) One year old diazinone preparation (a precipitate present was removed before use); (*D*) and (*E*) Pure diazinone treated with bromine for 3 h (*D*) and tested immediately after the addition of bromine (*E*). pI = — log of molar inhibitor concentration (based on pure diazinone, mol. weight 303·35). Values inserted refer to 50% enzyme inhibition (pI_{50}).

of the S-ethyl isomer of diazinone. The *in vitro* inhibition was still more increased when pure diazinone was treated with dilute bromine water (1:2500). The reaction with bromine was instantaneous, and the greatest increase in inhibition was obtained when the mixture was tested as soon as possible after the addition of bromine. It is assumed that diazinone was thereby oxidised to its oxygen analogue, which is probably the active derivative *in vivo*. When diazinone was incubated with bromine water for longer periods of time, the inhibiting activity of the mixture was decreased. Incubation must be carried out in aqueous solution; in ethanol, diazinone was not oxidised by bromine. It is seen in Figure 2 that after the bromine treatment, the enzyme-inhibiting power was increased 10,000 times.

Paper chromatography. Pure and technical grade diazinone preparations as well as preparations heated or treated with bromine were analysed by paper chromatography. The chromatograms obtained with vaseline-treated paper were developed by spraying with N-bromosuccinimide-fluorescein, and those obtained with silicone-treated paper were evaluated by biochemical means. The results of some of these experiments are recorded in Figure 3. The R_f values obtained with the vaselin- and silicone-treated papers are not comparable, which may be noted when comparing the results shown in Figure 3 (*A* and *a*).

On a colour-developed chromatogram of pure (99·9%) diazinone preparation (*A*, *a*) only one spot was visible. Biochemical evaluation revealed two active constituents. On the other hand, six various constituents could be traced by the biochemical method on a chromatogram of an old preparation. It has not been possible to identify all these constituents, but some suggestions in this direction could be made, when the following experiments had been performed.

A pure diazinone preparation heated for 65 h at 140°C revealed only two spots (*C*; R_f 0·68 and 0·88). It is suggested that these correspond to the two fast-running constituents observed in the old preparation. One of the two constituents, probably that with R_f 0·88, is the S-ethyl isomer of diazinone; the other (R_f 0·68) may be the oxygen analogue of diazinone. The latter derivative was the predominant constituent in a mixture obtained after the treatment of diazinone with bromine water (*D*, Fig. 3).

We conclude, therefore, that the two constituents traced biochemically in a 'pure' diazinone preparation were diazinone (R_f 0·1) and its oxygen analogue (R_f 0·68), the latter probably being easily formed from the former even in contact with air. As to the identity of the three remaining constituents present in the old preparation (*B*, Fig. 3), no answer can be given before testing other possible analogues or isomers of diazinone, e.g., the S-pyrimidyl isomer, the bis-pyrimidyl analogue, and others.

K.-B. Augustinsson and G. Jonsson

Institute of Organic Chemistry and Biochemistry, University Stockholm (Sweden), May 3, 1957.

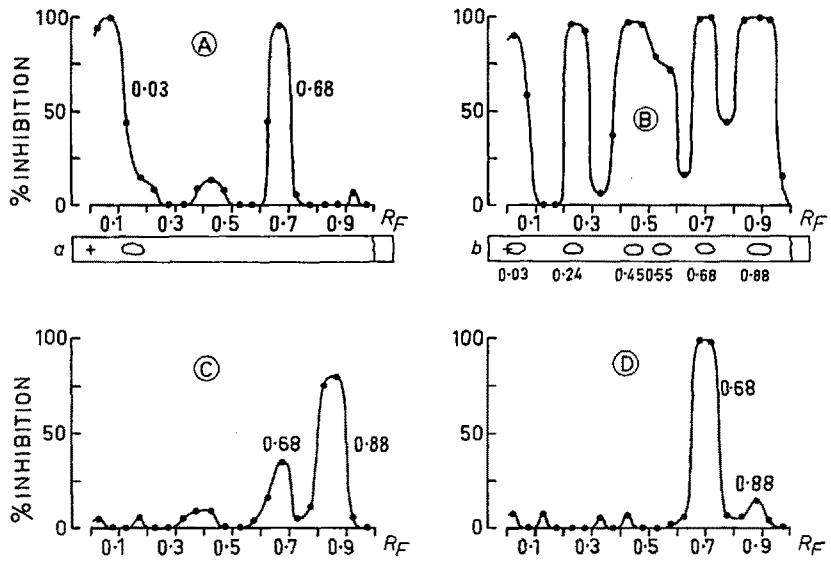

Fig. 3. — Cholinesterase inhibition by various sections of the paper chromatograms of pure (99·9%) diazinone (*A*), one year old diazinone (*B*), heated diazinone (65 h at 140°C) (*C*), and bromine-treated diazinone (*D*). Silicone treated paper used, except for the colour developed chromatogram (*a*) of pure diazinone when vaseline-treated paper was used instead. The R_f values of all constituents observed are noted on a 'hypothetical' chromatogram (*b*).

