amount of Gly$^1$-$\alpha^{1-18}$-ACTH amide was no longer detectable 2 hr after injection, although the maximum corticoid level was approximately the same. In a separate experiment, the effect of pretreatment with poly-L-aspartic acid on corticosteroidogenesis by Gly$^1$-$\alpha^{1-18}$-ACTH amide was examined. Hypophysectomized rats were given 5 μl of Gly$^1$-$\alpha^{1-18}$-ACTH amide (5 μg) in 20 mM phosphate buffer as described above, but 10 μl (500 μg) of poly-L-aspartic acid was pre-injected into a different site of the thigh muscle 10 or 60 min before the hormone was administered. The plasma 11-OHCS levels, 30 min, 1 and 2 hr after Gly$^1$-$\alpha^{1-18}$-ACTH amide injection, were found to be essentially identical with those in animals not pretreated, suggesting that complex formation is necessary to cause the depot-effect. Amino acids analysis showed that the ratio of polymer to ACTH in the complex described above was 1: 2. On the other hand, the ratio was 1: 4, if a polymer of molecular weight 4460 was used. The former polymer consisted of 20 aspartic acid residues whereas the latter consisted of 39 aspartic acids. It thus seems probable that the carboxyl groups of poly-L-aspartic acid interact chiefly with the amino groups of the 8—18 portion of Gly$^1$-$\alpha^{1-18}$-ACTH amide to form a complex which, after injection, dissociates gradually at a local site releasing ACTH into body fluid with a favourable rate to maintain a high plasma 11-OHCS level over a long period. Recent studies on the immunogenicity of polypeptide indicate that charged homopolymers such as poly-L-glutamic acid are usually less antigenic than hydrophobic polymers.[8] The immunological properties of ACTH-polymer complexes are under investigation.

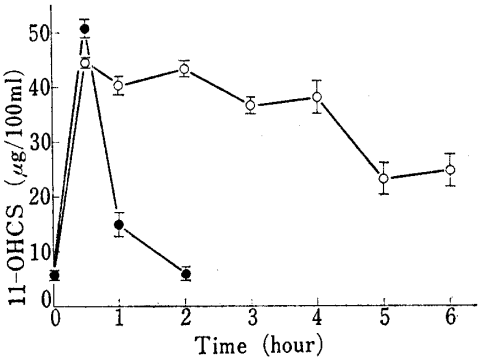


Fig. 1. Time Course of Plasma 11-OHCS Level in Hypophysectomized Rat Following Intra-muscular Injection of Gly$^1$-$\alpha^{1-18}$-ACTH Amide (●——●) and Poly-L-aspartate-Gly$^1$-$\alpha^{1-18}$-ACTH Amide Complex (○——○). Means ± S.E.

*Shionogi Research Laboratory*
*Shionogi & Co., Ltd.*
*Fukushima-ku, Osaka, 553, Japan*

MASAHARU HIRATA
AKIRA TANAKA

Received May 29, 1972

8) Y.J. Gill III., H.W. Kunz, and D.S. Papermaster, *J. Biol. Chem.*, **242**, 3308 (1967).

[Chem. Pharm. Bull. 20(8)1845—1847(1972)]

*UDC 547.29.08*

## Application of Dicyclohexyl Carbodiimide for Detection of Carboxylic Acids

Iron (III) complex of hydroxamic acid has long been used for the detection and determination of carboxylic acid derivatives. This reaction is simple and straightforward for carboxylic acid anhydrides, chlorides and esters.[1] However, the application to free carboxylic acids is cumbersome, because they should be first derivatized either to acid chlorides or esters. Furthermore, it is impractical to apply this reaction for the detection of carboxylic acids on paper or thin-layer.

1) D. Davidson, *J. Chem. Education*, **17**, 81 (1940).

Consequently, pH indicators has been used for that purposes.[2] But apparently pH indicators are sensitive to any acidic substances, the coloration is not specific for carboxylic acid.

In this communication, it is reported that dicyclohexyl carbodiimide is applied for detection of free carboxylic acid by converting them to hydroxamic acids in one step.[3] The reaction proceeds in a few minutes at room temperature. The presence of water does not interfere the reaction.

The hydroxamic acid formed is detected following known procedure as iron (III) complexes. An example of the procedure is as follows:

Take one drop of test solution which contains five micrograms or more carboxylic acid, add one drop of saturated alcoholic solution of hydroxylamine hydrochloride, then one drop of 1.0% dicyclohexyl carbodiimide in EtOH is added and the mixture is left about one minute or longer at room temperature. On addition of one drop of 1.0% solution of $FeCl_3 \cdot 6H_2O$ in acidic alcohol. The wine red color of iron (III) complex of hydroxamic acid develops instantaneously.

This method is also applicable to paper chromatography and thin-layer chromatography both on silica gel and crystalline cellulose. For the detection of individual carboxylic acids, paper or plates are sprayed with 10% methylene chloride solution of dicyclohexyl carbodiimide and 1% acidic alcoholic solution of $FeCl_3 \cdot 6H_2O$ saturated with hydroxylamine hydrochloride. The color develops in a minute. Since this method of detection is specific to carboxylic group, it is possible to use acidic or alkaline media to obtain better separation. Thus, thin-layer chromatography with $NaHCO_3$ impregnated crystalline cellulose reduces diffusion and tailing spots substantially (Table I). The limits of detection on the chromatogram are approximately several micrograms.

Table I. *Rf* Values of Some Carboxylic Acids on $NaHCO_3$ Impregnated Crystalline Cellulose

| Substance | Solvent system | *Rf* |
|---|---|---|
| Formic acid | A | 0.31 |
| Acetic acid | A | 0.37 |
| Propionic acid | A | 0.45 |
| n-Butyric acid | A | 0.52 |
| Cinnamic acid | B | 0.54 |
| Benzoic acid | B | 0.41 |
| N-Acetylglycylleucine | A | 0.49 |
| L-Proline | A | 0.22 |
| Citric acid | C | 0.39 |
| Malic acid | C | 0.40 |

solvent system: A, EtOH: $H_2O$=100:20; B, *n*-BuOH: $H_2O$=100:20; C, EtOH: $H_2O$=100:60

Among the carboxylic acids so far tested, oxalic acid is the single exception which is negative to the procedure. Oxalic acid is known to decompose quantitatively into carbon dioxide and carbon monoxide by the reaction with dicyclohexyl carbodiimide.[4] Amino

2) F. Bryant and B.T. Overell, *Biochim. Biophys. Acta*, **10**, 471 (1953).
3) J.C. Sheehan and G.P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955); C. Franzblau, P.M. Gallop and S. Seifter, *Biopolymers*, **1**, 79 (1963); D.G. Hoare, A. Olson and D.E. Koshland, Jr., *J. Am. Chem. Soc.*, **90**, 1638 (1968).
4) F. Zetzsche and A. Fredrich, *Chem. Ber.*, **72**, 363 (1939).

acids and hydroxy acids gives positive reaction as well. Esters are negative to the reaction, since the whole reaction proceeds at room temperature in the absence of strong alkali.

*Faculty of Pharmaceutical Sciences,*
*University of Tokyo*
*Hongo 7-3-1, Bunkyo-ku,*
*Tokyo*

TAKENORI TANIMURA
YASUHIKO KASAI
ZENZO TAMURA

Received February 1, 1972