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A STUDY OF THE EFFECT OF SOME DRUGS WHICH CAUSE AGRANULOCYTOSIS ON THE BIOSYNTHESIS OF PYRIMIDINES IN HUMAN GRANULOCYTES

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Abstract—The activities of the first five enzymes of *de novo* pyrimidine biosynthesis [carbamoylphosphate: L-aspartate transferase (EC 2.1.3.2), L-4,5-dihydroorotate amino-hydrolase (EC 3.5.2.3), L-4,5-dihydroorotate, O_2 oxidoreductase (EC 1.3.3.1), orotidine-5′-phosphate–pyrophosphate phosphoribosyltransferase (EC 2.4.2.10) and orotidine-5′-phosphate carboxy–lyase (EC 4.1.1.23)] have been measured in human granulocytes. All five enzymes can be measured in the granulocytes (approximately 5×10^7 cells) from 20 ml blood using the ultrasensitive radiochemical methods described. The effect of amidopyrine, carbimazole, chloramphenicol, chlorpromazine, phenylbutazone and methylthiouracil on the activity of these enzymes in human granulocyte lysates has been investigated. Phenylbutazone inhibited L-4,5-dihydroorotate, O_2 oxidoreductase (EC 1.3.3.1). Carbimazole either inhibited or stimulated carbamoylphosphate, L-aspartate transferase (EC 2.1.3.2) depending on the experimental conditions. Thus, when the substrate and inhibitor concentrations were held constant and the amount of the enzyme in the system varied, the drug was stimulatory at very low enzyme concentrations and inhibitory at higher enzyme concentrations. No other examples of enzyme inhibition or activation were encountered.

DEPRESSION of bone marrow function is a serious hazard of drug medication, and it occurs unpredictably in a small proportion of the population at risk. Drug induced autoimmunity does not explain all of the cases, and these considerations suggest that some patients may be biochemically predisposed to respond in this manner. If the biochemical lesion was such that nucleic acid synthesis is abnormally sensitive to inhibition by the drug or its metabolites, the production of granulocytes would be arrested. The work of Pisciotta and his colleagues[1,2] who introduced the concept of "the limited proliferative potential" of the bone marrow in chlorpromazine sensitive patients gives some general support to such a hypothesis.

The present work was undertaken to explore the action of six drugs which sometimes cause agranulocytosis, on the five enzymes responsible for the biosynthesis of uridine-5′-phosphate (UMP), this being the intermediate compound from which the pyrimidine nucleotides needed for DNA and RNA synthesis are formed. The enzymes which we have studied are; aspartate transcarbamylase (carbamoylphosphate, L-aspartate transferase, EC 2.1.3.2), dihydroorotase (L-4,5-dihydroorotate amidohydrolase, EC

3.5.2.3), dihydroorotate dehydrogenase (L-4,5-dihydroorotate, O_2 oxidoreductase, EC 1.3.3.1), orotate phosphoribosyltransferase (orotidine-5′-phosphate:pyrophosphate phosphoribosyltransferase, EC 2.4.2.10), and orotidine-5′-phosphate decarboxylase (orotidine-5′-phosphate carboxylyase, EC 4.1.1.23). The relevant metabolic interrelationships are shown in Fig. 1.

The present studies show that mature human granulocytes contain these enzymes. It was possible, therefore, by using ultra-sensitive assays, to study the human enzymes in cells which are part of the tissue system upon which the drugs exert their toxic action *in vivo*.

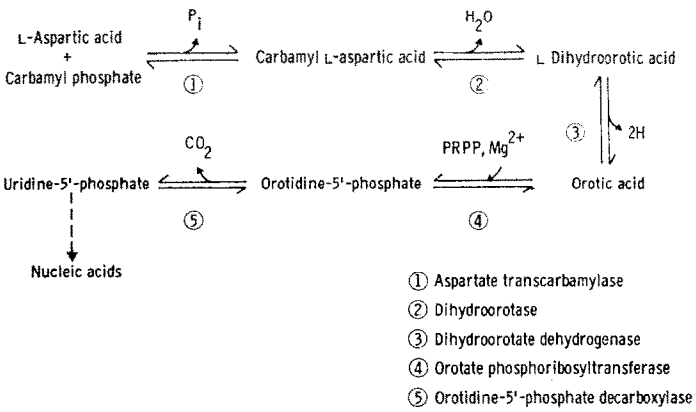


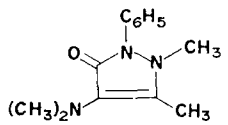
FIG. 1. The pathway for *de novo* pyrimidine biosynthesis.

