Acknowledgment

It is a pleasure to acknowledge the technical assistance of Mrs. Karen McCauley and Mr. Norman Argue.

References

Ailhaud, G., Samuel, D., and Desnuelle, P. (1963), *Biochim. Biophys. Acta 67*, 150.
Chung, A., and Law, J. H. (1964), *Biochemistry 3*, 967.
Creasey, W. A., (1962), *Biochim. Biophys. Acta 64*, 559.
Croom, J. A., and McNeill, J. J. (1961), Bacteriological Proceedings, Abstracts of the 61st Annual Meeting, Baltimore, p. 170.
Deuel, H. J. (1951), *in* The Lipids, New York, Interscience, p. 58.
Goldfine, H., and Bloch, K. (1961), *J. Biol. Chem. 236*, 2596.
Goldman, P., Alberts, A. W., and Vagelos, P. R. (1963), *J. Biol. Chem. 238*, 3579.
Gornall, A. G., Bardawill, C. J., and David, M. (1949), *J. Biol. Chem. 177*, 751.
Green, A. A., and Hughes, W. L. (1955), *Methods Enzymol. 3*, 76.
Green, D. E., and Wakil, S. J. (1960), *in* Lipide Metabolism, Bloch, K. ed., New York, Wiley, p. 10.
Hill, U T. (1947), *Anal. Chem. 19*, 932.
Kanfer, J., and Kennedy, E. P. (1964), *J. Biol. Chem. 239*, 1720.
Kornberg, A., and Pricer, W. E. (1953), *J. Biol. Chem. 204*, 329.
Lennarz, W. J. (1963), *Biochim. Biophys. Acta 73*, 335.
Lennarz, W. J., Scheuerbrandt, G., and Bloch, K. (1962), *J. Biol. Chem. 237*, 664.
Mahler, H. R., Wakil, S. J., and Bock, R. M. (1953), *J. Biol. Chem. 204*, 453.
Massaro, E. J., and Lennarz, W. J. (1964), *Federation Proc. 23*, 269.
Senior, J. R., and Isselbacher, K. J. (1960), *Biochim. Biophys. Acta 44*, 399.
Steinberg, D., Vaughan, M., Margolis, S., and Karmen, A. (1960), *Federation Proc. 19*, 227.
Weibull, C. (1957), *Acta Chem. Scand. 11*, 881.
Weibull, C., Beckman, H., and Bergström, L. J. (1959), *J. Gen. Microbiol. 20*, 519.
Zamenhof, S. (1957), *Methods Enzymol. 3*, 702.

Reaction of Ficin with Diisopropylphosphorofluoridate. Evidence for a Contaminating Inhibitor*

Norman R. Gould† and Irvin E. Liener

ABSTRACT: Commercial samples of diisopropylphosphorofluoridate (DFP) which inhibit ficin were found to contain a small amount of impurity which combines irreversibly with the SH groups of ficin, cysteine, and glutathione. The inhibition of ficin could be prevented by prior activation of the enzyme with cysteine; the latter presumably exerts this protective effect by reacting preferentially with the inhibitor. By means of fractional distillation DFP could be obtained free of the inhibitor which remained in the nondistillable residue. DFP which had been purified in this manner was capable of phosphorylating ficin without affecting its activity. The inhibitor has been further purified by thin-layer chromatography, but its chemical identity remains unknown

The inhibition of animal proteases and esterases by the organophosphorus compound, DFP, has provided an extremely useful technique for elucidating the chemical nature of the active site of these enzymes (see, for example, the review by Koshland, 1963). A decided lack of agreement exists in the literature, however, regarding the inhibitory effect of DFP on the proteases of plant origin, most of which are considered SH enzymes. Jansen *et al.* (1948) and Kimmel and Smith (1954) were unable to inhibit papain with DFP, contrary to the observations of Masuda (1959), Ebata *et al.* (1962), and Heinicke and Mori (1959), all of whom reported inhibition. Several papers (Heinicke and Mori, 1959; Ota *et al.*, 1961; Ebata *et al.*, 1962) have reported the

*From the Department of Biochemistry, University of Minnesota, St. Paul. *Received September 4, 1964.* Paper No. 5510, Scientific Journal Series, Minnesota Agricultural Experiment Station, University of Minnesota, St. Paul. This investigation was supported by research grants from the National Institutes of Health, U.S. Public Health Service (GM 04616) and from the National Science Foundation (G 13965).

† This report is based on a dissertation presented by one of us (N. R. G) in partial fulfillment for the degree of Doctor of Philosophy in the Department of Biochemistry, Institute of Agriculture, University of Minnesota.

inhibition of bromelain by DFP, which is at variance with the findings of Murachi and Neurath (1960). Inactive, crystalline preparations of DFP-treated papain (Masuda, 1959) and chymopapain (Ebata and Yasunobu, 1963) have been isolated. Murachi (1963), on the other hand, found that DFP could phosphorylate SH enzymes such as papain and bromelain without affecting their activity.

A short communication from this laboratory (Gould *et al.*, 1963) described some preliminary experiments which led us to conclude that some commercial lots of DFP contain an impurity which inhibits papain and ficin. Purified DFP, however, was completely devoid of inhibitory activity toward these enzymes although phosphorylation could still be effected. The present paper reports an extension of these studies which lend additional support to our original conclusions and provide a simple explanation for the conflicting reports which have appeared in the literature.