Zusammenfassung

Pflanzen, die mit Diazinon gespritzt wurden, enthalten biochemisch aktive Rückstände des Insektizids auch nach vergleichsweise langer Zeit; 50% der aktiven Stoffe waren vorhanden 25 Tage nach der Spritzung; 20% der ursprünglichen biochemischen Aktivität wurden noch nach 9 Wochen festgestellt.

Reines (99,9%) Diazinon enthielt ausser dem Diazinon eine andere cholinesterasehemmende Substanz, wahrscheinlich identisch mit dem Sauerstoffanalogon des Diazinons. In einem ein Jahr alten reinen Diazinonpräparat konnten 6 Komponenten papierchromatographisch nachgewiesen werden. 3 Bestandteile einer derartigen Mischung konnten identifiziert werden: Diazinon, sein Sauerstoffanalogon und sein S-ethylisomeres. Die beiden letzteren wurden wahrscheinlich bei der Behandlung von Diazinon mit Brom bzw. bei seiner Erhitzung auf 140°C als Hauptprodukte gebildet.

Demonstration of Specific and Non-specific Agglutinogens in the Normal Bone Marrow Erythroblasts

The presence in the bone marrow erythroblasts of agglutinogens (specific and non-specific) similar to those of the mature erythrocytes, though theoretically intuitive, has up to now been suggested only indirectly. For instance, BJÖRKMAN[1] reports the agglutinability of erythremic cells by influenza virus and by a serum containing cold agglutinins, and WAGNER[2] that of erythroblasts of the rat by an anti-rat-erythrocytes serum. More recently PISCIOTTA and HINZ[3], using circulating erythroblasts in a case of acquired haemolytic anaemia, have shown that they agglutinate with group-specific sera, and with sera containing autohaemoagglutinins.

The problem is especially interesting in relation to the suggested bone marrow involvement in course of immunohaemolytic syndromes: the bone marrow vulnerability in such pathological conditions, demonstrated by several different studies (GASSER[4], SACCHETTI, ROSSI, and DIENA[5]), would be supported by the finding of erythroblastic agglutinability.

The present work has been then directed to establish:

(1) whether group-specific antigens exist in the normal bone marrow erythroblasts, and the stage of their appearance in the cells; and

(2) whether the erythroblasts are agglutinated by non-specific agglutinating sera.

The erythroblasts of the bone marrow have been separated by a fractionated centrifugation technique (BRACCO, CURTI, and MASERA[6]), modified with the addition of Polyvinylpyrrolidone, which makes it possible to obtain in a short time (15 min from the bone marrow puncture) an erythroblastic suspension of 90–95%. For the agglutination tests with grouping sera, a drop of the agglutinating serum was added, in serological tubes, to a drop of an erythroblastic suspension at 40–50% in saline.

For sera containing auto-antibodies, the erythroblasts, at 2–4% in saline, were added to equal volumes of serum.

The readings of the agglutination, after incubation at the appropriate temperature for not longer than 30 min to avoid cellular damage, were taken macro- and microscopically.

In case of incomplete antibodies, compatible normal serum was added to the erythroblasts-serum-mixture, and the readings were taken as above.

All glassware used was siliconated.

Table I shows the results of some experiments of agglutination of normal erythroblasts by group-specific sera: the results obtained were in complete agreement with those obtained when mature erythrocytes of the same subjects were used instead of bone marrow erythroblasts.

Table I

Case No. 1		Case No. 2		Case No. 3	
Anti-A	–	Anti-A	+	Anti-A	–
Anti-B	–	Anti-B	–	Anti-B	+
Anti-M	+	Anti-M	–	Anti-M	+
Anti-N	–	Anti-N	+	Anti-N	+
Anti-D	+	Anti-D	–	Anti-D	+
Anti-Lewis Le^a	–	Anti-Lewis	+	Anti-Lewis	–
Anti-Lutheran Lu^a	+	Anti-Lutheran	–	Anti-Lutheran	+

+ denotes agglutination; – denotes no agglutination.

Inspection of the agglutinates, in freshly made preparations carefully observed with phase-contrast microscopy, or after staining of the slides, made it possible to recognize the presence of basophilic erythroblasts also in the earlier phases.

Table II

Agglutinating sera	Type of antibody	Optimal temperature	Agglutination
1	Incomplete	37	+
2	Incomplete	37	+ + +
3	Incomplete	20	+ +
4	Complete	4	+ + + +
5	Complete	20	+ + + +
6	Complete	4	+ + +

+, + +, + + +, + + + + denote varying degrees of agglutination.

Table II shows the results of erythroblastic agglutination by sera containing auto-antibodies, cold and warm type, complete and incomplete form.

Our observations indicate that the bone marrow erythroblasts possess group-specific antigens similar in all respects to those found on the mature erythrocytes. Such antigens are present also in the most immature basophilic erythroblasts: this fact demonstrates that the presence of antigens is unrelated to the appearance of haemoglobin in the cells. The demonstration that the erythroblasts are strongly agglutinated by sera containing auto-antibodies of different type seems to be significant: this suggests that, during the immunohae-

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