METHODS AND MATERIALS

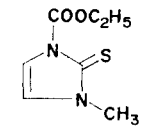
The reagents were the highest grade of purity available and aqueous solutions were prepared with deionised water. Bovine serum albumin solution (30% w/v) was purchased from Stayne Laboratories Ltd.* Non-radioactive biochemicals were obtained from Sigma (London) Chemical Co., Ltd. [U-^{14}C]L-aspartic acid (stated radiochemical purity >97 per cent) and [6-^{14}C]orotic acid (stated radiochemical purity >98 per cent) were purchased from the Radiochemical Centre, Amersham. [6-^{14}C]DL-dihydroorotic acid was synthesized by the hydrogenation of [6-^{14}C]orotic acid in the presence of a rhodium catalyst.[3] The product was approximately 94% [6-^{14}C]DL-dihydroorotic acid and there was negligible radioactive contamination with orotic acid or carbamylaspartic acid. [6-^{14}C]orotidine-5′-phosphate (OMP) was prepared enzymatically using a purified preparation of yeast orotate phosphoribosyltransferase, [6-^{14}C]orotic acid and α-D-ribofuranose-1-pyrophosphate-5-phosphate (PRPP). The method followed was essentially that of Lieberman, Kornberg and Simms,[4] and the final product contained approximately 90% [6-^{14}C]OMP.

* The bovine serum albumin solution contained 0·1% (w/v) sodium azide as a preservative. It was established that this did not interfere with any of the enzyme assays.

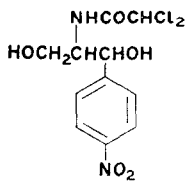
The six drugs used were: amidopyrine (Hopkin & Williams Ltd.), carbimazole (Nicholas Laboratories Ltd.), chloramphenicol (Carlo Erba, (U.K.) Ltd.), chlorpromazine hydrochloride (May & Baker Ltd.), methylthiouracil (Zimmerman & Co., Ltd.) and phenylbutazone (Geigy, (U.K.) Ltd.). The structural formulae of the drugs are shown below.



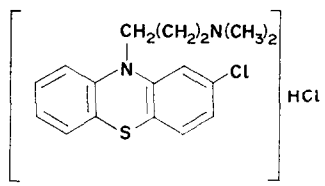
Amidopyrine



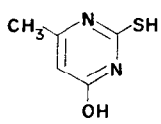
Carbimazole



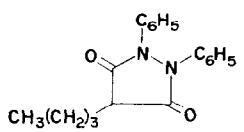
Chloramphenicol



Chlorpromazine Hydrochloride



Methylthiouracil



Phenylbutazone

Separation of granulocytes from blood

Plastic vessels or siliconized glass vessels were used for all of these operations. Heparinized venous blood was mixed with a solution of dextran (MW $1–2 \times 10^5$, concentration = 1·2% (w/v) in a buffered salt solution[5] containing Mg^{2+}, 2·5 mM) in the syringe and allowed to stand at the ambient temperature (approximately 20°) for 40 min.[6] Most of the erythrocytes sedimented to the bottom of the syringe leaving a leukocyte rich cell suspension which was layered onto bovine serum albumin (approximately 5 ml of a 27% (w/v) solution) in a 50 ml centrifuge tube (internal diameter 2·5 cm). The granulocytes and the few contaminating erythrocytes were centrifuged (400 *g*, 12 min, 17°) through the layer of bovine serum albumin, the lymphocytes and platelets remaining at the interface between the dextran and bovine serum albumin solutions. The granulocyte pellet was resuspended in the balanced salt solution[5] containing added Mg^{2+} and the contaminating erythrocytes lysed.[7] On repeating the layering process, the erythrocyte ghosts remained on the albumin surface, and the cell pellet at the bottom of the tube consisted almost entirely of granulocytes. Sometimes, there was still a slight degree of contamination by erythrocytes but these could be removed by a second haemolytic shock. The granulocytes were washed twice with NaCl (0·9% w/v) containing glucose (0·1% w/v), centrifuged for 4 min at 100 *g* and finally resuspended in the NaCl–glucose solution. The final cell suspension

consisted of 98% granulocytes, 90–95 per cent of which were viable as judged by the trypan blue exclusion test.[8] The yield of cells was usually about 50 per cent, and the separated cells were kept at 0° until required, this period of storage never exceeding 2 hr.

Disruption of the cells

The cells were disrupted at 0° using an ultrasonic disintegrator (Measuring and Scientific Equipment Co., Ltd.) operating at 8 μ peak amplitude, 20 *K* cycles/sec. The length of time for which the cells were exposed to the ultrasonic vibrations was found to be important, different enzymes requiring different times (2–15 sec) to give optimum activities.

