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The Alkaloids of Hemlock (*Conium maculatum* L.)

DISTRIBUTION IN RELATION TO THE DEVELOPMENT OF THE FRUIT

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Much of the work published on the formation and role of the alkaloids in the plant has been concerned with the problem of the site of synthesis (whether root or leaf) and the determination of possible precursors by suitable feeding experiments. We have investigated the composition and quantity of the alkaloids of a given plant during a critical stage in its life history and have correlated our findings with physiological age, anatomical changes and histochemical tests. The phase of the development of the flower to the mature fruit was selected for study.

Conium maculatum L. (family Umbelliferae) was chosen because (*a*) there was already evidence that the quantity of alkaloid varied markedly as the fruit developed, (*b*) the alkaloids present were relatively simple ones and few in number and (*c*) the alkaloids are said to be mainly restricted to a special layer of the fruit (the coniine layer), which would give scope for anatomical and histochemical studies. Very few members of the Umbelliferae produce alkaloids, the majority producing volatile oils in clearly delimited tissues of the fruit, known as vittae; in *Conium*, vittae occur in the young fruits but almost disappear during maturation.

During the course of our work a paper on the distribution of alkaloids in *Conium maculatum* was published by Cromwell (1956). While Cromwell's work anticipated some of our techniques, and we have made use of some of those which he published, it has not affected the main body of our work. Some of our findings confirm those of Cromwell but since we have made a more detailed study of a restricted part of the life history of the plant, we can make a more complete report of this aspect.

EXPERIMENTAL

Initially two separate samples of seeds were sown in the School of Pharmacy Medicinal Plants Garden at Myddelton House, Enfield, Middlesex; one sample was derived from plants already growing at Myddelton House and the other was obtained from Chelsea Physic Garden, London. It soon became obvious that these two samples represented two varieties of *C. maculatum* which differed slightly in fruit and leaf morphology. More important still we found that one variety (that from Chelsea) produced little or no methylconiine whereas the other one did. To avoid complication in our work, we decided to use only the Chelsea variety of *C. maculatum*.

Sampling techniques. Careful sampling techniques are essential but previous work on alkaloids in plants has often shown a lack of appreciation of this requisite (Dawson, 1948). As our studies were restricted to the flower and fruit we made a careful study of the distribution of the flowers on the growing plant. The inflorescence is a compound umbel consisting of 10–15 rays, and each ray bears a secondary umbel with 8–18 flowers. It was noticed that all the flowers on a given compound umbel developed at practically the same time but that the stage of development varied from compound umbel to compound umbel on the same plant or even on the same branch. It was therefore decided to use the compound umbel, containing on an average about 150 flowers, as the 'unit'. These compound umbels are first recognized as small greenish masses of

tightly coiled buds which soon developed into a flat head of fully opened white flowers. This stage was easily recognized and it was assumed that pollination took place then; this stage was therefore called week 1. In a normal season it took 7–8 weeks for these pollinated flowers to develop into fully ripened, almost dehiscent, fruits. At the beginning of this period 250 compound umbels at the stage of week 1 were tagged by tying a white card just below the point where the rays arise, and 25 of them were collected between 9 and 10 a.m. (G.M.T.) the same day each week during the developing period. From these weekly samples, containing about 3000–4000 fruits, two groups of 800 fruits were placed in tared stoppered flasks as follows: (*a*) with 50 ml. of 70% ethanol (for quantitative chemical work); (*b*) for oven-dry weight determination. The remainder of the fruits were reserved for qualitative chemical examination and for anatomical studies.

The flasks were then re-weighed and the following information was obtained for each weekly collection: (i) the average fresh weight of one fruit; (ii) the average dry weight of one fruit; (iii) the amount of alkaloids, both total and individual, per fruit; (iv) the percentage of alkaloids on a dry-weight and a fresh-weight basis. For the quantitative chemical work the fruits were allowed to macerate in the ethanol for at least 7 days before assay and allowance was made for the amount of water contained in the 800 fruits added to each 50 ml. of 70% ethanol.

By using this sampling technique we obtained a variety of reference bases for our quantitative data and were able to select the most suitable for our purposes and at the same time compare our results with those of other workers even when they had used less suitable sampling methods.

The following methods for the determination of total alkaloids and of individual alkaloids in the fruits were used.