Experimental

Materials. The purification and characterization of the ficin used in these studies have already been described (Liener, 1961).[1] The mercury derivative of ficin (mercurificin) was prepared by adding a 1.2 molar excess of $HgCl_2$ to an aqueous solution of ficin, followed by dialysis and lyophilization. Carboxymethylated ficin (CM-ficin) was prepared by adding 4 ml. of 0.8 mM solution of iodoacetic acid, adjusted to *p*H 5, to 1 ml of a solution containing 41.8 mg ficin (1.6 μmoles). After standing at room temperature for 30 minutes, the solution was dialyzed and lyophilized. Analysis of the acid hydrolysate of CM-ficin on the Spinco Model 120 automatic amino acid analyzer (Spackman *et al.*, 1958; Brigham *et al.*, 1960) revealed CM-cysteine to be the only amino acid which had been carboxymethylated. Values ranging from 0.6 to 0.8 mole of CM-cysteine per mole of protein were obtained for different preparations, indicating that the one -SH group known to be readily reactive in the ficin molecule (Liener, 1961) may have undergone partial oxidation. The concentration of protein was calculated from nitrogen determinations (Lanni *et al.*, 1950) and a nitrogen content of 15.1% for ficin on a dry weight basis, or from absorbance measurements at 280 mμ using a $E^{1\%}_{1cm}$ value determined to be 22.4. Protein-to-mole conversions were based on a molecular weight of 26,000 reported by Hammond and Gutfreund (1959).

α-Chymotrypsin was a crystalline product purchased from Worthington Biochemicals, Freehold, N.J. Samples of DFP used during the course of this study were purchased or were gifts from the following firms: Aldrich Chemical Co., Milwaukee, Wis.; Merck and Co., Rahway, N.J.; Sigma Chemical Co., St. Louis, Mo.; and Calbiochem, Los Angeles, Calif. L-Cysteine, free base, was purchased from Mann Research Laboratories, New York City, and glutathione was a product of General Biochemicals, Chagrin Falls, Ohio.

Methods. Proteolytic activity was assayed on casein by the method of Kunitz (1947) in which the absorbance of the digestion products in the trichloracetic filtrate is measured at 280 mμ. Thiol groups were determined by the colorimetric method of Ellman (1959) or as CM-cysteine after alkylation with iodoacetate (Brigham *et al.*, 1960). Protein-bound phosphorus was determined by the method of Bartlett (1959). The technique of Hanes and Isherwood (1949) was used to detect phosphorus-containing components on paper or thin-layer chromatograms. Other experimental details are described in the appropriate tables or figures.

Results

Experiments with Aldrich DFP

Reaction with SH Groups of Ficin. Since a thiol group is believed to be an essential component of the active site of ficin (Liener, 1961), it was of interest to ascertain whether the inhibitory effect which DFP might have on this enzyme would involve the thiol group. Initial studies along these lines were carried out with a sample of Aldrich DFP (lot A)[2] which completely inhibited ficin at a mole ratio of 13:1. The results of a study of the effect of DFP on the activity of ficin when added before and after treatment with cysteine or KCN, known activators of SH enzymes, are shown in Table I. These data show that inhibition of DFP was effectively counteracted when the enzyme was pretreated with cysteine but not with KCN. When cysteine or KCN was added subsequent to DFP treatment of the enzyme, only partial restoration of the activity was obtained. The level of activity attained in these instances was approximately equivalent to the increment in activity produced by cysteine or KCN alone. Mercurificin, treated first with DFP and then reactivated with cysteine, was fully as active as the control from which DFP had been omitted or in which the system was preactivated with cysteine. These results suggest that DFP must be combining irreversibly with the SH group of ficin, a reaction which can be prevented by prior treatment of the enzyme with cysteine but not with KCN.

As shown in Figure 1A, Aldrich DFP did in fact cause a loss in the SH content of ficin which paralleled the loss in activity. Accompanying these losses in activity and SH titer was a corresponding uptake in phosphorus by the protein. Similar studies to verify the protective effect of cysteine are shown in Figure 1B where the

[1] Following the completion of this work, Sgarbieri *et al.* (1964) reported on the heterogeneity of preparations of ficin. Although the preparation used in our studies was homogeneous by moving-boundary electrophoresis and ultracentrifugation (Liener, 1961), it is quite possible that the chromatographic techniques described by Sgarbieri *et al.* may have disclosed heterogeneity. This heterogeneity, however, in our opinion, should not invalidate any of the major conclusions which have been made from the results of this study.

[2] Since the various batches of Aldrich DFP were not identified by lot numbers by the manufacturer, these have been lettered alphabetically in the order in which they were received and studied in the laboratory.

TABLE I: Effect of DFP on the Activity of Ficin or Mercurificin before and after Treatment with Cysteine or KCN.[a]

System[b]	Absorbancy at 280 mμ	Relative Activity (%)[c]
Ficin	0.450	100
Ficin + DFP	0.022	5
Ficin + cysteine	0.640	142
Ficin + cysteine + DFP	0.620	138
Ficin + DFP + cysteine	0.194	43
Ficin + KCN	0.820	182
Ficin + KCN + DFP	0.082	18
Ficin + DFP + KCN	0.360	80
Mercurificin + cysteine	0.604	134
Mercurificin + cysteine + DFP	0.610	135
Mercurificin + DFP + cysteine	0.590	130

[a] To 1 ml of solution containing 0.037 μmole of ficin or mercurificin in 0.1 M borate buffer, *p*H 8.6, was added 0.5 ml of 0.25 M cysteine or KCN in the same buffer. After 2 minutes 0.5 ml of 0.002 M DFP (Aldrich, lot A) was added and the mixture was allowed to stand at room temperature for 30 minutes. Reaction mixtures were also prepared in which DFP was added prior to cysteine or KCN. Enzyme controls with DFP and activators alone were also included. Activity was measured on a 0.1-ml aliquot of each mixture. Absorbancy of trichloroacetic acid filtrate of casein digest measured at 280 mμ is recorded in the table. [b] Reactants are given in the sequence with which they were introduced into the reaction mixture. [c] Activity of ficin in absence of DFP and activator taken as 100%.