Protein determinations

The protein content of the disrupted cell preparations was measured colorimetrically.[9]

Enzyme assays

Ultramicroradiochemical assays were developed for each enzyme, the optimum substrate concentration and pH being established experimentally. In each assay, the amount of product formed was proportional to the time of incubation and to the enzyme concentration (measured as the total cell protein). Non-enzymatic synthesis of the products was measured using either boiled cells or water, only the aspartate transcarbamylase assay gave any significant non-enzymic reaction, although traces of ^{14}C-impurities in the substrates gave a blank value in most cases.

The drugs were dissolved in water or the appropriate tris–HCl buffer and either used immediately or stored at −20° for periods not exceeding 5 days. The drugs did not alter the pH of the assay systems. In most cases the concentrations of drugs used were within the range of the therapeutic plasma levels of the free drug, i.e. 1 μM − 0·1 mM.[10] Much higher drug concentrations were used in the aspartate transcarbamylase assays, because the optimum substrate concentrations for this enzyme are high.

The radioactive products of the reactions were separated from the radioactive substrates by either high voltage paper electrophoresis on Whatman's 3 MM paper (Miles Hivolt Ltd., 10 kv electrophoresis unit) or ion exchange paper chromatography using Whatman DE81 paper. Portions of the samples were co-electrophoresed or co-chromatographed with the corresponding non-radioactive markers (0·3 μmoles) so that the product could be located on the paper. The relevant areas of paper were cut out and immersed in a vial containing a toluene solution of 2,5-bis [5′-t-butylbenzoxazolyl(2′)]-thiophene (0·5% w/v) and the radioactivity measured in a liquid scintillation spectrometer (Nuclear Chicago Mark 1), 10^4 counts being collected on each occasion. The counting procedures were calibrated with each batch of determinations by counting replicate portions of the ^{14}C-labelled substrate on the type of paper used for the electrophoretic or chromatographic separation. The substrate and product are equivalent to one another on a molar basis in each case; hence, the amount of product formed during the incubation was calculated from the specific radioactivity of the substrate and the radioactivity of the product bearing area of the electrophoretogram or chromatogram.

Aspartate transcarbamylase. The assay mixture, total volume 250 μl, contained: 15 mM [U-^{14}C]L-aspartate (1 μci), 10 mM carbamyl phosphate, 0·1 M tris–HCl (pH 9·2) and granulocytes (0·2–0·6 mg protein) which had been sonicated for 10 sec. The drugs were studied at a final concentration of 1 mM except for chlorpromazine which was used at 0·1 mM. The system was incubated for 30 min at 37° and the reaction stopped with $HClO_4$ (25 μl 40% w/v solution) followed by KOH (25 μl 40% w/v solution) and cooling to 0°. The precipitated protein and potassium perchlorate were removed by centrifugation at 4°. The addition of the KOH solution not only precipitated $KClO_4$, but hydrolysed any [^{14}C]dihydroorotic acid formed by dihydroorotase back to [^{14}C] carbamylaspartate. Portions of the supernatant (50 μl) were electrophoresed in 0·25 M acetic acid (pH 2·7 for 45 min at 160 V/cm). Two portions of each sample were electrophoresed, one being used to locate the carbamylaspartate by dipping the paper strip in 4-dimethylaminobenzaldehyde (1% w/v solution in ethanol containing 1% v/v HCl) and heating at 80° for a few minutes, after which carbamylaspartate appeared as a yellow spot.

Dihydroorotase and dihydroorotic dehydrogenase. These enzymes were assayed simultaneously using [6-^{14}C]L-dihydroorotic acid as substrate. The dehydrogenase did not require added NAD^+. The assay system, total volume 150 μl, contained: 0·5 mM [6-^{14}C]L-dihydroorotic acid (0·15 μc), 0·1 M tris–HCl (pH 8·5) and granulocytes (0·2–0·5 mg protein) which had been sonicated for 2 sec. Phenylbutazone was used at a final concentration of 0·01 mM and the other drugs were studied at 0·05 mM. The system was incubated for 1 hr at 37°, and the reaction stopped by adding absolute ethanol (100 μl) followed by cooling to −10°. $HClO_4$ was not used because perchlorate interfered with the subsequent electrophoretic separation. The precipitated protein was removed by centrifugation. [^{14}C]carbamylaspartic acid was separated from [^{14}C] dihydroorotic acid and [^{14}C]orotic acid using an aqueous buffer (pH 6·1) containing 2% (v/v) pyridine and 0·16% (v/v) glacial acetic acid by high voltage electrophoresis (160 V/cm for 18 min). Using this buffer, it was possible to locate the carbamylaspartate by its quenching of U.V. light at 254 nm. [^{14}C]orotic acid was separated from [^{14}C]dihydroorotic acid and [^{14}C]carbamylaspartate by high voltage electrophoresis at pH 2·4 in a formic–acetic acid buffer (2·5% v/v glacial acetic acid in 0·32% v/v formic acid) at 160 V/cm for 30 min. The orotic acid spots were located by their quenching of U.V. light (254 nm).