Measurement of the areas of spots on paper chromatograms. The methods described by Fisher, Parsons & Morrison (1948) and Fisher, Parsons & Holmes (1949) for amino acids was applied to the determination of the hemlock alkaloids. The results were insufficiently accurate for our purposes but the method was found to be valuable for preliminary investigations, especially when dealing with small quantities of plant material.

Spectrophotometric methods. The gravimetric method of Farr & Wright (1904) and the titrimetric method of Janot & Fabre (1929) were considered unsuitable as large quantities of material were required. Accordingly, a number of colour reactions reported for the hemlock alkaloids were investigated with a view to devising suitable spectrophotometric methods. The sodium β-naphthaquinone-4-sulphate method of Euler (1945) for piperidine, the chloranilic acid method of Barreto (1954) for coniine, the α-nitroso-β-naphthol method of Reichard (1905) and the carbon disulphide method of Dilling (1909) were all tried but found to be unsuitable. Cromwell (1956) used two colour reactions, based on reactions reported by earlier workers, and we found his methods the most suitable so far reported. Certain alterations were made and the following is an account of the methods we used, together with reasons for changing those described by Cromwell.

Preliminary extraction of the alkaloids from the fruits. A suitable volume (40 ml.) of the 70% ethanolic extract prepared from the weekly collection of fruits was transferred to an evaporating basin, acidified with 5 ml. of N-H_2SO_4 and evaporated to small volume on a water bath to remove most of the ethanol. The contents of the dish were transferred to a separator, with several rinsings of water and a final one of chloroform. The aqueous solution was extracted with chloroform to remove coloured impurities and the chloroform extracted with 2 × 5 ml. portions of 0·1 N-H_2SO_4, which were added to the original aqueous solution. The solution was then made alkaline to litmus with 10% NaOH solution and extracted with successive portions of chloroform. The mixed chloroform fractions were shaken with a few drops of concentrated HCl (A.R.) and the chloroform was removed by evaporation on a water bath. The mixed hydrochlorides were dried in an oven at about 90° to remove excess of acid and were dissolved in water to 100 ml. This solution was used for further determinations, each of which was made in duplicate. In no instance did the two results differ by more than 3%. Cromwell recommended micro-steam-distillation for the preliminary extraction of the alkaloids: we found this method inconvenient as it was difficult to prevent overflow of froth into the condenser and at the same time maintain an adequate pressure of steam through the mixture.

Determination of the total alkaloids in the fruit. The following solutions were mixed together in a 50 ml. separator: a suitable volume (0·5–15·0 ml.) of the solution of alkaloidal hydrochlorides already referred to, 1 ml. of phosphate buffer [0·1 M-KH_2PO_4 adjusted to pH 7·0 with 0·1 M-KOH and Universal indicator (B.D.H. Ltd.)], 5 ml. of a saturated solution of bromothymol blue in water. The mixture was then extracted with three 5 ml. quantities of benzene; each extract was successively dried with 0·5 g. of anhydrous Na_2SO_4, transferred to a 20 ml. measuring flask and made up to volume with further benzene which had been washed with the Na_2SO_4 previously used. The extinction of the yellow solution in benzene was then determined, with dry benzene as blank, in 0·5 cm. tubes, at 440 mμ with the Unicam D.G. spectrophotometer. The amount of total alkaloid present was calculated from a calibration curve prepared by using pure coniine hydrochloride.

We found that it was necessary to use a larger amount of bromothymol blue than that stated by Cromwell, as this indicator must be present in excess in order to complete the reaction. Moreover, two or three shakings with benzene are necessary in order completely to extract the coloured complex from the aqueous layer; furthermore the Na_2SO_4 used for drying the benzene extract adsorbs an appreciable amount of the coloured complex, and has therefore to be extracted with benzene and this fraction added to the others.

Determination of γ-coniceine. To a 20 ml. volumetric flask were added, in the following order: a suitable volume (0·5–15·0 ml.) of the solutions of alkaloidal hydrochlorides already referred to, aqueous 1% sodium nitroprusside solution (0·6 ml.), aqueous 10% (w/v) Na_2CO_3 solution (0·4 ml.). The mixture was made up to volume with water and the red colour produced was measured at 495 mμ between 7 and 10 min. after the addition of the alkali. Preliminary work with pure γ-coniceine showed that the maximum colour intensity occurred between 7 and 10 min. and the peak of the absorption curve was at 495 mμ. The amount of γ-coniceine present was calculated from a calibration curve prepared from pure γ-coniceine as follows.