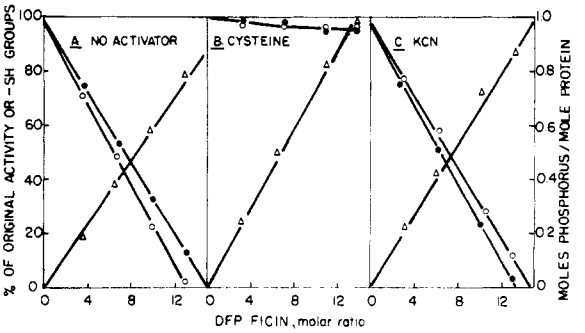

FIGURE 1: Effect of Aldrich DFP on activity, SH content, and uptake of phosphorus of ficin in the presence and absence of cysteine and KCN. To solutions containing 23.4 mg ficin (0.9 μmole) were added graded levels of Aldrich DFP (lot A) in a final volume of 10 ml in 0.1 M borate buffer, *p*H 8.0. Where activation was desired cysteine or KCN was included at a final concentration of 0.05 M. After 30 minutes at room temperature solutions were assayed for activity, and, where no activator was present, analyzed directly for SH content by the method of Ellman (1959). Where activator was present, a 4-molar excess of iodoacetate (with respect to ficin plus cysteine or KCN) was added, and the solutions were dialyzed and analyzed for CM-cysteine after acid hydrolysis. Phosphorus was determined on reaction mixtures after exhaustive dialysis to remove excess DFP. Activity and SH content are expressed as per cent of control solutions from which DFP had been omitted. ○—○, activity; ●—●, SH content; △—△, phosphorus incorporated into protein.

SH content of ficin was measured as CM-cysteine.[3] It may be noted that, although cysteine protects ficin from inactivation by DFP, the enzyme is nevertheless phosphorylated to the extent of about 1 mole phosphorus per mole at the highest level of DFP employed. Figure 1c shows that KCN was completely ineffective in protecting ficin from inactivation, decrease in SH content, and phosphorylation by DFP.

Reaction with Cysteine and Glutathione. When cysteine was treated with a 100 molar excess of Aldrich DFP SH groups could no longer be detected. When the DFP-treated cysteine was placed on the automatic amino acid analyzer, only 20% of the cysteine could be accounted for as cystine; no other reaction product was noted. When glutathione was similarly treated and acid hydrolyzed (6 N HCl, 110°, 22 hours), glutamic acid, glycine, and cystine were recovered in a mole ratio of 1.00:0.98:0.22. The recovery of these amino acids from untreated glutathione was 1.00:0.98:0.90. DFP thus appears to react with the SH group of either free or peptide-bound cysteine, but the reaction product unexplainably escapes detection under the usual conditions of amino acid analysis.

It was of interest to ascertain whether glutathione itself would yield a reaction product with DFP which could be observed on the analyzer. The chromatographic analysis of glutathione treated with DFP produced the pattern shown in the lower part of Figure 2; glutathione itself is shown in the upper part of this figure. Oxidized glutathione, if present, would have appeared as a very broad peak underlying the positions extending from aspartic acid to proline.[4] It is evident that DFP treatment of glutathione has resulted in the formation of a new compound which appears in a position which immediately follows the expected location of phenylalanine.

[3] Measurement of the SH content of ficin under these conditions requires the prior removal of cysteine or KCN. Dialysis led to a significant decrease in the SH titer of the protein, presumably owing to spontaneous oxidation. This problem was avoided by determining the SH content of ficin as CM-cysteine without removing the cysteine or KCN from the reaction mixture as described in the legend to Figure 1.

[4] Instruction Manual, Beckman/Spinco Model 120 amino acid analyzer, p. 7-5.

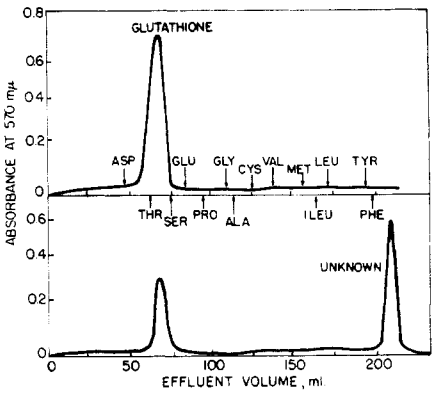

FIGURE 2: Chromatographic analysis of reaction mixture of glutathione and Aldrich DFP. To 1.2 μmoles of glutathione dissolved in 10 ml 0.1 M pyridine–acetic acid buffer, *p*H 6.5, was added 0.1 ml Aldrich DFP (lot A). After 2 hours at room temperature, the mixture was analyzed on the Spinco amino acid analyzer under accelerated conditions (see Beckman Technical Bulletin A-TB-005, May, 1963). The relative positions of other amino acids are shown for reference. Upper curve, control without DFP; lower curve, in the presence of DFP.