Orotate phosphoribosyltransferase. The assay system, total volume 75 μl contained: 60 μM [6-^{14}C]orotic acid (0·27 μc), 1·0 mM PRPP, 2·5 mM $MgCl_2$, 0·1 M tris–HCl (pH 8·0) and granulocytes (0·1–0·3 mg protein) which had been sonicated for 10 sec. All of the drugs were used at a final concentration of 0·05 mM. The system was incubated for one hour at 37°, and the reaction was stopped by adding 50 μl of absolute ethanol followed by cooling to −10°. This procedure stopped the reaction effectively without interfering with the chromatography on ion-exchange paper as did perchlorate and EDTA.

In this assay the initial product OMP, is rapidly decarboxylated to UMP by the next enzyme in the pathway, and the sum of the radioactivity in both nucleotides was therefore used to measure the phosphoribosyltransferase activity. The [^{14}C]nucleotides, were separated from [^{14}C]orotic acid by descending chromatography on Whatman DE81 paper for 28 hr, the chromatogram being developed with ammonium formate (0·025 M). The nucleotides were located by their quenching of U.V. light (254 nm).

Orotidylic decarboxylase. The final assay system, 100 μl total volume, contained; 40 μM [6-^{14}C]OMP (0·25 μci), 0·1 M tris–HCl (pH 8·5) and granulocytes (20–50 μg protein) which had been sonicated for 15 sec. The samples were incubated for 45 min at 37°, and 50 μl 15% (w/v) $HClO_4$ were added to stop the reaction. After removing the protein precipitate, portions of the supernatants were electrophoresed using 0·015 M trisodium citrate solution (pH 4·05) containing 0·04% (w/v) EDTA for 30 min at 150 V/cm. The UMP was located by its quenching of U.V. light (254 nm).

Subjects. The subjects were healthy staff members who had no history of granulocytopoenia in response to the drugs which were being investigated. Blood samples were obtained from approximately equal numbers of men and women.

RESULTS

Normal values

The values for the activities of the enzymes are shown in Fig. 2. We have not given means and standard deviations because although probability plots show the aspartate transcarbamylase and dihydroorotic dehydrogenase activities to have normal frequency distributions, the data for the dihydroorotase and orotate phosphoribosyltransferase are log normally distributed, and those for orotate decarboxylase appear to have a bimodal frequency distribution.

The effects of the drugs on the activity of the enzymes

Aspartate Transcarbamylase. Figure 3 shows the results obtained in the presence of each of the six drugs. With the exception of carbimazole none of the drugs had any marked effect, though chloramphenicol, methylthiouracil and phenylbutazone appeared to be very slightly inhibitory under these conditions.

The effect of carbimazole was very variable and results ranged between 50–200 per

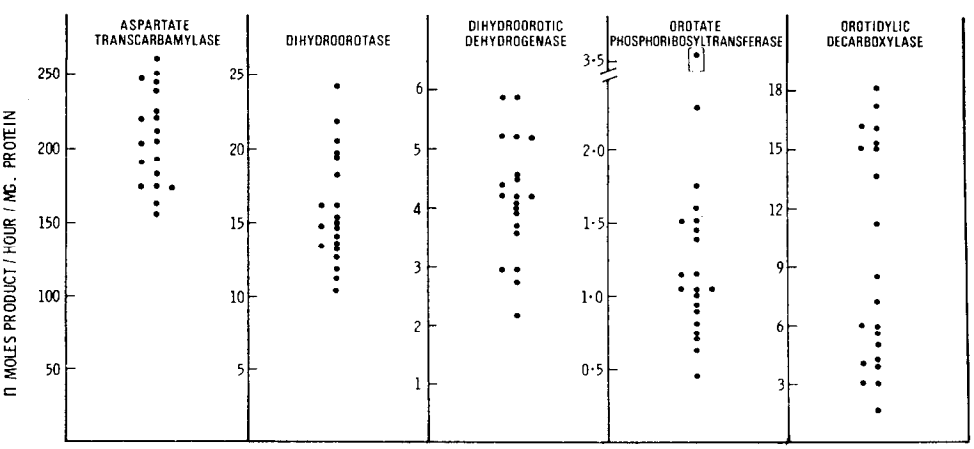


FIG. 2. The range of values for the first five enzymes of the pyrimidine biosynthetic pathway measured in granulocytes isolated from normal subjects. The values for orotate phosphoribosyltransferase were plotted on "log normal-probability" graph paper and the value in square brackets is that which lay far from the line defined by the rest of the sample.