Preparation of pure γ-coniceine standard solution. The leaves of second-year *Conium* plants, collected in the spring, were found to be good sources of γ-coniceine. Steam-distillation in the presence of alkali was found unsuitable as the oily distillate was black. The dried leaf was therefore extracted in the cold with chloroform–aqueous NH_3 solution (sp.gr. 0·880) (25:1, v/v). The chloroform solution was washed with water, and was then extracted with 5% (w/v) tartaric acid solution (we found that γ-coniceine turned pink in mineral acid). The pale-yellow acid solution was made alkaline to litmus with aqueous KOH and re-extracted with chloroform; this chloroform solution was then extracted with N-HCl. At this stage we found it impossible to prepare pure crystalline γ-coniceine hydrochloride owing to its deliquescent nature and to the discoloration already referred to. We found, however, that the reineckate was the most suitable salt for further purification. Accordingly a saturated aqueous solution of ammonium reineckate was immediately added to the solution of γ-coniceine in HCl until no further precipitation occurred. The pink flocculent precipitate was dried in a desiccator and pure γ-coniceine reineckate (m.p. 143° uncorrected) obtained by several recrystallizations from acetone–water (25:75, v/v). A portion (20 mg.) of this reineckate was dissolved in 10 ml. of methanol by warming and the solution diluted with an equal volume of water. The reineckate ion was precipitated by titration with aqueous 1% $AgNO_3$ solution until no further precipitate formed. The mixture was filtered, the precipitate well washed with water and the filtrate and washings were made up to 100 ml. This solution contained γ-coniceine (70 μg./ml., calculated as the hydrochloride).

Determination of individual alkaloids (other than γ-coniceine). The method of band chromatography devised by Cromwell was applied to the solution of alkaloidal hydrochlorides already referred to. As this method is laborious it was used only when information on the quantities of alkaloids other than γ-coniceine and coniine was required.

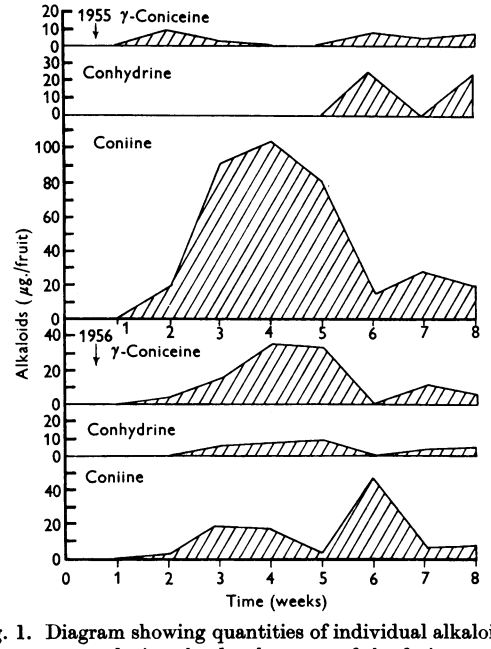

Fig. 1. Diagram showing quantities of individual alkaloids present during the development of the fruit.

RESULTS

Assay of alkaloids

The results are expressed as weight of alkaloid per fruit, rather than percentage of dry or fresh weight, as this gave a more accurate picture of the changes taking place within an individual fruit. The results for 1955 and 1956 are summarized in Fig. 1.

Effect of rainfall and sunshine. The 1955 season was sunny and dry with a total sunshine of 349 hr. and rainfall of 1·16 in. during the 8 weeks of the experiment. In 1956 the weather was less sunny and wetter with a total sunshine of 221 hr. and rainfall of 8·66 in. during the corresponding 8 weeks. In 1955 the average dry weight of the fruits and the amount of alkaloid per fruit was about twice that of the 1956 season. Other effects on the composition of the alkaloids are discussed below.