Evidence for an Inhibitory Impurity in Aldrich DFP. All of the experiments which have thus far been described involved the use of a single batch of Aldrich DFP, namely, lot A. As this work progressed, however, it became necessary to renew our supply of DFP, and it soon became apparent that subsequent batches of DFP from Aldrich, as well as from other sources, varied widely in their ability to inhibit ficin (see Table II). This was true despite the fact that all of these samples of DFP markedly inhibited chymotrypsin at a mole ratio slightly in excess of 1. This marked variation in the capacity to inhibit ficin, coupled with the fact that the mole ratio of DFP to enzyme necessary to effect this inhibition was frequently 100 times that usually required to inhibit the animal proteases, led us to suspect that the DFP might contain an impurity which is the actual inhibitory agent rather than DFP per se.

In order to dissociate the ficin-inhibitory effect from the effect of DFP itself, a sample of Aldrich DFP (lot G) was treated with a mixture of cupric ions and histidine, a procedure reported to catalyze the hydrolysis of DFP (Wagner-Jauregg *et al.*, 1955). During the course of this hydrolysis the reaction mixture was assayed for its ability to inhibit chymotrypsin as well as ficin. From the results shown in Table III it is apparent that, although DFP rapidly lost its capacity to inhibit chymotrypsin, its inhibitory action toward ficin was only slightly diminished.

By means of fractional distillation as described in Table IV, it was possible to effect a separation of the ficin inhibitor from the DFP itself. The bulk of the DFP could be distilled at a temperature of 36° *in*

TABLE II: Inhibitory Potency of Various Commercial Samples of DFP.

Source of DFP[a]	Mole Ratio DFP to Ficin Producing Total Inactivation[b]
Aldrich A	13
B	118
C	171
D	112
E	86
F	315
G	467
H	500
I	672
Merck (lot 62360)	No inhibition at 500
Calbiochem	157
Sigma (lot 44B-0450)	No inhibition at 300

[a] See text footnote 2. [b] Solutions of 0.1 mM ficin in 0.1 M phosphate buffer, *p*H 7.9, were treated with 0.1 ml 2-propanol containing various levels of DFP. Activity was measured after 10 minutes at room temperature. The mole ratio DFP to ficin producing total inactivation was obtained by extrapolation to zero of curves relating per cent of activity in absence of DFP to mole ratio DFP to ficin (see, for example, activity curve in Figure 1A obtained with Aldrich DFP, lot A).

TABLE III: Inhibition of Ficin and Chymotrypsin by Aldrich DFP Subjected to Catalytic Hydrolysis by the Method of Wagner-Jauregg *et al.* (1955).[a]

Reaction Time (min)	Inhibition (%)[b]	
	Ficin	Chymotrypsin
	Trial 1	
2	64	100
5	53	64
60	42	0
	Trial 2	
2	54	86
30	45	13
60	42	8

[a] To 8 ml of a solution containing 625 μmoles each of $CuSO_4$ and histidine was added 0.025 ml Aldrich DFP (lot G) and the *p*H was maintained at 7.6 in a *p*H-stat at 30°. At various intervals of time 0.1-ml aliquots were added to 10 mg EDTA (to chelate excess cupric ions), and 0.025 ml of this solution was added to 0.2 ml of 0.01 mM solution of ficin or chymotrypsin (final mole ratio of DFP to enzyme, 195). Activity was measured after 30 minutes at room temperature. [b] Relative to controls from which DFP had been omitted.

TABLE IV: Inhibition of Ficin and Chymotrypsin by Fractions Distilled from Aldrich DFP.[a]

Fraction No.	Temperature Range	Inhibition (%)[b]	
		Ficin	Chymotrypsin
I	20–36°	0	100
II	37–46°	0	100
III	47–56°	0	100
IV	Residue	95	82

[a] One g of Aldrich DFP (lot G) was distilled under vacuum (0.06 mm mercury) and the distillates were collected in receivers immersed in dry ice–acetone. Fractions II and III were trapped in 4 ml 2-propanol. Residue remaining in distillation flask, fraction IV, was dissolved in 1 ml 2-propanol. [b] Each fraction was tested at a level which would have given 100% inhibition of ficin if it contained all of the original ficin inhibitory activity. The same levels were tested for their ability to inhibit chymotrypsin. Details of assay are described in Table II.

vacuo (fraction I), with smaller amounts of DFP distilling at temperature ranges of 37–46° (fraction II) and 47–56° (fraction III). All these fractions markedly inhibited chymotrypsin but had no measurable effect on ficin. The residue that remained in the distillation vessel (fraction IV) contained about 95% of the ficin-inhibitory activity in the original sample of DFP, although this fraction still contained some DFP as evidenced by its ability to inhibit chymotrypsin. Fractions I and IV will hereafter be referred to as "distilled DFP" and "inhibitor," respectively.

Comparison of Distilled DFP and Inhibitor

Reaction with SH Groups. The distilled DFP and the inhibitor derived therefrom were compared with respect to their action on the SH groups of ficin, cysteine, and glutathione. The data in Table V show that the inhibitor reacts with the SH groups of cysteine and ficin, causing inactivation in the latter case. Distilled DFP had no significant effect on the proteolytic activity of ficin nor the SH groups of ficin and cysteine. Parallel experiments with benzoyl-L-arginine ethyl ester as the substrate, using the method previously described (Liener, 1961), also showed that the esterolytic activity of ficin was not affected by distilled DFP. Table V also shows that Merck DFP, which had previously been noted to be noninhibitory toward ficin (see Table II), behaved in essentially the same fashion as distilled DFP. When the inhibitor was added to glutathione and the mixture was placed on the amino acid analyzer, the same unknown reaction product which had been obtained previously with unpurified Aldrich DFP (see Figure 2) was again noted in the chromatogram. Glutathione treated with distilled DFP under the same conditions was unchanged.