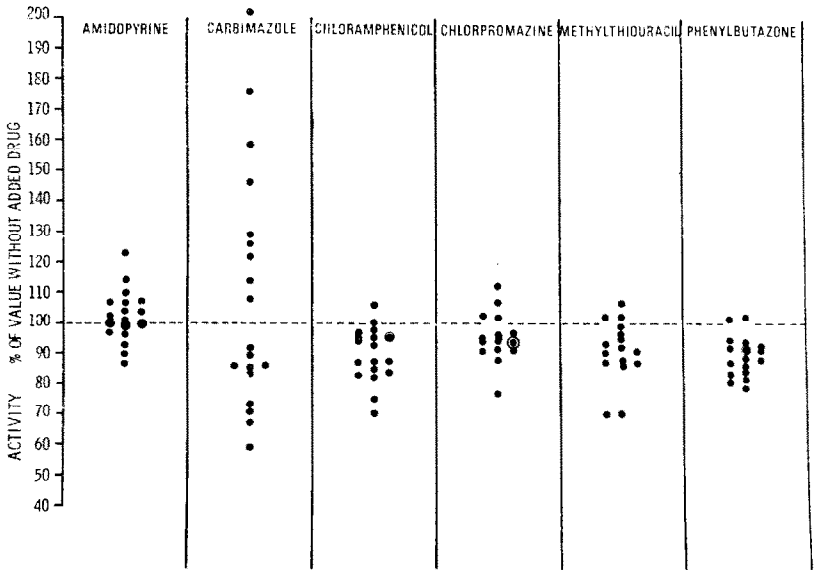


FIG. 3. The *in vitro* effect of drugs on aspartate transcarbamylase activity in disrupted granulocytes. The final concentration of each drug was 1 mM except for chlorpromazine which was 0·1 mM. Substrate concentrations were: 15 mM L-aspartate and 10 mM carbamylphosphate.

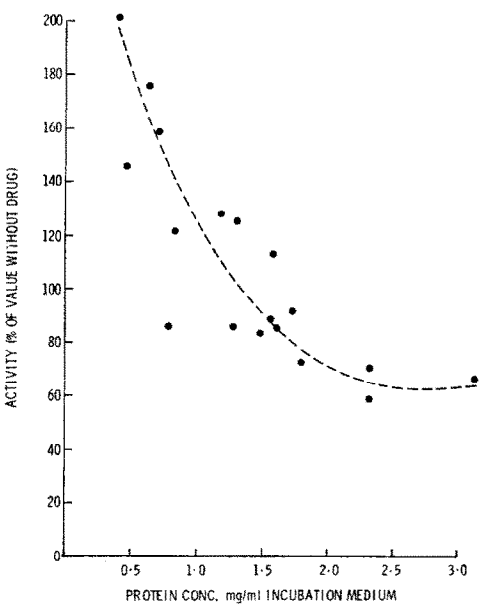


FIG. 4. The effect of 1 mM carbimazole on the aspartate transcarbamylase activity in normal subjects plotted as a percentage of the control value without added drug against the concentration of cell protein used in the final incubation medium.

cent of the control value. It was found that this effect depended on the concentration of cell protein used in the assay (Fig. 4).

Carbimazole was the only drug to have any effect on the nonenzymic reaction and at a concentration of 1 mM caused an almost 3-fold increase over the value without added carbimazole. The results which are presented in Fig. 4 have been corrected for for this effect.

Dihydroorotase. None of the drugs had any marked effect on the activity of this enzyme (Fig. 5).

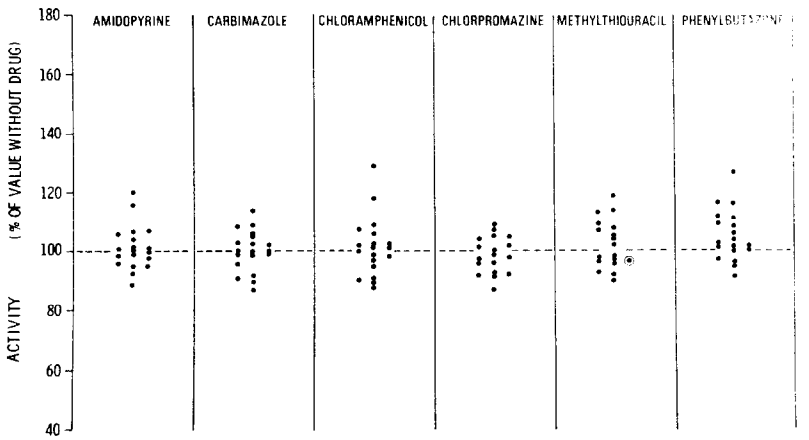


FIG. 5. The *in vitro* effect of drugs on dihydroorotase activity in disrupted granulocytes. The final concentration of each drug was 0·05 mM except for phenylbutazone which was 0·01 mM. L-dihydroorotate concentration was 0·5 mM.