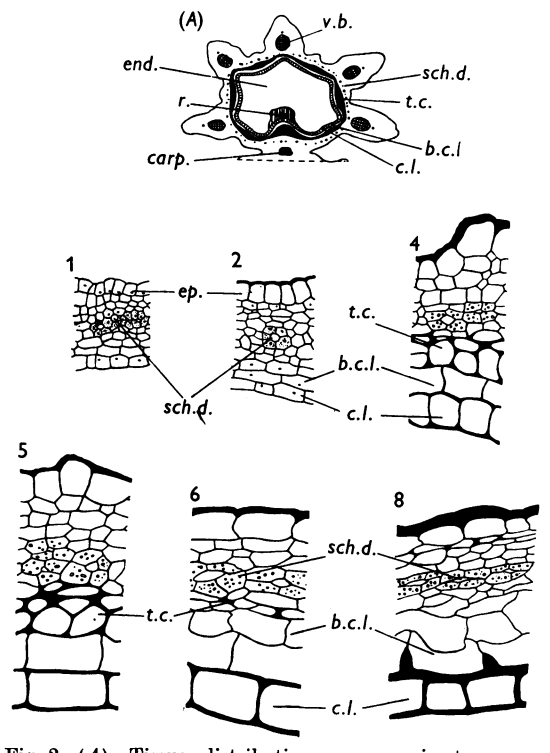

Fig. 2. (*A*). Tissue distribution, as seen in transverse section, of a mericarp at week 5 (×15). 1, 2, 4, 5, 6, 8, (numbers indicate the week of collection): tissue details of the pericarp (×200). *b.c.l.*, Beaker-cell layer; *carp.*, carpophore; *c.l.*, coniine layer; *end.*, endodermis; *ep.*, epidermis; *r.*, raphe; *sch.d.*, schizogenous duct; *t.c.*, temporary collenchyma; *v.b.*, vascular bundle.

Table 1. *Summary of anatomical changes during development of the fruits in* 1955

Week no.	Coniine layer	Beaker-cell layer	Temporary collenchyma	Schizogenous ducts	Endosperm
1, 2	Walls not thickened	Walls not thickened	Absent	Present	Translucent
3	Inner tangential walls partially thickened	Walls not thickened	Absent	Present	Translucent
4	Both tangential walls partially thickened	Inner tangential walls partially thickened	Developing	Present	Translucent
5	Radial and tangential walls thickened	Inner tangential walls partially thickened	Fully developed	Fully developed	Becoming opaque; minute crystals appear
6	Radial and tangential walls thickened	Inner tangential walls partially thickened	Partially lost. Some distortion	Tangential compression begins	Opaque
7	Walls completely thickened	'Beaker-shaped' thickening	Lost and tissues distorted	Distorted	Opaque; crystals at maximum size
8	Walls completely thickened	'Beaker-shaped' thickening	Lost and tissues distorted	Distorted	Opaque

Developmental anatomy of the fruit

The general arrangement of the tissues, as seen in transverse section, is shown in Fig. 2. The endocarp (coniine layer) and the layer of pericarp cells immediately within (which we have called the 'beaker-cell layer') have long been known to contain alkaloids (Goris, 1914). Both develop at approximately the same rate but the ultimate thickening and pitting of the walls indicate that diffusion of substances from these layers would be outwards towards the mesocarp rather than inwards towards the seed (Fig. 2, 8). In the inner region of the mesocarp, temporary collenchyma arises in week 4 and disappears in week 7. During this same period the endosperm tissues reach maturity both in size and cell contents. There are numerous schizogenous ducts (vittae) in the mesocarp but at about week 4 they cease to develop and never form well-marked vittae which are so characteristic of most umbelliferous fruits.

A summary of these changes is given in Table 1 and some of the stages are illustrated in Fig. 2.

Histochemical studies

We used a solution of potassium bismuth iodide (Munier, 1951) as a general histochemical reagent for alkaloids, and an alkaline solution of sodium nitroprusside for piperideines. The latter was used by placing a thick section (about 0·5 mm.) in a drop of freshly prepared 1 % sodium nitroprusside solution on a slide, followed by the addition of a drop of aqueous 10 % NH_3 solution; if piperideines, such as γ-coniceine, were present a blood red developed. Fully saturated alkaloids like coniine and conhydrine do not give red.