TABLE V: Effect of Inhibitor, Distilled Aldrich DFP, and Merck DFP on the SH Content of Ficin and Cysteine.[a]

System	SH Content (mole/mole ficin or cysteine)	Activity (%)
Ficin	0.60	100
Ficin + inhibitor	0.15	10
Ficin + distilled DFP	0.58	98
Ficin + Merck DFP	0.53	95
Cysteine	0.79	
Cysteine + inhibitor	0.05	
Cysteine + distilled DFP	0.75	

[a] To a 2-ml solution of 1 μmole ficin or cysteine was added 0.25 ml of inhibitor, or 0.5 ml distilled Aldrich DFP or Merck DFP. The *p*H was adjusted to 7.0 and the volume was brought to 5 ml with distilled water. SH content and activity were determined after the solutions had been allowed to stand at 30° for 30 minutes. Controls consisted of ficin or cysteine alone under the same experimental conditions.

Phosphorylation. To determine whether the inhibitor or distilled DFP was responsible for the phosphorylation of ficin as depicted in Figure 1, ficin was treated with each of these components at various *p*H values, and the amount of phosphorus incorporated into the protein was measured. The results of this study are shown in Figure 3, where striking differences may be noted with respect to activity and uptake of phosphorus. Although incapable of inhibiting ficin over a *p*H range of 4–9, the distilled DFP nevertheless phosphorylated the enzyme in a *p*H-dependent manner (Figure 3A). Phosphorylation was enhanced at the higher *p*H values, and, at *p*H 9, as many as 2 moles of phosphorus were introduced into the ficin molecule. Conversely, the inhibitor inactivated ficin by a process which was also *p*H dependent but which was not accompanied by any significant uptake of phosphorus (Figure 3B).

Phosphorylation experiments were also conducted with the noninhibitory sample of Merck DFP. As the data in Table VI show, Merck DFP likewise phosphorylated ficin without affecting its activity. Furthermore, blockage of the SH group of ficin with mercury or by carboxymethylation did not interfere with phosphorylation, thus indicating that the SH group cannot be the site of phosphorylation.

Possible Sites of Phosphorylation

Other possible sites of phosphorylation by DFP are the aliphatic hydroxyl (Cohen *et al.*, 1959), free amino (Viswanatha, 1957), and phenolic (Ashbolt and Rydon, 1952; Jandorf *et al.*, 1952) groups. Which of these

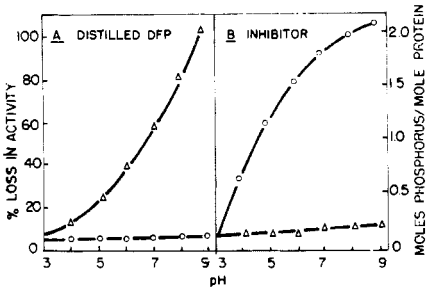

FIGURE 3: Comparison of distilled DFP and inhibitor with respect to their effect on the activity and uptake of phosphorus by ficin as a function of *p*H. Solutions containing 21.3 mg ficin (0.82 μmole) and 0.05 ml distilled DFP or inhibitor solution were prepared in a final volume of 10 ml in 0.1 M borate buffer, *p*H 8.5. After the solution had stood at 37° for 90 minutes, activity was measured, and the protein was precipitated by adding an equal volume of saturated $(NH_4)_2SO_4$. Phosphorus determinations were made on the dialyzed protein solution. ○—○, activity; △—△, phosphorus incorporated into protein.

groups might be phosphorylated in the ficin molecule was determined indirectly by measuring the content of these functional groups before and after treatment with distilled DFP. Mercurificin was used in these studies to block the activity of the SH group.

Phenolic Groups. Unmodified tyrosine residues were determined on the intact protein with Folin's phenol reagent under mild alkaline conditions which do not lead to the hydrolysis of any *O*-acyl linkages of tyrosine that might be present (Herriott, 1935). The data in Table VII show no significant decrease in tyrosine content despite the fact that almost 6 moles of phosphorus had been introduced into the molecule.

Aliphatic Hydroxyl Groups. For measuring nonacylated aliphatic hydroxyl groups, advantage was taken of the fact that such groups can be acetylated with

TABLE VI: Inhibition and Phosphorylation of Ficin, CM-Ficin, and Mercurificin by Merck DFP.[a]

	Mole Ratio DFP/ Enzyme	Activity (%)[b]	P Incorporated (mole/mole enzyme)
Ficin	0	100	0.07
	19	99	0.20
	38	100	0.33
	75	100	0.50
	150	95	0.69
	300	97	1.01
CM-ficin	300		1.00
Mercurificin	300		1.58

[a] Various levels of Merck DFP were added to 21.3 mg ficin or derivative (0.82 μmole) in a final volume of 10 ml 0.1 M borate buffer, *p*H 8.5. After the solution had stood at 37° for 90 minutes, activity was measured and protein was precipitated by adding an equal volume of saturated $(NH_4)_2SO_4$. Phosphorus determinations were made on the dialyzed protein. [b] Activity in absence of DFP taken as 100%.