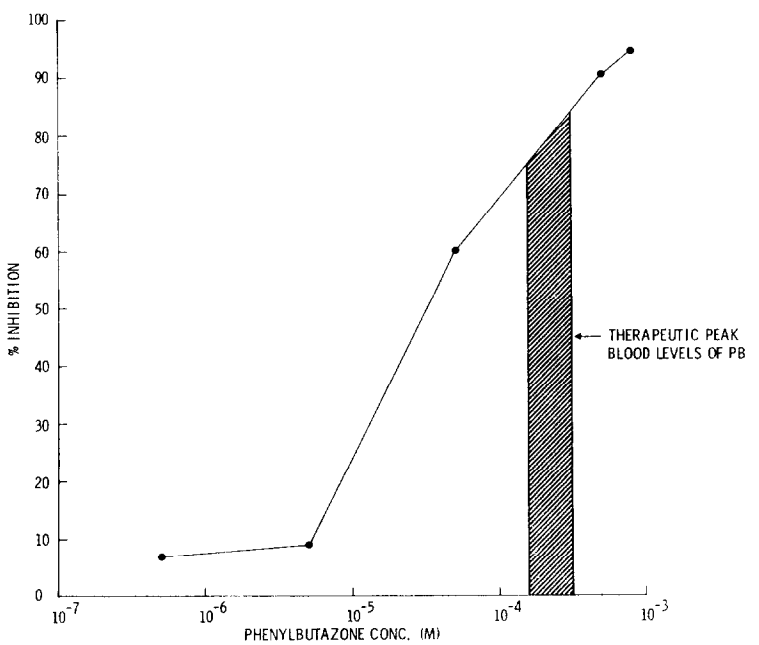


FIG. 6. The *in vitro* inhibition of dihydroorotic dehydrogenase by various concentrations of phenylbutazone. The final concentration of L- dihydroorotate used in the assays was 0·5 mM. The hatched area, ▨, represents the range of peak blood—levels of phenylbutazone with routine therapy.[11]

Dihydroorotic dehydrogenase. Phenylbutazone was a potent inhibitor of this enzyme (Fig. 6). At the concentration of phenylbutazone chosen for routine studies, the average value was 68 per cent of the control value without drugs, (Fig. 7). Lymphocytes separated from the blood of five of the normal subjects showed a similar degree of inhibition at this concentration of phenylbutazone i.e. 30–40 per cent inhibition.

None of the other drugs used appeared to have any effect. Neither did they have any effect in one case when they were used at 10 times the concentration i.e. at 0·5 mM.

Orotate phosphoribosyltransferase. Figure 8 shows that the drugs had no effect on the activity of this enzyme.

Orotate decarboxylase. Figure 9 shows that the drugs had no effect on the activity of this enzyme.

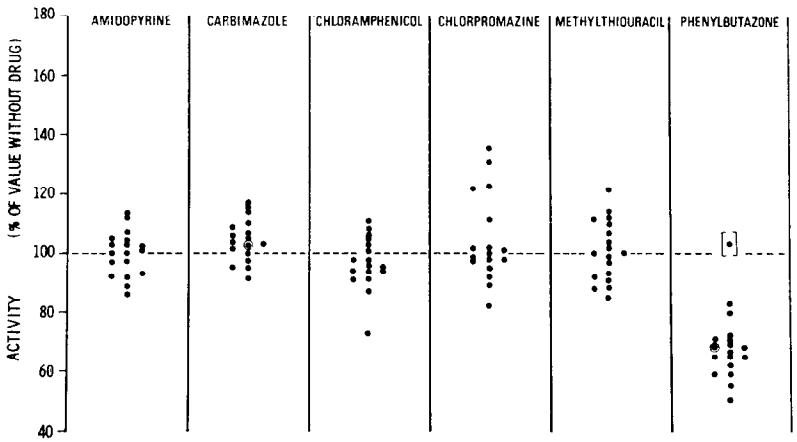


FIG. 7. The *in vitro* effect of drugs on dihydroorotic dehydrogenase activity in disrupted granulocytes. The final concentration of each drug was 0·05 mM except for phenylbutazone which was 0·01 mM. L-dihydroorotate concentration was 0·5 mM. The values with added phenylbutazone were plotted on "normal–probability" graph paper and the value in square brackets is that which lay far from the line defined by the rest of the sample.