Alkaloid was found in the epidermis and in the coniine layer at all stages of development, being particularly abundant about week 5, but its presence was doubtful in all other tissues. This observation conflicts with that of earlier workers, whose results are reviewed by Goris (1914). He states that coniine occurs in the epidermis, subepidermal collenchyma, parenchyma adjoining the vascular bundles and schizogenous ducts and in the coniine layer and its adjacent one (the beaker-cell layer). It is completely absent from the seed. Our own results do not prove that coniine is totally absent from the other tissues, as Tunman (1931) reports that potassium bismuth iodide is not sensitive to *Conium* alkaloids in concentrations of less than 0·1 %. They do indicate, however, that the alkaloids are concentrated in the peripheral tissues of the pericarp, that is, the epidermis and the endocarp.

The distribution of piperideines showed considerable variation. Well-marked reactions were obtained in the vascular bundles of the carpophore and pericarp during the entire development of the fruit. In the raphe and the schizogenous ducts piperideines were present in the early stages, being particularly intense in the ducts in week 4, but only traces were present in the later stages. None occurred in the coniine and beaker-cell layers until week 6 and even then the reaction in the coniine layer was doubtful. This appearance of piperideines in the beaker-cell layer coincided with a general diffusion of piperideines throughout the pericarp tissues from week 6 onwards (Table 2).

Aborted mericarps. Abnormal fruits occurred fairly frequently; in these, one mericarp developed normally and the other became aborted. Histochemical tests showed that piperideines were always much more abundant in the aborted mericarp than in the normal one.

Table 2. *Distribution of piperideines in fruits collected in* 1956, *as shown by the nitroprusside test*

Colours: ±, doubtful red; +, blood red. The tissues referred to are shown in Fig. 2.

Week no.	1	2	3	4	5	6	7	8
Epidermis	.	.	.	.	.	.	+	+
Schizogenous ducts	+	+	+	+	+	+	±	±
Collenchyma	.	.	.	.	.	+	+	+
Beaker-cell layer	.	.	.	.	.	+	+	+
Coniine layer	.	.	.	.	.	+	±	±
Vascular bundles in pericarp and carpophore	+	+	+	+	+	+	+	+
Raphe and adjoining parenchyma	.	+	+	+	+	+	±	±

DISCUSSION

Piperideines as precursors of the saturated alkaloids

The results illustrated in Fig. 1 show that in both seasons a peak of γ-coniceine content nearly always preceded a peak of coniine content. This happened twice in 1955 and shows particularly well in 1956, between weeks 5 and 6, when the γ-coniceine dropped rapidly and the coniine increased correspondingly. These results are in agreement with those of Cromwell (1956), who found that in growing seedlings and young leaves γ-coniceine predominated but that as the vegetative parts matured so the content of coniine increased. Similarly, in the development from flower to fruit the overall picture was of the γ-coniceine content decreasing as the coniine content increased. Therefore γ-coniceine may well be the precursor of coniine. The results of our histochemical studies are also in agreement with this hypothesis, since they indicate that piperideines enter the fruit by the conducting tissues and the schizogenous ducts and that in the latter they appear to reach a maximum concentration in week 4, the same week as that in which total alkaloids are also at a maximum.

Interchange between saturated and unsaturated alkaloids

γ-Coniceine seems to be the predominant alkaloid during rainy weather. Thus in both seasons the maximum alkaloidal content was at week 4, but in the comparatively dry conditions of 1955 coniine was the predominant alkaloid whereas in the wetter season of 1956 γ-coniceine predominated at the corresponding stage. Further, between weeks 5 and 6 in 1956 there was a marked reduction in the γ-coniceine content (and a corresponding increase in coniine). This was paralleled by a very marked reduction of rainfall. Since the overall effect of the dry 1955 season was an increase in growth it may well be that the change from γ-coniceine to the saturated alkaloids is associated with active growth. This is supported by a consideration of the anatomical studies, which also indicate that this change is reversible.