TABLE VII: Phosphorus and Tyrosine Content of Mercurificin Treated with Distilled DFP.[a]

Mole Ratio DFP/Ficin	Tyrosine Residues (mole/mole protein)	P Incorporated (mole/mole protein)
0	15.1	0
45	15.2	2.5
90	15.2	3.3
180	15.0	5.4
360	14.8	5.7

[a] Various levels of distilled DFP were added to solutions containing 3 μmoles mercurificin in a final volume of 10 ml 0.2 M borate buffer, *p*H 8.2. After 18 hours at room temperature, 0.5-ml aliquots were analyzed for tyrosine with Folin's reagent. The remainder of the reaction mixture was dialyzed and assayed for phosphorus.

TABLE VIII: Phosphorus and Aliphatic Hydroxyl Content of Mercurificin Treated with Distilled DFP.[a]

Mole Ratio DFP/Ficin	*O*-Acetyl Groups (mole/mole protein)		P Incorporated (mole/mole protein)
	Total	Net Loss	
0	5.23	0	0
16	4.91	0.48	0.32
32	4.32	0.87	0.91
65	4.22	1.28	1.01
130	3.93	1.64	1.30

[a] Various levels of distilled Aldrich DFP were added to solutions containing 1.2 μmoles mercurificin in a final volume of 10 ml 0.2 M borate buffer, *p*H 8.2. After the solution had stood at room temperature for 18 hours, the protein was precipitated by half-saturation with $(NH_4)_2SO_4$, dialyzed, and acetylated with acetic anhydride in acetate buffer according to Sri Ram *et al.* (1954). The volume of the acetylated protein solution after dialysis was brought to 10 ml with distilled water and aliquots were removed for the determination of *O*-acetyl groups (Uraki *et al.*, 1957), protein nitrogen, and phosphorus.

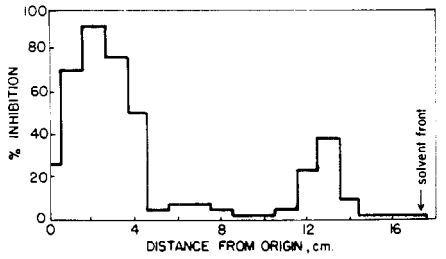

FIGURE 4: Thin-layer chromatography of inhibitor. Fraction IV (0.25 ml) obtained by the fractional distillation of Aldrich DFP (lot G) was applied to a plate (20 × 20 cm) coated with silica gel G and developed with 1.4% acetone in petroleum ether for 2 hours at room temperature. A 13-mm lateral strip of the gel was sprayed for phosphorus. The remainder of the gel was divided into 1-cm horizontal segments and scraped into tubes containing 1 ml 2-propanol. After thorough shaking, 0.1 ml aliquots were added to an equal volume of a solution containing 0.02 μmole ficin per ml and the mixture was tested for activity. Per cent inhibition was calculated by comparison with enzyme controls without inhibitor. The origin gave the strongest test for phosphorus although a weak response was also given by the segment located 15 cm from the origin.

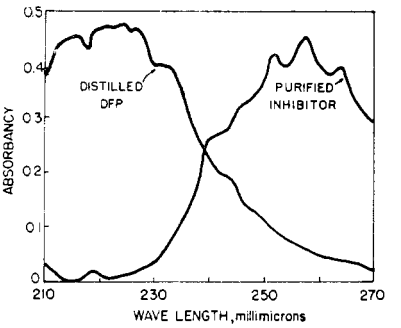

FIGURE 5: Ultraviolet spectrum of inhibitor purified by thin-layer chromatography and distilled DFP. Solvent, 2-propanol; concentration of distilled DFP, 5 mM. Concentration of inhibitor selected to give an absorption maximum essentially equivalent to that of the DFP.

acetic anhydride in acetate buffer (Sri Ram *et al.*, 1954; Uraki *et al.*, 1957). The *O*-acetyl groups, as determined with hydroxylamine (Uraki *et al.*, 1957), may then be taken as a measure of the unblocked hydroxyl groups of serine or threonine. Such data (Table VIII) show that increasing concentrations of DFP caused a decrease in the number of aliphatic hydroxyl groups which could be acetylated and a concomitant increase in the amount of phosphorus which had been introduced into the protein. Since there are 10 threonine and 16 serine residues in ficin,[5] it is evident that only one-fifth of the total number of alkylhydroxyl groups had been acetylated even in the absence of DFP. Nevertheless, the close correspondence between the decrease in *O*-acetyl groups and the amount of phosphorus incorporated would suggest that those aliphatic hydroxyl groups which are most readily acetylated are also those which are most easily phosphorylated. Since the amount of phosphorus taken up by the protein may be very nearly accounted for by the decrease in free aliphatic hydroxyl groups, it may be assumed that the phosphorylation of amino groups could not have occurred to any significant extent.

Attempts to Further Purify and Characterize the Inhibitor

When the inhibitor, as represented by fraction IV obtained by fractional distillation, was chromatographed on paper in a variety of solvent systems, the ficin-inhibitory activity invariably migrated with the solvent front, and at least three phosphorus-containing components could be detected, one of which also migrated with the solvent front. Thin-layer chromatography proved more effective in resolving the crude inhibitor fraction. When the latter was chromatographed on a thin layer of silica gel G (Stahl) with a solvent system composed of 1.4% acetone in petroleum ether (Kovac, 1963), the bulk of the inhibitory activity migrated a short distance (3–4 cm) from the origin, leaving most of the phosphorus-containing material at the origin (Figure 4). A small amount of inhibitory material and phosphorus could also be located about 13 and 15 cm from the origin, respectively. A sufficient amount of material could be recovered from the main inhibitor component by elution with 2-propanol to permit a determination of its ultraviolet spectrum. Figure 5 shows a striking difference between the ultraviolet spectrum of the purified inhibitor and that of the distilled DFP.