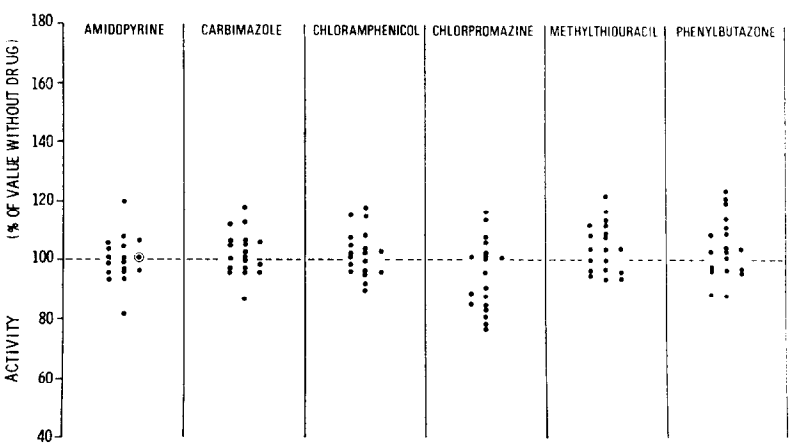


FIG. 8. The *in vitro* effect of drugs on orotate phosphoribosyltransferase activity in disrupted granulocytes. The final concentration of all drugs was 0·05 mM. The substrate concentrations were: 60 μM orotic acid, 1·0 mM PRPP and 2·5 mM $MgCl_2$.

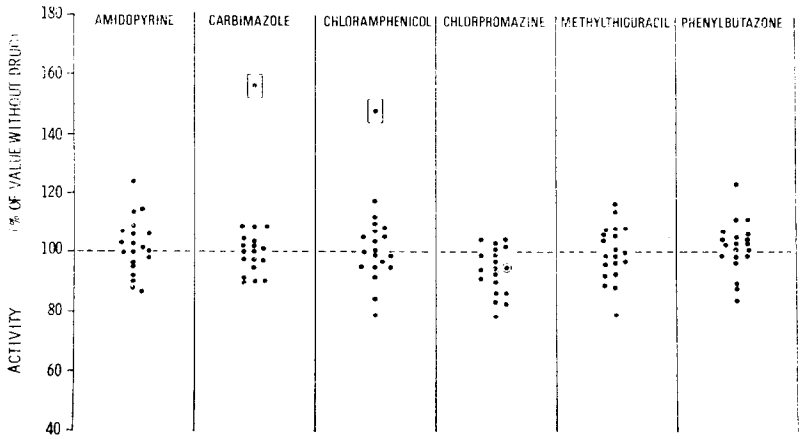


FIG. 9. The *in vitro* effect of drugs on orotidylic decarboxylase activity in disrupted granulocytes. The final concentration of all drugs was 0·05 mM. The substrate concentration was 40 μM. The values with added carbimazole and added chloramphenicol were each plotted on "normal-probability" graph paper and the values in square brackets are those which lay far from the corresponding line defined by the rest of the sample.

DISCUSSION

Smith *et al.*[12,13] made the only previous systematic study of all five enzymes in human blood cells, and found that the erythrocytes lack dihydroorotic dehydrogenase. These investigators did not differentiate between granulocytes and other types of white blood cells and related their values for the enzyme activities of the mixed leukocytes to a specified number of cells. We have related our results to the protein content of the disrupted cell preparation, because separated cells form clumps and are difficult to count accurately. The present results cannot be compared directly with those of Smith and his colleagues, although there is general agreement between the two sets of data. These workers[14] commented on the low and variable orotate phosphoribosyltransferase values for mixed leukocytes, and it appears from the present work that the range of the phosphoribosy transferase values may be wider in women than in men. The leukocyte dihydroorotic dehydrogenase does not require added NAD^+ unlike the purified enzyme from *Zymobacterium oroticum*.[15]

The inhibition of dihydroorotic acid dehydrogenase by phenylbutazone was specific to this drug among those tested and indicates that particular attention should be paid to the activity of this enzyme and its susceptibility to phenylbutazone in future studies with cells from patients in whom this drug has produced agranulocytosis. This specificity with respect to both drug and enzyme is in contra-distinction to the observations of McCurragh, Park and Perry[10] who studied the effect of amidopyrine, chlorpromazine, chloramphenicol, phenylbutazone and thiouracil on the oxygen consumption, lactate production and glucose uptake by granulocytes *in vitro*, and found that concentrations of amidopyrine, chlorpromazine and thiouracil which were equal to the therapeutic plasma levels all depressed the oxygen consumption of the cells. Although these results showed that the drugs can have a toxic action on granulocytes, the site of action of this effect could be distinct from that of a metabolic lesion which predisposes to granulocytopoenia. In the absence of any better guide, we also chose

drug concentrations which were similar to the therapeutic plasma levels, except in the case of aspartate transcarbamylase. It should, however, be emphasised that these are no indication of the concentration of a drug within the cells, or at the active sites on enzyme molecules *in vivo*. Thus, they may be far from the optimum values for demonstrating an inhibitory effect.