Anatomical studies of the 1955 samples showed that the rapid increase in alkaloidal content occurred while the pericarp was increasing in size, up to weeks 4 and 5. During this time piperideines were abundant in the schizogenous ducts (which are possibly important sources of alkaloid precursors) and vascular tissue; the coniine and beaker-cell layers increased in size but gave reactions for saturated alkaloids only. Presumably piperideines are transported to the pericarp during this period of rapid development, are hydrogenated or hydrated and then deposited in the coniine and beaker-cell layers as saturated alkaloids. In the aborted mericarp, on the other hand, no such rapid development takes place and the piperideines accumulate to a much greater extent than in the corresponding normal mericarp. After week 4 the alkaloidal content falls. This is paralleled by the gradual distortion of the schizogenous ducts and a reduction in their piperideine content. At the same time the seed begins to mature and it may be in connexion with this further metabolic activity that additional changes take place in the alkaloidal content. Piperideines begin to appear in the coniine and beaker-cell layers and become generally diffused throughout the pericarp (Table 2). Chemical analyses confirm that there is an increase in γ-coniceine from weeks 5 to 6 (Fig. 1). These results indicate that the 'piperideine pool' associated with this second phase of metabolic activity is partly derived from the stores of coniine in the coniine and beaker-cell layers. It has already been pointed out that the thickening of the walls of these cells would favour a diffusion from them into the pericarp. The conversion of piperideines into saturated alkaloids may therefore be reversible. A similar situation has been shown to occur in certain *Senecio* species, where the alkaloids occur either in an oxidized or a reduced form according to reversible reactions of hydrogenation and varying degrees of hydration (Areschkina, 1957). We intend investigating further this possibility that the hemlock alkaloids are connected with oxidation–reduction mechanisms.

Assay figures for total alkaloids

We found that in both seasons the maximum percentage of alkaloids (dry-weight basis) was about 3%. This is considerably higher than the figures usually quoted (Farr & Wright, 1904; Madaus & Schindler, 1938; Cromwell, 1956), and is probably due to the careful sampling technique we employed. A sample collected haphazardly from several plants on any given day will contain fruits at many stages of development and this would lead to a lowering of the assay value. Farr & Wright (1904) state that the highest percentage of alkaloids occurs when the fruit is green, and the results given by Cromwell (1956) indicate a maximum in mature green fruits. Our results show that these statements should be modified, for the maximum occurred in 1955 when the fruits were just beginning to turn yellow, whereas in 1956 the fruits were still quite green after the period of alkaloidal maximum. In 1955 the total alkaloid per fruit was twice that of the fruit in 1956; at the same time the dry weight was also doubled so that the assay figures for the two seasons were similar. Increased alkaloidal production is therefore associated with the general improvement in growth and vigour.

SUMMARY

1. A study of the changes occurring during the development of the flower to the fruit of *Conium maculatum* L. has been made.

2. The changes in the composition and quantity of alkaloids were investigated simultaneously with the changes in anatomical structure and in reactions to histochemical tests.

3. A carefully chosen sample of seeds was used and crops from two successive seasons were harvested. Special sampling techniques and methods of analysis were devised.

4. The climatic conditions in the two seasons differed; in the sunnier season the average weight of the fruits and amount of alkaloids was twice that of the wetter season.

5. In both seasons the maximum alkaloidal content occurred 3 weeks after fertilization of the ovule; in the wet season, however, γ-coniceine (an unsaturated alkaloid) predominated at this stage; in the dry season coniine (a saturated alkaloid) predominated.

6. In both seasons a peak of γ-coniceine content preceded a peak of coniine content.

7. It is suggested that γ-coniceine and other piperideines are the precursors of the saturated alkaloids such as coniine and conhydrine and this change is associated with active growth. This change may be reversible.

The work described in this paper forms part of a thesis presented by one of us (S. B. Challen) for the award of a Ph.D. degree of the University of London. We would like to thank Mr J. F. Rogers, Head Gardener at Myddelton House, for his valuable assistance in the cultivation of the hemlock plants.

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The Point of the Aerobic Inhibition of Glycolytic Activity Associated with Brain Mitochondria

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One of the oldest-known controlling mechanisms of metabolic processes at the cellular level is the aerobic sparing effect on glucose utilization. Warburg (1926) defined this effect as follows: 'Respiration and glycolysis are linked by a chemical reaction which I call the Pasteur reaction after its discoverer.' This reaction has been the subject of a large number of investigations (cf. reviews by Burk, 1937, 1939; Dixon, 1937; Lipmann, 1942; Dickens, 1951; Krebs, 1957). However, the elucidation of the mechanism and of the point of inhibition in the glycolytic chain has been hampered by the fact that the effect was obtained only in whole-cell preparations, where permeability factors