Discussion

The recognition that there exists in some commercial preparations of DFP an impurity which reacts with SH groups provides a reasonable explanation for many of the controversial issues that have arisen in connection with studies of the effect of DFP on SH enzymes. Simply stated, whether the inactivation of an SH enzyme will be observed or not depends on (a) how much of this impurity is present in the particular lot of DFP being studied, and (b) the presence or absence of SH compounds as activators. The fact that commercial samples of DFP may vary in their ability to inhibit SH enzymes had previously been noted by Ebata and coworkers (Ebata *et al.*, 1962; Ebata and Yasunobu, 1963), and is amply attested to by the data presented in this paper.

The protection which cysteine, but not KCN, affords against inactivation of SH enzymes by DFP has prompted some rather bizarre explanations by other investigators who have also made this observation (Masuda, 1959; Ota *et al.*, 1961; Ebata *et al.*, 1962; Ebata and Yasunobu, 1963). Masuda (1959) postulated

[5] N. R. Gould and I. E. Liener, unpublished data.

that, since KCN appeared to be essential for the inactivation of papain by DFP, one of the potential active sites of this enzyme is a carbonyl group which forms a cyanohydrin when activated by cyanide; the hydroxyl component of this new group then becomes the site of phosphorylation by DFP. He explained the protective effect of cysteine by stating that "cysteine reacts with cyanide-activated papain and makes it unsusceptible to DFP." Ebata and Yasunobu (1963), working with chymopapain, theorize that the effect of activators is to reduce a critical disulfide bond which serves to expose the DFP binding site which they believe to be serine. No explanation is offered, however, for the fact that cysteine, in sufficiently high concentration, actually prevents DFP from inactivating chymopapain.

Based on the evidence presented here, another explanation for the protective effect of cysteine would be that an impurity of DFP preferentially reacts with the thiol group of cysteine, especially when it is present in large excess compared to the SH content of the enzyme, thus rendering the impurity ineffective. Because cysteine did not protect the proteinase of *Bacillus subtilis* N′ from inactivation by DFP, Masuda (1959) did not believe that DFP reacts directly with cysteine. It is clear now that the reason cysteine did not prevent DFP from inhibiting this bacterial proteinase is simply because it is not an SH enzyme and is therefore inhibited by DFP rather than the impurity. In this regard it will be recalled that, although cysteine prevented the inactivation of ficin by Aldrich DFP, phosphorylation of the enzyme nevertheless still occurred (Figure 1B). The explanation we propose for inactivation in the presence of KCN is that this compound activates SH enzymes by reducing oxidized thiol groups, thus making a higher level of thiol groups available for reaction with the impurity and consequent inactivation of the enzyme. The data of Masuda (1959) and Ebata and Yasunobu (1963) indicate that their inactivated enzymes were essentially devoid of activity so that the requirement for cyanide prior to inactivation by DFP simply reflects the fact that an active enzyme with free SH groups is necessary before inhibition can be demonstrated.

Although our data clearly imply an irreversible combination of the impurity with the SH group of ficin, it was not possible to demonstrate the formation of a reaction product with cysteine itself on the amino acid analyzer. It is conceivable that the impurity might have reacted with the amino group as well as, or instead of, the thiol group of cysteine, thus yielding a product which would have escaped detection by failing to react with ninhydrin. This question was partially resolved by treating glutathione with impure DFP and analyzing the hydrolyzed product. Although the recovery of glutamic acid and glycine was quantitative, only 20% of the cysteine could be accounted for, thus implicating the SH group of the cysteine residue as the site of reaction. On the other hand, when the reaction mixture of glutathione and the impurity was analyzed directly a new unidentified component appeared on the chromatogram in a position readily differentiated from reduced or oxidized glutathione. It would appear, therefore, that, although the impurity reacts with the SH group of peptide-bound cysteine, subsequent release of this modified residue from its peptidic environment renders it nondetectable under conditions conventionally employed for the analysis of amino acids by ion-exchange chromatography.

Wholly unrelated to the action of the impurity is the observation that DFP which had been purified by distillation phosphorylates ficin without affecting its SH content or its activity. This confirms the findings of Murachi (1963) who likewise observed that his particular sample of DFP, a product of Mann Research Laboratories, New York City, was capable of phosphorylating a number of SH enzymes without affecting their activity. Again in agreement with Murachi (1963), phosphorylation was found to be *p*H dependent, a greater incorporation of phosphorus being obtained under alkaline conditions. Quite coincidentally, the inhibition of ficin by the impurity also increases as the *p*H is raised. Those who have reported SH enzymes to be inhibited by DFP have also noted this inhibition to be *p*H dependent (Heinicke and Mori, 1959; Ebata and Yasunobu, 1963). It is not surprising therefore that phosphorylation and inactivation have been assumed to bear a cause-and-effect relationship to each other, an assumption which appeared to be supported by the isolation of crystalline phosphorylated derivatives of DFP-inhibited papain (Masuda, 1959) and chymopapain (Ebata and Yasunobu, 1963). From our present vantage point, it would now appear that such preparations had indeed been phosphorylated by DFP but were inactive as a consequence of the action of the inhibitor on their SH groups.