The type of inhibition of dihydroorotic dehydrogenase which phenylbutazone produced was not investigated, because this enzyme is assayed in the presence of dihydroorotase, which simultaneously hydrolyses the substrate to carbamylaspartate. This precluded any studies which involve investigating the effect of substrate concentration on initial reaction rates. Furthermore, the effect of protein–protein interaction and the effect of ions and other small molecular compounds on the activity of the enzymes could not be controlled in the impure enzyme system used for this work. We would emphasise that mechanistic studies should only be undertaken with adequately purified enzymes.

The present observations confirm the relatively high activity of aspartate transcarbamylase in human granulocytes. The allosteric properties of this regulatory enzyme have been widely studied in microorganisms so that the possibility that it might be subject to inhibition by drugs was of particular interest. However, other investigators[16,17] failed to demonstrate feed back inhibition of human leukocyte and rabbit erythrocyte aspartate transcarbamylase by a series of pyrimidine ribonucleotides. The results obtained in the present studies with aspartate transcarbamylase were clearly influenced by the total concentration of the cell preparation within quite a narrow range, this might be due to the effect of dilution on the concentration of other components of the cell preparation which can affect the activity of aspartate transcarbamylase or to conformational changes in the protein molecule related to the degree of dilution. Further studies with purified granulocyte aspartate transcarbamylase are needed to elucidate this phenomenon.

Pisciotta and Hinz[18] reported that chlorpromazine inhibited deoxythymidine and deoxythymidilate kinases and DNA polymerase in regenerating rat liver extract. It is of interest that in contra-distinction to this we found no effect of chlorpromazine on the five enzymes which catalyse the earlier steps of pyrimidine biosynthesis. Pisciotta and his colleagues[2] reported that patients who had had agranulocytosis showed a limited "proliferative potential" in their marrow cells even in the absence of drugs possibly due to an enzyme deficiency. Unlike normal non-sensitive subjects, these patients may be unable to overcome the inhibitory effect of a drug and so cell production ceases. Studies on patients are now in progress to determine if such a hypothesis applies to any of the enzymes concerned in *de novo* pyrimidine biosynthesis.

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REFERENCES

1. A. V. Pisciotta and J. Kaldahl, *Blood*, **20**, 364 (1962).
2. A. V. Pisciotta, *J. Lab. clin. Med.* **65**, 240 (1965).
3. M. Green and S. S. Cohen, *J. biol. Chem.* **225**, 397 (1957).
4. I. Lieberman, A. Kornberg and E. S. Simms, *J. biol. Chem.* **215**, 403 (1955).
5. P. Grabar, N. M. Seligman and J. Bernard, *Ann. Inst. Pasteur.* **88**, 548 (1955).
6. W. A. Skoog and W. S. Beck, *Blood*, **11**, 436 (1956).

7. H. J. FALLON, E. FREI, J. D. DAVIDSON, J. S. TRIER and D. BURK, *J. Lab. clin. Med.* **59,** 779 (1962).
8. J. H. CUTTS in *Cell Separation Methods in Haematology*, p. 182. Academic Press, New York (1970).
9. O. H. LOWRY, W. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
10. P. H. MCCURRACH, J. K. PARK and W. L. M. PERRY, *Br. J. Pharmac.* **38,** 608 (1970).
11. E. G. C. CLARKE (Ed.), *Isolation and Identification of Drugs*, p. 493. The Pharmaceutical Press, London (1969).
12. L. H. SMITH JR. and F. A. BAKER, *J. clin. Invest.* **38,** 798 (1959).
13. L. H. SMITH JR., M. SULLIVAN and C. M. HUGULEY JR. *J. clin. Invest.* **40,** 656 (1961).
14. L. H. SMITH JR, C. M. HUGULEY JR. and J. A. BAIN in *The Metabolic Basis of Inherited Disease* (Eds. J. B. STANBURY, J. B. WYNGAARDEN and D. S. FREDRICKSON) p. 746. McGraw-Hill, New York (1966).
15. I. LIEBERMAN and A. KORNBERG, *Biochim. biophys. Acta* **12,** 223 (1953).
16. M. D. PRAGER, J. E. YOUNG and I. C. ATKINS, *J. Lab. clin. Med.* **70,** 768 (1967).
17. M. R. CURCI and W. D. DONACHIE, *Biochim. biophys. Acta* **85,** 338 (1964).
18. A. V. PISCIOTTA and J. E. HINZ, *Fedn Proc.* **25,** 195 (1966).