Indirect evidence has been obtained to indicate that the serine (and/or threonine) residues of ficin are the most likely sites of phosphorylation. Although this finding should obviously be confirmed by more direct techniques, it is, nevertheless, of interest to note that Masuda (1959) was able to isolate a phosphopeptide from DFP-treated papain that was composed of glycine, alanine, serine, and glutamic or aspartic acid. Although they did not identify the specific amino acid which had been phosphorylated, the serine residue would be the most logical one to expect. Murachi and Inagami (1963), on the other hand, have reported that the tyrosine residues of stem bromelain are alkylphosphorylated by DFP. This lack of agreement may simply be a reflection of intrinsic differences in the nature of the enzymes.

As to the possible origin of this contaminant, it does not appear to be a decomposition product of DFP since prolonged storage of DFP at elevated temperatures did not produce any appreciable increase in its ficin-inhibitory activity. It seems more likely that this impurity probably arises during the course of the preparation of DFP. It would be helpful to know which of the several methods which have been described for the preparation of DFP (McCombie *et al.*, 1945; Chapman and Saunders, 1948; Saunders and Stacey, 1948; Monard and Jean, 1952) had been used to prepare the various samples used in this study. Through the cooperation of the manufacturers and distributors of DFP we are at-

tempting to correlate such information with the ficin-inhibitory activity of samples which have been made available to us.[6]

References

Ashbolt, R. F., and Rydon, H. N. (1952), *J. Am. Chem. Soc., 74*, 1865.

Bartlett, G. R. (1959), *J. Biol. Chem. 234*, 466.

Brigham, M. P., Stein, W. H., and Moore, S. (1960), *J. Clin. Invest. 39*, 1633.

Chapman, N. B., and Saunders, B. C. (1948), *J. Chem. Soc.*, 1010.

Cohen, J. A., Oosterbaan, R. A., Jansz, H. S., and Birends, F. (1959), *J. Cell. Comp. Physiol. 54, Suppl. 1*, 231.

Ebata, M., Tsunoda, J. S., and Yasunobu, K. T. (1962), *Biochem. Biophys. Res. Commun. 9*, 173.

Ebata, M., and Yasunobu, K. T. (1963), *Biochim. Biophys. Acta 73*, 132.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.

Gould, N., Wong, R. C., and Liener, I. E. (1963), *Biochem. Biophys. Res. Commun. 12*, 469.

Hammond, B. R., and Gutfreund, H. (1959), *Biochem. J. 72*, 349.

Hanes, C. W., and Isherwood, F. A. (1949), *Nature 164*, 1107.

Heinicke, R. M., and Mori, R. (1959), *Science 129*, 1678.

Herriott, R. M. (1935), *J. Gen. Physiol. 19*, 283.

Jandorf, B. J., Wagner-Jauregg, T., O'Neill, J. J., and Stolberg, M. A. (1952), *J. Am. Chem. Soc. 74*, 1521.

Jansen, E. F., Nutting, M. D. F., and Balls, A. K. (1948), *J. Biol. Chem. 175*, 975.

Kimmel, J. R., and Smith, E. L. (1954), *J. Biol. Chem. 207*, 515.

Koshland, D. E., Jr. (1963), *Science 142*, 1533.

Kovac, J. (1963), *J. Chromatog. 11*, 412.

Kunitz, M. (1947), *J. Gen. Physiol. 30*, 291.

Lanni, F., Dillon, M. L., and Beard, J. W. (1950), *Proc. Soc. Exptl. Biol. Med. 74*, 4.

Liener, I. E. (1961), *Biochim. Biophys. Acta 53*, 332.

McCombie, H., Saunders, B. C., and Stacey, G. J. (1945), *J. Chem. Soc.*, 380.

Masuda, T. (1959), *J. Biochem. (Tokyo) 46*, 1569.

Monard, C., and Jean, H. (1952), *Bull. Soc. Chim. France*, 544.

Murachi, T. (1963), *Biochim. Biophys. Acta 71*, 239.

Murachi, T., and Inagami, T. (1963), Abstracts, 19th International Congress of Pure and Applied Chemistry, p. 303.

Murachi, T., and Neurath, H. (1960), *J. Biol. Chem. 235*, 99.

Ota, S., Fu, T.-H., and Hirohata, R. (1961), *J. Biochem. (Tokyo) 49*, 532.

Saunders, B. C., and Stacey, G. J. (1948), *J. Chem. Soc.*, 695.

Sgarbieri, V. C., Gupte, S. M., Kramer, D. E., and Whitaker, J. R. (1964), *J. Biol. Chem. 239*, 2170.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Sri Ram, J., Terminiello, L., Bier, M., and Nord, F. F. (1954), *Arch. Biochem. Biophys. 52*, 464.

Uraki, Z., Terminiello, L., Bier, M., and Nord, F. F. (1957), *Arch. Biochem. Biophys. 69*, 644.

Viswanatha, T. (1957), *Compt. Rend. Trav. Lab. Carlberg Sér. Chim. 30*, 183.

Wagner-Jauregg, T., Hackley, B. E., Jr., Lies, T. A., Owens, O. O., and Proper, R. (1955), *J. Am. Chem. Soc. 77*, 922.

[6] We are particularly grateful to Dr. Louis Berg, Sigma Chemical Co., Dr. Alfred Bader, Aldrich Chemical Co., and Mr. Earl Pierson, Merck and Co., for samples of DFP and information pertaining thereto.